Strategies for the Direct Coupling of Solid Phase Microextraction to Mass Spectrometry

by

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A thesis

presented to the University of Waterloo

in fulfillment of the

thesis requirement for the degree of

Doctor of Philosophy

in

Chemistry

Waterloo, Ontario, Canada, 2017

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Statement of contributions

Chapter 2 was already published in three articles. Section 2.2 includes the following manuscript "Solid phase microextraction (SPME)-transmission mode (TM) pushes down detection limits in direct analysis in real time (DART)" (Chem. Commun., **2014**, 50, 12937-12940) which was co-authored/supervised by J. Pawliszyn. In all cases, experimental planning and design, experimental work conducted in the laboratory, data analysis, interpretation, and writing were performed by the author of the thesis. Section 2.3 includes the following manuscript "Ultrafast Screening and Quantitation of Pesticides in Food and Environmental Matrices by Solid-Phase Microextraction–Transmission Mode (SPME-TM) and Direct Analysis in Real Time (DART)" (Anal. Chem., **2017**, 89 (13), 7240–7248) which was co-authored by E. Gionfriddo and J. Poole, and supervised by J. Pawliszyn. In all cases, experimental planning and design, experimental work conducted in the laboratory, data analysis, interpretation, and writing were performed by the author of the thesis. Section 2.4 includes the following manuscript "Towards on-site analysis of complex matrices by solid-phase microextraction-transmission mode coupled to a portable mass spectrometer via direct analysis in real time" (Analyst, **2017**, 142, 2928-2935) which was co-authored by T. Vasilejvic, E. Gionfriddo and M. Yu and supervised by J. Pawliszyn. In all cases, experimental planning and design, experimental work conducted in the laboratory, data analysis, interpretation, and writing were performed by the author of the thesis.

Chapter 3 was already published in four articles. Section 3.2 includes the following manuscript "Biocompatible Solid-Phase Microextraction Nanoelectrospray Ionization: An Unexploited Tool in Bioanalysis" (Anal. Chem., **2016**, 88 (2), 1259–1265) which was co-authored by N. Reyes-Garcés and B. Bojko, and supervised by J. Pawliszyn. In all cases, experimental planning and

design, experimental work conducted in the laboratory, data analysis, interpretation, and writing were performed by the author of the thesis. Section 3.3 includes an excerpt (1700 words) that is published in the following manuscript "Fast Quantitation of Target Analytes in Small Volumes of Complex Samples by Matrix-Compatible Solid-Phase Microextraction Devices" (Angew. Chem. **2016**, 128, 7636-7640) which was co-authored by H. Piri-Moghadam, H., F. Ahmadi, E. Boyacı, N. Reyes-Garcés, A. Aghakhani, and B. Bojko, and supervised by J. Pawliszyn. In all cases, experimental planning and design, experimental work conducted in the laboratory, data analysis, interpretation, and writing of the excerpt were performed by the author of the thesis. Section 3.4 includes the following manuscript "Open Port Probe Sampling Interface for the Direct Coupling of Biocompatible Solid-Phase Microextraction to Atmospheric Pressure Ionization Mass Spectrometry" (Anal. Chem., **2017**, 89 (7), 3805–3809) which was co-authored by C. Liu, M. Tascon, N. Reyes-Garcés, D.W. Arnold and T. Covey, and supervised by J. Pawliszyn. In all cases, experimental planning and design, experimental work conducted in the laboratory, data analysis, interpretation, and writing were performed by the author of the thesis. Section 3.5 includes the following manuscript "Fast quantitation of opioid isomers in human plasma by differential mobility spectrometry/mass spectrometry via SPME/open-port probe sampling interface" (Anal.Chim. Acta. *article in press*; **2017**, DOI: 10.1016/j.aca.2017.08.023) which was co-authored by C. Liu, B. Schneider, Y. Le Blanc, Reyes-Garcés, D.W. Arnold and T. Covey, and supervised by J. Pawliszyn. In all cases, experimental planning and design, experimental work conducted in the laboratory, data analysis, interpretation, and writing were performed by the author of the thesis.

Chapter 4 was already published in four articles. In addition, chapter 4 contains two manuscripts submitted and one manuscript in preparation. Section 4.2 includes the following manuscript "Development of coated blade spray ionization mass spectrometry for the quantitation of target

analytes present in complex matrices" (Angew. Chem. **2014**, 53, 14503-14507) which was co-authored/supervised by J. Pawliszyn. In all cases, experimental planning and design, experimental work conducted in the laboratory, data analysis, interpretation, and writing were performed by the author of the thesis. Section 4.2 also includes an excerpt (500 words) that is published in the following manuscript "Fast Quantitation of Target Analytes in Small Volumes of Complex Samples by Matrix-Compatible Solid-Phase Microextraction Devices" (Angew. Chem. **2016**, 128, 7636-7640) which was co-authored by H. Piri-Moghadam, H., F. Ahmadi, E. Boyacı, N. Reyes-Garcés, A. Aghakhani, and B. Bojko, and supervised by J. Pawliszyn. In all cases, experimental planning and design, experimental work conducted in the laboratory, data analysis, interpretation, and writing of the excerpt were performed by the author of the thesis. Section 4.3 includes the following manuscript "Ultra-fast quantitation of voriconazole in human plasma by coated blade spray mass spectrometry" (J Pharmaceut. Biomed., **2017**, 144, 106-111) which was co-authored by M. Tascon, N. Reyes-Garcés, E. Boyacı and J. Poole, and supervised by J. Pawliszyn. In all cases, experimental planning and design, experimental work conducted in the laboratory, data analysis, interpretation, and writing were performed by the author of the thesis. Section 4.4 includes the following manuscript "High-Throughput Screening and Quantitation of Target Compounds in Biofluids by Coated Blade Spray-Mass Spectrometry" (Anal. Chem., **2017**, 89 (16), 8421-8428) which was co-authored by M. Tascon, N. Reyes-Garcés, E. Boyacı and J. Poole, and supervised by J. Pawliszyn. In all cases, experimental planning and design, experimental work conducted in the laboratory, data analysis, interpretation, and writing were performed by the author of the thesis. Section 4.5 includes the following manuscript "Rapid determination of immunosuppressive drug concentrations in whole blood by Coated Blade Spray-Tandem Mass Spectrometry (CBS-MS/MS)" (*manuscript submitted*, **2017**) which was co-authored by M.

Tascon, N. Reyes-Garcés, E. Boyacı and J. Poole, and supervised by J. Pawliszyn. In all cases, experimental planning and design, experimental work conducted in the laboratory, data analysis, interpretation, and writing were performed by the author of the thesis. Section 4.6 includes the following manuscript "Quantitative analysis of biofluid spots by coated blade spray mass spectrometry, a new approach to rapid screening" (*manuscript submitted*, **2017**) which was co-authored by M. Tascon, N. Reyes-Garcés, E. Boyacı and J. Poole, and supervised by J. Pawliszyn. In all cases, experimental planning and design, experimental work conducted in the laboratory, data analysis, interpretation, and writing were performed by the author of the thesis. Section 4.6 also includes an excerpt (1000 words) of the manuscript in preparation entitled "Lab-on-a-blade" which was co-authored by M. Tascon, N. Reyes-Garcés, V. Aquaro, D. Rickert, A. Kasperkiewicz, and supervised by J. Pawliszyn. In all cases, experimental planning and design, experimental work conducted in the laboratory, data analysis, interpretation, and writing were performed by the author of the thesis.

Abstract

In recent years, advances in direct sample to mass spectrometry (MS) techniques have allowed for the application of these methods towards quantitative analysis in complex matrices such as biofluids and tissue. However, the predictable limitations of these technologies, such as ionization suppression, poor sensitivity at trace levels, and narrow linear dynamic range, have been the driving force toward the development of methods that efficiently integrate sampling, sample cleanup, and analyte collection and ionization. In this context, the direct interface of microextraction technologies and MS has undoubtedly revolutionized the speed, efficacy, and robustness with which complex matrices can be scrutinized. In this thesis, numerous strategies recently developed for the direct and efficient coupling of Solid Phase Micro Extraction (SPME) and MS are presented towards the analysis of complex matrices. Aiming to supply a range of technologies suited for diverse applications, different SPME geometries such as coated fibers, blades and meshes, as well as ionization approaches, such as atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI), were studied. In addition, these workflows are compatible with SPME devices that undergo either sampling of tissue or direct immersion into liquid samples. The strategies developed as part of this doctoral dissertation include the following: SPME-Transmission Mode coupled to MS via Direct Analysis in Real Time (SPME-TM-DART-MS), SPME coupled to MS via nano-ESI (SPME-nanoESI-MS), SPME coupled to MS via Open Port Probe (SPME-OPP-MS), and Coated Blade Spray-MS (CBS-MS). In most of the applications herein compiled, total analysis time does not exceed 5 minutes, while sample volumes ranging between 1 and 1500 μL can be utilized for analysis. Sampling/sample-preparation is performed

either by spotting the sample onto the SPME-device, or by immersing the SPME-device on a vessel containing the sample. Despite short extraction times, limits of detection in the pg/mL to sub-ng/mL range were obtained, while good accuracy (*i.e.* 80-120%) and linearity (*i.e.* ppt to ppm) were attained for all studied probes (*i.e.* therapeutic drugs, drugs of abuse, pharmaceuticals, and pesticides) in the diverse sample matrices (*e.g.* phosphate buffer saline, urine, plasma, blood, grape juice, orange juice, milk, and ground water). Lastly, this work describes exemplary cases in which the mere coupling of SPME to MS is not sufficient to answer relevant analytical questions, and the use of a chromatographic step is justified. Hence, supplementary instrumental strategies that allow for removal of co-extracted interferences or source artifacts, such as Differential Mobility Spectrometry (DMS), tandem MS in time (MS^n), and Multiple Reaction Monitoring with Multistage Fragmentation (MRM^3), are also discussed in this dissertation.

Although the body of this work is chiefly focused on biofluid analysis, the attained results certainly support the implementation of this group of technologies towards the analysis of diverse complex matrices of environmental, biological, food, clinical, military, forensic, and pharmaceutical significance. We are confident that in a foreseeable future, this work will encourage readers around the globe towards the use of SPME-MS as a workhorse for on-site and benchtop analysis. In few words, SPME-MS technologies appear poised to shift the paradigm of direct sample introduction to MS.

Acknowledgment

I would like to take this opportunity to express my appreciation to all who have supported and assisted me in the completion of my PhD.

First of all, I would like to thank my supervisor and mentor, Professor. Janusz Pawliszyn, for giving me the opportunity to be part of his research group - first as a Masters student, and now as a PhD candidate. I am sincerely grateful for being given the opportunity to participate in diverse exciting projects, for having been allowed to attend important international conferences and scientific meetings where I could present my results and meet other fellow scientists.

I would like to thank my committee members, Professor Michael Palmer and Professor Scott Hopkins, for the time and efforts they have spent reading my thesis, and for their guidance and advice. I would also like to thank my external examiner, Professor Zoltan Takáts, and my internal examiner, Professor Brendan McConkey, for accepting the invitation to be part of the evaluation committee of my work, and for the time and patience they spent in examining my thesis. Thanks to the examining committee for nominating my thesis to the Pearson Medal award. In addition, I would like to thank Professor Wojciech Gabryelski and Professor Marcin Wasowicz who, although could not attended my defense due to work constrains, were part of my PhD advisory committee and I am grateful for their valuable time and support during the last four years.

I would like to extend my gratitude to my University of Waterloo colleagues that helped me in different ways during my doctoral studies including Dr. Marcos Tascon, .Dr. Ezel Boyacı, Dr. Emanuela Gionfriddo, Justen Poole, Daniel Rickert, Alexander Kasperkiewicz, Sofia Lendor, Vinicius Aqcuaro, Tijana Vasiljevic, Jonathan Grandy, Dr. Miao Yu, Dr. Varoon Sigh, Dr. Erica Souza-Silva, Dr. Barbara Bojko and Dr. Hamed Piri-Moghadam.

Likewise, I would like to thank all the industrial research collaborators that I met during the last four years for sharing of their knowledge and life experience, particularly to Dietmar Hein, Dr. Mike Morris, Dr. Emrys Jones, John Vukovic, Dr. Chang Liu, Dr. Tom Covey, Bradley Hart, Marta Kozak, Dr. Mary Prieto-Conaway, Dr. Edward Goucher, Clay Campbell, Kristine Van Natta, Sam Scozzaro, Jason Lui, Olivier Collin, Dr. Brian Musselman, Dr. Dragan Vuckovic, Joseph Tice, Robert Goguen, Gary Stidsen, Bob Shirey, Dr. Paul Ross, Craig Aurand, Len Sidisky, Dr. Marcelo Cypel, Dr. Pedro Reck dos Santos, Dr. Mauricio Pipkin, Mustansir Diwan and Dr. Clement Hamani.

I would like to express my gratitude to the Science Shop of the University of Waterloo, especially to Krunomir Dvorski, Harmen Vander Heide, Andrew Dube and Hiruy Haile. Thanks for your friendship and outstanding help and service whenever me or my team was in need.

I would like to express my love and appreciation to Emanuela Gionfriddo, Ezel Boyacı, Erica Souza-Silva, Erasmus Cudjoe, Marcos Tascon and Ruifen Jiang, who are not only excellent scientists but also great friends that make the last seven years of my life easier.

I am honored to thank my colleague and wife, Nathaly Reyes Garcés, for her love, support, help and advice through the last seven years. Thanks for bringing so much joy to my life.

Lastly, I owe my deepest gratitude to my family and the Reyes-Garcés family, their encouragement and unconditional love means everything to me.

Dedication

I dedicate this thesis to

my parents, German and Carmen Cecilia

my sister, Viviana Lucia

my aunties, Maria Elena, Beatriz and Rosita

and my wife, Nathaly

for their love, understanding and support through all my career

Always keep your dreams alive, because the best things in life take time

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List of Abbreviations

µe	Microextraction
AMS	Ambient Mass Spectrometry
APCI	Atmospheric Pressure Chemical Ionization
C18	Octadecyl silica particles
CBS	Coated Blade Spray
Da	Dalton
DART	Direct Analysis in Real Time
DESI	Desorption Electrospray Ionization
DI	Direct immersion
DMS	Differential Mobility Spectrometry
EI	Electron impact
ESI	Electrospray Ionization
GC	Gas Chromatography
HLB	Hydrophilic-Lipophilic Balance particles
HRMS	High Resolution Mass Spectrometry
HS	Head Space
IMS	Ion Mobility Spectrometry
ISD	Immunosuppressive drugs
IT	Ion trap analyzer
K_{fs}	SPME affinity constant
LC	Liquid Chromatography
LLE	Liquid Liquid Extraction
LOD	Limit of Detection
LogP	Logarithm of the partition coefficient
LOQ	Limit of Quantitation
m/z	Mass to charge ratio
MRM	Multiple Reaction Monitoring
MRM3	Multiple reaction monitoring with multistage fragmentation
MS	Mass Spectrometry

MS/MS	Tandem Mass Spectrometry in space
MSn	Tandem Mass Spectrometry in time
MW	Molecular weight
nanoESI	Nano Electrospray Ionization
OPP	Open Port Probe
PAN	Polyacrylonitrile
POC	Point-of-care
PBS	Phosphate Buffer Saline
QqQ	Triple Quadrupole analyzer
QTOF	Quadrupole - Time of Flight analyzer
RMSE	Root Mean Square Error
SAMS	Stand Alone Mass Spectrometry workflow
SPME	Solid Phase Micro Extraction
SPME-TM	Solid Phase Micro Extraction-Transmission Mode
SRM	Single Reaction Monitoring
SSP	Simplistic Sample Preparation workflow
TDM	Therapuetic Drug Monitoring
TOF	Time of Flight analyzer
TSP	Thorough Sample Preparation workflow

Chapter 1: Introduction

1.1. Bioanalytical chemistry: targeted analysis of small molecules in biofluids

Bioanalytical chemistry is a sub-discipline of analytical chemistry that involves the development of methods and instruments that enable sensitive, reproducible, and selective analysis of xenobiotics and biotics in biological samples.[1] In order to attain this objective, a typical bioanalytical workflow integrates multiple technologies, including sampling, sample preparation (*i.e.* enrichment of analytes of interest and clean-up of undesired matrix components), analyte separation (*i.e.* discrimination between analyte and potential matrix interferences such isomers, isobars, in-source fragmentation products), and analyte detection (*e.g.* via tandem mass spectrometry).[2] Given that bioanalytical chemistry encompasses both the analysis of small and large molecules, it should be noted at this juncture that this doctoral dissertation is fundamentally focused on the development of methods and devices for analysis of small molecules; hence, all topics discussed hereafter are strictly related to the analysis of compounds with molecular weights (MW) ranging between 100 and 1250 Daltons (Da). Furthermore, most of the embodiments of this thesis describe technologies chiefly used for the analysis of biofluids; likewise, this introductory chapter is centered on a discussion of technologies capable of providing quantitative and qualitative information of compounds present in biological fluids.

In definition, a biofluid is a biological liquid that either: a) flows through certain compartments in the body and can be accessed with a needle or similar instrumentation (*e.g.* cerebrospinal fluid or blood); b) is excreted/secreted from the body (*e.g.* urine, saliva or sweat), or c) develops in response to a medical condition (*e.g.* blister). Inspections of biofluids, such as of urine,[3] have been

carried out since ancient times to diagnose diseases.[4] Thanks to outstanding breakthroughs in the fields of analytical and bioanalytical chemistry, clinicians possess nowadays tools that provide them with more accurate means of understanding the efficacy of a given treatment (*e.g.* up or down-regulation of a biomarker),[5] or the plausible negative impact such a treatment might have on a patient (*e.g.* drug concentration above or below its therapeutic range).[6] Undeniably, the state of the art of mass spectrometry (MS) has played a tremendous role in these advances; for instance, absolute compound sensitivity has increased several orders of magnitude since 1980.[7] Such improvements have enabled the introduction of sub-femtogram quantities of analyte(s) of interest for quantification into MS systems, instead of the nanogram quantities achieved years ago [8,9]

Currently, as shown in Figure 1.1, the bioanalytical workflow for inspection of biological fluids via mass spectrometry can be divided into three main strategies. The first strategy, herein called the "*thorough sample preparation*" (TSP) approach, is perhaps the most widely used strategy. Basically, it consists of taking an aliquot of the biofluid under investigation, then carrying out one or multiple sample preparation steps (*e.g.* liquid-liquid extraction, LLE; protein precipitation, PP; solid-phase extraction, SPE; or a combination of those technologies) with aims of extracting and enriching analyte(s) of interest from the sample matrix. Subsequently, the sample-extract is injected into the analyzer (*e.g.* mass spectrometer) via a separation system, gas and liquid chromatography (GC and LC, respectively) being the most common techniques.[10] The second approach, herein named the "*simplistic sample preparation*" (SSP) approach, encompasses the use of methods aimed at inducing the release of analytes bound to proteins prior to subsequent introduction of the extract into the detection system (*e.g.* MS) via a chromatographic separation method.

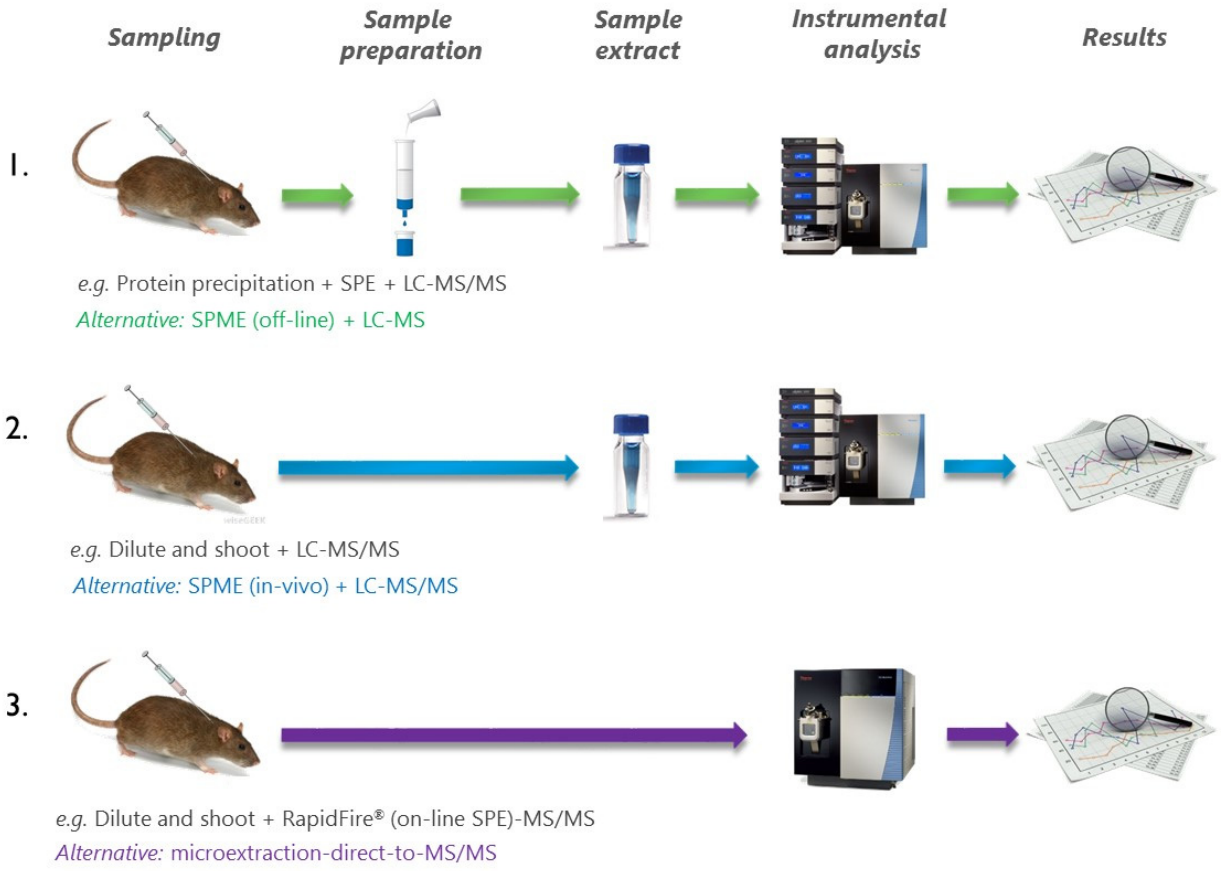

Figure 1.1 Schematic representation of most common bioanalytical workflows currently used for the analysis of small molecules in biofluids via mass spectrometry. **1.** *Thorough Sample Preparation (TSP) approach:* one or multiple sample preparation steps, plus separation, plus detection approach ("*gold standard*"); **2.** *Simplistic Sample Preparation (SSP) approach:* rapid sample dilution/extraction, plus separation, plus detection approach; **3.** *Stand Alone MS (SAMS) approach:* direct interface of the sample extract to the MS system (might or might not include and on-line sample preparation step).

Such methods are generally carried out via dilution of the matrix (*e.g.* dilute-and-shoot (DS), frequently used in analysis of less viscous biofluids such as urine[11]) with a mixture of organic-solvent/water, or through quick precipitation of matrix proteins (*e.g.* protein precipitation (PP), frequently used for analysis of blood, plasma, serum). The third approach, so-called the "*Stand Alone MS*" (SAMS) strategy, completely eradicates the chromatographic separation step inherent in the first two workflows by directly injecting the sample extract into the mass spectrometer.[12]

Given the relevance of this approach to the work herein presented, pros and cons of this methodology will be thoroughly discussed in the following section.

Succinctly, the TSP strategy is unquestionably the most comprehensive in terms of sample clean-up and analyte enrichment. TSP workflows prevent matrix interference with adequate isolation of analyte(s) of interest from matrix interferences via chromatographic separation and their subsequent selective detection through specific mass spectrometry experiments (*e.g.* high resolution mass spectrometry, HRMS; tandem mass spectrometry, MS/MS; or multistage fragmentation, MS^n), and they are therefore used as gold standards in the development of novel bioanalytical methodologies.[13] Nevertheless, such technologies are not without drawbacks; the excessive labor, as well as the multiple and tedious steps typically required in such workflows have been the driving force towards the development of methods that are capable of providing comparable analytical answers while requiring shorter periods of time and less sample handling. Recent advances in instrumental automation[14,15] permitted the development of unmanned TSP methodologies capable of determining small molecules in record times.[16–18] Yet, the most efficient TSP technologies developed to date are prohibitively expensive for small/mid-size research laboratories; thus, the routine use of these technologies is limited to well-funded clinical laboratories and pharmaceutical companies of a scale that necessitates the analysis of thousands of samples per day, a practice which significantly decreases the cost of analysis per sample and affords enough margin to justify their use.[13,19,20]

Consequently, in recent years, researchers have also developed workflows, such as SSP, that require fewer steps and resources. In spite of the speed, simplicity, and ease of automation of SSP protocols,[21,22] in certain cases, such methods fail to eliminate sample constituents such as salts or phospholipids that maydramatically increase or suppressionization efficiency, thus distorting

sample detection.[11] Thus, SSP strategies are mainly appropriate for molecules that are easily-ionizable (*e.g*. those containing a basic nitrogen atom) and not present at ultra-low concentrations (*e.g.* sub-nanogram mL^{-1}), as such workflows offer limited sensitivity due to the absence of a preconcentration step.[11,23]

1.2. Bioanalysis: towards rapid and reliable determination of small molecules in biofluids

Commerical MS instruments arrived in routine labs in the 90s[7]. Their limited sensitivity necessitated the use of relatively large samples, which also had to be cleaned up by chromatography to prevent matrix interference. The much improved sensitivity of recent instruments has made viable the use of SAMS methods, which has greatly decreased sample processing time.[24] By removing the separation step (*i.e.* classical LC/GC-MS methods range between 10 and 30 min,[25] while ultrafast separations can be as fast as 0.5-5 min [26–28]), SAMS strategies allow for outstanding reductions in total analysis times (≤ 1 min).[29,30] The workflow in SAMS typically consists of three steps. First, the sample extract is introduced into a "mixing chamber", either by liquid or thermal desorption (see Figure 1.2). [31–33] Subsequently, a pressurized liquid or gas carries the introduced analytes towards the ionization chamber (*e.g.* ESI, APCI, EI), where analytes become molecular species suitable for mass spectrometric analysis. Once ionized, compounds can then be submitted to detection by a mass analyzer, although certain mass analyzer configurations also enable the introduction of additional steps prior to ion detection that aid in improvements in the selectivity/sensitivity of the final data (*e.g.* ion-mobility[29] or MS^n[34]). Given that in SAMS all analytes are introduced into the mass spectrometer at once, the signal that is generated for each analyte/feature by the MS has the shape of a peak or a band (see Figure 1.3A).

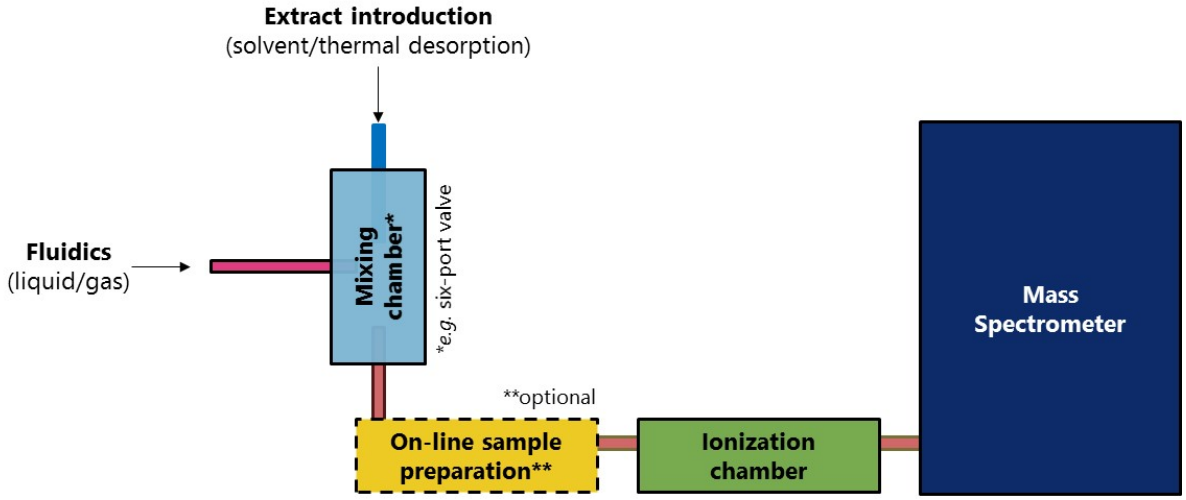

Figure 1.2 Schematic representation of a SAMS workflow from extract introduction to analyte detection by mass analyzer.

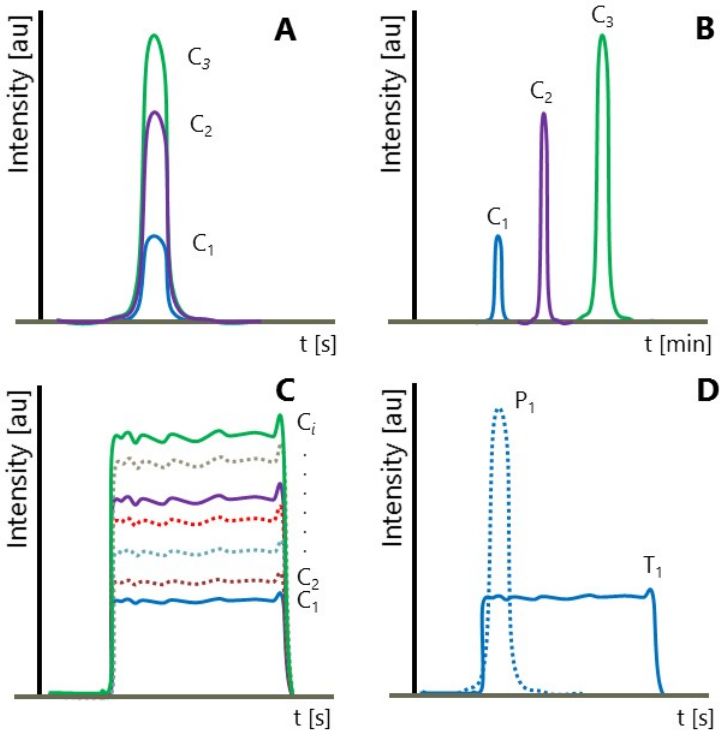

Figure 1.3 Schematic representation of instrumental signals obtained via mass spectrometry according to the analytical workflow. **A.** single peak signal obtained via SAMS approach; **B.** multiple peaks obtained via chromatographic separation after SSP or TSP; **C.** transient signal obtained by employment of the direct-to-MS approach, without a liquid or gas flow pushing the analytes towards the MS inlet; **D.** comparison between transient signal (T1) and peak signal (P1).

In this context, the presence of a single band brings along a series of advantages and disadvantages. First of all, by excluding the separation step from their workflows, SAMS methods completely[35,36] or partially[29] bypass the removal of compounds capable of causing matrix effects, which in result compromise the sensitivity of the method, particularly for poor ionizers. As shown in Figure 1.2, contingent on the sample matrix (*e.g.* whether it is urine[37] or plasma[38]), the physicochemical properties of the analyte(s) of interest (*e.g.* protein-binding and octanol-water partition coefficient, LogP), the ionization chamber used (*e.g.* on-line SPE-ESI[39], ESI[7], or nano-ESI[37]), and the sensitivity required, SAMS strategies may or may not include an on-line analyte enrichment step prior to the introduction of analyte into the MS analyzer.[7,40] Undeniably, technologies that include an on-line pre-concentration step such as RapidFire™,[41] TurboFlow™[38] and the column-switching approach[42] are more robust and less prone to matrix effects. Conversely, aiming to reach desired LOQ values, off-line analyte enrichment steps, such SPE, have also been implemented prior to direct introduction of sample extract into the MS system.[43,44] Indisputably, on-line methodologies are faster and maximize the utilization of MS instruments; however, in this case, gains in speed of analysis are certainly linked to the cost of the instrumentation being used. For instance, studies using the RapidFire™ system by Agilent Technologies (~300-400k CAD) have claimed instrumental analysis times in the order of 8-15s per sample,[41] whereas similar experiments on the cheaper TurboFlow™ system by Thermo Fisher (~90-100k CAD) allow for analysis times in the order of 1-2 min per sample.[38] Although the analysis speed provided by these systems is hardly unbeatable, and certainly unthinkable two decades ago, this doctoral dissertation presents several technologies that could make us reconsider whether this is the fastest speed of analysis that can be achieved with respect to bioanalytical measurements. Second, the peak width in SAMS is mainly governed by the injection band diffusion; mixing occurs as the sample extract travels from the

injection chamber to ionization chamber. Hence, the shape of a given analyte peak is dependent on the selected instrumental conditions (*e.g.* carrier flow rate and injection volume[43]) and the configuration of the system (*e.g.* connecting capillaries and ionization source dimensions[45]). For instance, signal intensity increases as the diameter of the capillary connecting the desorption and ionization chamber decreases, or when the fluidics flow rate increases, due to the reduction of injection band diffusion. Whatever the peak shape, at least 10 data points, which may not be achievable if the peak widths are too narrow. Certainly, narrow peaks also limit the total number of compounds that can be analyzed, especially in tandem mass spectrometry applications, as this number is dependent on the selected amount of transitions per compound (*i.e.* single reaction monitoring, SRM) as well as the designated dwell time for each transition. Accordingly, optimal parameters should allow for sufficient analyte sensitivity while maintaining adequate peak width for data collection. In terms of analytical figures of merit and sample analysis throughput (see Figure 1.4), the performance of SAMS technologies fall somewhere between those afforded by chromatography-MS and ambient mass spectrometry (AMS). While SAMS offers a higher sample analysis throughput and simpler methods than chromatography-MS, AMS, a subset of SAMS, utterly eradicates from its workflow the sample preparation and separation steps, thus allowing for the fastest throughput at the expense of the figures of merit. The following section explores the most recent advances in AMS towards the analysis of biofluids.

1.3. Ambient mass spectrometry: a novel avenue for rapid analysis of complex matrices

The term ambient mass spectrometry, or ambient ionization mass spectrometry, was proposed for the first time by professor Zoltan Takáts and his former collaborators at University of Purdue more than 10 years ago, aiming to describe a family of techniques that allow for the generation of ions

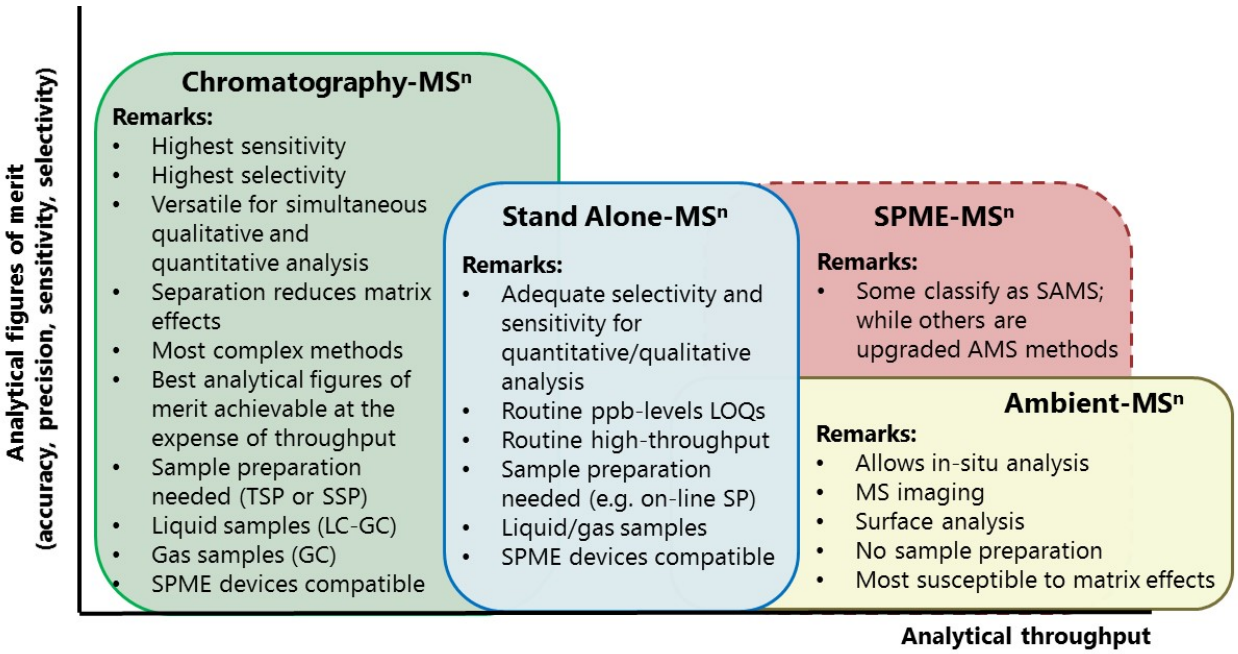

Figure 1.4 Position of SPME-MS relative to chromatography-MS, stand-alone MS, and ambient MS in terms of analytical throughput and figures of merit. Figure was adapted from Nanita *et al.*[7]

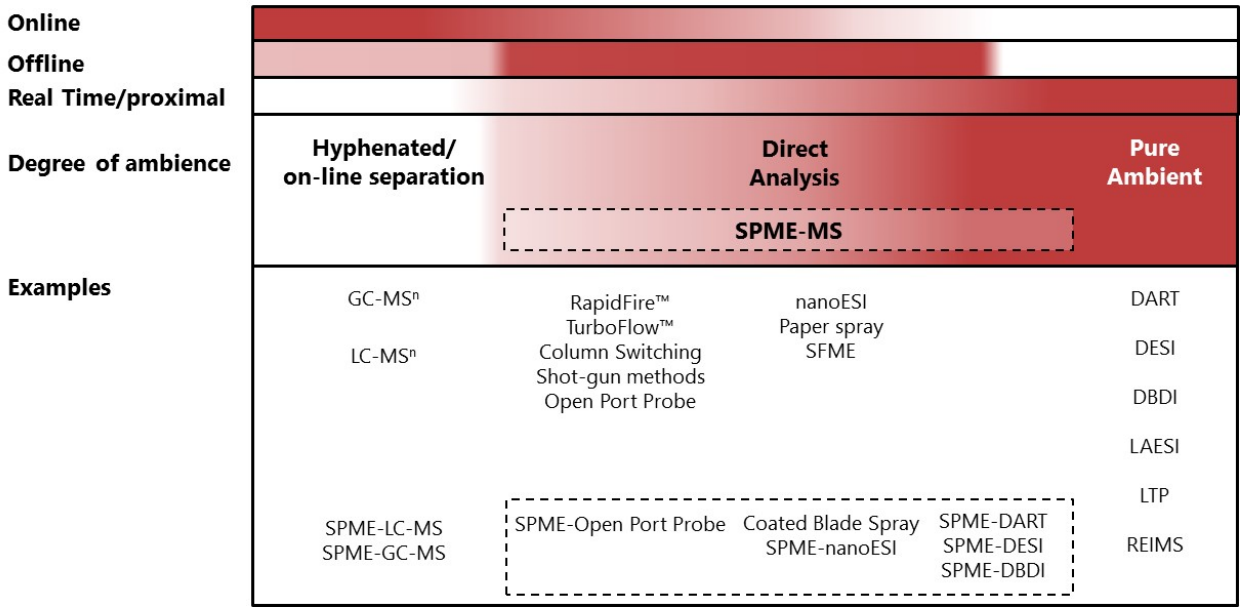

Figure 1.5 Color scale presenting the relative scale of ambience and how different SPME-MS technologies fit on it. Figure was adapted from Venter *et al*.[46]

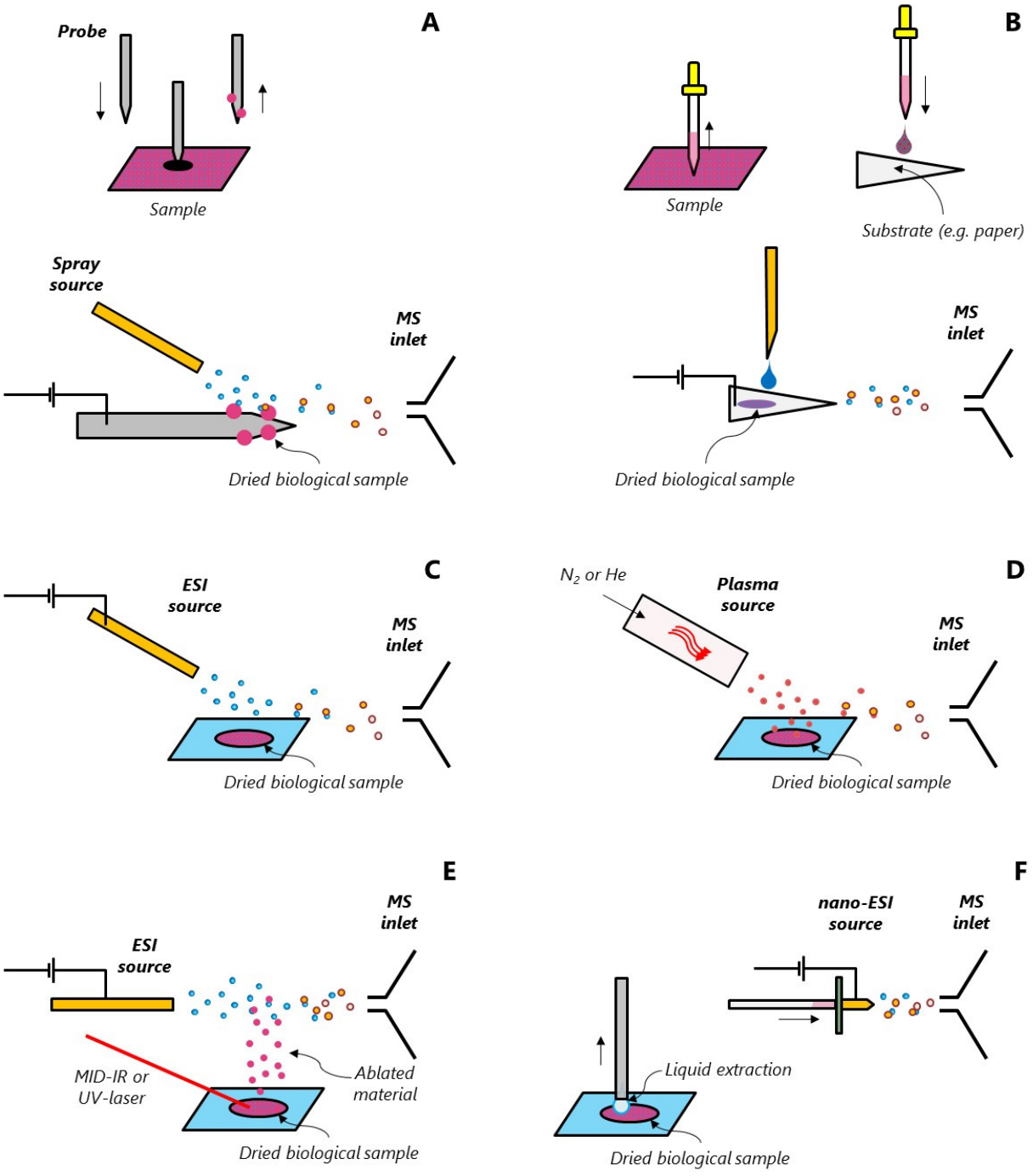

Figure 1.6 Schematic diagrams of some of the most relevant ambient ionization methods suitable for analysis of biofluids. **A.** Probe electrospray ionization (PESI)[47] or touch-spray;[48] **B.** Substrate electrospray ionization (e.g. paper spray, PS; leaf-spray or wooden-tip spray);[49–51] **C.** Desorption electrospray ionization (DESI)[52] and the set-up typically used for MSI; **D.** Plasma-based desorption source (*e.g.* direct analysis in real time, DART; low temperature plasma, LTP);[53,54] **E.** Laser ablation electrospray ionization (LAESI);[55] **F.** Liquid extraction surface analysis (LESA).[56]

under ambient conditions from ordinary samples in their native environment, without the need for sample preparation.[52,57] As depicted in Figure 1.5, ambient technologies fundamentally differ from any other technology in their ability to measure a given compound(s) in real time,[52,53,58] and in their proximity to the system under study (*e.g.* in-vivo or in-situ analysis).[59–65] Over the last decade, more than forty ambient ionization methods (*i.e.* comprising pure ambient and direct analysis) have been introduced for the analysis of complex matrices. These methods can be classified according to the manner in which analytes are extracted from the matrix (*i.e.* liquid and thermal desorption, or laser ablation), the ionization mechanism employed,[8,9,46,58,66] and whether the process includes more than one step (*e.g.* mechanical extraction and subsequent ionization via electrospray ionization).[67] Some of these methods might include a combination of features as well, such as thermal desorption from a solid substrate and subsequent ionization via ESI.[31] Figure 1.6 illustrates the methods that are most useful for biofluid analysis.

Undeniably, the introduction of pure ambient technologies has been a game changer in diverse fields such clinical,[5,68] food,[69,70] and forensic[71,72] analysis, given the offered throughput capabilities, their simplified workflow, and their easy operation — a particularly useful attribute, given that it facilitates operation of this technology by personnel not specialized in analytical chemistry.[46] Some of the most relevant AMS technologies developed to date include desorption electrospray ionization (DESI),[73] direct analysis in real time (DART),[74] rapid evaporative ionization mass spectrometry (REIMS),[75] laser ablation electrospray ionization (LAESI),[55] low temperature plasma (LTP),[54] liquid extraction surface analysis (LESA),[56] and resonant infrared laser ablation (RIR-LA).[62] Nonetheless, given that pure ambient ionization methods do not offer any analyte enrichment, these technologies are chiefly suited for surface characterization applications (*e.g.* mass spectrometry imaging, MSI[76]) and sample classification by chemometric

tools[77–79] via the detection of effortlessly ionizable compounds.[80] Therefore, applications of such methods towards the quantitation of target compounds present at trace levels (*e.g.* sub-nanogram per milliliter) in biofluids are rarely found in scientific publications, unless such technologies are combined with an efficient sample preparation step, as presented in the following sections.[81,82]

With respect to the second group, while such technologies allow for the direct analysis of samples without prior sample preparation and under ambient conditions, as the analytical process of such technologies occurs off-line, these techniques are unable to provide real-time data.[83] Such technologies, classified in Figure 1.5 as direct analysis technologies, essentially discern from SAMS approaches due to their analysis being performed without the use of elaborate equipment (*e.g.* fluidic pumps, pressurized gas, or heating, or laser devices). For instance, substrate spray technologies[46] such as paper spray (PS)[49], probe electrospray ionization (PESI),[84,85] and wooden-tip electrospray ionization (WT-ESI)[51] have been proven by scientists as suitable for analysis of target analytes in biofluids.[58,86] Succinctly, the substrate can be made of any material (*e.g.* metal, wood, paper), and charged droplets can be produced directly from substrates with sharp tips.[87] In this context, substrate-spray devices function as sample collection devices, where biofluids can be either pipetted onto the substrate (*e.g.* PS[8]), or the substrate dipped into the biofluid (*e.g.* PESI[47]), as shown in Figure 1.6. After application, liquid samples are usually allowed to dry on the substrate. Subsequently, a droplet of solvent is added onto the substrate to extract the target analytes from the dried biofluid. After sufficient time has elapsed so as to allow the substrate to become fully wetted with desorption/ionization solvent, a high-voltage is applied to the substrate so as to generate an electrospray from the tip of the substrate. Unlike most SAMS approaches, substrate-spray technologies typically generate a transient signal rather than a peak (see Figure 1.3C) that may last for several seconds, provided the substrate does not dry up, and that compounds

still remain on the substrate. Employment of transient signals for quantitation offers advantages such as the ability to monitor multiple compounds, or perform multiple MS experiments in a single analysis; conversely, this approach is limited by loss of sensitivity, since the signal-to-noise ratio is sacrificed in such cases (see Figure 1.3D).

Among the diverse substrate-spray technologies available to date,[50] PS is indisputably the most popular and widely accepted technique due to its simple operation (*i.e.* sample is spotted on the paper as in dried blood spots);[88] low cost (*i.e.* each device is comprised of just a tiny piece of inexpensive filter paper);[89] suitability for therapeutic drug monitoring (TDM), point-of-care (POC) and pharmaceutical applications (*i.e.* PS uses small sample volumes, ≤ 20μL);[90] and verified capabilities for quantitative analysis of target compounds in diverse biofluids at part-per-billion levels (*e.g.* plasma, serum, blood).[83,91,92] Furthermore, PS is commercially available from Prosolia,[93] while devices that allow for the automated coupling of multiple PS to MS[94,95] can also be purchased by laboratories where high-throughput is needed.[96] Certainly, the implementation of direct analysis of biofluids via PS-MS leads not only to savings in chromatographic time, but also in sample preparation costs. In spite of the multiple advantages of PS and all the fascinating applications that can be developed with this technology (*e.g.* discrimination of bacteria samples and quantitation of drugs of abuse in whole blood),[97–100] few scientist have objectively discussed the drawbacks associated with PS.[83,101,102] For example, PS applications lack sufficient sample clean-up; accordingly, matrix components soluble in the desorption/ionization solvent can be sprayed onto the MS system, which can thwart long-term instrument operation (*e.g.* due to contamination of transfer-tube or exit lenses). Furthermore, due to the lack of analyte enrichment, PS offers poor limits of quantitation in the sub nanogram per milliliter range, while also being limited to the analysis of small sample volumes.[102] Yet, it is important to bear in mind that the

problems observed in PS applications are common to any direct-to-MS technology where the sample clean-up step is neglected.[81] Because they are aware of these limitations, former pure ambient mass spectrometrists are moving towards technologies where at least one extraction step precedes direct interface to MS.[82,102] The following section discusses recently developed novel technologies that have as focus the direct interface of sample preparation technologies with mass spectrometry.

1.4. Methods based on microextraction (μe) technologies and its direct coupling to mass spectrometry

Although some works claim to not incorporate sample preparation in ambient mass spectrometry, it would be more precise to say that some of these methods require no *additional* sample preparation, since the analyte extraction process takes place during the analysis.[46] For instance, the physical process happening during DESI at the millisecond scale can be considered as a micro-liquid extraction from the analytes present on the surface of the system under investigation (*i.e.* sampling-spot size is typically less than 150 μm, with a few microliters of solvent per square mm performing the extraction). Similarly, an extraction takes place when liquid microjunction (LMJ)[24] devices such as LESA[56], nano-DESI,[103] or the open port probe (OPP)[36,45] are employed. Given that the extraction process in ambient ionization occurs directly from the matrix in the majority of cases, and that such process is rarely selective or enriching, it did not take several years after its conception for many users to realize the quantitative limitations of these technologies when applied to trace level detection.[104] Besides, since extraction typically occurs from very small sample areas (≤ 1 mm), it is quite hard to couple such technologies with well-known, yet cumbersome and cumbersome sample preparation technologies such as SPE or LLE. As a consequence,

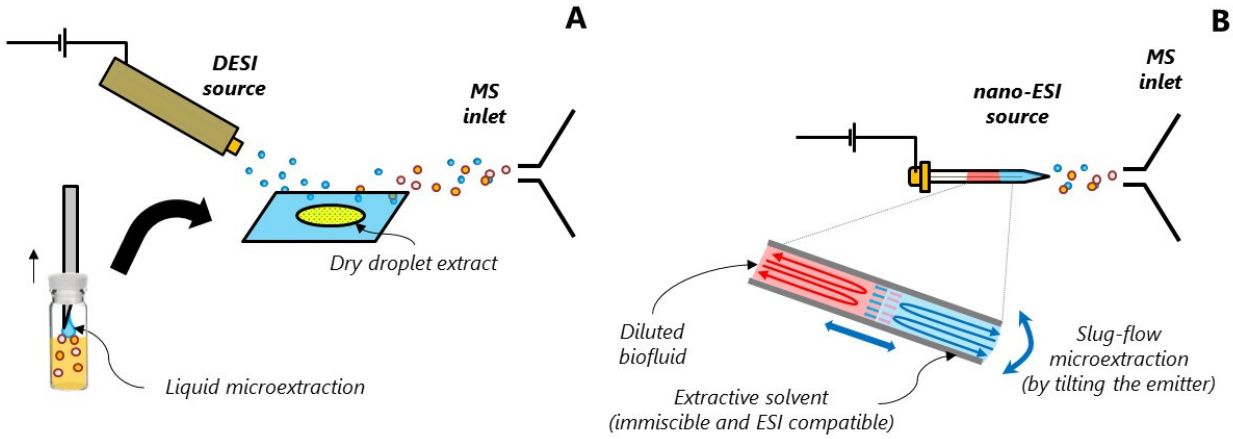

Figure 1.7 Schematic diagram of selected liquid phase microextraction (LPME) approaches. **A.** Large container vessel (*e.g.* single droplet microextraction, SDME with DESI[105,106]) *versus* **B.** Small container vessel (*e.g.* slug flow microextraction on a nanoESI emitter[82]).

technologies have been introduced as the most logical and suitable technology to overcome the encountered issues.[104]

Microextraction can be performed with the use of either a liquid (liquid phase microextraction, LPME) or a solid sorbent (solid phase microextraction, SPME). As solid phase microextraction technologies will be covered in the following sections, a brief discussion of LPME technologies directly coupled to MS is presented in this section. Essentially, the workflow in LPME-MS consists of 2 simple steps. First, a small amount ($\leq 20\mu L$) of liquid extractive phase is exposed to the matrix under study to enrich analytes of interest; classically, a liquid organic phase of sufficient elutrophic strength.[106,107] In the extraction process, analyte partitioning between the biofluid and the solvent droplet occurs according to the affinity of the analyte and the set extraction conditions (*e.g.* temperature, convection, time). After extraction, the droplet can be either transferred to a device for ESI or APCI (*e.g.* gas pressure assisted microliquid-liquid extraction via nanoESI[108] or micro-liquid extraction followed by venture-ESI[14,109]), or placed in-between an ambient ionization

source and the entrance of the mass spectrometer (*e.g.* microextract is placed and dried on a surface for DESI analysis,[106] or the extractive droplet is exposed to the jet of DESI micro-droplets[105]). As shown in Figure 1.7, in both scenarios, the extraction process occurs on a large sample container. A second option consists of enclosing the liquid extraction phase in a small chamber such as a microfluidic device or a nanoESI emitter, where extraction is carried out by fast interactions between the liquid phase and the biofluid. For instance, Kirby *et al.* demonstrated this principle by performing analysis of drugs of abuse on dried urine via a microfluidic device directly coupled to a portable MS.[110] Likewise, Ren *et al.* have described diverse technologies which involve liquid micro-extraction inside a nanoESI emitter.[82,89,111] For example, this group of authors reported on the quantitative determination of drugs of abuse through extraction of target compounds from a piece of paper containing a small dried blood spot,[111] or through extraction from diluted blood via slug-flow mechanism (*i.e.* slug-flow microextraction, SFME).[82] Certainly, scientific literature published to date supports LPME as a great alternative to enrich analytes of interest present in biofluids prior to direct-coupling to mass spectrometry. Definitively, a great many novel applications can be developed with respect to LPME-MS, and one could foresee a great future in workflows that involve novel advances on unmanned systems[20] and acoustically levitated micro droplets.[112] However, liquid extraction methods lack the selectivity provided by tailored coating chemistries,[113] and are unable to match the stiffness and endurance afforded by SPME-based devices; qualities that are highly desired in applications that involve extractions from very complex matrices, or when handling extremely small biofluid volumes such as droplets without sample dilution.[114]

1.5. Solid Phase Microextraction in bioanalytical chemistry

SPME is a solventless sampling/sample preparation technology conceived in the late eighties by Professor Janusz Pawliszyn and collaborators at University of Waterloo.[115] While this technology was originally envisaged for equilibrium-based extractions of volatile and semi-volatile compounds present on the headspace of liquid/solid samples enclosed in a vessel,[115–117] over the last two decades, SPME has undergone continuous reinventions that have expanded its applicability to the direct extraction of target analytes from complex liquid and solid matrices such as biofluids and tissue.[2,118–121] Furthermore, in recent times, and thanks to dramatic improvements in both coating technologies[122–125] and mass spectrometer sensitivity,[80,81] the use of pre-equilibrium SPME has gained more relevance, allowing for faster extractions with acceptable limits of detection.[126–128]

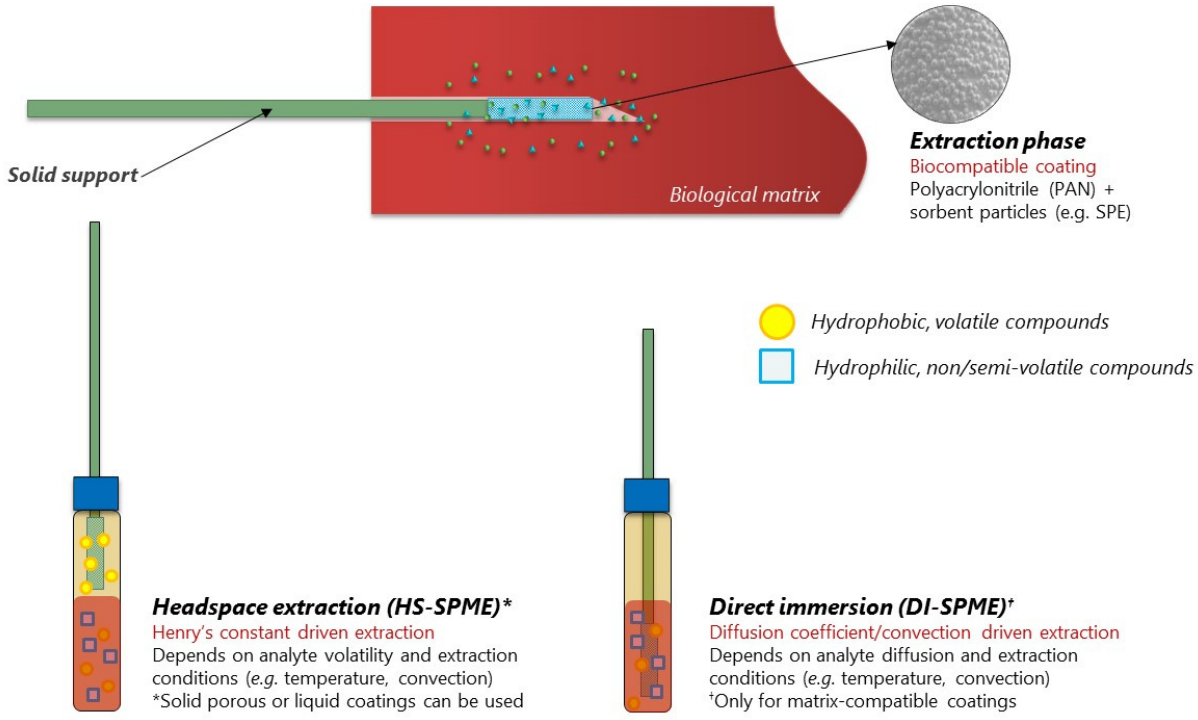

Figure 1.8 Schematic representation of SPME device and different extraction options, such as headspace extraction (HS) and direct immersion (DI) SPME.

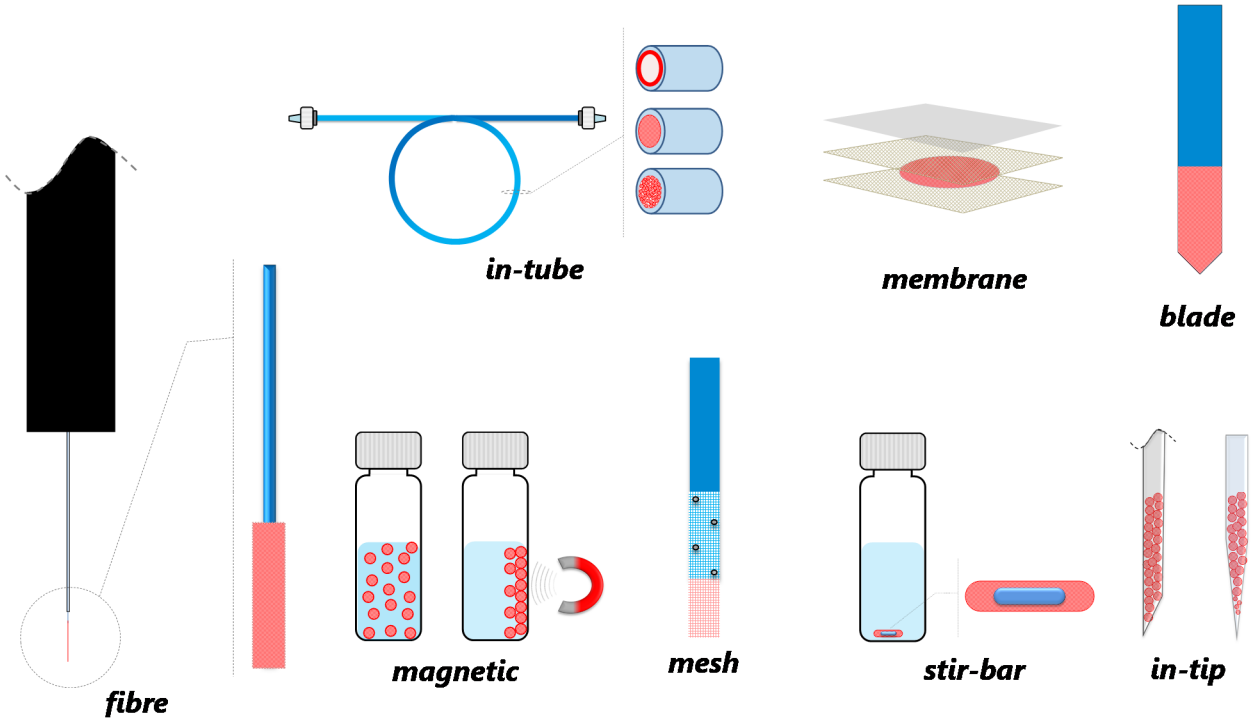

Figure 1.9 Schematic diagram presenting the different geometrical shapes the SPME concept embraces. Coating is indicated in red, while solid support is indicated in medium blue.

Conceptually, an SPME device consists of a solid support where a solid or liquid polymer phase is coated on a portion of the support (see Figure 1.8). The solid substrate can be made of any material (*e.g.* plastic[123], metal[129], wood[130]); however, matrix-compatible supports are generally preferred in most applications, as their employment aids in the prevention of potential biofouling of matrix components on the surface of the coating.[123] Thus, the SPME concept comprises a wide group of microextraction technologies with different geometrical configurations[113] that efficiently integrate sampling and sample clean-up, while also allowing for enrichment of the molar fraction of a given analyte in a single step (see Figure 1.9).

The analytical workflow of SPME typically consists of 3 to 5 steps. First, following their manufacture, coatings are cleaned either thermally[122] or with solvents,[131] depending on the manufacturing process used.[124] The cleaning of coatings aids in the removal of any byproducts of

the manufacturing procedure (*e.g.* particle polymerization process) that may cause instrument contamination or potential interferences during analysis. While the cleaning process is typically performed by the manufacturer, given that commercial SPME devices are generally stored in a box or a container prior to their use, most coatings need to be conditioned in order to remove substances that may have been extracted via passive sampling during their storage.[132] Similar to the cleaning step, conditioning is performed according to the intrinsic characteristics of the coating.[2,115,118,133] After their conditioning, coatings can then be exposed to the sample of interest for a given period of time to collect analytes from the sample matrix. Finally, analytes are desorbed either thermally or in a solvent mixture, this selection being dependent on the coating characteristics and the inherent physicochemical properties of the compound(s) under investigation.

Several calibration approaches have been developed for SPME, while equilibrium extraction is historically the most used method. Essentially, after a certain period of time has elapsed during the extraction process, concentration equilibrium is established between the sample matrix and the extraction phase; consequently, exposing the fiber for a longer period will not result in the accumulation of more analytes. Under equilibrium conditions and, provided the analyte has not been depleted from the sample or that the coating has not become saturated, the number of moles of analyte extracted (n) by the coating at equilibrium can be expressed by Equation 1.1.

Equation 1.1

$$n = \frac{K_{fs} \boldsymbol{V_e} V_s C_o}{K_{fs} V_e + V_s}^{\#}$$

where V_e is the fiber-coating volume, V_s the sample volume, C_o the initial concentration of the analyte in the sample, and K_{fs} is the extracting phase/sample matrix distribution constant.

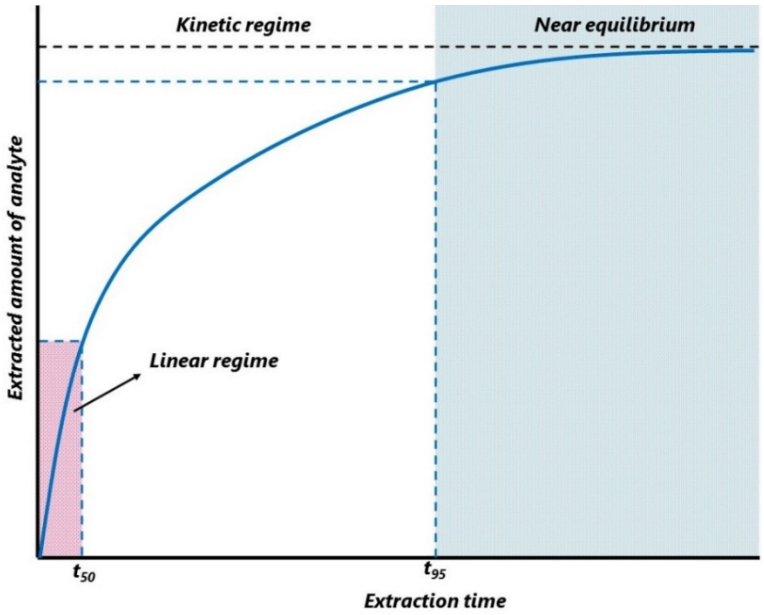

Figure 1.10 Typical extraction time profile for SPME

Moreover, Equation 1.1 indicates that the amount of analyte extracted in the coating (n) is linearly proportional to the analyte concentration in the sample (C_o), which is the analytical basis for quantification using SPME. Additionally, when the sample volume is very large, $V_s >> K_{fs}V_e$, Equation 1.1 can be simplified to:

$$n = K_{fs}\boldsymbol{V_e}C_o\# \qquad \text{Equation 1.2}$$

While straightforward in theory, equilibrium extraction may be rendered impractical by the potentially very long time required for equilibration. A more expedient alternative is the measurement under pre-equilibrium conditions[131], which can provide total concentration of a given compound in a biofluid via matrix-matched calibration approach. Indeed, equilibrium extraction time is influenced by the hydrophobicity of a compound,[134] where less polar compounds exhibit the largest affinities for SPME coatings, and accordingly the longest equilibration times.[135] Such long extraction times have unsurprisingly given SPME a reputation as a tedious technology, unable to compete with novel workflows such SAMS and AMS.[83] Yet, the extraction process in SPME

generally follows the profile shown in Figure 1.10. Thus, when sampling is longer than t_{95}, extraction nearly reaches equilibrium. If the sampling time is less than t_{95}, the extraction is a kinetic process; as such, under t_{50}, the mass uptake rate is considered to be linear. Undeniably, linear mass uptakes are more beneficial in applications that require fast analysis, and a feature to be targeted when developing novel SPME methods for rapid diagnostics. Certainly, the amounts extracted under the linear regime are less than those otherwise obtained through equilibrium extraction (see Figure 1.10); nonetheless, improvements in sensitivity currently offered by state-of-the-art mass spectrometry have certainly facilitated pre-equilibrium applications.[136] As discussed in the following section, further time reductions in the extraction step have been made possible thanks to recent advances in SPME-MS technologies.[80]

As briefly mentioned before, the extraction process in SPME can take place either by direct immersion (DI) or by headspace (HS) sampling (see Figure 1.8). Although HS-SPME is perhaps the most well-known approach of SPME, it is certainly not the best configuration of SPME for determination of drugs and metabolites in biofluids (*i.e.* since many analytes of interest will have low volatility and thus not be efficiently transported into the headspace.), or for applications that require balanced coverage.[137] Essentially, HS-SPME is better suited for extraction of compounds with low hydrophilicity and large Henry constants. Such compounds prefer to partition to the headspace due to their lower solubility in water-based matrices, and have shown great affinity towards commonly used coatings such as PDMS or its combinations with solid adsorbents (*e.g.* PDMS-carboxen, PDMS-Car; or PDMS-divinyl benzene, PDMS-DVB). In addition, as recently revised by Souza-Silva *et al.*[138] and Gionfriddo *et al.,*[139] most of the coatings commercially available for HS analysis are not matrix compatible[140–142] due to their propensity to incur biofouling when directly immersed in complex matrices such as biofluids (*i.e.* highly porous, non-

smooth surface). Thanks to recent advances in coating technology, and more precisely matrix-compatible coatings, DI-SPME has gained more relevance in recent years. Recent developments in matrix-compatible coatings include the PDMS over-coated fibers,[143,144] the polyacrylonitrile (PAN) based coatings,[1,123,125] and the Polytetrafluoroethylene (PTFE) based coatings,[122] among others. PAN-based coatings were introduced approximately 10 years ago as biocompatible coatings, meaning the coatings do not induce or cause toxic reactions to the system under study. Consequently, fouling or adsorption of proteins are not incurred by the coating when the extraction phase surface is exposed to a complex matrix such a tissue or plasma.[113,124] As an additional benefit, the extraction efficiency of SPME for semi-volatile and polar compounds improves significantly when DI-SPME is performed, as the diffusion coefficients of the analytes in the biofluid, that define the mass transfer properties of the extraction mode, are similar for all small molecules present in the system.[122,137]

Among the diverse SPME geometries developed to date, fiber (*i.e.* tiny coated wire, typically with a diameter of less than 500µm),[115] in-tube (*i.e.* coating inside a capillary),[114,117] membrane (*e.g.* polydimethylsiloxane – PDMS –embedded membrane),[145,146] and blade (*e.g.* rectangular piece of coated metal or plastic)[123,147,148] configurations are the most well-known and used worldwide. Certainly, selection of SPME geometry is dependent on the application of interest. For instance, small devices (*i.e.* tips or fibers) are a better fit for analysis of samples that must be obtained with minimal invasiveness, such as tissue.[149–153] On the other hand, in analysis of relative large sample volumes (*e.g.* ≥ 100 µL) that seek fast enrichment, devices with larger surface areas (*e.g.* blades) are preferred, as they provide much faster extraction rates due to the larger surface area to extraction-phase volume ratio of these geometries (see Equation 1.3).[154]

$$\frac{dn}{dt} = \frac{D_s \boldsymbol{A}}{\delta} C_0 t \qquad \text{Equation 1.3}$$

In the equation, n is the amount extracted at a given time t, D_s is the diffusion coefficient of the analyte, C_0 is the initial total concentration on the sample, and A is the coated surface area of the device.

In addition, SPME devices that provide larger extraction volumes are useful in certain applications in that they allow for larger amounts of analyte extracted, which leads to better sensitivity (see Equation 1.1). Yet, as presented in the following chapters, selection of SPME geometry, particularly with respect to direct-to-MS analysis, will also be dependent on factors such as the geometry of the interface (*e.g.* injection port or ambient interface), the transition of the analytes from the interface to the MS system (*e.g.* transmission device or continuous liquid fluidics), and the analyte desorption operation principle (*i.e.* liquid, laser, or thermal desorption).

As an additional feature, SPME provides balanced coverage of analytes, that is extracts/enriches compounds from a broad range of polarities.[131] Fundamentally, SPME extracts via free-concentration, and most SPME coatings have a larger affinity for compounds with medium hydrophobicity and above (*i.e.* LogP $\geq$ 2)[1,2]. However, in complex matrices, non-polar compounds are likely to be heavily bound to proteins or partition into cells.[123,155] Therefore, since the free amount of hydrophobic compounds available for extraction is generally lower in complex matrices, the coating is unlikely to become saturated by these compounds. On the other hand, polar compounds (*i.e.* LogP $\leq$ 2) are neither bound to proteins, nor partitioned into cells, and thus, in comparison to hydrophobic compounds, are commonly present in high concentrations in complex matrices. Yet, since SPME coatings (K_{fs}) have a lower affinity for polar compounds, the coating does not suffer saturation from these compounds either. SPME devices offer an additional advantage, particularly with respect to devices with large surface areas such as the blade

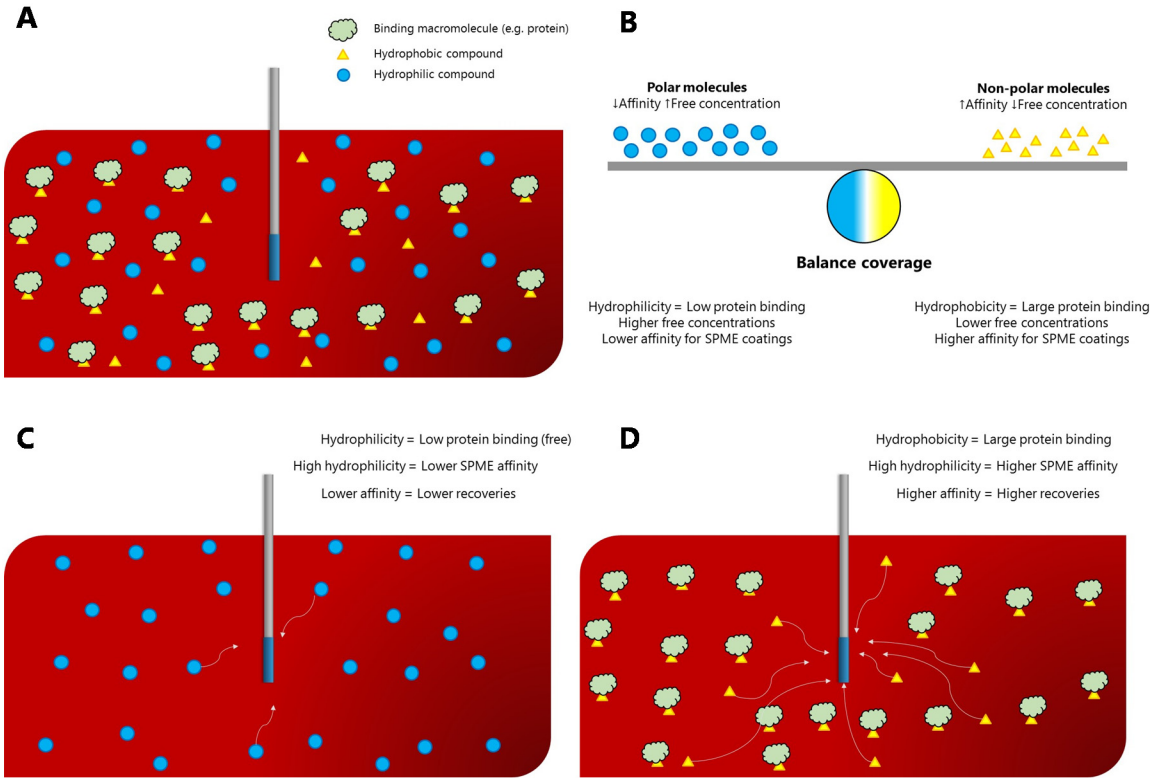

Figure 1.11 Schematic representation of. **A.** Extraction from complex matrix. **B.** Balanced coverage concept. **C.** Extraction of polar compounds, and **D.** Extraction of hydrophobic compounds.

configurations: in comparison to the 'gold standard' technology SPE, SPME does not suffer from cartridge clogging or compound breakthrough. Since the geometry of the SPME device is open, and the principle of extraction is based on equilibration principles,[156] SPME devices are not susceptible to either of these commonly observed limitations of SPE. Furthermore, the automation of an analytical workflow has several advantages, such as faster sample throughput, greater reproducibility, and reduced analyst time for both method development and routine analysis.[157,158] Presently, in cases where multiple analyses have to be carried out within a short period of time, traditional sample preparation methods such SPE become the bottleneck in the throughput of the analysis, owing to the multiple steps that need to be conducted (*e.g.* conditioning of the cartridge, enriching step, washing step, elution step, evaporation of the extract and reconstitution of the extract).[159] Following the initial disclosure of SPME, the fiber arrangement was shown to be

suitable for automation with GC autosamplers due to its similarity to traditional GC syringes used for liquid injection.[133,160,161] While this original automation was meant for single devices,[161] in the last ten years, concentrated efforts have been focused on the development of devices that allow for automated parallel processing (*e.g.* 96-well configuration).[123,131,162] The development of such approaches has allowed for dramatic reductions in total analysis time. Hence, the automation of SPME for 96 samples (or more) eliminates time-related drawbacks of traditional techniques, rendering SPME an efficient approach towards integration of sample preparation with GC or LC. In addition, methods that adapt automated SPME afford several advantages over manual SPME methodology. For instance, superior extraction time reproducibility allows for the development of multiple, faster non-equilibrium extractions, which are otherwise unattainable through non-automated methods.[133]

To date, the majority of methods involving couplings between SPME and mass spectrometry follow the same workflow as that used for TSP approaches. Hence, after extraction, analytes are released from the SPME coating either via either thermal or solvent desorption. In the case of LC analysis, the desorption step is performed on a vessel (*e.g.* vial or well plate),[131,148,162] while in GC, the desorption process classically occurs in a desorption chamber.[146,163,164] Subsequently, the extracted analytes are transported from the injection port (*e.g.* six-port valve in the case of an LC-experiment[117]) and into the chromatographic column for analyte purification and subsequent ionization (see Figure 1.1). Certainly, when aiming for faster analysis times, more efficient ways of coupling SPME devices to MS must be further investigated by the scientific community. The following section discusses the most relevant advances made towards the interface of SPME and MS (see Figure 1.4).

1.6. Brief overview on direct coupling of SPME to mass spectrometry (SPME-MS)

The coupling of SPME directly to MS eliminates the separation step, which greatly reduces the time needed for analysis. In addition, since the dilution step (inherent of most SPME-LC applications) is removed from the workflow, higher sensitivity and faster analyses can be attained.[136,151] Furthermore, owing to its intrinsic features (*i.e.* sample extraction, clean-up, enrichment, and matrix normalization), low matrix-suppression effects can be attained via SPME, an advantage that is highly beneficial for detection of trace compounds in complex matrices. The direct coupling of MS to SPME-based devices dates back more than 20 years, and is under continuous exploration. While these advances have been interrupted by pauses, great breakthroughs have been made in the last 5 years, driven in part by the advancing state of the art in mass spectrometry (see Figure 1.12).[80,81] SPME-MS technologies can be categorized according to the ionization strategy employed, with the following as the most common approaches: inductively-coupled plasma (ICP)-MS;[165,166] electron impact (EI)-MS;[164,167–170] and atmospheric pressure ionization (API, via either ESI or APCI mechanisms)-MS.[171,172] In addition, the direct coupling of SPME to MS can also be categorized by the desorption mechanism employed, with solvent,[130,173] thermal,[164,174] or laser [175–177] desorption being the three most commonly used. Finally, as shown in Figure 1.5, SPME-MS technologies can also be classified according to their degree of ambience: SAMS (*e.g.* open port probe[178]), direct-analysis (*e.g.* SPME as substrate-ESI[130,136]) or AMS (*e.g.* via desorption electrospray ionization, DESI[179]). Given the multiple ways of categorizing these couplings, and aiming to have better flow with previous sections, the latter classification was selected.

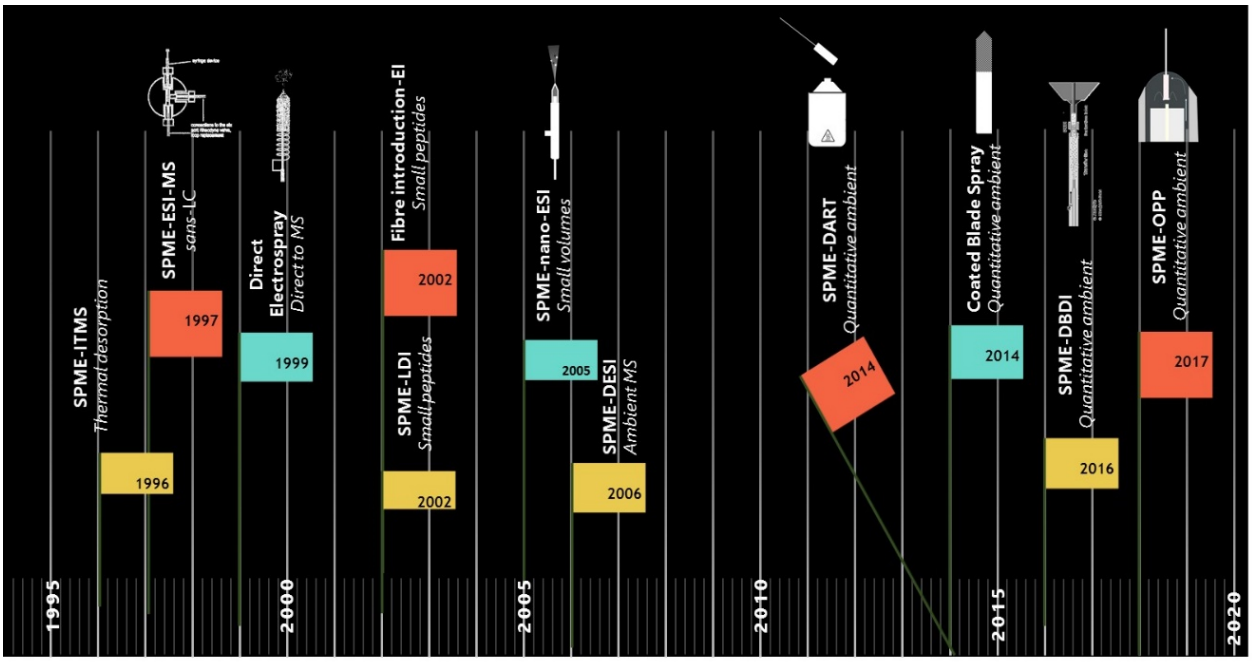

Figure 1.12 Timeline event of relevant SPME-Mass Spectrometry developments[80]

1.6.1 SPME-SAMS technologies

The first SPME-SAMS technology based on liquid desorption was described by Möder *et al.* in 1997[180] (see Figure 1.12). In this approach, an SPME fiber, used for extraction of acyl carnitines from urine, was statically desorbed on a sealed chamber. Subsequently, by switching a six-port-valve, the mechanically pumped solvent carried the desorbed analytes towards the ionization source.[117] Since this first development, in the last twenty years, several publications have followed the SAMS-liquid desorption concept, using either ESI or APCI commercial sources.[114,172,181,182] Recently, the use of an open chamber that allows for effortless desorption of SPME devices and rapid transfer to the ionization source was reported in the literature. This interface, known as Open Port Probe (OPP), was developed by Van Berkel and collaborators[36,45] at Oak Ridge National Laboratory (ORNL). The first direct coupling of MS to SPME devices that uses this system is fully described on Chapter 3 of this thesis.

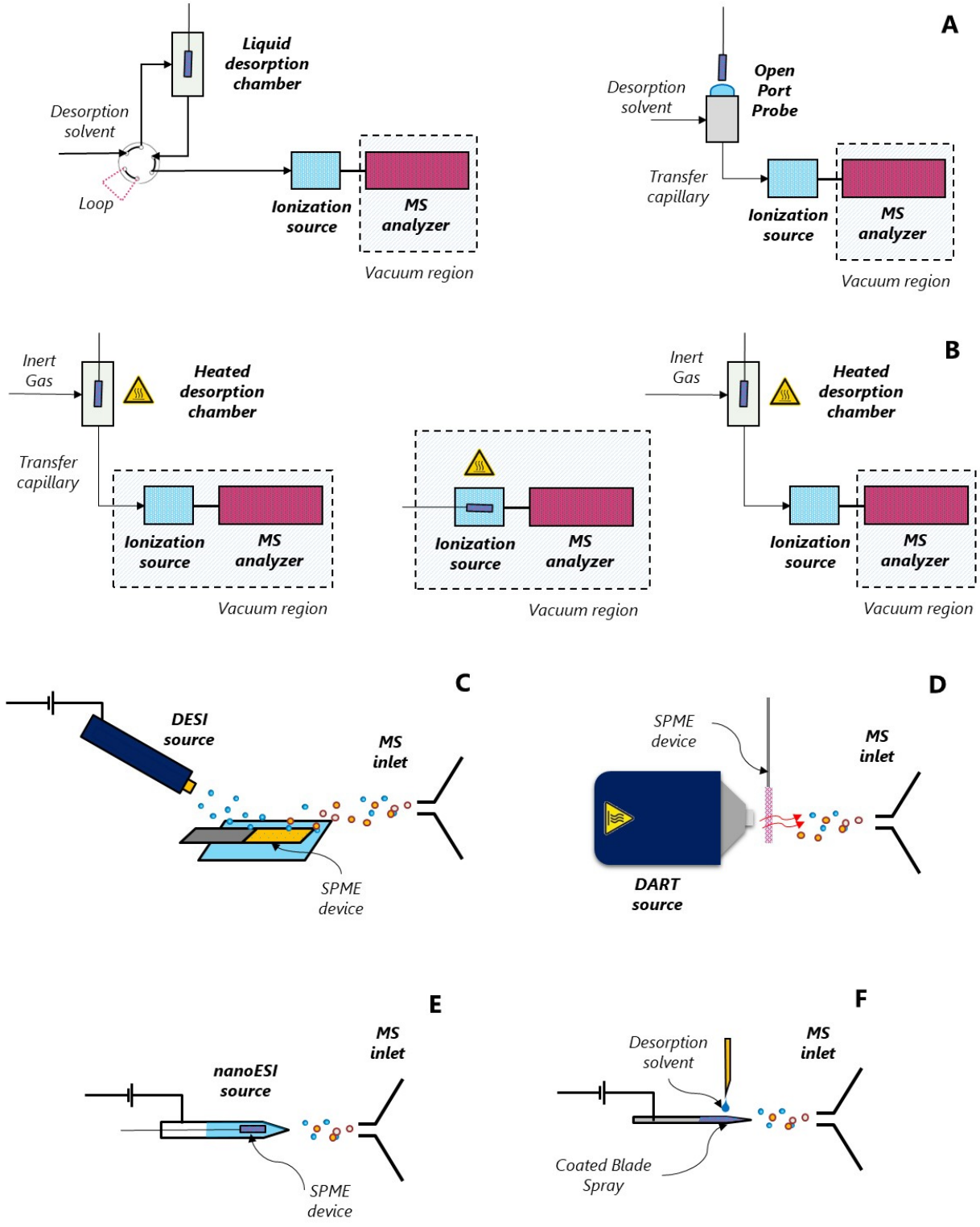

Figure 1.13 Schematic diagrams of some of the most relevant SPME-MS technologies suitable for analysis of biofluids. **A.** SAMS technologies via liquid desorption; **B.** SAMS technologies via thermal desorption; **C.** SPME-DESI **D.** SPME-DART; **E.** SPME-nanoESI; **F.** SPME as a substrate spray device.

However, it is worth noting that, chronologically speaking, the first coupling to MS in a SAMS configuration was performed via thermal desorption (circa 1996,[183,184] Figure 1.12). In this approach, SPME devices can be desorbed on the injection port of the GC system, where analytes are subsequently moved towards the MS system with the use of carrier gas. Instead of the classical chromatographic process, where separation of analytes occurs on a long coated column, a short and non-coated capillary is used to link the injection port with the MS analyzer.[30,32,163,167,168,185,186] Although this configuration has been mainly used for rapid qualitative analysis (*e.g.* profiling), Boyacı *et al.* recently reported its applicability towards quantitative analysis.[164] A second arrangement of SPME-SAMS via thermal desorption consists of circumventing the transfer capillary by performing the desorption of the SPME device directly at the ionization chamber of the mass spectrometer.[87,169,170] Although this approach allows for higher sensitivity, its configuration cannot be easily set up without affecting the instrument vacuum. Further, the technique cannot be easily automated, nor does it allow for easily reproducible results. The last SPME-SAMS strategy via thermal desorption does not require gas chromatography hardware. In addition, the ionization step, unlike the two previous strategies, occurs under ambient conditions (*i.e.* outside the instrument). As shown in Figure 1.13, thermal desorption takes place on a custom-made chamber, where analytes are driven towards the ionization chamber prior to MS analysis. Analyte ionization can be performed either by ICP,[187] Dielectric Barrier Desorption Ionization (DBDI),[174] or Low Temperature Plasma (LTP),[188,189] among others.[80]

Similar to SAMS via liquid desorption, one of the greatest advantages of the thermal desorption approach lies on its capability to introduce into the MS all analytes extracted onto the SPME device in a single band or peak. This feature affords better signal-to-noise ratios in comparison to substrate-spray methods and, as a result, outstanding sensitivity.[174] Yet, three factors should be

kept in mind when selecting SAMS technologies for direct coupling. First of all, depending on the materials chosen for the construction of the device, including materials for the desorption chamber, transfer tubes, and capillaries that move the analyte towards the MS system, it is possible that analyte carry-over may occur as a result of non-specific attachments. Therefore, if analytes are not efficiently desorbed/transferred, the analyst should wait until the entire system is purged, either by the desorption solvent or the carrier gas, before a second "injection" is made.[174] Undoubtedly, such an issue might lead to a dramatic reduction in the analytical throughput. This problem can be overcome by the use of silanized materials, which are also used in GC and LC to similar effect. The second factor, on the other hand, relies on the inherent ionization weaknesses of each technology.[190] Given that some technologies might be intrinsically unable to ionize certain compounds, this factor must be kept in mind with respect to technique selection. In this line, a third factor to consider involves the versatility (*i.e.* capability to rapidly interchange between ESI or APCI modes), and the simplicity of set-up (*i.e.* rapid installation and operation with minimal modifications to the system) of a given technology. Based on the last factor, OPP is certainly one of the SPME-SAMS technologies with the greatest potential to be further developed in the upcoming years.

1.6.2 SPME-AMS technologies

As mentioned above, most AMS approaches seek to record mass spectra with minimal or no sample preparation/analyte pre-concentration.[191] Due to the absence of sample pre-treatment, the linear dynamic range (*e.g.* diminished sensitivity by ion suppression[81]) as well as the selectivity of such methods are likely to be sacrificed. Therefore, modifications in the experimental workflow, such as addition of an efficient enrichment step,[78,192,193] are often needed in order to obtain better

analyte quantitation. Given the multiple advantages already mentioned and the easiness of its coupling, SPME has aided well-known AMS technologies such as DART and DESI in reaching required limits of quantitation.[104,144,194–197]

Undeniably, DESI is one the most successful ambient ionization techniques developed to date.[52,198] DESI's advantage lies in its capability to scrutinize analytes on surfaces, with its high-throughput imaging-MS capabilities being one of its major attractions.[66,199–201] As suggested by its name, in DESI, analytes present on a given surface are initially desorbed/swept, and subsequently ionized.[52] DESI is equally suitable to the desorption/ionization of analytes previously concentrated on SPME devices. Accordingly, the first SPME-DESI applications were disclosed only a few years following the initial publications on DESI-MS by Cooks and co-workers.[76,202,203] Expectedly, authors have reported that SPME fibers greatly improved the quantitation capabilities of DESI towards the analysis of anabolic steroids spiked on raw urine[203] or drugs of abuse (DoA) on real urine samples.[179] In addition, data showed good agreement between the results obtained by DESI and those reported using traditional confirmation methods (*i.e.* LC-MS/MS and GC/MS protocols).[179] Recently, Strittmatter *et al.* reported for the first time the direct coupling of blade geometry to DESI-MS.[104] In this study, devices coated with C_{18} and a strong cation exchanger were used for the targeted and untargeted determination of pharmaceuticals and personal-care product components in wastewater samples. One of the greatest advantages of the blade-DESI-MS coupling entails the possibility of performing both steps, extraction/pre-concentration and desorption/ionization, in an automated fashion.[104,118] In addition, one could foresee in the near future a combination of SPME fibers and DESI-MS/MS for the determination of the spatial distribution of targeted analytes in living samples such as tissues.[152,200] While the development of new SPME-DESI applications has been pursued vigorously, fundamental studies are still needed

in order to fully understand the desorption/ionization process from particle coatings used on SPME devices, as well as to determine ideal experimental conditions such as desorption solvent flow,[200] sprayer position,[204] and coating thickness.

At the same time that DESI was developed, Cody and collaborators originated DART, an atmospheric pressure chemical ionization (APCI)-based ambient ionization technique.[53] In applications that couple SPME to DART, analytes are first thermally desorbed from the coating surface by a heated gas, and subsequently ionized by excited-state species created in the atmosphere proximal to the coating surface.[192,205] Initial experiments to couple SPME fibers to DART-TOF-MS by Cajka *et al.* have shown SPME to be a promising tool for determination of chemical profiles that allow for rapid authentication of food commodities.[206] Due to the large flow of heated gas (~ 3L/min), a necessary precaution is that the SPME fiber needs to be properly secured when passed in front of the DART source so as to avoid severe fiber swinging and, consequently, irreproducible desorption/ionization of extracted analytes.[207,208] Recently, Wang *et al.* reported the coupling of IT-SPME with DART for the determination of trace pesticides in liquid food matrices.[81] In spite of the great sensitivity attained by this method, the proposed system was very intricate, and required solvent assistance to move analytes from the coating for ionization by the DART stream. Likewise, due to the inherent design of the IT-SPME device used, the sample required centrifugation and filtration to prevent clogging of the extraction material with fibers or particulates from the matrix. Concomitantly, Pawliszyn's group reported the first combination of thin-film SPME with DART-MS/MS for the determination of cocaine and diazepam in urine and whole blood, respectively.[144,194] Essentially, the device consisted of a stainless steel mesh coated with a matrix-compatible coating. The idea behind this work was to emulate the transmission mode (TM) configuration reported by the Fernandez group.[77,209] Yet, while the device had the

extraction/enrichment features of SPME, the methodology used during the coating process (*i.e.* brush painting) covered not only the strands, but also the mesh openings.[194] Aiming to improve ion transmission, tiny, randomly selected holes were placed on the coated mesh to allow the gas stream to flow through it. Unfortunately, results were not as good as expected. As a result, Gómez-Ríos and Pawliszyn redesigned the SPME-TM.[192] One distinctive characteristic of the new device is that the mesh is exclusively coated on the strands with a thin-layer of particles ($Ø \leq 20$ μm).[195,196] Therefore, the stream of gas with metastable helium atoms easily flows through the mesh, and efficient desorption and ion transmission can be attained.[74,205] In this context, the use of a thin coating allowed for faster extraction/enrichment, rapid desorption, and minimal carry-over to be achieved. Chapter 2 of this thesis presents the latest developments on SPME-TM configurations, as well as its application to the determination of target compounds in complex matrices.

1.6.3 Direct SPME to MS

Unlike other approaches, direct SPME-to-MS technologies do not require intricate equipment to interface to the MS system. These technologies can be separated into two groups: those using nanoelectrospray (nanoESI) emitters,[210] and those operating as substrate spray devices.[46] SPME-nanoESI-MS, first explored by Walles *et al.,*[210] has been assessed for its qualitative and quantitation capabilities in several publications to date.[151,173,211–213]. The main goal behind the interfacing of SPME with MS via nanoESI is to desorb devices on a chamber with very small volumes ($V_{des} \leq 10$ μL) so to have the best enrichment factor (*i.e.* highest volume of extraction/volume of desorption) and fully utilize the molar enrichment factor offered by SPME.[151,173] Furthermore, nanoESI not only yields higher ionization efficiency when compared to ESI, it also allows for longer electrospray events that permit a far greater number of MS

experiments. For instance, a single nanoESI emitter could be used for targeted analysis (MS^n), high-resolution MS experiments (HRMS and HRMS/MS), as well as for its interface with other on-line technologies such as ion mobility (IMS).[214] However, due to the inner diameter of the emitters (Ø ≤ 1000 μm), nanoESI technologies are suitable only for SPME devices with small diameters (Ø ≤ 500 μm). Recently, Pawliszyn and collaborators have explored the use of nanoESI as a means to improve the quantitation capabilities offered by commercial BioSPME fibers,[152,215] as well as miniature devices manufactured in-house.[151] Succinctly, when employed in tandem with nanoESI, SPME is capable of reaching sub-part per billion LOQs, even when small sample volumes (≤ 20 μL) and short extraction times (≤ 2 min) are used. As a matter of fact, most of these developments are described in detail on Chapter 3 of this thesis.

As shown on Figure 1.12, substrate spray technologies based on SPME devices were described prior to the invention of AMS (circa 1999).[85] Indeed, Prof. Shiea and his group of collaborators were the first to report the use of this technology; by spraying directly from wetted SPME fibers, researchers could detected sub-ppb levels of Triton X-100 in aqueous samples. Surprisingly, after this breakthrough, almost 15 years passed without major advances in these technologies until 2014, when novel technologies such as surface coated wooden-tip ESI,[130,216] coated membrane spray,[217,218] and Coated Blade Spray (CBS)[136,151,219,220] were disclosed. Essentially, this group of technologies integrates sample preparation and sample introduction into the MS system on a single device. Thus, such technologies not only simplify the analytical workflow, they also decrease the cost per analysis. Given the material used for its construction (*i.e.* stainless steel), as well as the geometrical characteristics of the substrate (*i.e.* ~ 300 μm flat sheet, sword-like shape), CBS would appear to be not only the best substrate spray strategy developed to date, but also a disrupting technology that may shift the paradigm of direct sample introduction to MS. Chapter 4 of this

thesis summarizes the development of CBS, as well as its application to the determination of exogenous substances in biofluids.

1.7. Research objectives

In critical care settings (emergency and surgery units), the ability to deliver a rapid prognostic metric of a given clinical condition plays a critical role in the successful and timely treatment of patients.[221] The amount of time spent choosing and implementing a therapeutic strategy could be the difference between life and death. For this reason, molecular diagnostic and prognostic instruments, which are able to provide doctors with fast and reliable results, are highly desired in such facilities for the personalized diagnosis and treatment of patients. Ideally, assessments of such molecules (drugs, metabolites, and biomarkers) before, during, and after surgery and/or emergency, should be performed in real or close to real time. Nevertheless, such metrics are limited by a number of challenges which cannot be easily overcome by standard high-throughput assays, such as compound selectivity, space resolution, and analysis of unstable/short-lived metabolites. AMS and direct-sample-to-MS methods are advantageous for point of care (POC) and therapeutic drug monitoring (TDM) due to their speed, simplicity, and ability to monitor multiple analytes simultaneously.[8] However, as discussed above, most of these techniques are chiefly limited in selectivity and sensitivity due to the lack of an appropriate sample preparation step. Accordingly, the performance of modern mass spectrometry instruments in the clinical environment can be enhanced with the use of fast, simple, and efficient sample preparation technologies.

The main objective of the currently presented PhD work encompassed the development of novel SPME-MS methods that can be used for rapid diagnosis throughout the patient journey.[222] These

workflows needed to be suitable for the analysis of SPME devices that undergo either sampling of tissue (*e.g. in vivo* studies) or direct immersion into biofluid samples (*e.g.* blood, plasma, and urine). As a result, the currently presented work can be divided in two lines of research: workflows that can be used for either biofluids or tissue analysis (*i.e.* those involving SPME fibers/tips), and methods chiefly focused on the examination of biofluids (*i.e.* any SPME geometry). Aiming for a better understanding of the analytical capabilities of each direct-to-MS technology, this research was also focused on assessing the diverse technologies herein described, including: SAMS, AMS, and direct SPME to MS. This dissertation compiles my contributions to SPME-MS during the last 4 years, including: SPME-TM, SPME-nanoESI, SPME-OPP, and CBS. The majority of this work is already published in peer-review journals including Analytical Chemistry (3),[173,195,219] Angewandte Chemie (2),[136,151] Analytica Chimica Acta (1)[223], Chemical Communications (1),[192] Analyst (1)[196] and Journal of Pharmaceutical and Biomedical Analysis (1).[220] As a result, each chapter of this thesis is composed of at least two publications, with the breakdown of the thesis as follows. Chapter 2 is chiefly focused on the development of SPME-TM and its application to the determination of xenobiotics in biofluids via DART-MS/MS.[192] Furthermore, this chapter also presents the implementation of this technology to other fields such as food and environmental analysis, as well as the coupling of these devices with portable mass spectrometers for on-site semi-quantitative determinations and molecular profiling.[195,196] Chapter 3 describes work performed on commercial BioSPME fibers coupled to nanoESI-MS/MS for examination of target compounds on biofluids,[173] and discloses the first coupling of SPME-tips to MS via nanoESI for the analysis of ultra-small volumes of biological samples (≤ 5 µL).[151] Moreover, aiming to offer an interface that requires minimal modifications to the front-end of the instrument, and that is suitable for desorption of small SPME devices, the direct coupling to MS of BioSPME via OPP

^{178} is also presented in this chapter. Since some target compounds cannot be quantitatively determined at low concentration levels due to the presence of a co-extracted interference, in this chapter, the suitability of differential mobility spectrometry (DMS) and multistage fragmentation (MRM^3) were also assessed as on-line MS tools to enhance the selectivity of the workflow without affecting the total analysis time. Finally, Chapter 4 introduces CBS and its application towards the rapid and high-throughput determination of diverse analytes in biofluids. In addition, Chapter 4 discloses for the first time the use of an SPME-based technology for the TDM of compounds heavily bound to red-blood cells such immunosuppressive drugs. Further, this chapter also reveals the suitability of this technology for the quantitative analysis of target substances in biofluid droplets, and a novel strategy to dramatically enhance the LOQ of heavily bound compounds. In summary, the data herein gathered demonstrates the great potential of SPME-MS in the area of rapid analyte detection, particularly in fields that require robust, inexpensive, simple, sensitive, and rapid methodologies, such as clinical, drug screening, and forensic sciences. Confidently, in a foreseeable future, this work will encourage readers around the globe towards the use of SPME-MS as a laboratory workhorse.

Chapter 2 Development of Solid Phase Micro Extraction Transmission Mode (SPME-TM) and its application towards the qualitative and quantitative analysis of complex matrices via Direct Analysis in Real Time-Mass Spectrometry (DART-MS)

2.1 Preamble

Chapter 2 consists of 3 sections that correspond to three manuscripts already published in Chemical Communications, Analytical Chemistry, and Analyst. Most of the data, tables, and text presented within this chapter have already been incorporated in the aforementioned manuscripts, the details of which are listed below. Section 2.2 describes the development of Solid Phase Micro Extraction-Transmission Mode (SPME-TM) and its direct coupling to tandem MS via DART towards the quantitative analysis of cocaine and diazepam in urine and plasma samples. Section 2.3 describes the application of SPME-TM-DART-MS/MS for the quantitation of eighteen pesticides with different physicochemical properties in food and environmental matrices such as grape juice, orange juice, milk, and surface water. Finally, section 2.4 presents the direct coupling of SPME-TM to a portable MS system via DART. In this subdivision, the suitability of the proposed workflow is discussed for semi-quantitative analysis of seven pesticides in grape juice, and for the molecular profiling of milk samples from assorted animal and vegetal sources.

Section 2.2 includes the following manuscript:

1. Gómez-Ríos, G.A., Pawliszyn, J., *Solid phase microextraction (SPME)-transmission mode (TM) pushes down detection limits in direct analysis in real time (DART)*, Chem. Commun., 2014, 50, 12937-12940, DOI: 10.1039/C4CC05301J (open access) This article is licensed under a Creative Commons Attribution 3.0 Unported Licence.

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I participated at all stages of the manuscript preparation process: the planning of experiments, manufacturing of devices, method development, sample preparation and analyte extraction procedures, data processing, manuscript writing and submission, as well as in manuscript replies to reviewers.

Section 2.3 includes the following manuscript:

2. Gómez-Ríos, G.A., Gionfriddo, E., Poole, J., Pawliszyn, J., *Ultrafast Screening and Quantitation of Pesticides in Food and Environmental Matrices by Solid-Phase Microextraction–Transmission Mode (SPME-TM) and Direct Analysis in Real Time (DART)*, Anal. Chem., 2017, 89 (13), 7240–7248, DOI: 10.1021/acs.analchem.7b01553

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I participated at all stages of the manuscript preparation process: the planning of experiments, manufacturing of devices, method development, in sample preparation and analyte extraction procedures, data processing, manuscript writing and submission, as well as in manuscript replies to reviewers. The contributions of co-author Justen Poole included the manufacture of SPME-TM devices, execution of selected experiments, and data processing. Dr. Emanuela Gionfriddo was involved in the planning of experiments, writing of the manuscript, extraction execution, and data processing.

I, Justen Poole, authorize German Augusto Gómez-Ríos to use this material for his thesis.

I, Emanuela Gionfriddo, authorize German Augusto Gómez-Ríos to use this material for his thesis.

Section 2.4 includes the following manuscript:

3. Gómez-Ríos, G.A., Vasilejvic, T., Gionfriddo, E., Yu, M., Pawliszyn, J., *Towards on-site analysis of complex matrices by solid-phase microextraction-transmission mode coupled to a portable mass spectrometer via direct analysis in real time*, Analyst, 2017, 142, 2928-2935, DOI: 10.1039/C7AN00718C

I participated at all stages of the manuscript preparation process: the planning of experiments, manufacturing of devices, method development, sample preparation and analyte extraction procedures, data processing, manuscript writing and submission, as well as in manuscript replies to reviewers. The contributions of co-author Tijana Vasilejvic included the manufacturing of SPME-TM devices, execution of selected experiments, and data processing. Dr. Emanuela Gionfriddo was involved in the planning of experiments, execution of extractions, and data processing. Dr. Miao Yu was involved in data processing for metabolic profiling experiments.

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I, Emanuela Gionfriddo, authorize German Augusto Gómez-Ríos to use this material for his thesis.

I, Miao Yu, authorize German Augusto Gómez-Ríos to use this material for his thesis.

Section 2.2 Solid phase microextraction (SPME)-transmission mode (TM) pushes down detection limits in direct analysis in real time (DART)

2.2.1 Introduction

In the last decade, ambient ionization methods have changed the way samples are analysed by mass spectrometry (MS).[57] Several techniques, such as direct analysis in real-time (DART), desorption electrospray ionization (DESI), and paper spray (PS) have provided the scientific community with key tools for screening, pass/fail analysis, fingerprinting, and native sample imaging applications.[46,53,58,74,81,224,225]Today, most ambient MS approaches seek to record mass spectra without the need for sample preparation.[46,53,57,58] Hence, the scientific community, usually trained on standard sample preparation/separation methods, may have numerous inquiries regarding ambient MS techniques, including their performance in terms of accurate and fast quantitative analysis. This includes inquiries over the suitability of such methods for trace analysis (*e.g.* low pg mL^{-1}) in complex matrices, circumventing all sample preparation steps. With the use of such methods, analyses cannot always be performed in exceptionally short periods of time (*i.e.* $\leq$ 1 minute).[49,225,226] Generally, given that there is no sample pre-treatment, both the analysis time (*i.e.* time required to dry the sample onto the paper substrate)[49] and the linear dynamic range (*i.e.* diminished sensitivity by ion suppression) are likely to be sacrificed.[77,227] As recently reviewed by Monge *et al.* and Venter *et al.*,[3-4] improvements in the experimental workflow are still needed in order to obtain better *in situ* analyte quantitation. Therefore, rather than subscribing to a no sample treatment technique, the use of minimal sample preparation could result in lower detection limits and more efficient analysis. To address the shortfalls described above, a solid phase microextraction (SPME)[115,124,228] device that can be coupled as a

transmission mode (TM)[209,229,230] substrate to DART was developed. Succinctly, SPME-TM has been devised to act as an effective integration between sample preparation and ambient ionization.[81] Essentially, the device consists of a stainless steel mesh (74 x 74 in^{-1} wires, wire diameter 0.004 in) precisely coated on the strands with a biocompatible polymer (C_{18}-PAN).[133] As an SPME approach, the coated mesh (Ø ≤ 20µm, Figure 2.1) concurrently isolates and enriches the analytes of interest present in the sample matrix.[228] Additionally, given that undesirable interferences that might provide ionization suppression/enhancement are excluded from the sample during extraction (*i.e.* sample clean-up), detection limits are significantly enhanced.[133,144]As a TM substrate,[77] the coated mesh is positioned between the DART source and the mass spectrometer inlet (with all three coaxial to one another, 0º angle); the stream of gas with metastable helium atoms flows through the mesh performing simultaneous desorption/ionization of the compounds sorbed on the surface of the coating particles.[74] Subsequently, ions of the extracted/pre-concentrated analytes are transported into the atmospheric pressure interface (API) and analysed by tandem mass spectrometry (MS/MS).[194]

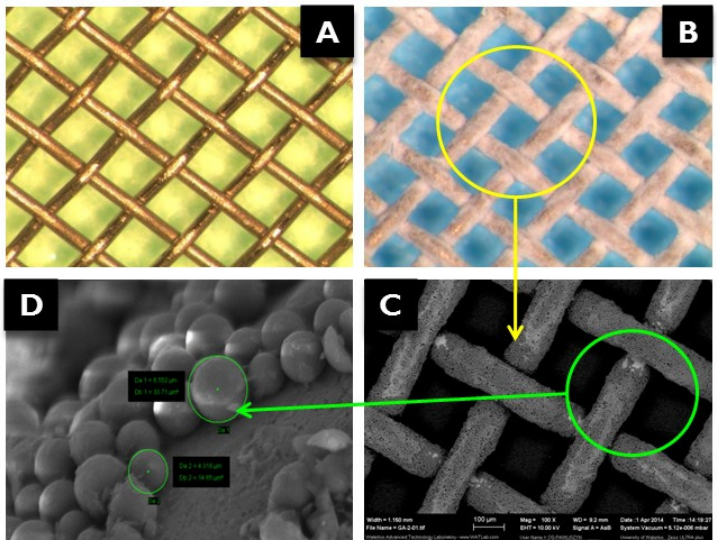


Figure 2.1 SPME-TM coating characteristics: **A.** Microscope image of a bare SS mesh; **B.** Microscope image of a mesh coated with C18 particles; **C.** SEM image of the same mesh; **D.** SEM image of layer coating particles on the mesh (particle size ~ 5 µm).

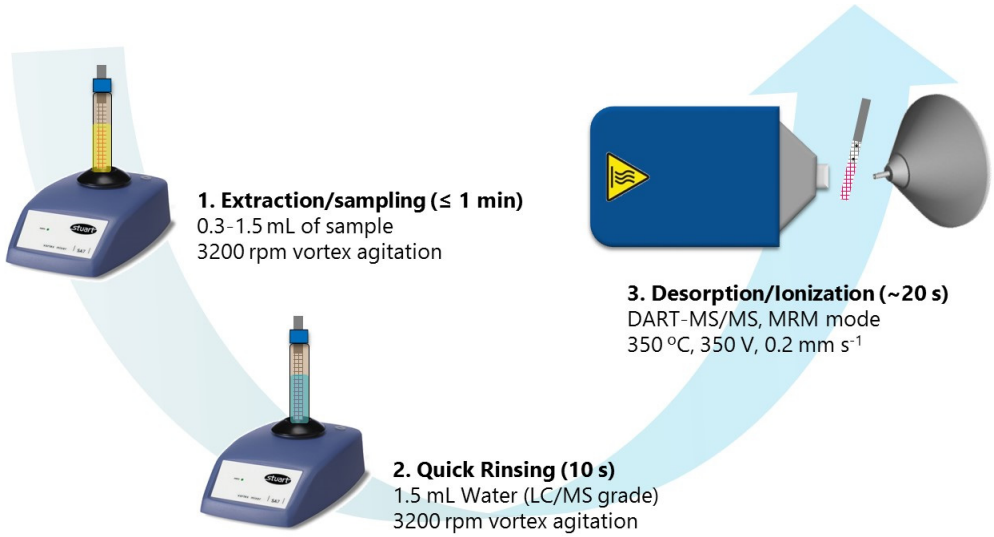


Figure 2.2 Experimental set up for SPME-TM extraction from complex matrices and desorption/ionization using DART-MS/MS.

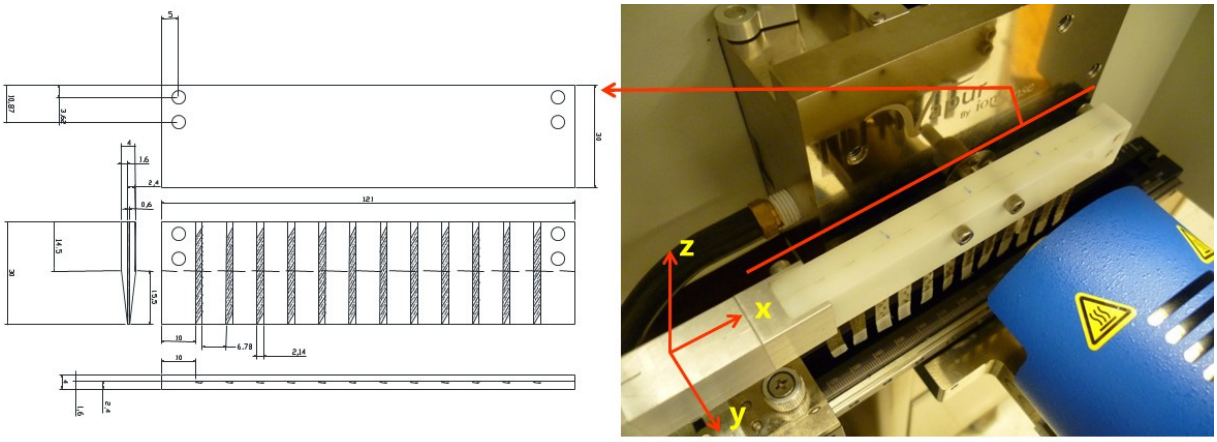


Figure 2.3 Schematic of UW-12 SPME-TM holder for DART. The holder was developed at the machine shop of the University of Waterloo. It can be used to perform concomitant extractions on a 96 well-Concept autosampler (PAS technologies)[131], as well as automated and stable desorption/ionizations. The system is compatible with the automated rail commercialized by IonSense. Up to 12 SPME-TM devices can be easily installed/removed from the holder, and spatial position can be accurately adjusted on the Z and Y axis.

As illustrated on Figure 2.2, the analytical process by SPME-TM-DART-MS/MS consists of three simple steps. First, a pre-conditioned coated mesh is immersed in a vial containing the sample matrix (300-1500 µL) and fast enrichment is performed by agitating the sample at a high

speed (*e.g.* vortex agitator; $t \leq 1$ min). Afterwards, the mesh is rapidly rinsed in a vial containing water ($t \leq 10$s) to remove potential artefacts (contaminants/particulates) adhered to the coating surface. Lastly, the mesh is installed on a mesh-holder that allows for easy and fast replacement of up to twelve SPME-TM devices, and is adapted in an automatic linear rail that moves the mesh to the front of the DART source (Figure 2.3).

To date, different geometries of SPME (*i.e.* fibre, in-tube, and thin-film) have been coupled to DART.[144,194,197,206] However, most of them have evident drawbacks (Figure 2.4). For example, as described by Cajka *et al.*,[206] the traditional SPME fibre requires cautious adjustment in front of the source to avoid severe fibre swinging and, consequently, irreproducible desorption/ionization of the analytes extracted. Although the early thin-film configuration reported by Mirnaghi *et al.* and Rodriguez-Lafuente *et al.* provided most SPME benefits,[144,194] it was not an ideal TM substrate, since the coating, applied using brush painting[144], covered not only the strands but also the mesh openings. Although tiny random holes were placed on the coated mesh to allow the gas stream to flow through it, ion transmission was unfavourably affected (Figure 2.4).[194] Thus, the potential of the combination SPME-TM was not fully realized. Recently, Wang *et al.* disclosed the first on-line coupling of the in-tube (IT) configuration to DART.[197] In this work, the authors demonstrated that IT-SPME is a sensitive method for the determination of trace pesticides in juice/water (Limit of quantitation (LOQ) ~ 0.2 ng mL^{-1}). However, the operation of the system is cumbersome and requires extra instrumentation (*i.e.* syringe pump to control solvent desorption flow). In addition, prior to the IT-SPME sampling, the sample requires centrifugation and filtration; otherwise, the extraction material might get clogged with particulates, fibres, or proteins from the matrix.[197] In contrast to the applications listed above, the present contribution describes multiple SPME-TM devices that can be easily and steadily installed on a 12-position holder and accurately positioned

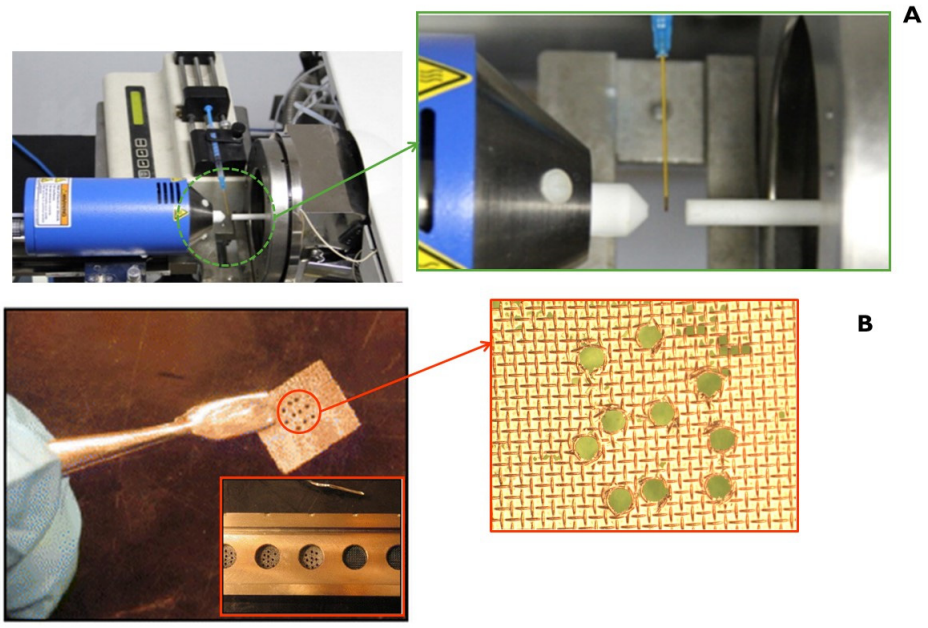


Figure 2.4 A. In-Tube Solid-Phase Microextraction with Direct Analysis in Real Time Mass Spectrometry; image was adapted from the original source published by Wang and collaborators [197]. **B.** Thin-film solid-phase microextraction and direct analysis in real time; image was adapted from the original source published by Rodriguez-Lafuente and collaborators. [144,194]

between the DART source and the mass spectrometry inlet (Figure 2.3). In addition, given that the mesh is exclusively coated on the strands, efficient desorption/ionization and ion transmission is obtained. Finally, unlike IT-SPME, SPME-TM requires neither additional apparatus nor sample pre-treatment. Although the method herein described is not the first coupling of SPME to DART-MS, it is undeniably the most comprehensive and simple approach proposed to date.[81]

2.2.2 Experimental Section

Biological samples

A phosphate-buffered saline solution (PBS) (pH 7.4) was prepared by adding 8.0 g of sodium chloride, 0.2 g of potassium chloride, 0.2 g of potassium phosphate and 1.44 g of sodium phosphate to 1 L of nanopure water. Pooled human plasma and whole blood from healthy donors in potassium ethylenediaminetetraacetic acid (EDTA) were purchased from Lampire Biological Laboratories

(Pipersville, PA, USA). Urine samples were collected from one healthy volunteer. Collection of urine from healthy volunteer for this particular study was under the approval of the Office of Research Ethical Board of University of Waterloo.

Mass Spectrometry

Experiments were performed with the use of a triple quadrupole mass spectrometer TSQ Vantage (Thermo Scientific, San Jose, USA). A DART-Standardized Voltage and Pressure (DART-SVP) model ion source (IonSense, Saugus, MA USA) was coupled to the MS system via a Vapur® interface (IonSense, Inc.). The DART-SVP was fitted with a single dimensional motorized linear rail controlled through the DART-SVP web-based software so as to reproducibly and consecutively automatically position the SPME-TM devices in front of the DART source (speed of 0.2 mm s^{-1} was used). The DART source was operated using the following conditions: positive ion mode; high voltage (HV) electrode, −3000 V; discharge electrode, +350 V; grid voltage, +350 V. The gas heater was optimized at 350 °C, which yielded the optimum intensities for the selected analytes.

Materials and supplies

Cocaine and diazepam were selected as model analytes to evaluate SPME-TM. Deuterated analogues of each analyte were used for correction of intra- and inter-experiment variability. Further details about compounds properties and SRM transitions are provided on Table 2.1. LC-MS grade solvents (acetonitrile, methanol, and water) used in all the experiments were purchased from Fischer Scientific. Calibration curves of each analyte were prepared on each matrix between 0.01 and 100 ng mL^{-1}.

Table 2.1 SRM parameters used for monitoring diverse controlled substances[131] in positive mode with C_{18}-PAN SPME-TM devices

#	Compound name	Log P	Parent ion (m/z)	Product ion (m/z)	S-lenses	Collision energy	Polarity	LOD* [pg/mL]
1	Amphetamine	1.76	136.099	91.114	17	36	+	112
2	Methamphetamine	2.07	150.112	91.120	19	45	+	20
3	Nikethamide	0.33	179.100	108.102	18	76	+	17
4	Salbutamol	0.64	240.143	148.103	18	59	+	1474
5	Propranolol	3.48	260.123	116.138	17	89	+	31
6	Metoprolol	1.60	268.140	116.146	18	94	+	108
7	Trenbolone	2.27	271.133	165.106	56	97	+	31
8	Clenbuterol	2.61	277.068	203.049	15	70	+	13
9	Diazepam	2.91	285.050	193.113	32	102	+	19
10	Testosterone	3.32	289.157	97.123	21	91	+	10
11	Exemestane	3.70	297.173	121.118	19	72	+	17
12	Codeine	1.20	300.105	152.092	64	104	+	46
13	Cocaine	2.30	304.142	182.173	18	87	+	2
14	Bisoprolol	2.14	326.160	116.135	17	102	+	45
15	6-acetylmorphine	0.42	328.126	165.092	37	122	+	21
16	Stanozolol	5.53	329.229	81.108	44	130	+	22
17	Strychnine	1.93	335.155	184.129	36	136	+	33
18	6-acetylcodeine	2.08	342.124	165.092	45	165	+	7
19	Formoterol	2.20	345.133	121.090	32	85	+	831
20	Heroin	1.52	370.133	165.097	48	119	+	13
21	Toremifene	6.80	406.210	72.167	24	108	+	42
22	GW501516	6.29	454.091	257.068	29	108	+	352

LOD*, limit of detection estimated based on the blank signal. Results are based on the integrated peak area obtained for a 20 ng mL-1 solution in PBS.

SPME-TM manufacturing

The SPME-TM device was prepared as follows: a stainless steel mesh (74×74 wires/in, wire diameter 0.004 in) with a length of 30 mm and width of 4 mm was etched for 5 min in concentrated hydrochloric acid (37% vol/vol), washed with water, and cleaned by sonication in methanol (see Figure 2.1). The coating was applied on an etched mesh by dipping the mesh into a vessel containing a suspension of extraction particles in a biocompatible coating solution (*i.e.* 0.18 wt/wt PAN/C18 particles ratio and 1% wt/wt PAN/DMF ratio). Essentially, 10 mm of the mesh were immersed in this solution for 15 seconds and then removed at a speed of about 0.5 mm per second. Then, a flow of nitrogen of 1.5 L/min was used to remove the excess of coating slurry accumulated on the openings of the mesh. After applying one layer of coating, the mesh was passed through a heater at an elevated temperature (*i.e.* 1 min at 125 °C). The steps noted above were repeated five or less times until a thickness ≤ 20 µm was attained (see Figure 2.1). Finally, the non-coated area of the mesh was arc welded to a support handle made of a stainless steel sheet that is 42×4 mm (L×W). In order to provide a strong attachment between the mesh and the solid substrate, the mesh was welded on 6 points (as illustrated in Figure 2.5).

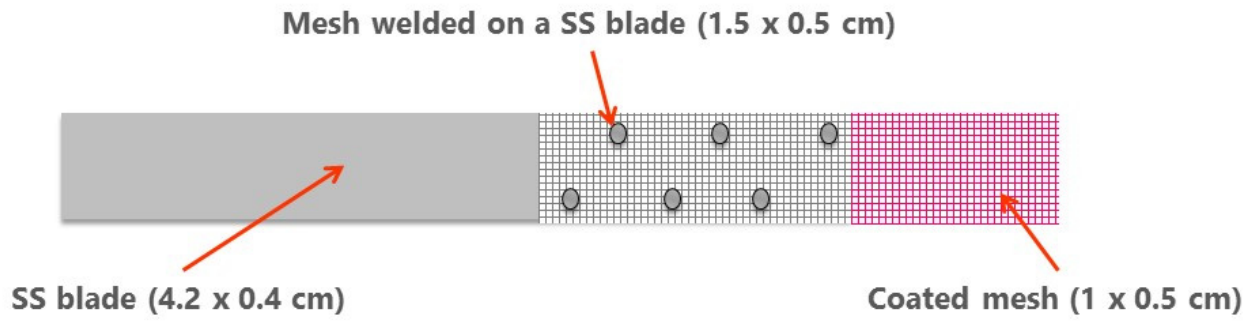


Figure 2.5 Scheme of the SPME-TM mesh-blade arrangement

2.2.3 Results and discussion

In the past, it has been incorrectly assumed by scientists not familiar with SPME that extraction/enrichment cannot be performed in short periods of time[53,57,58], and that extractions exclusively performed at equilibirum can achieve low LOD/LOQ.[115,231] However, contrary to general assumptions, direct coupling of SPME to mass spectrometry easily outperforms traditional detection limits with remarkably brief extraction times due to several reasons. First, the dilution factor inherent in most SPME-LC methods is removed from the analytical procedure.[25] Second, a large contact surface area between the extraction phase and matrix facilitates high mass transfer rates, while thin coatings ensure rapid equilibration times and efficient desorption to the mass spectrometer. Hence, the LOD is mainly constrained by the detection capabilities of the MS system rather than by the built-in features of the coating.[124] Preliminary experiments in our laboratory using thin-film microextraction devices (TFME, blade geometry[231]) showed that 15 seconds is sufficient to extract a quantifiable amount of analyte at the low ppb level even when using the traditional LC/MS approach (Figure 2.6). Indeed, if lower LODs are required, the interaction time between the coating and the sample matrix can be increased.[115] For instance, LOQs as low as 2 and 19 pg mL^{-1} were reached when performing 1 minute extractions from 1.5 mL of phosphate buffered saline (PBS) spiked with cocaine and diazepam (DZP), respectively.

Furthermore, the linear dynamic range of the method, evaluated from 10 pg mL^{-1} up to 50 ng mL^{-1}, showed astounding linearity (Figure 2.7) It is worth emphasizing that higher concentration levels are not a limitation for SPME;[115] thus, the scope of this report is to introduce the remarkable quantitation capabilities at trace levels of the SPME-TM-DART-MS/MS. However, in cases where a compound is present at a high concentration (*i.e.* ppm levels) and the affinity of the coating for the analyte is strong, even shorter extraction times (*e.g.* $\leq$ 30 s) can be performed.

A noteworthy feature SPME-TM devices in comparison with other ambient mass spectrometry apparatus is their reproducibility and potential for reusability.[144] Extractions performed with 9 independent SPME-TM devices (n=36) from 1.5 mL of PBS solution spiked with cocaine and diazepam showed intra-/inter-device reproducibility lower than 4.7 and 3.2 %, respectively (Tables 2.2 to 2.4). In addition, the extraction phase is able to withstand well the extraction/desorption cycles. However, although a decrease in the peak area signal use for quantitation after four consecutive uses was not observed, further experiments are required to determine the long-term durability of the SPME-TM devices.

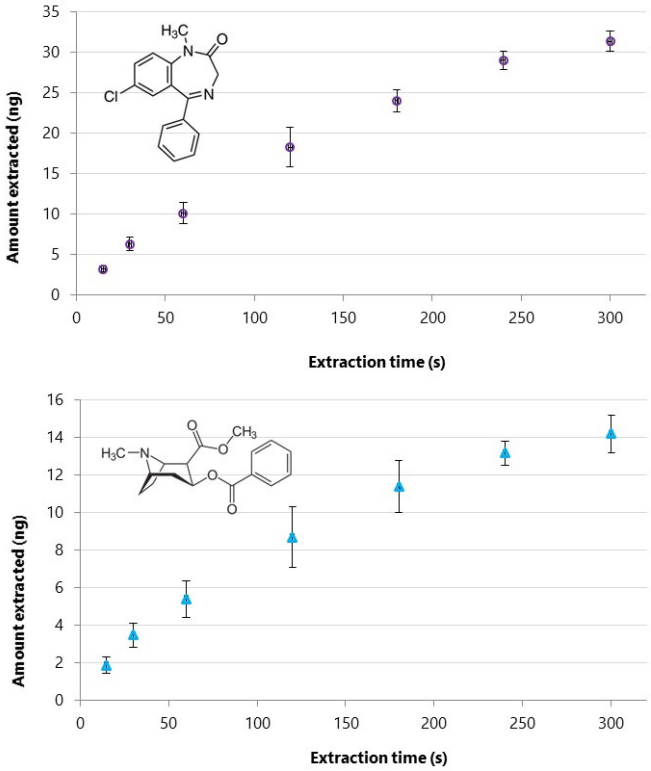


Figure 2.6 Extraction time profiles for **A.** diazepam and **B.** cocaine, respectively. Extractions were performed using a vortex agitator set-up at maximum speed (3200 rpm). Extractions from 1.5 mL of PBS spiked with 50 ng mL^{-1} of each analyte with 3 different TFME devices (n = 6). Extracts were analyzed using Thermo TSQ LC-MS/MS on SRM mode.

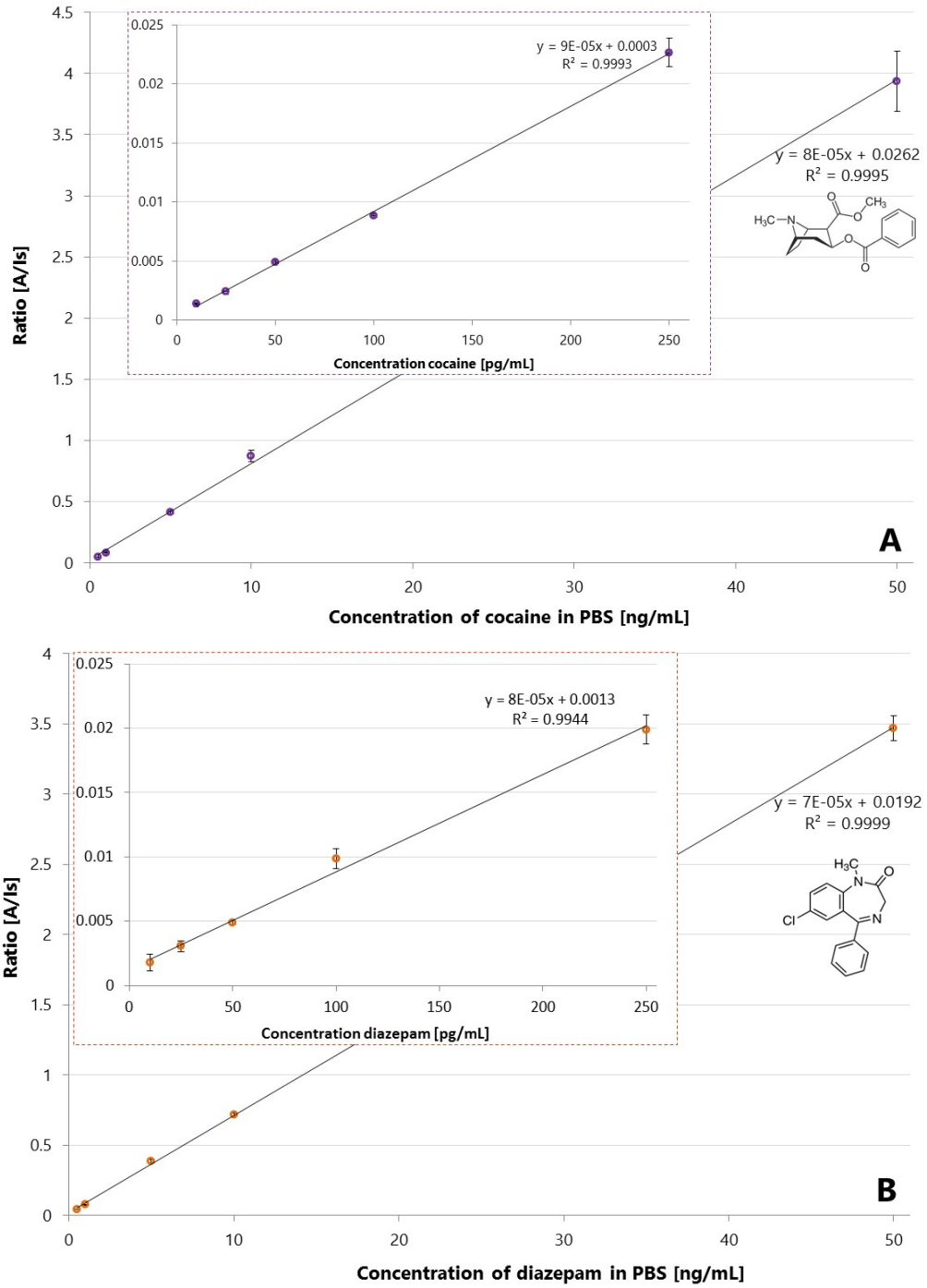


Figure 2.7 A. Quantitative analysis of PBS spiked with cocaine (10 pg mL^{-1} to 50 ng mL^{-1}) and its isotopologue [D_3] cocaine (12 ng mL^{-1}). **B.** Quantitative analysis of PBS spiked with diazepam (10 pg mL^{-1} to 50 ng mL^{-1}) and its isotopologue [D_5] diazepam (12 ng mL^{-1}).

Table 2.2 SPME-TM inter-device reproducibility; SD, standard deviation; RSD, relative standard deviation. Ratio results correspond to the average of extractions performed with 9 independent devices (n=36) from a PBS solution spiked with 20 ng mL^{-1} of each analyte.

Experiment	Ratio [A/Is]	SD	RSD [%]	% Carryover DART [A_c/A_i]	% Carryover solvent [A_c/A_i]
Diazepam	1.8	0.05	3	5.0	0.3
Cocaine	1.6	0.05	3	2.4	0.2

Certainly, it has been confirmed that by using thin-coatings, efficient mass transfer of analytes (fast extractions) and effective desorptions can be achieved.[228] Furthermore, while the signals obtained on a second desorption/ionization cycle (carry-over) were approximately 5% of the signal used for quantitation of DZP (Figure 2.8) it is important to highlight that detection of DZP and cocaine was performed concomitantly. Thus, DART experimental conditions were not exclusively optimized for DZP, which could explain why a small fraction of the analytes still remained after the first desorption/ionization cycle.[232] Nevertheless, by implementing a cleaning step shortly after the desorption/ionization cycle (*i.e.* mixture of methanol, isopropanol and acetonitrile; 50:25:25) negligible carry-over was attained (≤ 0.4%). Undeniably, the cleaning step should be optimized according to both the chemistry of the coating and its affinity towards the analyte of interest.[115] In cases where there is an extensive variation in analyte concentration among samples (*i.e.* low ppt to ppm levels), SPME-TM devices should be restricted to a single use. Otherwise, a small portion of the analytes could remain on the coating, even after the cleaning cycle, which could lead to potential false positives.[25] A possible solution while working with compounds at concentrations greater than 50 ppb and with high affinity towards the coating would be to perform shorter extractions (≤ 30s). Thus, the amount of analyte enriched would be diminished and the exhaustive removal of analytes non-desorbed by DART would be plausible with a cleaning step.

Table 2.3 Intra-mesh reproducibility (n=4). Results are reported as ratio of analyte (diazepam) versus internal standard isotopologue [D_5] diazepam. 1 min extractions were performed using vortex agitator set-up at maximum speed (3200 rpm). Extraction from 1.5 mL of PBS spiked with 20 ng mL^{-1} of diazepam. Analyses were performed using Thermo TSQ on SRM mode. SD, standard deviation. RSD, relative standard deviation.

Experiment	Mesh_1	Mesh_2	Mesh_3	Mesh_4	Mesh_5	Mesh_6	Mesh_7	Mesh_8	Mesh_9
Replicate 1	1.91	1.84	1.87	1.77	1.79	1.75	1.75	1.78	1.78
Replicate 2	1.87	1.80	1.77	1.80	1.80	1.80	1.75	1.77	1.71
Replicate 3	1.76	1.84	1.76	1.81	1.77	1.82	1.76	1.83	1.77
Replicate 4	1.80	1.84	1.94	1.86	1.83	1.82	1.77	1.74	1.78
Summary	1.83	1.83	1.84	1.81	1.80	1.80	1.76	1.78	1.76
SD	0.07	0.02	0.09	0.04	0.02	0.04	0.01	0.03	0.03
RSD	3.65	1.15	4.65	2.07	1.36	1.99	0.71	1.93	1.86

Table 2.4 Intra-mesh reproducibility (n=4). Results are reported as ratio of analyte (cocaine) versus internal standard isotopologue [D_3] cocaine 1 min extractions were performed using vortex agitator set-up at maximum speed (3200 rpm). Extraction from 1.5 mL of PBS spiked with 20 ng mL^{-1} of cocaine. Analyses were performed using Thermo TSQ on SRM mode. SD, standard deviation. RSD, relative standard deviation.

Experiment	Mesh_1	Mesh_2	Mesh_3	Mesh_4	Mesh_5	Mesh_6	Mesh_7	Mesh_8	Mesh_9
Replicate 1	1.48	1.51	1.53	1.62	1.53	1.58	1.59	1.61	1.62
Replicate 2	1.54	1.54	1.59	1.57	1.62	1.58	1.59	1.64	1.59
Replicate 3	1.51	1.50	1.56	1.52	1.57	1.59	1.67	1.55	1.50
Replicate 4	1.55	1.52	1.47	1.54	1.45	1.53	1.61	1.55	1.53
Summary	1.52	1.52	1.54	1.57	1.54	1.57	1.62	1.59	1.56
SD	0.03	0.02	0.05	0.04	0.07	0.03	0.04	0.04	0.05
RSD	2.07	1.00	3.29	2.77	4.69	1.73	2.23	2.76	3.32

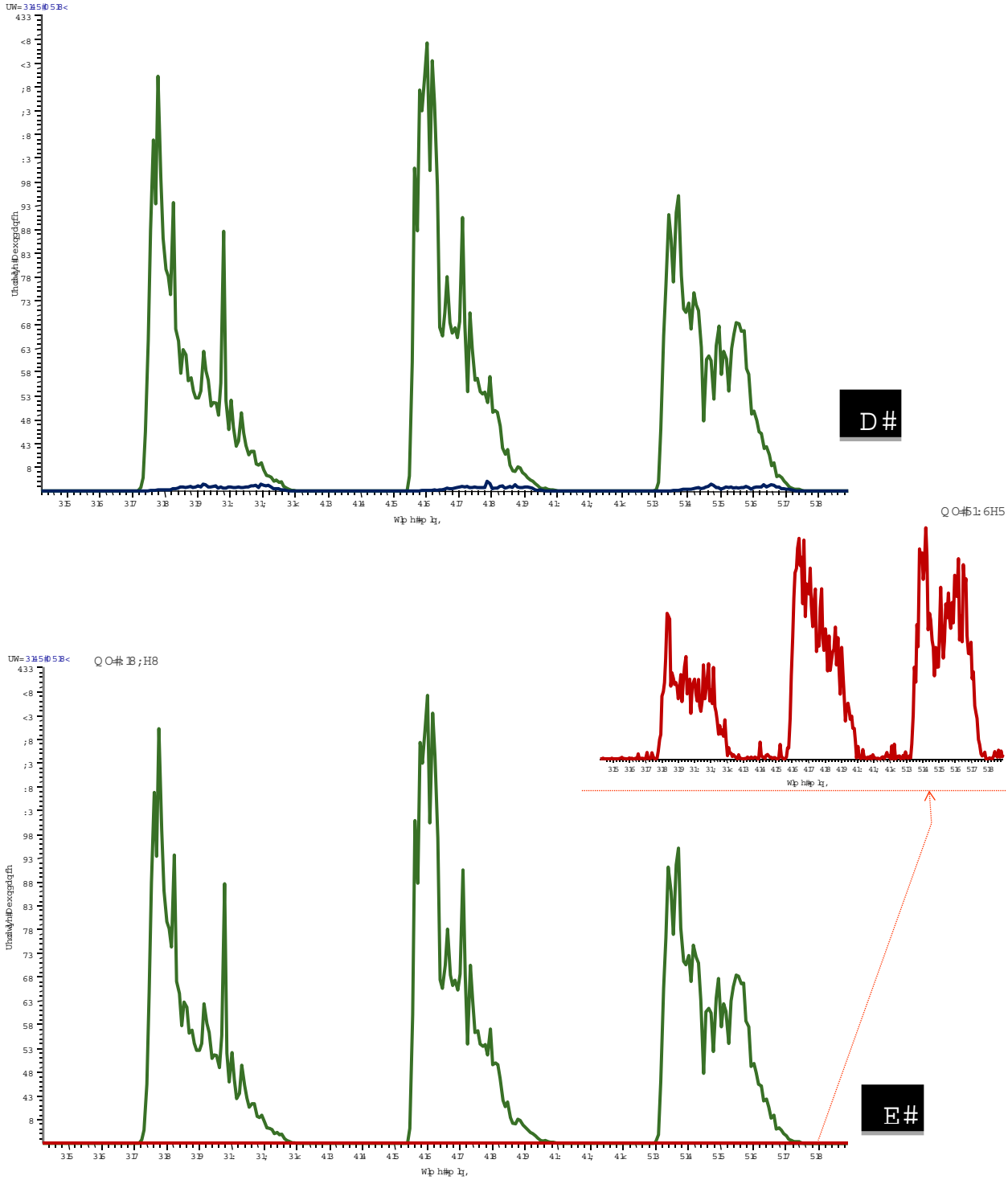


Figure 2.8 SPME-TM inter-mesh reproducibility; ion chronograms (non-smoothed) obtained after 1 min extraction from a solution spiked with 20 ppb of cocaine (green line) versus **A.** carry-over measured subsequently after the desorption/ionization cycle (blue line) and **B.** carry over measured after cleaning the SPME-TM device (red line) on 1.5 mL of a mixture of methanol, isopropanol and acetonitrile (50:25:25) for 30 minutes.

Despite the advantages of paper spray (PS) for the analysis of small volumes of untreated samples, Li *et al*. and Espy *et al.* reported that the use of paper substrate requires a drying step, either with air or electromechanical assistance, prior to desorption/ionization, which extends the total time of analysis.[100,226] In contrast to PS, analytes extracted by SPME-TM can be desorbed immediately after a quick rinse in water ($t \leq 10$ s) and gentle removal of excess water with a cleaning tissue ($t \leq 2$ s). An exceptional characteristic of SPME-TM is the mechanical strength provided by the mesh-blade arrangement used to build its structure (Figure 2.5) As a result, deformation/damage of the device hardly occurs regardless of the sample dimensions or its characteristics. Thus, SPME-TM could be used to perform extractions from limited sample volumes (*e.g.* extractive blood spot[144]) up to large volumes (*e.g.* on-site monitoring of a creek/river[228]).

MS analysis provides significant amounts of information about complex samples.[57] However, sample pre-treatment required before MS analysis not only is labour-intensive and time-consuming, but also intricate.[46,58] Contrastingly, due to its speed and ease of use, SPME-TM is an ideal device for the screening of controlled substances in biological samples as well as for therapeutic drug monitoring (TDM).[25,144,194] To demonstrate its applicability, SPME-TM was used for the quantification of cocaine and DZP in urine and plasma. Figures 2.9 and 2.10 summarize the exceptional linearity achieved in both matrices. Similar to PBS, LOQs of 2 and 5 pg mL^{-1} were determined for cocaine in urine and plasma, respectively. Thus, matrix effects are significantly minimized by the sample clean-up provided by SPME-TM, and analytes with low binding present comparable detection limits independently of the matrix.[115] By removing salts and biomolecules that mechanically attach to the coated strands during the extraction, the rinsing step extends the operative time of the mass spectrometer, providing reliably high instrumental sensitivity as well as minimizing instrument maintenance.[194]

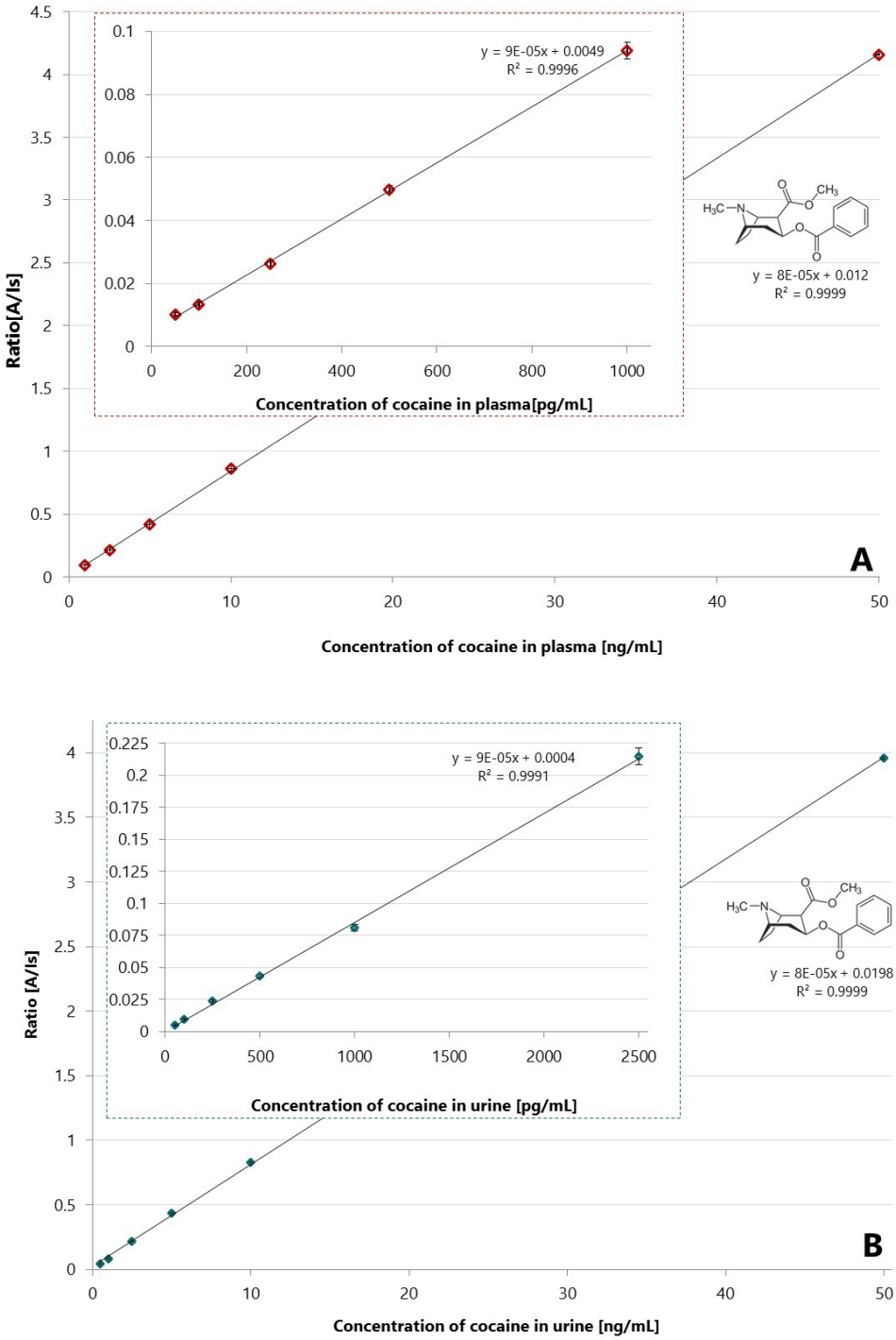


Figure 2.9 A. Quantitative analysis of plasma spiked with cocaine (50 pg mL^{-1} to 50 ng mL^{-1}) and its isotopologue [D_3] cocaine (12 ng mL^{-1}). **B.** Quantitative analysis of urine spiked with cocaine (50 pg mL^{-1} to 50 ng mL^{-1}) and its isotopologue [D_3] cocaine (12 ng mL^{-1}).

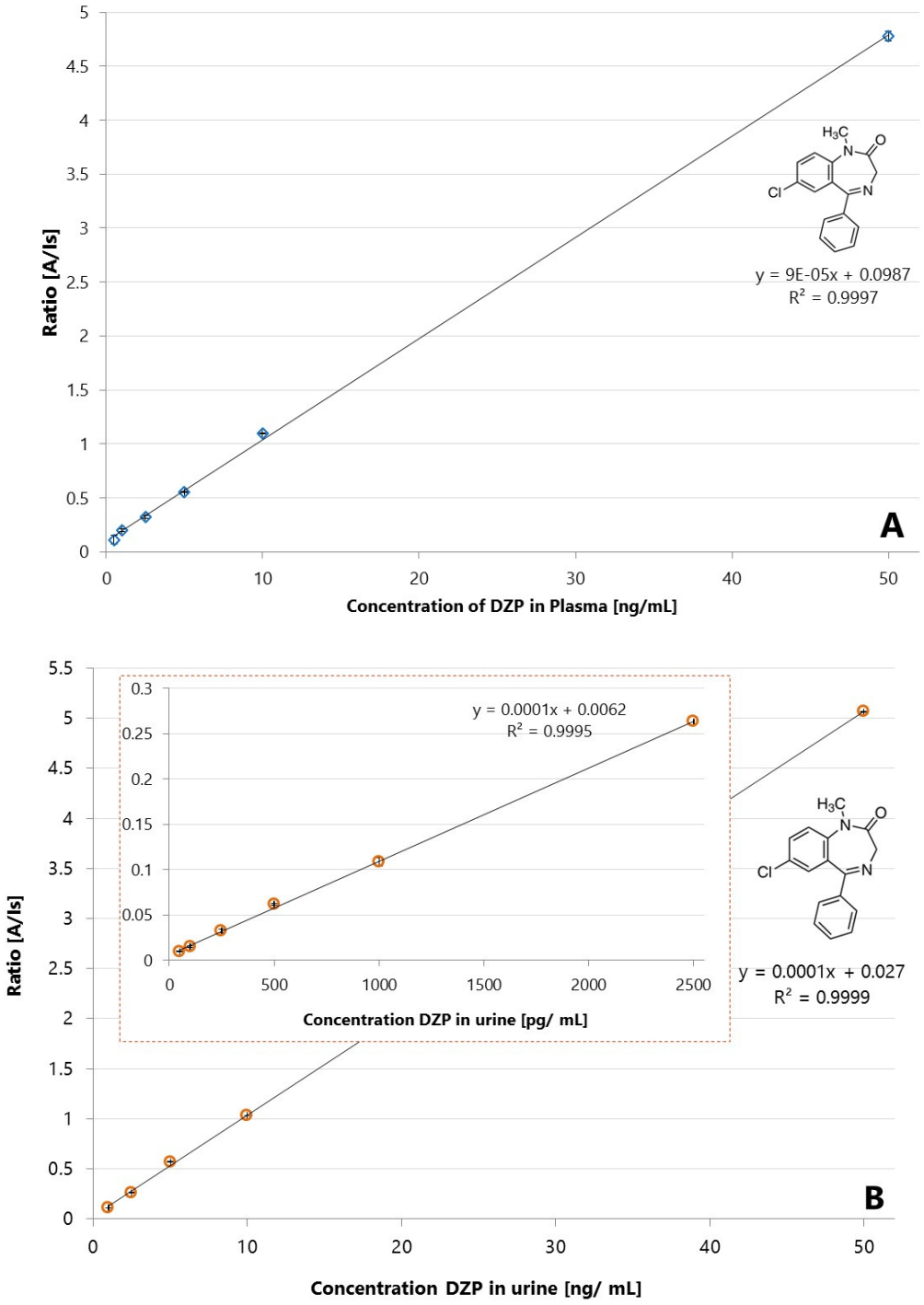


Figure 2.10 A. Quantitative analysis of plasma spiked with diazepam (500pg mL^{-1} to 50 ng mL^{-1}) and its isotopologue [D_5] diazepam (12 ng mL^{-1}). **B.** Quantitative analysis of urine spiked with diazepam (50 pg mL^{-1} to 50 ng mL^{-1}) and its isotopologue [D_5] diazepam (12 ng mL^{-1}).

Unlike cocaine, the LOQ for DZP in plasma (497 pg mL^{-1}) was significantly higher in comparison to urine and PBS (19 and 28 pg mL^{-1}, respectively). However, it is worth mentioning that DZP is 98% bound to plasma proteins and, as an SPME-based approach, the TM configuration only extracts the free-portion of analytes in the sample.[133] In addition, since the TM configuration guarantees homogeneous interaction between extracted and ionizing species, standard-free quantitation is also feasible with SPME-TM. Nevertheless, given that extraction is not performed at equilibrium ($t \leq 1$ min), precise variables should be cleverly controlled in order to obtain reproducible and repeatable results, namely sampling time, convection, as well as coating thickness homogeneity.[133] Definitely, devices having consistent coating distribution, composition, and thickness are needed to ensure reproducible extraction of the analytes to the coating and desorption of the analytes from the coating. Different to other direct ionization techniques, given that no sample matrix is placed in front of the mass spectrometer, homogeneous coatings are required to normalize the sample matrix by always extracting the same amount of analyte of interest independently of the device used. Hence, reproducible coatings not only minimize sample interferences, but also provide reproducible instrumental response and no internal standard is required to achieve reproducibility below 15 % RSD (Figure 2.11)

As a proof-of-concept, SPME-TM was used to simultaneously monitor 21 prohibited substances spiked on PBS at 20 ng mL^{-1}.[233] Selected reaction monitoring (SRM) was used to exclusively identify each compound. LOD were tentatively predicted based on the results obtained for cocaine and DZP in PBS. Even though DART source parameters were not optimized for each analyte, all substances were detected, and 16 compounds provided hypothetical detection limits lower than 50 pg mL^{-1} (*e.g.* heroin [Log P 1.52], propranolol [Log P 3.48], and stanozolol [Log P 5.53]; Figure 2.12, Table 2.1).

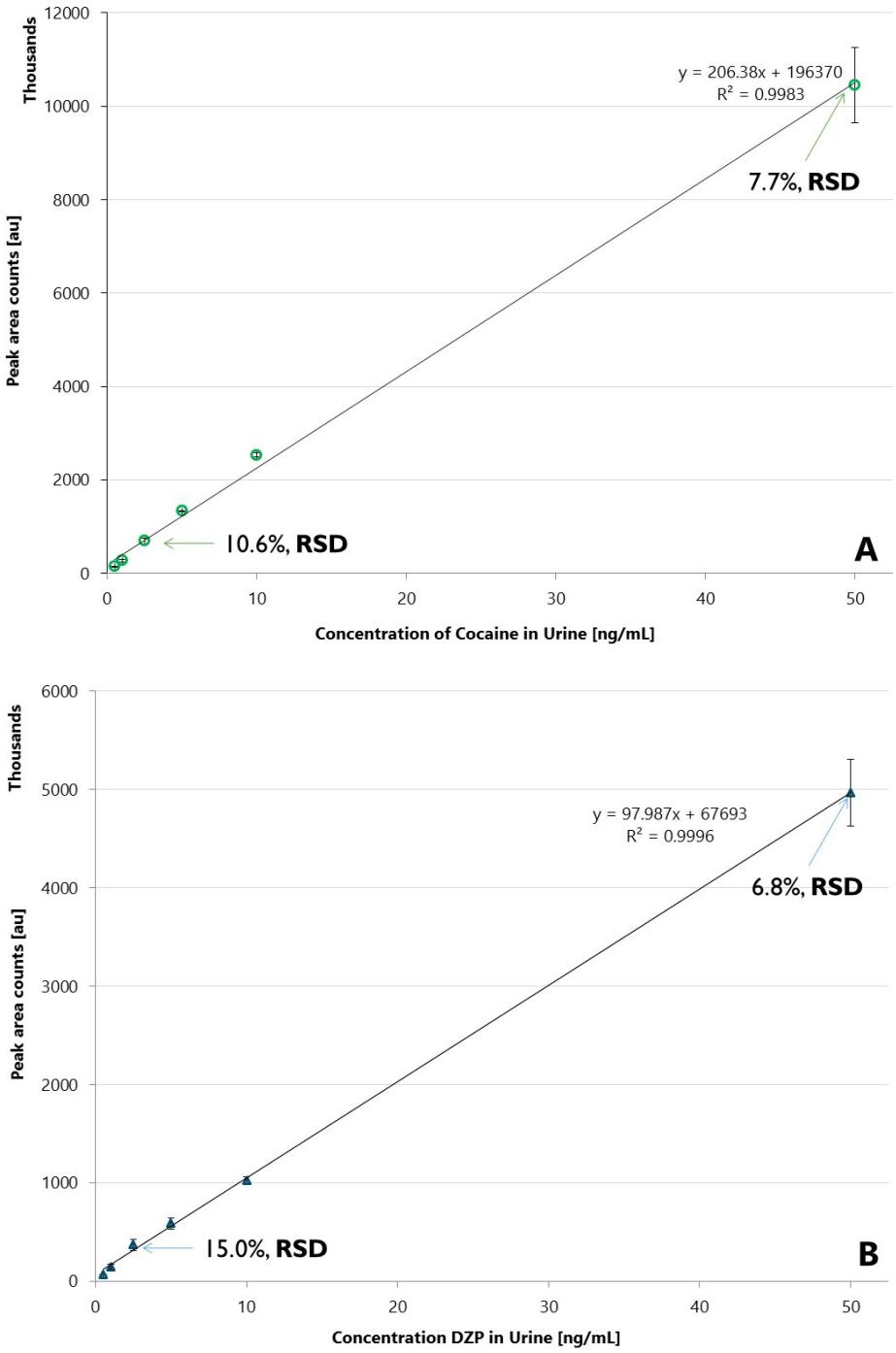


Figure 2.11 SPME-TM internal standard free calibration (n=3). **A.** Quantitative analysis of urine spiked with cocaine (500 pg mL^{-1} to 50 ng mL^{-1}) **B.** Quantitative analysis of urine spiked with diazepam (500 pg mL^{-1} to 50 ng mL^{-1}).

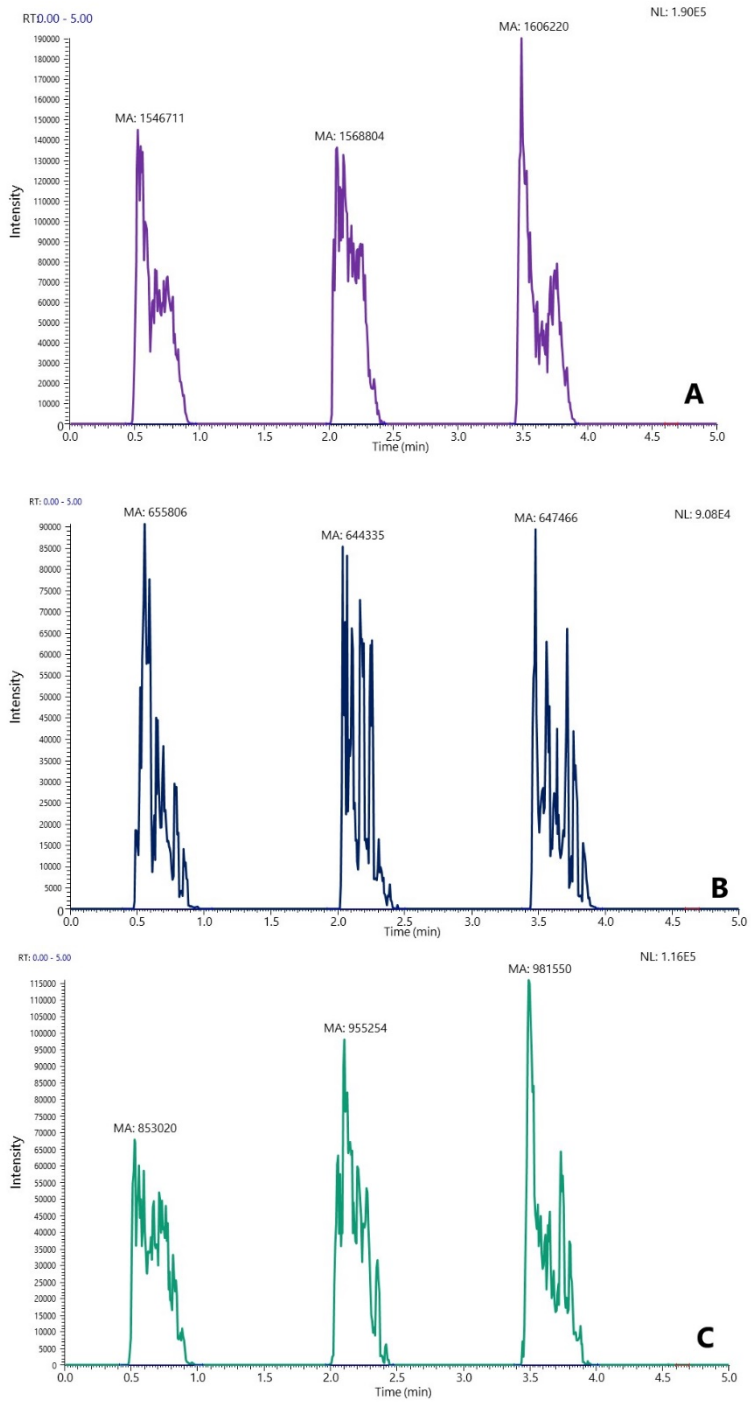


Figure 2.12 Ion chronograms of three controlled substances: heroin (**A**), propranolol (**B**), and stanozolol (**C**). 1 min extractions were performed using vortex agitator set-up at maximum speed (3200 rpm). Simultaneous extraction from 1.5 mL of PBS spiked with 20 ng mL^{-1} of 21 substances described on Table 2.1. Analyses were performed using a Thermo TSQ on MRM mode.

Insofar as SPME-TM derives its sensitivity and selectivity from the physicochemical properties of the extraction phase, current research is focused on the development of new devices with greater affinity towards specific target compounds. Certainly, the ability of SPME-TM to screen numerous substances in a single analysis, without forfeiting sensitivity or quickness, is a noteworthy characteristic of this technique, which could be used in other applications such as monitoring of personal care products in waste water or pesticides in food commodities.[228]

An asset of the mesh-blade arrangement of SPME-TM is that it can be used to perform either individual extractions (*i.e.* from a vial containing sample) or high-throughput extractions in a multi-well plate format (Figure 2.13). Similar to other SPME geometries, SPME-TM can be adapted to concurrently analyse up to 96 samples in a single run.[133,231] Hence, by automating the extraction/rinsing step, as well as the desorption step with the aforementioned system, a total analysis time of 60 seconds or less could be attained per sample. Undoubtedly, the multiple benefits of SPME-TM, such as low detection limits and minimal matrix interferences, should stimulate the scientific community to use a swift sample preparation approach prior to direct introduction to MS analysis.

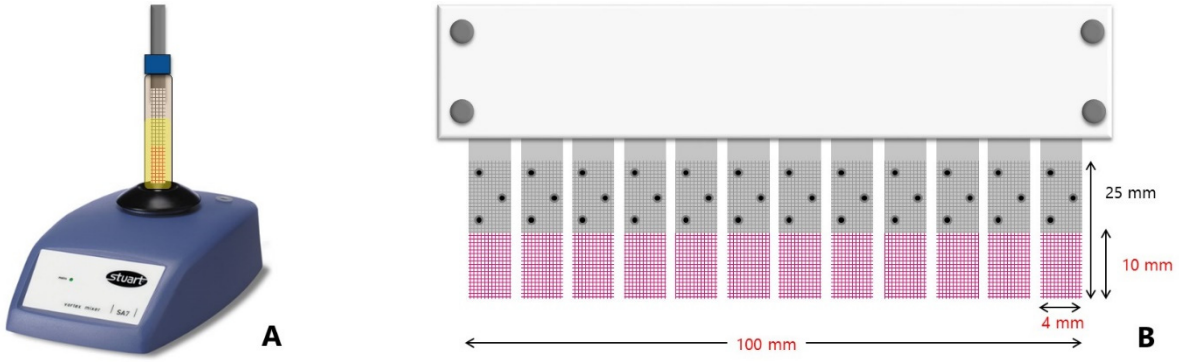


Figure 2.13 SPME-TM configuration for individual extractions and **B.** SPME-TM 12-strips configuration for high-throughput analysis using 96-Concept autosampler (PAS Technologies).

2.2.4 Summary

In summary, a novel SPME device that can be easily coupled to DART for targeted and quantitative trace analysis (ppt to ppm levels) was developed. Given the structural configuration of the apparatus, it can be used to perform extractions independently of the sample complexity and its dimensions. Contrary to popular belief,[57,58]analyte-enrichment and sample-clean-up with SPME-TM can be performed in less than 1 minute, with total analysis time not exceeding 3 minutes. In addition, since no solvent is required in the entire process, and the device can be used on-site, the whole analytical process with SPME-TM is "green". Unquestionably, better understanding of the fundamentals driving the extraction, as well as ion transportation would lead to lower the detection limits further currently attained by SPME-TM.[232] Continuous improvement in design of instrument sensitivity will aid this goal. Moreover, sensitivity can be enhanced by precisely tuning features of both techniques such as: a. the substrate characteristics (*i.e.* mesh material type, empty space diameter, consecutive hole to hole distance, and strand size);[234] b. the coating features (*i.e.* polymeric phase chemistry, particle size, porosity, thermal conductivity, thermal stability, and affinity for the analyte of interest),[124] and c. the position of the substrate in which there is a balance between efficient neutral generation by thermal desorption and transport into the mass spectrometer (*i.e.* DART source operative conditions such as: gas temperature and flow, discharge voltage, grid electrode voltage, spatial position of the mesh in relation to the ion source nozzle).[209,232] A comprehensive optimization of the variables described above will certainly boost the performance of SPME-TM technique herein discussed.

Although SPME-TM reusability is advantageous for high-throughput applications in which a large number of samples should be processed daily, it is also envisaged as a disposable device for *in situ* trace analysis and this research is currently undergoing in our research group.[13] By coupling

SPME-TM to deployable MS/MS systems, truly "real-time" and quantitative analysis of complex mixtures will be delivered on-site (see Section 2.4).[226,235] Therefore, due to the unique combination of speed, selectivity, sensitivity, reproducibility and simplicity, SPME-TM-DART-MS/MS is a suitable rapid screening and quantitation technique not only for point-of-care TDM, but also in numerous environmental, food and forensic applications, as presented in Section 2.3.[152]

Section 2.3 Ultrafast Screening and Quantitation of Pesticides in Food and Environmental Matrices by Solid-Phase Microextraction–Transmission Mode (SPME-TM) and Direct Analysis in Real Time (DART)

2.3.1 Introduction

To date, among the different analytical methods used for analysis of pesticides in food commodities, QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) has gained the broadest acceptance within the scientific community[236]. The success and widespread use of QuEChERS can be attributed to its convenient approach; as it combines well-known extraction and clean up methods,[237] trained analysts are not required to learn new procedures to utilize this method. However, the Achilles heel of this sample preparation strategy lies in its extensive number of steps, and in most cases, the various challenges associated with its automation. Thus, QuEChERS is not only tedious, but its use may also lead to large systematic errors.[120] Furthermore, the extensive use of organic solvents and the production of waste from the salts and sorbents employed in this method are additional factors that would make an alternative, comparably reliable, and sensitive green sample preparation technique desirable for multiresidue analysis of pesticides.

Solid phase microextraction (SPME) has been widely reported as a convenient alternative method for the analysis of pesticides in food and environmental matrices.[119,120] Historically, the use of SPME for the analysis of foodstuffs was confined to headspace (HS) sampling due to the short lifespan of commercially available coatings submitted to direct immersion in complex sample matrices[139]. Moreover, the results and limits of quantitation achievable by HS-SPME were limited by the vapor pressure of the pesticide analyzed; very often, further matrix modifications were

needed to promote the mass transfer of the analytes from the matrix to the HS. In addition, sensitive and accurate analysis could not be carried out by HS-SPME for polar and non-volatile analytes due to their low Henry's law constant. To address the shortcomings of HS-SPME, several attempts towards an effective direct immersion (DI) method were carried out; however, all developed methodologies included sample pre-treatment prior to SPME extraction.[238–240] These limitations were addressed with the introduction of new matrix-compatible SPME coatings (in fiber format) able to endure direct immersion in complex matrices for repetitive cycles of extraction/desorption.[241,242] This new coating opened the door to applications of SPME for multiresidue analysis in complex matrices, greatly improving the throughput of the methods and minimizing, or completely avoiding in most cases, the need for sample pre-treatment. In addition, comparative studies showed the ability of these novel SPME coatings to reach lower limits of detection than those attained by QuEChERS, with the further advantage of cleaner chromatograms being attained by such SPME coatings in comparison to QuEChERS as well.[138,243] However, in spite of all the benefits offered by matrix compatible SPME devices, the fastest throughput achievable was 30 minutes per sample (including an extraction time of 15 minutes, followed by 15 minutes of gas-chromatographic separation).[243] Undoubtedly, the long extraction-separation procedure may have thwarted the practice of this method for the expeditious determination of pesticides in food/environmental samples.

Not long ago, the concept of an analytical method capable of assessing the concentration of agrochemicals in food commodities with minimal sample handling and without a chromatographic separation step would have been regarded as chimerical by most food analysts.[244] However, recent developments in ambient mass spectrometry (AMS) technologies,[65,232,245] characterized as a group of technologies that allow for the generation of ions under atmospheric conditions from untreated

samples, have challenged whether the long and cumbersome protocols typically used for the determination of pesticides/contaminants in foodstuffs are necessary.

As shown in Figure 2.14, AMS allows for the rapid analysis of food samples; this can be accomplished by placing the untreated material between a jet of charge species[31] and the mass spectrometer (*e.g.* desorption electrospray ionization, DESI[246–250]; direct analysis in real time, DART[69,74,78,193,249,251–253]; atmospheric pressure solid analysis probe, ASAP[254]; or low-temperature plasma ambient ionization, LTP[54,255,256]), by spiking/spotting a portion of the sample on a solid substrate that is interfaced with the mass spectrometer to generate ionic species (*e.g.* paper spray, PS[257–261]; probe electrospray ionization, PESI[262]; touch spray ionization[263]; thermal-desorption-ESI[264]), or by sampling the surface with a continuous flowing liquid (*e.g.* liquid extraction surface analysis, LESA[56]; nanoextractive electrospray ionization, nanoEESI[265]). Although pure direct coupling methods have demonstrated fast and simple identification/quantitation of agrochemicals in several untreated matrices, their suitability for the determination of these analytes at trace-levels in complex food matrices still remains to be demonstrated. Consequently, the direct coupling of sample preparation technologies and mass spectrometry[151,173,174,197,266,267] has emerged as a solution to these unmet needs. Undeniably, microextraction-based approaches have outclassed other sample preparation technologies due to their intrinsically small configuration, rendering its direct coupling simpler and, in most cases, tremendously efficient.[136] SPME is not the exception to this trend, and multiple direct couplings of SPME to MS have shown its great potential in significantly shortening the total sample analysis time of complex matrices, as well as in diminishing potential matrix effects and instrument contamination, owing to the ability of SPME to provide clean sample extracts.[151,174]

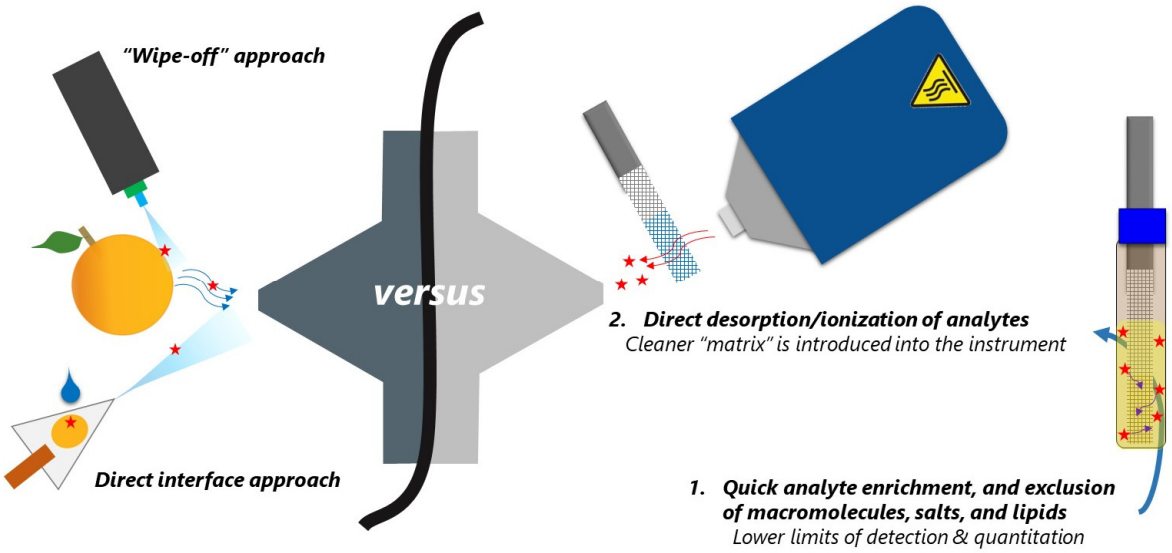


Figure 2.14 Schematic representation of ambient mass spectrometry (AMS) technologies versus direct coupling of SPME to mass spectrometry (SPME-MS) for the analysis of pesticides in food/environmental samples.

Recently, an alternative geometry of SPME was introduced for the direct coupling to mass spectrometry: a metallic mesh coated with adsorbent particles glued together by polyacrynonitrile (PAN). The device was successfully coupled as a transmission mode substrate (i.e. SPME-TM) for DART.[205,267] This approach drastically lowered the analysis time for determination of drugs in biofluids as well as considerably pushed down the limits of detection achievable by traditional sample spot DART determination.[58,74,268] The challenge taken up in this study encompassed the application of SPME-TM for the quantitative multiresidue analysis of pesticides in food and environmental matrices without any sample pre-treatment (e.g. filtration or solvent extraction)[197,269], with the additional goal of minimizing total analysis time[269]. In order to set a representative system and thoroughly evaluate the performance of the developed SPME-TM-DART/MS system, the matrices under study, namely Concord grape juice, orange juice, cow milk, and surface water, were selected on the basis of specific characteristics representative of the different challenges associated with complex matrix analysis, and their varied nutritional and

physical attributes. Analytes of interest, in turn, were selected from various classes of pesticides, covering a broad range of polarities, molecular weight, and functional groups. In addition to demonstrating the suitability of SPME-TM-DART/MS for quantitative analysis of multiresidue pesticides in complex matrices, the presented work also demonstrates the aptness of single SPME-TM devices to perform targeted quantitation as well as retrospective analysis of the same sample.

2.3.2 Experimental section

Materials and supplies

The analytes used in this investigation were purchased from Sigma Aldrich, and are listed in Table 2.5 alongside relevant information pertaining to their physical chemical properties, pesticide classification, and monitored SRM transitions. Individual stock solutions were prepared in acetonitrile, methanol, acetone, and dichloromethane at concentrations ranging from 9 to 15 mg/ml. Working solutions at 100, 10, and 1 µg/ml were prepared by mixing the analytes in acetonitrile. The internal standards used, namely atrazine-D5, metalaxyl-D6, and cyprodinyl-D6, were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Stock solutions of the internal standards were prepared in acetonitrile at a concentration of 1 mg/ml for atrazine-D5 and metalaxyl-D6, and at a concentration of 5mg/ml for cyprodinyl-D6. A working solution comprising all the internal standards was prepared in acetonitrile at 10 µg/ml. Samples of concord grape juice, orange juice (with pulp), and cow milk (2%, Milk Fat, M.F.) were purchased from local grocery stores (Waterloo, ON, Canada). Surface water samples were retrieved from Grand River (Waterloo Region, ON, Canada). Samples were prepared by spiking appropriate amounts of analytes and internal standards, with care given so that the total amount of organic solvent added never exceeded 1% (v/v) of the total amount of matrix. The spiked samples were equilibrated for at least two hours

Table 2.5 Target analytes, class type, molecular weight (MW), molecular structure, hydrophobicity (expressed as LogP), and monitored SRM transitions for each model compound in positive ionization mode with DART-MS/MS.

Compound	Class	Type	MW (g/mol)	LogP (pH=7, 20°C)	Parent ion [m/z]	Product ion #1 [m/z]	Product ion #2 [m/z]
Acephate	Organophosphate	I	183.17	-0.85	184.031	95.065	143.056
Omethate	Organophosphate	I, A	213.20	-0.74	214.038	125.045	183.048
Dimethoate	Organophosphate	I, A	229.26	0.70	230.014	125.057	199.006
Metalaxyl	Phenylamide	F	279.33	1.65	280.157	192.167	220.181
Azoxystrobin	Strobilurin	F	403.40	2.50	404.131	344.095	372.114
Imazalil	Imidazole	F	297.18	2.56	297.055	159.013	201.007
Atrazine	Triazine	H	215.68	2.70	216.112	68.158	174.112
Malathion	Organophosphate	I, A	330.36	2.75	331.054	99.101	127.113
Pyrimethanil	Anilinopyrimidine	F	199.11	2.84	200.130	107.144	168.129
Cyproconazole	Triazole	F	291.78	3.09	292.121	70.158	125.062
Tebuconazole	Triazole	F	307.82	3.70	308.162	70.166	125.078
Cyprodinil	Anilinopyrimidine	F	225.29	4.00	226.133	77.137	93.139
Pyraclostrobin	Strobilurin	F	387.82	4.25	388.114	149.073	194.095
Quinoxyfen	Quinoline	F	308.13	4.29	308.003	162.039	197.002
Difenoconazole	Triazole	F	406.26	4.36	406.070	188.038	250.984
Trifloxystrobin	Strobilurin	F	408.37	4.50	409.140	145.060	186.069
Pyriproxyfen	Unclassified	I	321.37	5.37	322.154	78.139	96.126
Fenproprathrin	Pyrethroid	I	349.42	6.04	350.167	97.273	125.163
Pyridaben	Pyridazinone	I, A	364.93	6.37	365.154	147.160	9.098

at 1500 rpm to guarantee that binding equilibria between analytes and matrix components were established, thus obtaining samples that closely represent real contamination scenarios. SPME-TM devices were manufactured in-house using stainless steel mesh (74 × 74 wires per in, wire diameter 0.004 in). The mesh's strands were precisely coated with a biocompatible polymer (slurry of 5 μm hydrophilic-lipophilic balance (HLB) particles and polyacrylonitrile as a binder) at a thickness of less than 20 μm and a length of 1 cm following the protocol described in section 2.2. As previously reported by our group, HLB particles were selected with the objective of providing wider analyte balance coverage, which is critical when performing multiresidue analysis[123,151].

DART-MS set up

A DART-Standardized Voltage and Pressure (DART-SVP) model ion source (IonSense, Saugus, MA USA) was coupled to either a triple quadrupole mass spectrometer (TSQ Vantage) or an Orbitrap (Exactive) by Thermo Scientific (San Jose, California, USA) via a Vapur® interface (IonSense, Inc.). The DART-SVP was fitted with a single dimensional motorized linear rail controlled through the DART-SVP web-based software so as to reproducibly and consecutively automatically position the SPME-TM devices in front of the DART source (speed of 0.2 mm s^{-1} was used). In order to guarantee good reproducibility and higher throughput of the desorption process, a custom-made holder (UW-12) able to allocate up to twelve SPME-TM devices was developed.[267] The DART source was operated using the following conditions: positive ion mode; high voltage (HV) electrode, −3000 V; discharge electrode, +350 V; grid voltage, +350 V. The gas heater was optimized at 350 °C, which yielded the optimum intensities for most of the analytes.

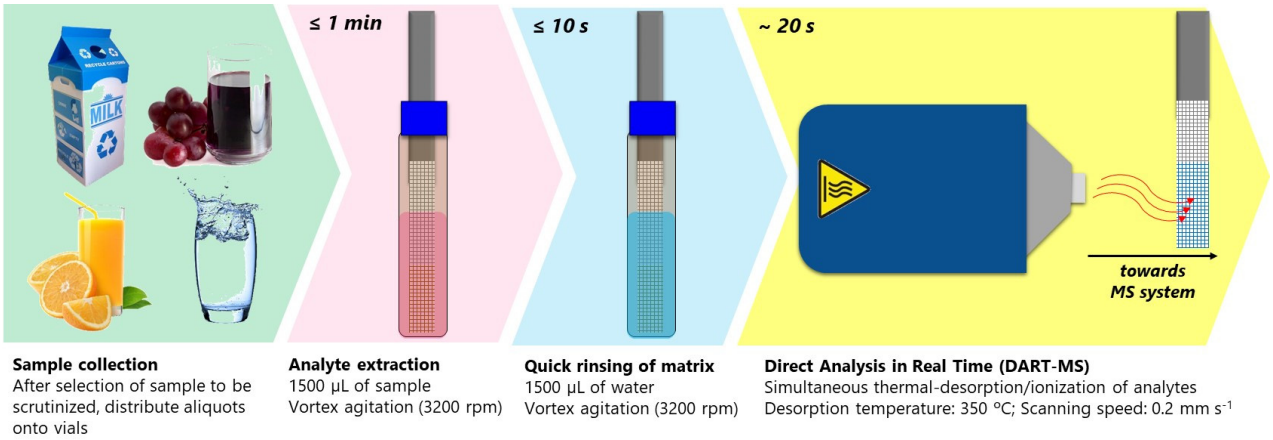


Figure 2.15 Schematic representation of the analytical workflow for the ultrafast determination of pesticides in food and environmental matrices by Solid Phase Microextraction-Transmission Mode (SPME-TM) and Direct Analysis in Real Time (DART).

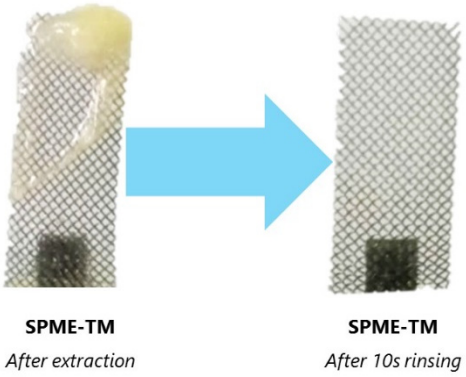


Figure 2.16 Relevance of the rinsing step on SPME-TM devices for the quantitative determination of pesticides in food/environmental matrices. Left picture portrays the SPME-TM after extraction from orange juice with pulp, while the left picture shows the mesh after subsequent 10 seconds rinsing in LC/MS water.

Sample preparation/analyte enrichment

As shown in Figure 2.15, the analytical procedure consisted of the following steps: 1. SPME-TM devices were rinsed (10 s) in water in order to eliminate any organic solvent residue (from the preconditioning step) that could potentially affect the extraction capability of the devices. 2. SPME-TM devices were exposed to the sample matrices (1.5 ml), and extraction was carried out for 1 minute at 3200 rpm. 3. SPME-TM devices were then quickly rinsed (10 s) in water in order to remove any loosely attached matrix constituents that could induce ion

enhancement/suppression, be irreversibly carbonized onto the coating surface during the desorption process, or cause instrumental contamination (see Figure 2.16, relevance of rinsing step). Finally, SPME-TM devices were positioned in the UW-12 holder and thermally desorbed. The total run time for 12 samples was approximately 4 minutes. It is worth mentioning that SPME-TM devices were used as consumables in this study (single use). However, as reported by Gomez-Rios et al[267], the devices can be reused by implementing a thorough cleaning step after the desorption/ionization cycle (e.g. immersing them on a mixture of methanol, isopropanol, and acetonitrile; 50:25:25 for half-hour).

2.3.3 Results and discussion

A novel technology for rapid screening and quantitative analysis of food and environmental samples

Aiming to thoroughly investigate the suitability of SPME-TM-DART-MS for quick multiresidue analysis of pesticides, four matrices with completely different nutritional and physical characteristics were carefully chosen. First, Concord grape juice was selected due to the high amount of anthocyanin pigments and sugars (20% w:w) present in this commodity. Previous studies have reported that the co-extraction of these compounds by SPME might induce the production of artefacts during thermal desorption on the GC injector (*i.e.*, Maillard reactions[139]). The second matrix, orange juice (10% w:w of sugar) composed of a substantial amount of pulp, was selected owing to the membranous content of its pulp, which could be easily entangled onto the coated mesh after analysis, leading to non-reproducible results. Cow milk (2%, M.F.), as a third matrix, was selected due to its composition as a matrix rich in fat and proteins, which can irreversibly deposit onto the SPME coating, thus compromising the mass transfer of the analytes.

Finally, surface water from a local river was selected so as to represent an environmental matrix where the presence of pesticides would be highly relevant in real world applications. All of these matrices present different challenges for chemical analysis, and many reported methods employ extensive sample preparation procedures in order to obtain concentrated extracts that can be introduced into the analytical system without the risk of instrumental contamination and matrix interferences. With the purpose of developing a fast and simple method for multiresidue analysis in complex matrices, no sample pre-treatment was carried out for the matrices studied. This is possible in view of the intrinsic nature of SPME, which allows for simultaneous isolation and concentration of analytes from a given matrix, thus providing a "clean-extract" for introduction into the analytical system. In addition, the TM geometry of SPME not only offers faster throughout in comparison to automated SPME-GC/LC methods[161], it also offers increased sensitivity, as analytes can be introduced onto the MS system without further dilution. Moreover, in comparison with SPME's fibre geometry,[207] the mesh configuration provides a larger surface area available for extraction, leading to dramatic improvements in enrichment kinetics and the limits of quantitation (LOQ) attainable. Furthermore, coating fouling and deterioration, due to irreversible attachment of matrix components onto the coating surface, could be avoided owing to the short extraction time needed to achieve the desired method sensitivity for the analytes of interest.[270]

SPME-TM for pesticide determination: method validation.

Validation of the method was carried out using the matrix-matched calibration approach, with calibration functions constructed on the basis of the signal ratio of the analyte and the internal standard (A/Is) for twelve points in three independent experiments, covering a range between 0.1 and 100 ng g^{-1}.

Table 2.6 Figures of merit for the determination of multiresidue pesticides in four different matrices via SPME-TM-DART-MS/MS.

Compounds	LogP	Concord Grape juice			Orange juice			Surface water			Cow milk		
		LDR (μg/kg)	R^2	LOQ (μg/kg)	LDR (μg/kg)	R^2	LOQ (μg/kg)	LDR (μg/kg)	R^2	LOQ (μg/kg)	LDR (μg/kg)	R^2	LOQ (μg/kg)
Acephate	-0.9	5-100	0.9988	5	0.5-50	0.9997	0.5	5.0-25	0.9996	5	1.0-50	0.9916	1
Pyrimethanil	-0.7	0.1-100	0.9997	0.1	0.25-100	0.996	0.25	0.25-10	0.9998	0.25	0.1-100	0.9995	0.1
Omethoate	0.7	0.1-100	0.9997	0.1	0.1-50	0.9905	0.1	0.1-100	0.9984	0.1	0.25-100	0.9974	0.25
Atrazine	1.7	0.1-100	0.9998	0.1	0.1-100	0.9981	0.1	0.1-100	0.9998	0.1	0.1-100	0.9996	0.1
Cyprodinyl	2.5	0.1-100	0.9987	0.1	0.1-100	0.9925	0.1	0.1-100	0.9983	0.1	0.25-100	0.9972	0.25
Dimethoate	2.6	0.25-100	0.9987	0.25	0.1-100	0.9975	0.1	0.25-100	0.9995	0.25	0.25-100	0.9982	0.25
Metalaxyl	2.7	0.25-100	0.9987	0.25	0.1-100	0.9989	0.1	0.1-100	0.9958	0.1	0.1-100	0.9997	0.1
Cyproconazole	2.8	0.1-100	0.9937	0.1	0.1-100	0.9999	0.1	0.25-100	0.9993	0.25	0.25-100	0.9948	0.25
Imazalil	2.8	0.25-100	0.9945	0.25	0.1-100	0.9999	0.1	0.5-100	0.9982	0.5	0.1-100	0.9997	0.1
Quinoxyfen	3.1	0.1-100	0.9995	0.1	0.25-100	0.9967	0.25	0.25-10	0.998	0.25	-	-	-
Tebuconazole	3.7	0.25-100	0.9994	0.25	0.1-100	0.9991	0.1	0.1-10	1	0.1	0.25-100	0.9975	0.25
Flusilazole	4.0	0.25-100	0.9981	0.25	0.1-100	0.9984	0.1	0.1-100	0.9972	0.1	0.25-100	0.9938	0.25
Pyriproxyfen	4.3	0.1-10	0.9964	0.1	0.25-100	0.9981	0.25	-	-	-	-	-	-
Malathion	4.3	0.1-100	0.9924	0.1	0.25-100	0.9981	0.25	0.25-100	0.9975	0.25	1-100	0.9979	1
Pyridaben	4.3	0.25-50	0.9974	0.25	0.25-100	0.9993	0.25	-	-	-	-	-	-
Pyraclostrobin	4.5	0.25-50	0.9874	0.25	0.1-100	0.9968	0.1	0.25-100	0.9774	0.25	-	-	-
Azoxystrobin	5.4	0.25-100	0.9929	0.25	0.25-100	0.9955	0.25	-	-	-	-	-	-
Difeconazole	6.0	0.25-100	0.9924	0.25	0.25-100	0.9959	0.25	-	-	-	-	-	-
Trifloxystrobin	6.4	0.1-50	0.9990	0.1	0.25-25	1	0.25	0.1-50	0.9975	0.1	-	-	-

LDR: lineal dynamic range; LOQ: limit of quantitation

Table 2.7 Multiresidue pesticide determination in orange juice by SPME-TM-DART-MS/MS. Method accuracy (%) and precision (Relative Standard Deviation, RSD, %) at three different validation levels.

	Accuracy, % (RSD, %; n=6)				
Compound	**0.3 µg/kg**	**7 µg/kg**	**75 µg/kg**	**IS**	**Weight**
Acephate	<LOQ	105 (8)	91 (8)	Atrazine d5	No weight
Pyrimethanil	109 (4)	88 (6)	100 (8)	Atrazine d5	1/X
Omethoate	128 (10)	101 (11)	-	Atrazine d5	1/X
Atrazine	118 (6)	108 (4)	105 (5)	Atrazine d5	1/X
Cyprodinil	110 (3)	100 (3)	96 (4)	Cyprodinyl d6	$1/X^2$
Dimethoate	116 (6)	117 (10)	105 (6)	Atrazine d5	1/X
Metalaxyl	116 (6)	99 (7)	97 (4)	Metalaxyl d6	1/X
Cyproconazole	89 (6)	90 (7)	100 (7)	Metalaxyl d6	1/X
Imazalil	121 (6)	118 (10)	102 (7)	Cyprodinyl d6	1/X
Quinoxyfen	111 (11)	88 (6)	101 (8)	Metalaxyl d6	1/X
Tebuconazole	97 (8)	90 (8)	99 (8)	Metalaxyl d6	1/X
Flusilazole	108 (9)	94 (7)	103 (6)	Metalaxyl d6	1/X
Pyriproxyfen	79 (13)	96 (7)	104 (10)	Atrazine d5	$1/X^2$
Malathion	109 (17)	113 (4)	111 (8)	Atrazine d5	1/X
Pyridaben	78* (17)	77 (9)	100 (6)	Atrazine d5	No weight
Pyraclostrobin	110 (13)	96 (8)	112 (6)	Atrazine d5	1/X
Azoxystrobin	130 (6)	117 (12)	99 (9)	Atrazine d5	1/X
Difeconazole	124 (3)	113 (8)	110 (3)	Atrazine d5	1/X
Trifloxystrobin	68 (22)	110 (9)	-	Metalaxyl d6	No weight

* Linear range 0.25-10 µg/kg

It is important to note that the calibration range brackets the maximum residue levels (MRLs) of 10 ng g^{-1}, imposed by the European Regulation (EC) N˚ 396/2005 of the European Parliament for most food commodities. All calibration curves were processed with each of the IS selected, using no weight, weight 1/X, and weight $1/X^2$. Limits of quantitation were calculated according the directives of the Food and Drug Administration (FDA), with the lowest calibration level providing less than 20% deviation from the nominal concentration in the constructed calibration curve, and with precision not exceeding 20% of the CV. Results obtained for all tested matrices, including linearity (R^2), linear dynamic range, and LOQs, are shown in Table 2.6.

Table 2.8 Multiresidue pesticide determination in Concord grape juice by SPME-TM-DART-MS/MS. Method accuracy (%) and precision (Relative Standard Deviation, RSD, %) at three different validation levels.

Accuracy, % (RSD, %; n=6)					
Compound	**0.3 µg/kg**	**7 µg/kg**	**75 µg/kg**	**IS**	**Weight**
Acephate	-	91 (10)	112 (12)	Atrazine d5	1/X
Pyrimethanil	96 (9)	77 (7)	100 (8)	Atrazine d5	1/X
Omethoate	97 (6)	117 (10)	110 (10)	Atrazine d5	1/X
Atrazine	95 (5)	96 (5)	97 (5)	Atrazine d5	1/X
Cyprodinyl	96 (4)	105 (3)	105 (2)	Cyprodinyl d6	$1/X^2$
Dimethoate	98 (7)	94 (9)	97 (5)	Atrazine d5	1/X
Metalaxyl	102 (6)	111 (6)	98 (6)	Atrazine d5	1/X
Cyproconazole	91 (7)	100 (5)	93 (9)	Atrazine d5	$1/X^2$
Imazalil	91 (8)	78 (10)	111 (10)	Atrazine d5	1/X
Quinoxyfen	97 (9)	122 (7)	98 (8)	Atrazine d5	1/X
Tebuconazole	80 (10)	103 (7)	103 (7)	Atrazine d5	1/X
Flusilazole	96 (10)	106 (9)	83 (8)	Atrazine d5	1/X
Pyriproxyfen	85 (8)	93 (7)	-	Atrazine d5	1/X
Malathion	77 (8)	95 (9)	102 (7)	Atrazine d5	1/X
Pyridaben	113 (10)	126 (12)	-	Atrazine d5	No weight
Pyraclostrobin	118 (15)	89 (12)	-	Atrazine d5	1/X
Azoxystrobin	126* (7)	69 (5)	87 (3)	Cyprodinyl d6	No weight
Difeconazole	108 (6)	125 (11)	89 (8)	Atrazine d5	1/X
Trifloxystrobin	116 (8)	123 (9)	-	Atrazine d5	$1/X^2$

* Linear range 0.25-10 µg/kg

Results pertaining to the precision and accuracy of the method for each of the examined matrices are summarized in Tables 2.7 to 2.10. As can be seen in Table 2.6, most of the target analytes provide outstanding linearity, and LOQs below the imposed MRLs for the majority of the matrices. Figure 2.17 succinctly presents calibration curves obtained for four different pesticides spiked into the four analysed matrices. Unlike grape and orange juice, particular analytes spiked in surface water (*i.e.*, Pyriproxyfen, Pyridaben, Azoxystrobin, and Difenoconazole) and cow milk (i.e., Quinoxyfen, Pyriproxyfen, Pyridaben, Pyraclostrobin, Azoxystrobin, Difeconazole, and Trifloxystrobin) did not provide satisfactory results (*i.e.* no detected at the lowest concentration levels).

Table 2.9 Multiresidue pesticide determination in surface water by SPME-TM-DART-MS/MS. Method accuracy (%) and precision (Relative Standard Deviation, RSD, %) at three different validation levels.

Accuracy, % (RSD, %; n=6)					
Compound	**0.3 µg/kg**	**7 µg/kg**	**75 µg/kg**	**IS**	**Weight**
Acephate	< LOQ	102	100*	Atrazine d5	No weight
Pyrimethanil	111 (4)	111 (6)	92* (7)	Atrazine d5	No weight
Omethoate	124 (4)	114 (13)	106 (10)	Atrazine d5	1/X
Atrazine	110 (3)	95 (4)	98 (3)	Atrazine d5	1/X
Cyprodinyl	106 (3)	100 (2)	99 (2)	Cyprodinyl d6	1/X
Dimethoate	81 (10)	101 (9)	97 (8)	Atrazine d5	1/X
Metalaxyl	104 (4)	94 (3)	97 (4)	Metalaxyl d6	1/X
Cyproconazole	114 (6)	93 (10)	102 (9)	Atrazine d5	1/X
Imazalil	< LOQ	74 (6)	96 (6)	Cyprodinyl d6	1/X
Quinoxyfen	92 (5)	93(2)	-	Atrazine d5	1/X
Tebuconazole	112 (9)	119 (12)	-	Atrazine d5	1/X
Flusilazole	93 (3)	72 (4)	71 (8)	Metalaxyl d6	$1/X^2$
Pyriproxyfen	91 (3)	84 (5)	110 (9)	Atrazine d5	1/X
Malathion	84 (13)	84 (11)	73 (8)	Atrazine d5	1/X
Pyridaben	102 (11)	115 (12)	-	Atrazine d5	1/X
Pyraclostrobin	< LOQ	102	100*	Atrazine d5	No weight
Azoxystrobin	111 (4)	111 (6)	92* (7)	Atrazine d5	No weight
Difeconazole	124 (4)	114 (13)	106 (10)	Atrazine d5	1/X
Trifloxystrobin	110 (3)	95 (4)	98 (3)	Atrazine d5	1/X

* Linear range 25-100 µg/kg

Certainly, this outcome can be explained by the intrinsic physicochemical characteristics of these analytes, and their interaction with the matrices/sample-containers. For instance, the abovementioned analytes possess medium to high hydrophobicities (expressed as LogP), and potentially demonstrate significant binding to the employed containers or the suspended matter present in these sample matrices.

Undoubtedly, milk represents a food matrix with a higher degree of complexity when compared to grape juice and orange juice due to its composition. Milk, is an emulsion of fat globules and casein micelles, which are suspended in an aqueous phase that contains solubilized lactose, whey proteins, and some minerals.

Table 2.10 Multiresidue pesticide determination in cow milk by SPME-TM-DART-MS/MS. Method accuracy (%) and precision (Relative Standard Deviation, RSD, %) at three different validation levels.

Compound	Accuracy % (RSD%, n=6)				
	0.3 µg/kg	7 µg/kg	75 µg/kg	IS	Weight
Acephate	<LOQ	92 (14)	-	Atrazine d5	No weight
Pyrimethanil	82 (8)	86 (3)	90 (8)	Atrazine d5	$1/X^2$
Omethoate	114* (10)	102 (4)	108 (10)	Atrazine d5	No weight
Atrazine	96 (6)	99 (4)	103 (5)	Atrazine d5	No weight
Cyprodinil	114 (10)	110 (6)	120 (6)	Cyprodinyl d6	1/X
Dimethoate	85 (5)	107 (8)	105 (10)	Atrazine d5	1/X
Metalaxyl	103 (3)	96 (5)	103 (4)	Metalaxyl d6	1/X
Cyproconazole	74 (5)	116 (7)	105 (8)	Atrazine d5	1/X
Imazalil	70 (12)	80 (9)	92 (15)	Atrazine d5	1/X
Tebuconazole	72 (8)	96 (12)	85 (8)	Atrazine d5	1/X
Flusilazole	70 (9)	100 (12)	82 (9)	Atrazine d5	1/X
Malathion	<LOQ	114 (7)	94 (9)	Atrazine d5	1/X

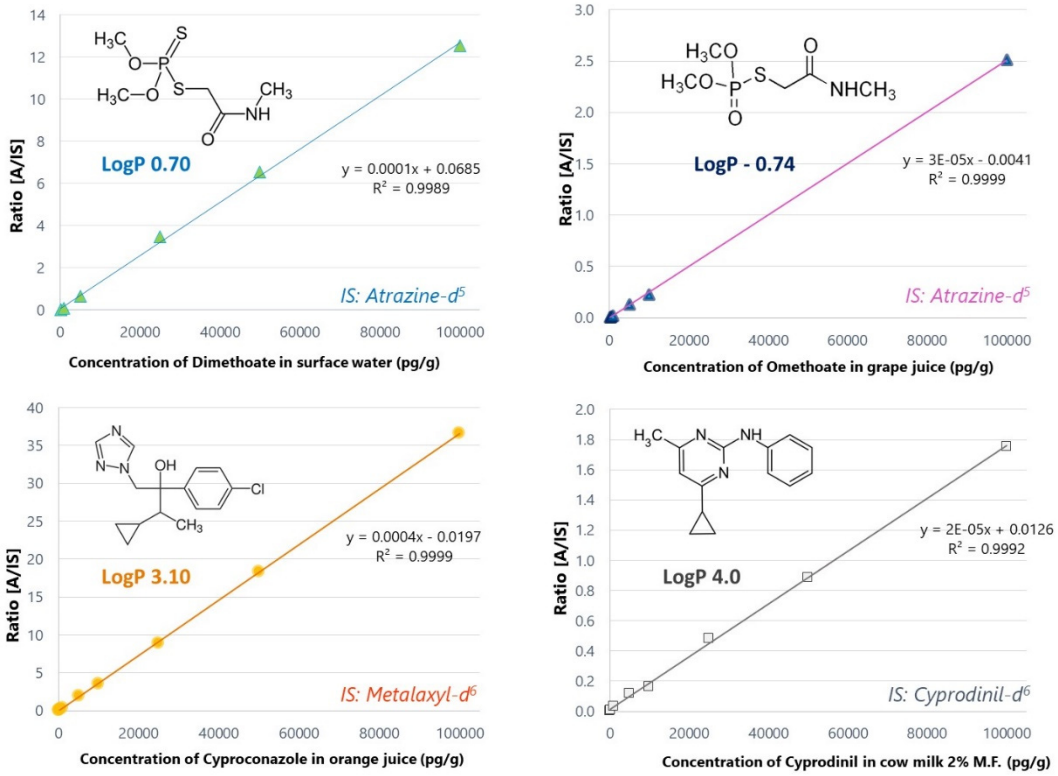


Figure 2.17 Quantitative analysis of surface water, Concord grape juice, orange juice, and cow milk spiked with four pesticides (100 pg ml^{-1} to 100 ng mL^{-1}). Internal standards (Metalaxyl-d6, Cyprodinil-d6, and Atrazine-d5) used for correction were spiked at a fixed concentration in all samples (10 ng mL−1). Only three deuterated standards were available for all target analytes.

Therefore, it is important to bear in mind that the various phases that constitute milk also participate in the partition equilibria established between the matrix and the extraction device. Consequently, considering the hydrophobicity of the matrix and the hydrophobicity of the aforementioned analytes, it is not surprising that these analytes are more likely to partition into the matrix rather than onto the extraction phase. Hence, the extraction of the above listed analytes for both matrices could have been improved by performing further matrix modifications with aims to either promote the release of the analytes from the sample, and preventing its adhesion to the glassware surface by using silanized equipment.[271]. However, this is out of the scope of the presented work, which had as a goal an assessment of system performance without further matrix modifications.

Towards highly reproducible devices, and the minimization of internal standard use

Previously, Gómez-Ríos *et al.*[267] demonstrated that suitable control of the manufacturing of SPME-TM devices plays a critical role in guaranteeing matrix normalization, which fundamentally translates into the increased ability of any SPME device to extract the same amount of analyte of interest, independently of the device being used. As a result, reproducible instrumental response and linear calibration curves can be attained without the need of the deuterated analogue of the targeted analyte. As shown in Figure 2, calibration curves for all studied analytes were built based on the best correction attained by one of the three internal standards spiked on the samples. Thus, in comparison to direct sample to MS technologies, the matrix normalization offered by SPME-TM is quite convenient. As a proof-of-concept, Figure 2.18 presents calibration curves obtained without the use of an internal standard for four diverse analytes (Metalaxyl, Pyrimethanil, Cyprodinyl, and Pyriproxyfen) extracted from Concord grape juice.

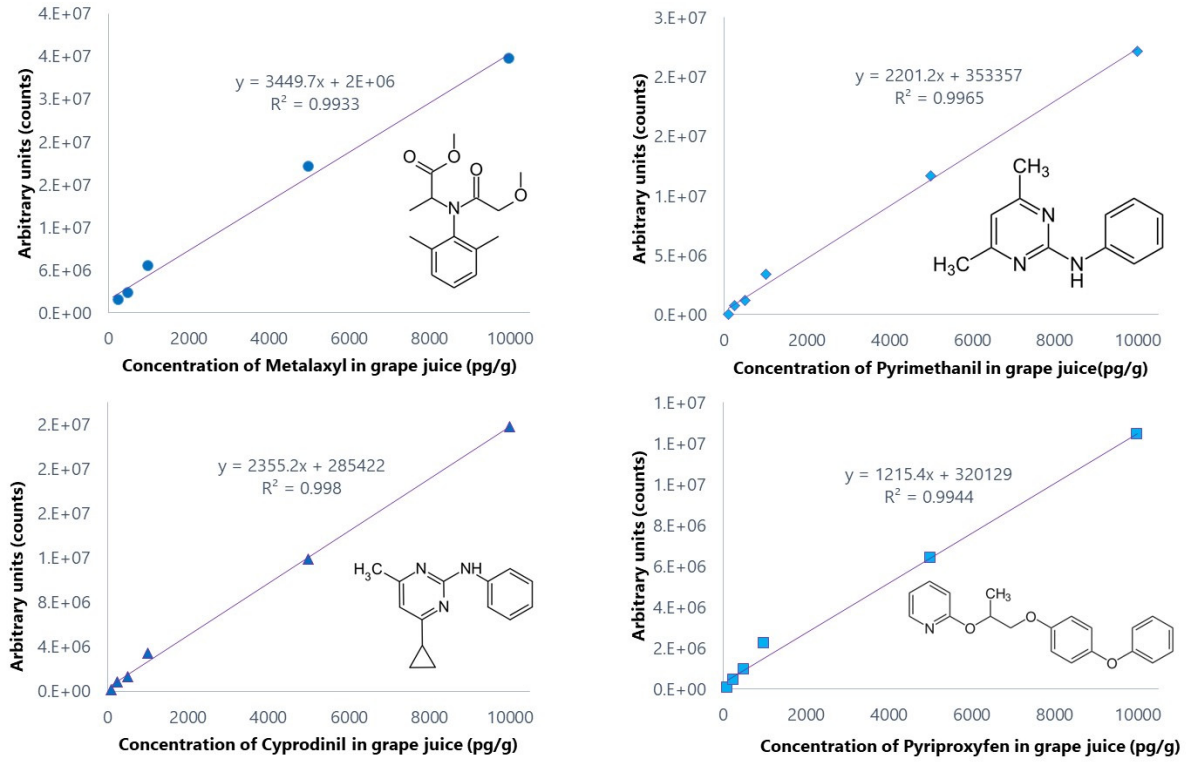


Figure 2.18 Quantitative analysis of Concord grape juice spiked with four pesticides (100 pg ml^{-1} to 10 ng mL−1). No internal standard was used for correction.

Despite the attainment of satisfactory results for the analytes of interest, it is important to highlight that a linear increment on the instrumental response was only observed for 2 orders of magnitude (i.e. 0.1-10 ng mL^{-1}). The lack of linearity obtained without the use of an internal standard above 10 ng mL^{-1} can be attributed to either source or MS detector saturation. Certainly, rather than bad news, it is quite impressive that a 1 minute extraction from a complex matrix is sufficient to reach required detection limits. Practically speaking, if the goal of a given application is to extend the linear dynamic range without the need of an internal standard, one can simply increase the scanning speed at which the mesh passes in front of the DART source, or decrease sampling time, or decrease the dwell time, or both of which can reduce the amount of analyte being introduced into the MS system.

Retrospective analysis by SPME-TM

Interest in retrospective analysis is continuously growing, as it allows agrochemicals to be investigated in samples after initial MS acquisition, and allows the user to screen sample sets at a later date when perhaps more information regarding the system is available.[272] Retrospective analysis, as a post-targeted analysis approach, is quite efficient for the screening of a larger number of compounds, such as those not listed by international regulation agencies at the time of initial analysis (e.g. transformation products of pesticides).[273] Yet, state-of-the-art retrospective analysis is chiefly focused on tedious sample preparation methods coupled to GC/LC-HRMS,[274,275] making this type of examination quite laborious. Therefore, methods that enable both retrospective and quantitative analysis in a "point-and-shoot" fashion are highly desirable in food analysis.[276]

In addition to the speed and sensitivity herein presented, an alluring feature provided by SPME-TM lies in the possibility of performing retrospective examinations of a given sample using the same sampling device. Given the size of the coated area on the SPME-TM device (5 mm wide by 15 mm length), more than one analysis can be performed by thermally-desorbing a different section of the coated mesh, as approximately only 25 mm^2 (area of 5 mm x 5 mm) of the mesh comes into contact with the stream of helium supplied by the DART source per scanning event. Consequently, an SPME-TM device primarily used for targeted determinations can be adequately stored (e.g. kept at -80 ˚C) so as to guarantee analyte stability for further retrospective studies using high-resolution mass spectrometry. Aiming to evaluate the appropriateness of SPME-TM for retrospective analysis, several devices previously used for extraction from grape juice spiked at 25 ng ml^{-1} were stored at -80°C for 4 months. Subsequently, DART analyses were carried out by means of an Orbitrap mass analyser. As can be seen in Figure 2.19, a second desorption of the stored devices revealed the presence of all pesticides spiked in the matrix.

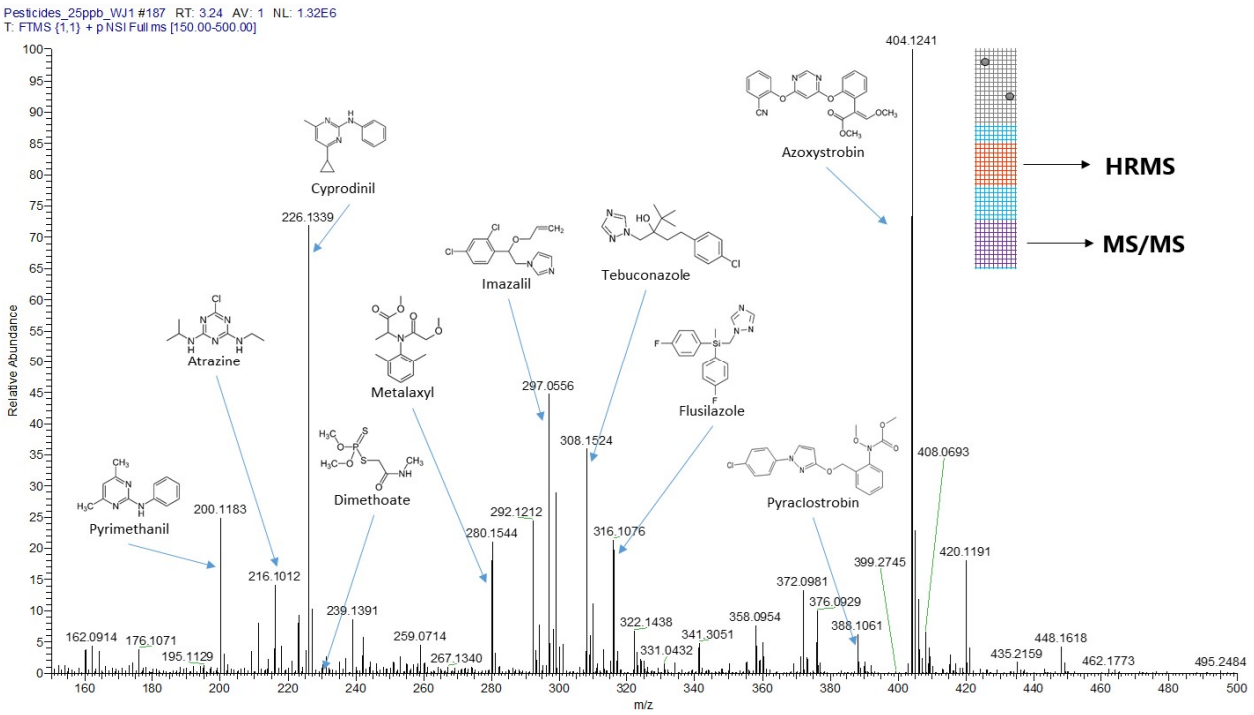


Figure 2.19 Retrospective analysis of an SPME-TM device stored at -80˚C for 4 months after prior targeted inspection of one segment. Mass spectra was attained using an Orbitrap-Exactive in full scan mode [m/z 100-500].

Certainly, these results are encouraging, as they pave the way for the implementation of SPME-TM for rapid on-site screening by means of portable instrumentation,[65,277] with the possibility of subsequent confirmation of results via benchtop instruments.[278]

SPME-TM versus direct-sample-to-MS

Although the direct interface of food samples to MS via DESI[279], LESA[56], LTP[255], REIMS[70], and DART[69] typically offers astonishingly fast results for the determination of pesticides, these technologies are truly useful only when the agrochemicals under study are present at high concentrations, typically above the MRLs set by food legislation[280]. In view of these limitations, sample pre-treatments prior to "direct" coupling to MS have been implemented to match the requirements enforced by regulatory authorities.[174,197,281] However, some of these sample

preparation approaches are inefficient and, in most cases, difficult to integrate with the ambient ionization process. In view of these facts, SPME-TM is undeniably a tool that not only provides the required sensitivity needed to attain the required levels of detection without sacrificing speed of analysis, it additionally minimizes the chance of instrument contamination due to its intrinsic ability to provide 'clean extracts'. Hence, the obtained results in this study can be posed as an early call to international legislating agencies towards the establishment of new guidelines that allow for the routine use of microextraction-MS technologies for the rapid screening of multiresidue pesticides in food commodities. In addition, we foresee SPME-TM as a promising new tool to tackle various analytical problems in other disciplines that require fast and clean analyte introduction.

To date, chromatographic separations, by either GC-MS or LC-MS, are still the platform of choice for the determination of pesticides, as the analysis specificity may suffer from the loss of a separation dimension. For instance, interferences may be observed when the target analyte is co-extracted from the matrix with isomeric species that share the same fragmentation pattern. Certainly, this is an issue inherent of any direct-sample-to-MS approach (not only SPME). Although SPME-TM devices are capable of performing ultra-fast extraction/enrichment from food matrices, it is important to highlight that the coatings developed up to date are not selective enough to discriminate between the compound of interest and a potential isobar or isomer. Hence, as recently demonstrated by our group[178], there is need of front-end separation technologies, such ion mobility spectrometry (IMS)[12] or differential mobility spectrometry (DMS)[178], that allow for analyte-interference separation without sacrificing the total analysis time[282]. Nevertheless, one should bear in mind that the main purpose of direct-to-MS technologies, including SPME-MS, is

not to fully replace chromatographic-based technologies, rather to alleviate the laboratories loads through a fast analysis.

2.3.4 Summary

The results herein presented evidence the great potential of SPME-TM as a tool for fast concomitant screening and quantitation of agrochemicals in complex matrices. Limits of quantitation in the sub-nanogram per millilitre range were attained. Further, total analysis time did not exceed 2 minutes per sample, a remarkable feat considering that SPME-TM probes were individually and sequentially introduced to the analysis platform. Unquestionably, further development of novel substrates geometries that allow for faster analysis by means of high-throughput devices[131] would reduce the total analysis time to less than 1 minute per sample. Although these results are essentially a proof-of-concept, as a next logical step, we foresee in the near future the undertaking of an inter-laboratory validation study of SPME-TM for the rapid determination of pesticides and others contaminants in food and environmental liquid matrices.[283] Compared with other currently used methods, SPME-TM offers significant advantages, including complete elimination of solvents, minimal use of sorbents, method simplicity, and tremendous time and labour savings. Certainly, SPME-TM is a technology not limited to DART and it could be easily interfaced to other ambient ionization approaches such DESI, LTP, Atmospheric Solids Analysis Probe (ASAP)[284] or Direct Surface Analysis (DSA)[58,209,229]. Our work currently focuses on the implementation of this technology to portable instrumentation that would allow for on-site screening of pesticides[65] as well as food profiling for food-fraud investigations.[70,285] Similarly, further research is focused on the evaluation of rapid on-mesh derivatization methods aimed at improving limits of quantitation for poorly-ionisable compounds.[286]

Section 2.4 Towards on-site analysis of complex matrices by solid-phase microextraction-transmission mode coupled to a portable mass spectrometer via direct analysis in real time

2.4.1 Introduction

Recently, remarkable research efforts are directed towards development of instruments and methods that facilitate the on-site analysis of a system[5,9,65]. On-site analysis helps to reduce the analytical errors associated with sample alteration during storage and transportation, as well as to eliminate the time delays and costs associated with these steps[287]. Preferably, the entire analytical process (sampling, sample preparation, and instrumental analysis) should be conducted on-site, as doing so favours repeated measurements in space and time and allows for the real time characterization of the processes occurring in the investigated system[5,288]. The ultimate goal of on-site analysis is to deliver accurate and precise measurements that allow quick decision in critical scenarios (e.g. point-of-care applications[8] or in-field chemical detection[276,289]). Undeniably, the miniaturization of mass spectrometry (MS) instrumentation has facilitated the development of multiple on-site methodologies that can rapidly characterize samples (*e.g.* profiling) and efficiently quantitate target compounds in clinical, forensic, food, and environmental applications[9,65,226,244]. Certainly, ambient ionization technologies, such as direct analysis in real time (DART)[53] and low-temperature plasma ionization (LTP)[52,290], have enabled simpler and quicker ways of interfacing the system under investigation with these portable mass spectrometers [65]. However, the inherent lack of sample preparation in these technologies restricts their use in quantitative trace analysis (low parts-per-billion, ppb)[82,291]. Therefore, in addition to instrument portability, another ideal feature of an on-site method is the ability to efficiently integrate the sampling strategies prior to instrumental quantification.

Solid-phase microextraction (SPME) is a technology that proficiently integrates sampling and sample preparation into a single step, while also allowing the analyte-enriched device to be introduced conveniently into the analytical equipment simply and conveniently via liquid, thermal, or laser desorption[80,228]. As a matter of fact, on-site analysis via SPME and portable instrumentation has been extensively explored for a broad variety of matrices over the past twenty years[292]. A number of different analysers have been tested for this purpose, including flame ionization detector (FID), electron capture detector (ECD), dry electrolytic conductivity detector (DELCD)[292,293], ion mobility[163,294], ion trap-MS[87] and toroidal-MS[146,295,296]. As recently shown[146,286], directly interfacing SPME-based devices (e.g. thin film membranes, needle traps or fibres) with portable gas chromatography-MS (GC-MS) systems offers on-site quantitation limits in aqueous and air samples (*i.e.* low/sub-nanogram per millilitre) that are well below what was thought possible for field instrumentation. Furthermore, recent results reported by Grandy *et al*. offer an accurate and complete on-site solution for determining contaminants in environmental water; however, the total analysis time for this method ranges between 20 and 25 min per sample[146]. Given this, there is still room for improvement in terms of reducing the total analysis time. Hence, the goal of this manuscript is to introduce solid-phase microextraction-transmission (SPME-TM)[192] coupled to a portable mass spectrometer (compact single quadrupole, Waters-QDA[297]), via DART, to enable the rapid profiling of samples and the semi-quantitation of trace analytes in complex matrices. Although this mesoscale MS system has previously been used with ambient ionization approaches in profiling or semi-quantitative applications[277,298,299], this is the first work where SPME-TM is evaluated as an efficient tool for rapid analyte enrichment ($t \leq 2$min) prior to analysis by portable mass spectrometers. Essentially, SPME-TM is a device based on the transmission mode concept originally reported by Chipuk *et al.* in which analytes are efficiently

transmitted into the MS system via DART and desorption electrospray ionization (DESI)[209,229]. Unlike the standard TM device developed by Fernandez's group (*i.e.* bare stainless steel mesh)[209,300], SPME-TM is a mesh with strands that are exclusively coated with an extractive composite phase (e.g. SPE particles, ≤ 10 μm) that can be used to enrich analytes that are present in either a gas or a liquid sample[192,195,205,301]. Our previous work has shown that, when used in combination with benchtop instruments (*e.g.* triple quadrupole and orbitrap), SPME-TM is suitable for quantitating target analytes in food, environmental, and biological matrices[192,195]. This study demonstrate that SPME-TM is a green analytical approach[195,302] that improves the simplicity, sensitivity, and speed of the on-site screening of pesticides in food (grape juice). Furthermore, we assess SPME-TM-DART-QDA's potential as a tool that can provide quasi-real-time molecularly-resolved information. This information would enable food samples to be examined more rapidly, and it could also potentially enable frauds to be identified more efficiently[70,276], while at the same time minimizing the risk of instrument contamination. As a proof-of-concept, our work focused on the analysis of milk samples from diverse animal (cow and goat) and vegetal (almond, coconut, and soy) sources[303].

2.4.2 Experimental section

Reagents and materials

The analytes used in this investigation were purchased from Sigma-Aldrich, and they are listed in Tables 2.11 alongside the relevant information pertaining to their hydrophobicity properties (LogP) and nominal mass (*m/z* ratio). Individual stock solutions were prepared in acetonitrile, methanol, acetone, and dichloromethane at concentrations ranging from 9 to 15 mg/ml. Working solutions at 100, 10, and 1 μg/ml were prepared by mixing the analytes in acetonitrile or methanol. The internal

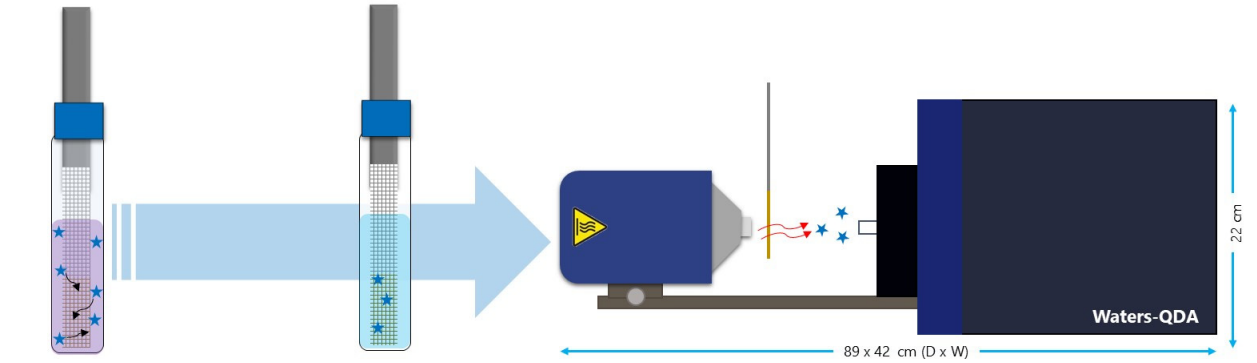
standards used for pesticide analysis—namely, atrazine-d5, metalaxyl-d6, and cyprodinyl-d6—were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Stock solutions of the internal standards were prepared in acetonitrile at a concentration of 1 mg/ml for atrazine-d5 and metalaxyl-d6, and at a concentration of 5mg/ml for cyprodinyl-d6. A working solution composed of all the internal standards was prepared in acetonitrile at 100 µg/ml. Samples of Concord grape juice, almond milk, coconut milk, soy milk, goat milk, and cow milk (2% , Milk Fat, M.F.) were purchased from local grocery stores (Waterloo, ON, Canada). The samples were prepared by spiking them with appropriate amounts of analytes and internal standards, while ensuring that the total amount of organic solvent added never exceeded 1% (v/v) of the total amount of matrix. The spiked samples were equilibrated for at least two hours at 1500 rpm to guarantee that equilibria between the analytes and matrix components were established, thus obtaining samples that closely represent real contamination scenarios. SPME-TM devices were manufactured in-house using stainless steel mesh (74 x 74 mesh per in, wire diameter 0.0037 in). The mesh's strands were precisely coated with a biocompatible polymer (slurry of 5 µm hydrophilic lipophilic balance (HLB) particles and polyacrylonitrile as a binder) at a thickness of less than 20 µm and a length of 1 cm. As previously reported in Section 2.2 [151,304], HLB particles were selected with the objective of providing wider analyte balance coverage.

Mass Spectrometry

A DART-Standardized Voltage and Pressure (DART-SVP) model ion source (IonSense, Saugus, MA USA) was coupled to a single-quadrupole mass spectrometer (ACQUITY QDa; Waters Corporation, Manchester, UK) via a Vapour® interface (IonSense, Inc.).The DART-SVP was fitted with a single-dimensional motorized linear rail that was controlled with the DART-SVP web-based

software so as to reproducibly and consecutively automatically position the SPME-TM devices in front of the DART source (a speed of 0.2 mm s^{-1} was used). To guarantee good reproducibility and higher throughput in the desorption process, a custom-made holder (UW-12) that could allocate up to twelve SPME-TM devices was used. The DART source was operated under the following conditions: positive ion mode; a high-voltage electrode set at -3000 V; a discharge electrode set at +350 V; and a grid voltage of +350 V. The gas heater was set to 350 °C, which yielded the optimum intensities for the majority of the analytes that were analysed.

Figure 2.20 Schematic representation of the analytical workflow for analysis of complex matrices by Solid-Phase Microextraction-Transmission Mode (SPME-TM) coupled to a portable mass spectrometer (Waters-QDA) via Direct Analysis in Real Time (DART).



Sample preparation

As shown in Figure 2.20, the analytical procedure consisted of four simple steps. First, the SPME-TM devices were rinsed (10 s) in water to eliminate any organic solvent residue (from the preconditioning step) that could potentially affect the enrichment capabilities of the devices. Second, the SPME-TM devices were exposed to the sample matrix (1.5 ml for grape juice or milk), and extraction was carried out for 1 min using vortex agitation (*i.e.* 3200 rpm). In the third step, the SPME-TM devices were quickly rinsed (10s) in water to remove any loosely attached matrix

constituents that could either be irreversibly carbonized on the coating surface during the desorption process, induce ion enhancement/suppression, or cause instrumental contamination. Lastly, SPME-TM devices were positioned in the UW-12 holder and thermally desorbed. The total analysis time for 1 sample was less than 2 min.

2.4.3 Results and discussion

Semi-quantitative analysis of pesticides in grape juice

Recently, we demonstrated that SPME-TM via DART is a suitable technology for rapidly quantitating pesticides in diverse liquid food matrices[195]. However, in spite of the speed and sensitivity attained, our method is not suitable to provide an analytical answer on-site, as the experiments were performed using high-end benchtop mass spectrometers such a triple quadrupole-MS or Orbitrap-MS. In this study, we explored whether a compact single-quadrupole mass spectrometer (Waters QDA) was a suitable tool for the semi-quantitative analysis of seven pesticides from different classes and with different molecular weights and polarities; namely: pyrimethanil, atrazine, cyprodinil, metalaxyl, imazalil, pyraclostrobin and azoxystrobin. Figure 2.21 depicts the typical mass spectrum attained after 1 min of extraction from 1.5 mL of grape juice that had been spiked with 100 ng mL^{-1} of each standard. The red colour bars identify the target analytes (7), while the blue colour bars identify the internal standards (3). It is important to note that isotopically-labelled internal standards for pesticides are either expensive or non-existent for each targeted pesticide. Hence, the calibration curves for all of the analytes were constructed using the 3 available internal standards on the basis of the signal ratio and the internal standard (A/Is) for 9 concentration levels in three independent replicates from 5 $ng \cdot mL^{-1}$ to 500 $ng \cdot mL^{-1}$.

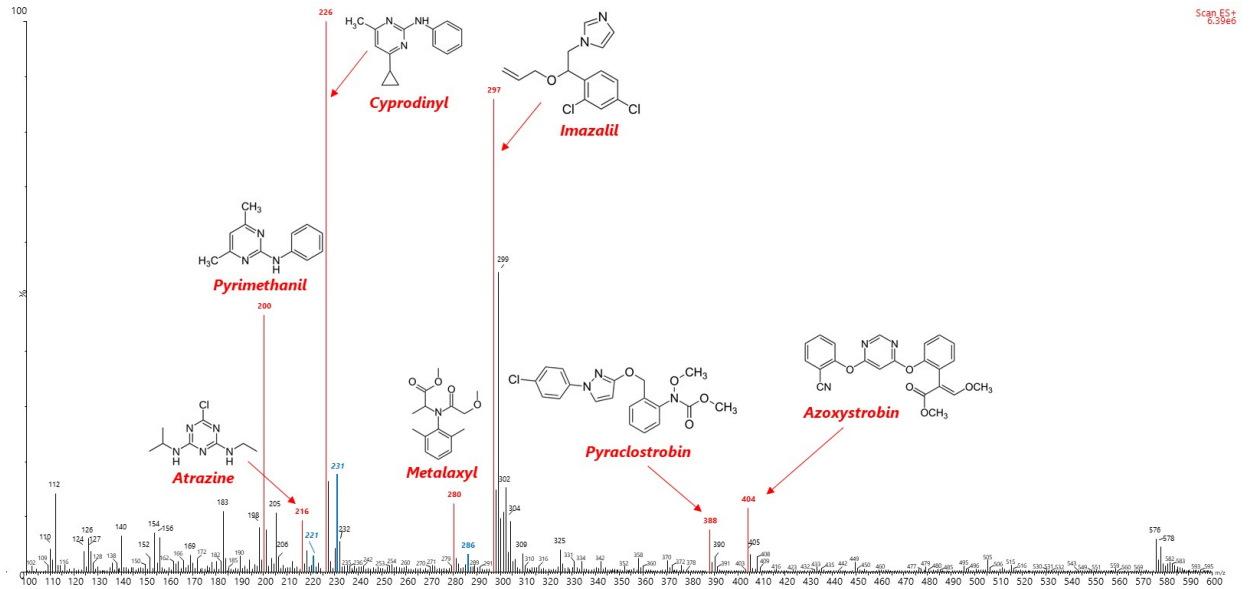


Figure 2.21 Mass spectrum profile obtained on a Waters-QDA after 1 min extraction from Concord grape juice spiked with pesticides at 500 ng mL^{-1}. Red bars represent the analytes of interest, while blue bars represent the internal standards listed in Table 2.11.

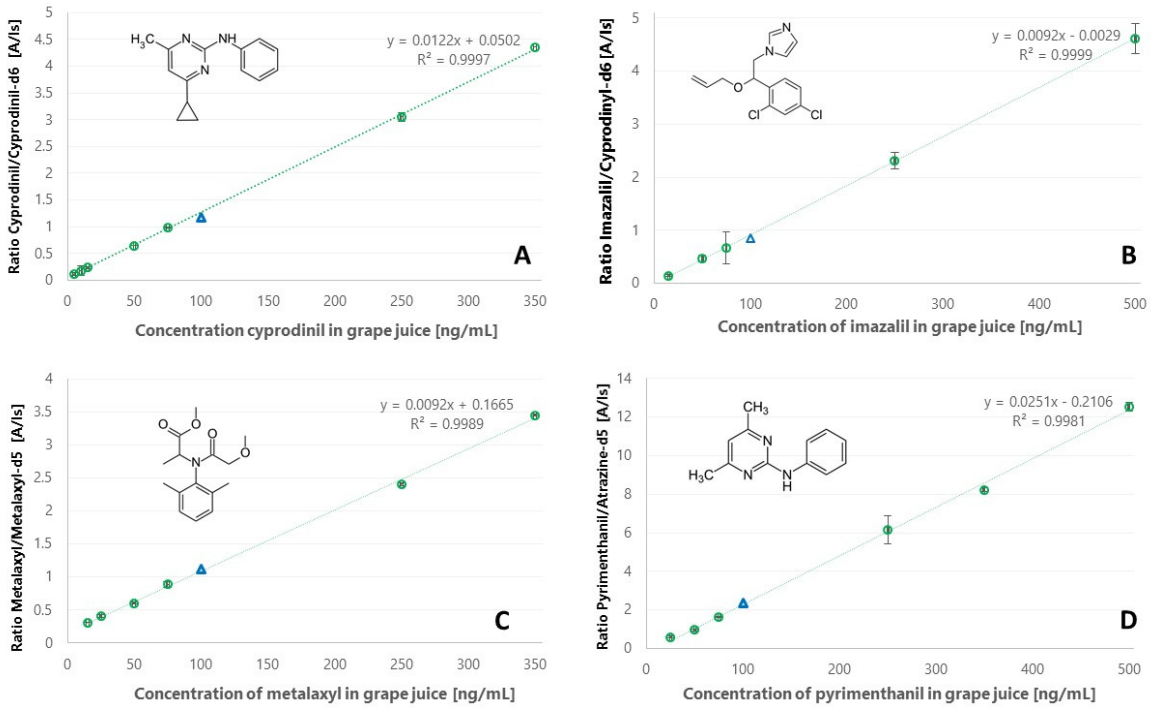


Figure 2.22 Semi-quantitative analysis of Concord grape juice spiked with the following pesticides: (**A**) cyprodinil (15 ng ml^{-1} to 350 ng mL^{-1}); (**B**) imazalil (15 ng ml^{-1} to 500 ng mL^{-1}); (**C**) metalaxyl (15 ng ml^{-1} to 350 ng mL^{-1}); and (**D**) pyrimenthanil (25 ng ml^{-1} to 500 ng mL^{-1}). Metalaxyl-d^6, cyprodinil-d^6, and atrazine-d^5 were used as internal standards and were spiked at a fixed concentration in all samples (100 ng mL^{-1}).

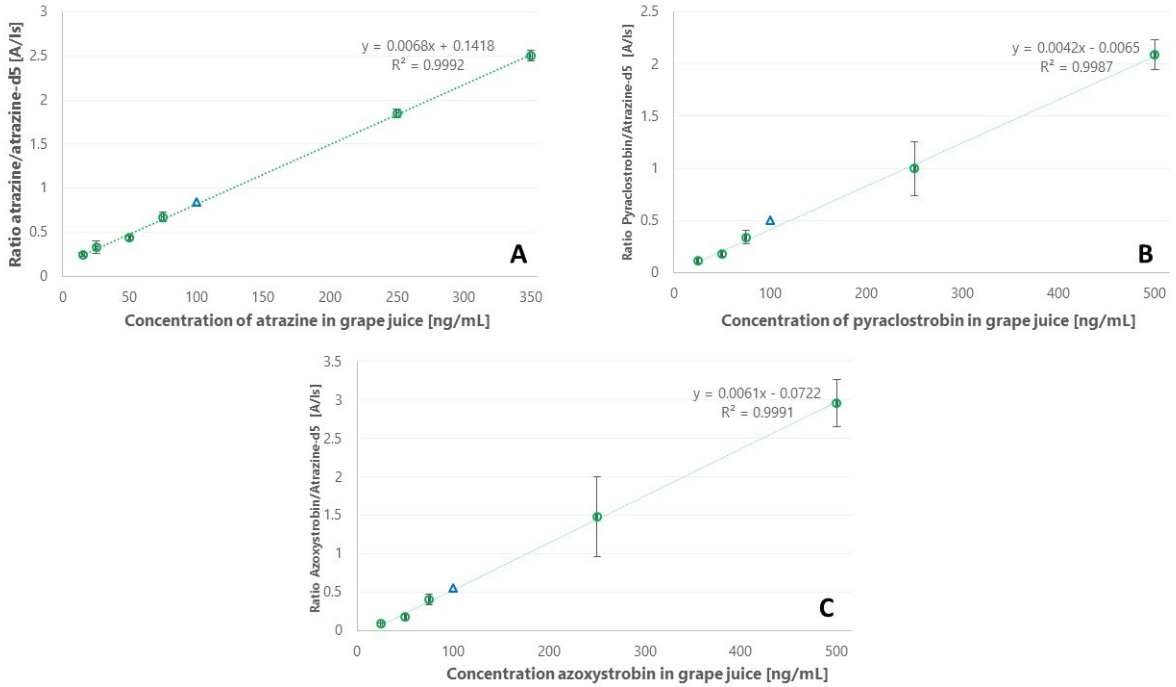


Figure 2.23 Semi-Quantitative analysis of Concord grape juice spiked with the following pesticides: (**A**) atrazine (15 ng ml^{-1} to 350 ng mL^{-1}); (**B**) pyraclostrobin (25 ng ml^{-1} to 500 ng mL^{-1}); and (**C**) azoxystrobin (25 ng ml^{-1} to 500 ng mL^{-1}). Atrazine-d^5 was used as the internal standard, and it was spiked at a fixed concentration in all samples (100 ng mL^{-1}).

Table 2.11 Figures of merit for the semi-quantitation of several pesticides in Concord grape juice by using Solid-Phase Microextraction-Transmission Mode (SPME-TM) coupled to a portable mass spectrometer (Waters-QDA) via Direct Analysis in Real Time (DART).

Compound	LogP*	Internal standard	MRLs** [ng/mL]	LOD [ng/mL]	LOQ [ng/mL]	Validation point [100 ng/mL]	
						Accuracy (%)	Precision (RSD, %)
Pyrimenthanil	2.8	Atrazine-d5	10	10	25	102	1.2
Atrazine	2.7	Atrazine-d5	10	5	15	106	2.3
Cyprodinil	4.0	Cyprodinil-d6	10	5	15	95	0.6
Metalaxyl	1.7	Metalaxyl-d5	10	5	15	103	1.2
Imazalil	2.6	Cyprodinil-d6	10	5	15	93	5.0
Pyraclostrobin	4.3	Atrazine-d5	10	10	25	122	15
Azoxystrobin	2.5	Atrazine-d5	10	10	25	103	28

* Data source: http://www.chemspider.com/

** REGULATION (EC) NO 396/2005 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council

As can be seen in Figures 2.22 and 2.23 good linearity ($R^2 \geq 0.998$) was attained for all the compounds studied in the range of 25 to 350 ng mL^{-1} after internal standard correction. Likewise,

adequate accuracy (93-122%) and precision (0.6-15 %) were attained for the majority of the compounds at the validation point of 100 ng mL^{-1} (see Table 2.11). Although the LOQ reached for all the pesticides was above 15 ng mL^{-1}, the limits of detection (LOD; 5-10 ng mL^{-1}) were less than or equal to the maximum residue levels (MRLs) allowed for most food commodities under the regulations set forth by the European Parliament (European Regulation (EC) N° 396/2005) (*i.e.* 10 ng mL^{-1}). Given that we were previously able to attain LOQ below 0.25 ng mL^{-1} for all the selected compounds while using the same enrichment time[195], the higher LOQ achieved in this study can be attributed to the lack of selectivity offered by a single MS event. However, taking into account the speed of analysis and the manoeuvrability offered by the DART-QDA system, the LOQ obtained in the present study can be considered satisfactory (*i.e.* 15-25 ng/mL). Thus, aiming to enhance LOQ offered by our prospective on-site protocol, the analyst could either increase the extraction time (*e.g.* 5 min), or the sample volume (*e.g.* 10 mL of sample), or both, as to assure that a larger amount of analyte is collected on the coating.

Nonetheless, it is important to keep in mind that lowering the LOQ may also result in lowering the upper limit of quantitation. For instance, as shown in Figure 2.24, our results showed that a continuous increase in the amount of target analyte extracted on the coating (*i.e.* increase on analyte signal) led to a continuous decrease in the internal standard signal, thus limiting the upper linearity of atrazine's calibration curve. These results suggest a potential saturation of the detector or ionization source. Hence, in developing a method for on-site analysis using the proposed system, one should be aware that there may be limitations in interpreting the data that will be generated. Furthermore, this observation suggests that the internal standard should be spiked on the sample in approximately the middle/upper part of the targeted concentration range to account for a decrease in signal.

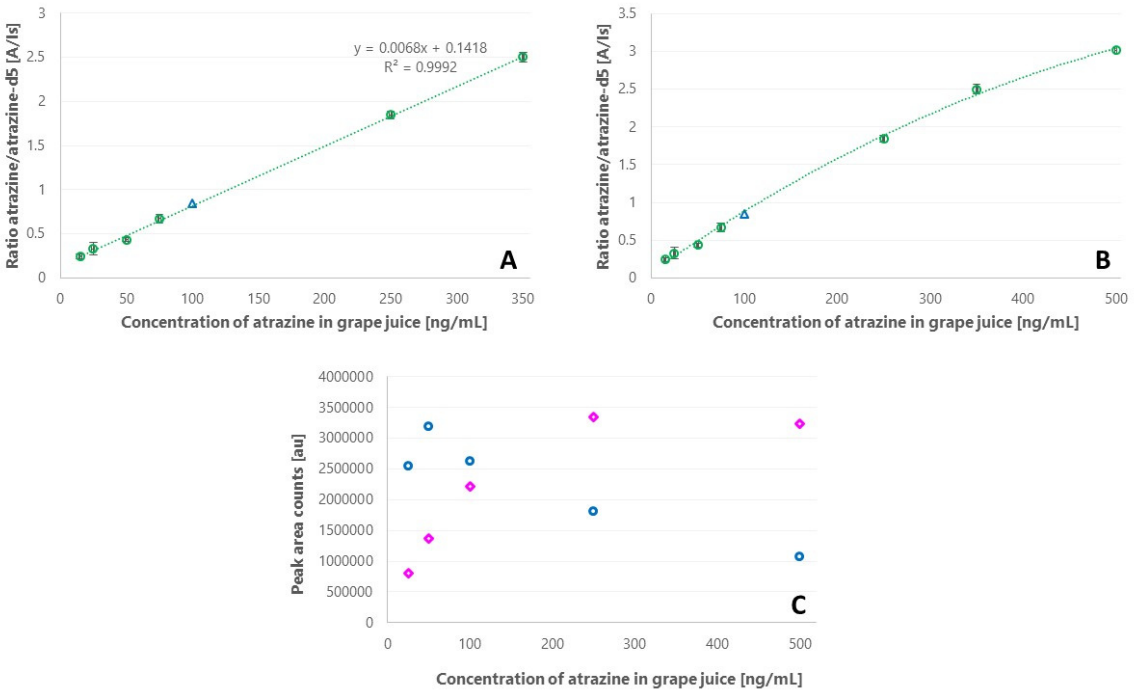


Figure 2.24 Semi-quantitative analysis of Concord grape juice spiked with atrazine: (**A**) atrazine (15 ng ml^{-1} to 350 ng mL^{-1}); (**B**) atrazine (15 ng ml^{-1} to 500 ng mL^{-1}); and (**C**) raw data of atrazine (pink diamonds) and atrazine-d^5 (blue circles). Atrazine-d^5 was used as the internal standard, and it was spiked at a fixed concentration in all samples (100 ng mL^{-1}).

Milk profiling

As recently demonstrated by Hajslova's and Fernandez's groups[77,300,305], one cannot place the matrix under study directly onto the transmission mode mesh for thermal desorption, as doing so could result in thermal degradation of the sample, mass spectrometer contamination, and dramatic ion suppression. Consequently, profiling studies that use TM-DART-MS typically require a sample pre-treatment approach such liquid-liquid extraction, lyophilisation, or protein precipitation. Nevertheless, the abovementioned steps take at least 15-20 min per sample. Given that SPME-TM efficiently integrates both the sample preparation device and the interface with the ambient mass spectrometry source, we propose SPME-TM-DART-QDA as a feasible approach for rapid, on-site, and high-throughput profiling.

Table 2.12 Nutritional facts of tested milks for untargeted molecular profiling.

Samples	Fat* (g)	Carbohydrate* (g)	Sodium* (mg)	Protein (g)
Coconut milk #1	4.5	1	35	0
Coconut milk #2	4.7	1	26	0
Coconut milk #3	5.2	1	156	0
Cow milk #1	5	13	110	8
Cow milk #2	5	12	100	5
Cow milk #3	5	12	100	9
Cow milk #4	5	12	110	9
Goat milk #1	5	11	100	7
Almond milk #1	2.5	<1	160	1
Almond milk #2	2.5	13	180	1
Almond milk #3	3	1	110	1
Soy milk #1	4	8	90	6
Soy milk #2	4	4	75	7
Soy milk #3	4	8	115	1

Since milk fraud is one of the most serious issues currently facing the dairy industry, we elected to evaluate the system's ability to discriminate between milk samples originating from different species and farming systems as proof-of-concept[303]. Essentially, we performed 1 min extraction from 1.5 mL of sample prior to DART analysis using the same instrumental conditions as in the previous two trials. Different kinds of milk samples (soy, almond, coconut, goat and cow milk) from diverse manufacturers were selected for this trial (see Table 2.12 for further details). After DART-QDA acquisition (see mass spectra profiles for each kind of matrix on Figure 2.25), the raw data was processed using XCMS. The peaks were extracted using the 'matchFilter' method and then grouped via the 'density' method[306–308]. Univariate data analysis was completed using MetaboAnalyst 3.0 and PCA plots were attained after the data had been normalized using the median, log transformation, and pareto scaling. As can be seen in Figure 2.26, our results showed that 1 min of extraction from each milk sample was sufficient to reach clustering according to the respective matrix category. Additionally, Figure 2.27 presents the random forest plot and its confusion matrix.

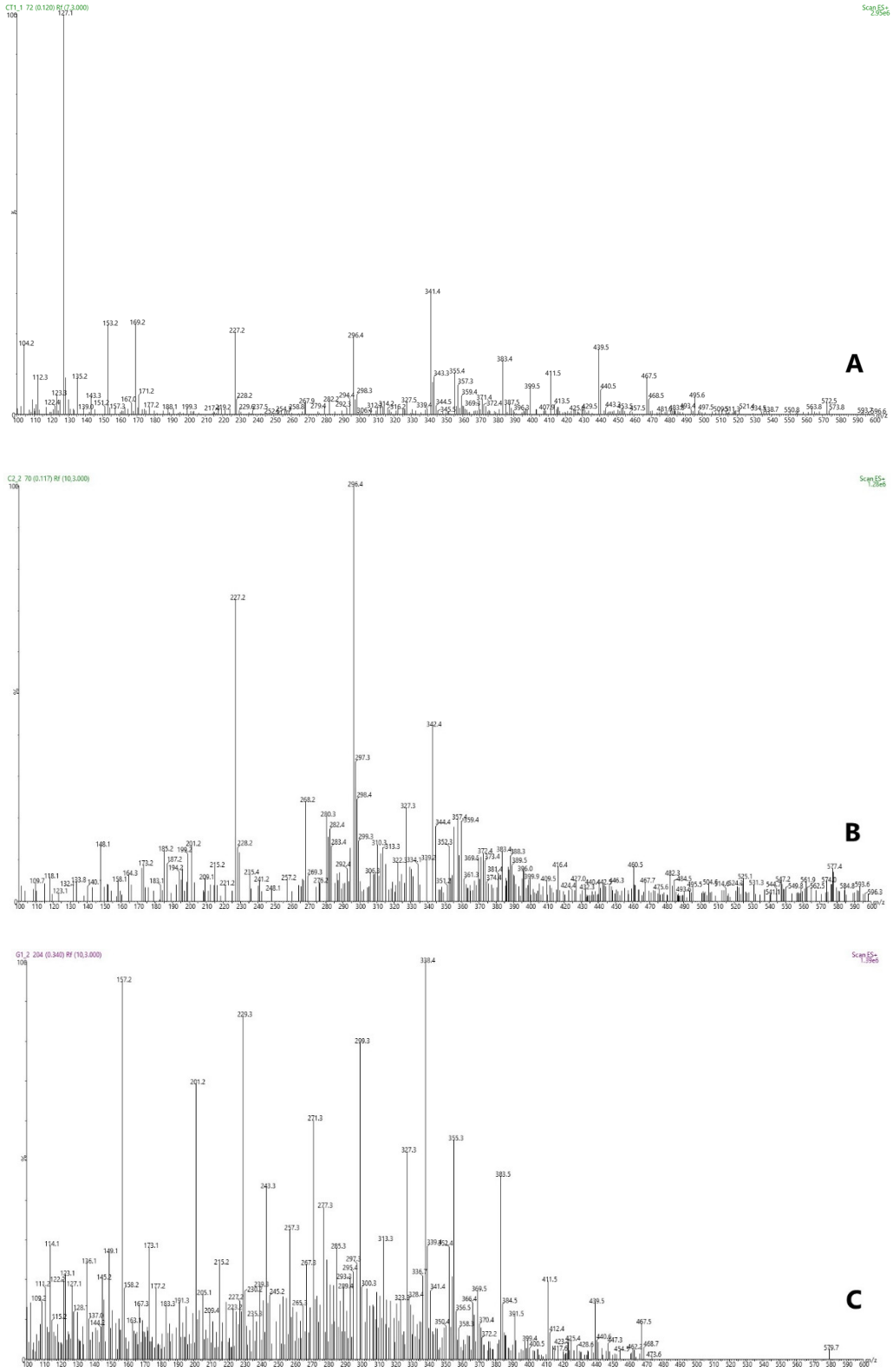


Figure 2.25 Mass spectra profile obtained after 1 min extraction from 1.5mL of (**A**) coconut, (**B**) goat and (**C**) cow milk.

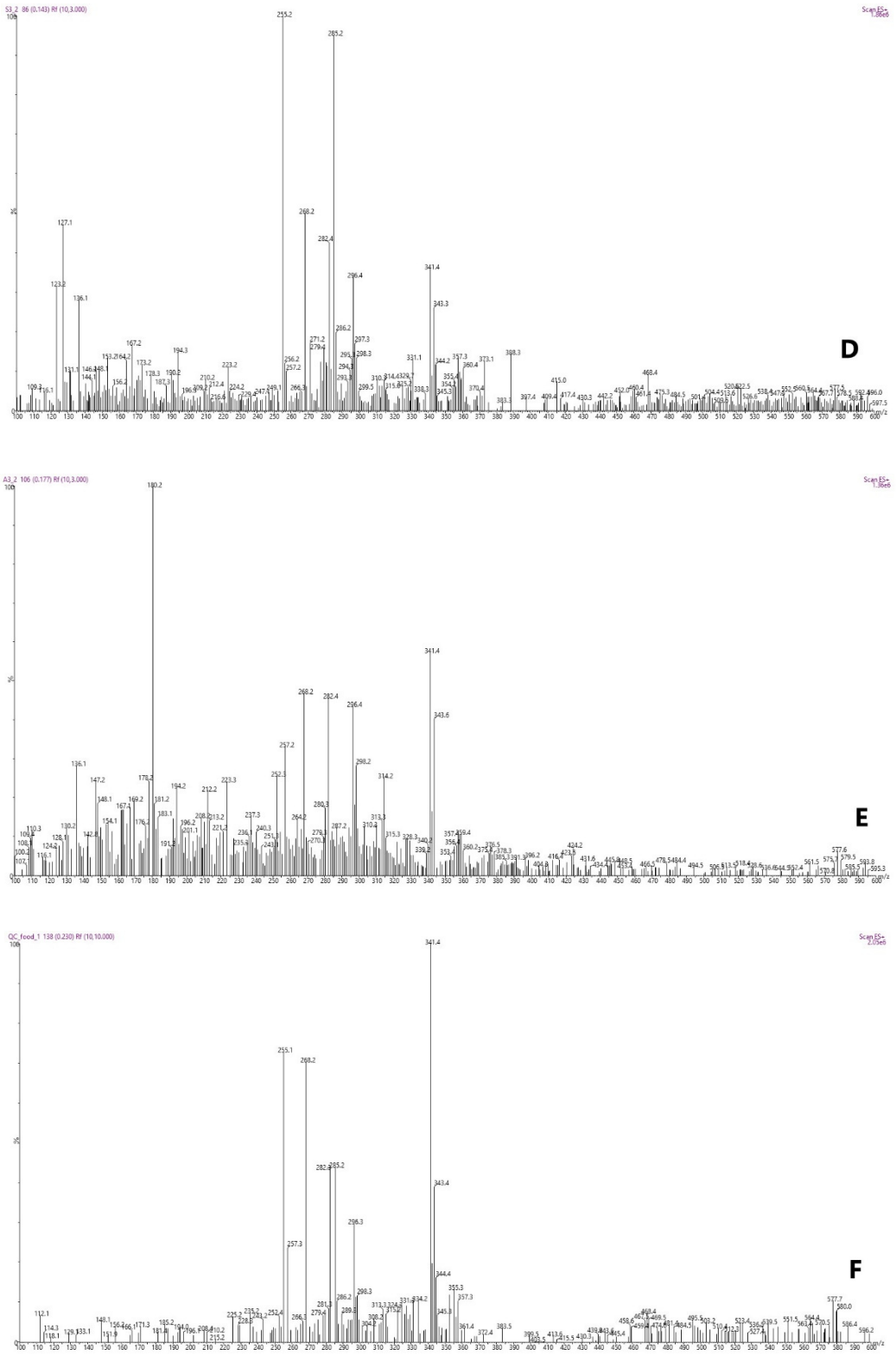


Figure 2.25 (continuation) Mass spectra profile obtained after 1 min extraction from 1.5mL of (**D**) soy, (**E**) almond and (**F**) QC milk (QC-milk is the mixture of all samples).

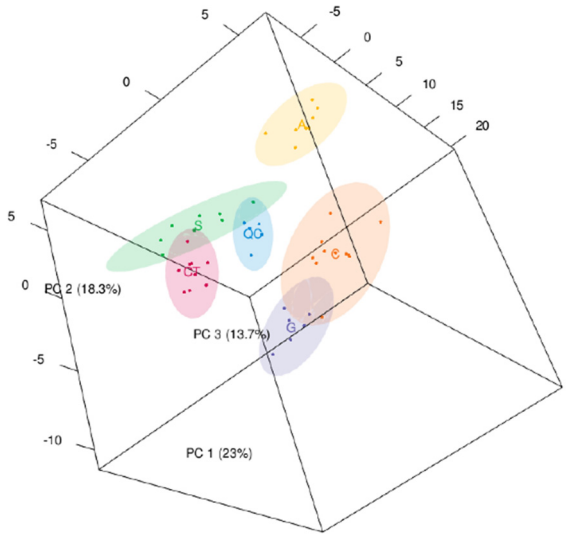
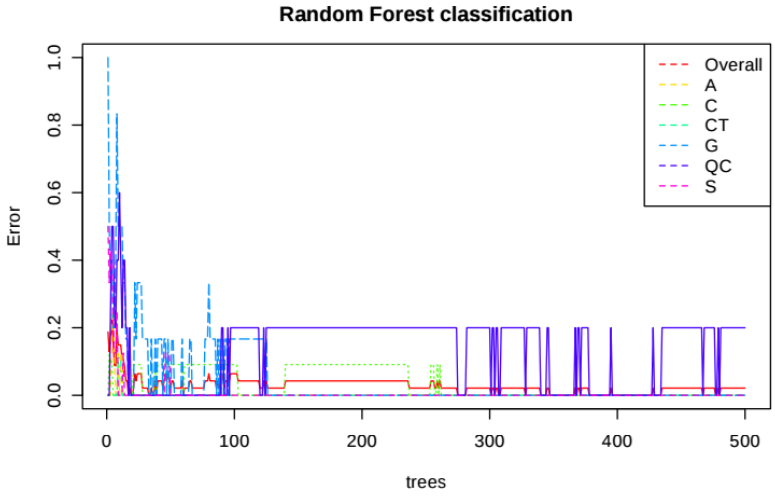


Figure 2.26 Three-dimensional PCA plot for identification of milk samples from different species and farming systems. Letters on the figure denote the milk type investigated: (A) almond milk, (C) cow milk, (CT) coconut milk, (G) goat milk, (QC) quality control-sample-mix, and (S) soy milk. Samples were acquired using SPME-TM coupled to a portable-single quadrupole (QDA) via DART.



Class	A	C	CT	G	QC	S	Class Error
A	8	0	0	0	0	0	0
C	0	11	0	0	0	0	0
CT	0	0	9	0	0	0	0
G	0	0	0	6	0	0	0
QC	0	0	0	0	4	1	0.2
S	0	0	0	0	0	8	0

Figure 2.27 Cumulative error rates by Random Forest classification. The overall error rate is shown as the black line; the red and green lines represent the error rates for each class. Table on figure presents the confusion matrix of random forest. The out-of-bag (OOB) error is 0.0213. Figures and table were generated using MetaboAnalyst 3.0.

Although these results prove this technology's feasibility for rapid sample identification (out-of-bag (OOB) error ≤ 0.0213), further work with larger data sets is required for it to be thoroughly validated.

2.4.4 Summary

Our experimental results demonstrate the suitability of SPME-TM for on-site semi-quantitative analyses of target analytes in complex matrices via DART coupled to portable mass spectrometry. In spite of lacking of a separation step (either chromatographic or ion-mobility) or MS/MS-fragmentation features (only in-source collision-induced dissociation feature is available) on this MS system, semi-quantitative results in the ranges required by the regulatory agencies were obtained when analysing pesticides in grape juice (LOD ~ 5 ng/mL and LOQ ~ 15 ng/mL). It is important to highlight that further improvements in LOQ can be attained by modifying the experimental conditions outlined in this section (*e.g.* using larger sample volumes or longer extraction times). Additionally, we demonstrated the suitability of SPME-TM-DART-QDA to perform profiling studies of complex matrices such as milk from different species and farming systems. Our preliminary results showed that, when combined with real-time recognition software, the proposed profiling methodology has great potential for field applications in which fast sample screening and identification with minimal sample preparation is required[68,79]. Undeniably, further improvements in MS miniaturization (*i.e.* smaller and more sensitive devices)[65], as well as in sample preparation devices that are more efficient and selective[113], will facilitate the transfer of these technologies from the hands of experts and into those of non-conventional users, such as medical doctors, farmers and police officers.

Chapter 3 Development of Novel SPME-MS interfaces suitable for in-vivo and on-site analysis

3.1 Preamble

Chapter 3 consists of 4 sections that correspond to an equal number of manuscripts published in Analytical Chemistry (2), Angewandte Chemie, and Analytica Chimica Acta. Most of the data, tables, and text presented within this chapter have already been incorporated in the aforementioned manuscripts, the details of which are listed below. For instance, section 3.2 describes the first coupling of Biocompatible SPME (Bio-SPME) fibers to MS via nanoelectrospray ionization (nanoESI) for the analysis of xenobiotics in diverse biofluids such urine, plasma, and blood. Section 3.3 introduces novel mini-SPME devices (SPME tips) used towards the analysis of sample volumes under 10 μL. In view of the current limitations of SPME-nanoESI, section 3.4 introduces SPME coupled to MS via open port probe (OPP) as a robust and easy-to-use interface. The technology was examined for the simultaneous quantitation of buprenorphine and fentanyl in urine samples. Aiming to improve the selectivity of this method, which is lost due to the absence of a chromatographic separation step, this section presents an assessment of Multiple Reaction Monitoring with Multistage Fragmentation (MRM3) for the quantitation of clenbuterol in urine samples. Finally, section 3.5 embraces the use of differential mobility spectrometry (DMS) as another on-line technology to enhance analyte selectivity and sensitivity in SPME-MS experiments. This technology was evaluated for the quantitation of codeine and hydrocodone, two isomers with shared fragmentation patterns, from plasma samples.

Section 3.2 include the following manuscript(s):

1. Gómez-Ríos, G.A., Reyes-Garcés, N., Bojko, B., Pawliszyn, J., *Biocompatible Solid-Phase Microextraction Nanoelectrospray Ionization: An Unexploited Tool in Bioanalysis*, Anal. Chem., 2016, 88 (2), pp 1259–1265, DOI: 10.1021/acs.analchem.5b03668 (open access) ACS AuthorChoice - This is an open access article published under an ACS AuthorChoice License.

I participated at all stages of the manuscript preparation process: the planning of experiments, manufacturing of the ionization source, method development, sample preparation and analyte extraction procedures, data processing, manuscript writing and submission, as well as in manuscript replies to reviewers. The contributions of co-author Nathaly Reyes-Garcés include the planning and execution of selected experiments. Dr. Barbara Bojko was involved in the original planning of experiments.

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Section 3.3 include the following manuscript(s):

2. Piri-Moghadam, H.*, Ahmadi, F.*, Gómez-Ríos, G.A.*, Boyacı, E., Reyes-Garcés, N., Aghakhani, A., Bojko, B., Pawliszyn, J. *Fast Quantitation of Target Analytes in Small Volumes of Complex Samples by Matrix-Compatible Solid-Phase Microextraction Devices*, Angew. Chem. 2016, 128, 7636-7640, DOI: 10.1002/ange.201601476. *Equal contribution

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I participated at all stages of the manuscript preparation process: the planning of experiments for nanoESI-MS/MS, manufacturing of the ionization source, manufacturing of CBS devices, method development for nanoESI-MS/MS and HRMS, sample preparation and analyte extraction procedures, data processing, manuscript writing and submission, as well as in manuscript replies to reviewers. Dr. Hamed Piri-Moghadam manufactured the polypyrrole coated SPME tips, planned experiments related to the evaluation and characterization of the polypyrrole tips, and contributed to the manuscript writing process. The contributions of co-author Fardin Ahmadi include the planning and performance of selected LC-experiments with polypyrrole tips (information not included in this thesis). Dr. Ezel Boyacı and Nathaly Reyes-Garcés participated in the manufacturing of CBS devices used in the manuscript, and in the execution of selected experiments for direct coupling. Dr. Ali Aghakhani planned and performed selected GC-experiments with carbon microfibers (information not included in this thesis). Dr. Barbara Bojko was involved in the original planning of experiments.

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I, Hamed Piri-Moghadam, authorize German Augusto Gómez-Ríos to use this material for his thesis.

I, Ali Aghakhani, authorize German Augusto Gómez-Ríos to use this material for his thesis.

Section 3.4 include the following manuscript(s):

3. Gómez-Ríos, G.A.*, Liu, C.*, Tascon, M., Reyes-Garcés, N., Arnold, D.W., Covey, T., Pawliszyn, J., Anal. Chem., 2017, 89 (7), pp 3805–3809, *Open Port Probe Sampling Interface for the Direct Coupling of Biocompatible Solid-Phase Microextraction to Atmospheric Pressure Ionization Mass Spectrometry*, DOI: 10.1021/acs.analchem.6b04737. *Equal contribution

I participated at all stages of the manuscript preparation process: the planning of experiments, method development for OPP, sample preparation and analyte extraction procedures, data processing, manuscript writing and submission, as well as in manuscript replies to reviewers. Dr. Chang Liu participated in the planning of experiments, preparation and installation of the OPP, method development for DMS and MRM^3 analysis at SCIEX facility, data processing, and manuscript writing. Nathaly Reyes-Garcés and Dr. Marcos Tascon performed selected experiments for OPP. Dr. Don Arnold and Tom Covey were involved in the original planning of experiments.

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I, Tom Covey, authorize German Augusto Gómez-Ríos to use this material for his thesis.

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I, Marcos Tascon, authorize German Augusto Gómez-Ríos to use this material for his thesis.

Section 3.5 include the following manuscript(s):

4. Liu, C.*, Gómez-Ríos, G.A.*, Schneider, B., Le Blanc, Y., Reyes-Garcés, N., Arnold, D.W., Covey, T., Pawliszyn, J., *Fast quantitation of opioid isomers in human plasma by differential mobility spectrometry/mass spectrometry via SPME/open-port probe sampling interface*, Anal. Chim. Acta (article in press), DOI: (10.1016/j.aca.2017.08.023). *Equal contribution

I participated at all stages of the manuscript preparation process: the planning of experiments, method development for OPP, sample preparation, analyte extraction, data processing, manuscript writing and submission, as well as in manuscript replies to reviewers. Dr. Chang Liu participated in the planning of experiments, preparation and installation of the OPP, method development for DMS and DMS research grade analysis at SCIEX facility, data processing, and manuscript writing. Dr. Bradley Schneider and Dr. Yves Le Blanc designed and manufactured the research grade DMS used in these experiments. Dr. Don Arnold and Tom Covey were involved in the original planning of experiments. Nathaly Reyes-Garcés performed selected experiments.

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I, Tom Covey, authorize German Augusto Gómez-Ríos to use this material for his thesis.

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I, Bradley Schneider, authorize German Augusto Gómez-Ríos to use this material for his thesis.

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Section 3.2 Biocompatible Solid-Phase Microextraction Nanoelectrospray Ionization: An Unexploited Tool in Bioanalysis

3.2.1 Introduction

The capability of scrutinizing samples without sample pre-treatment, or with minimum sample preparation, is the key feature of ambient mass spectrometry (AMS) that has promoted its use around the globe[46,58], comprising a variety of applications from monitoring chemical transformations[309] up to discerning between healthy and cancerous tissues at the surgery room[61]. However, no technique is perfect, and predictable limitations of pure AMS when analysing complex matrices such as blood and urine (*e.g.* ionization suppression, poor sensitivity at trace levels, and narrow linear dynamic range), prompted the development of methods that efficiently integrate sample clean-up, analyte extraction/enrichment, and ionization[82,102,136,218,267,310]. This modern era of mass spectrometry (MS), where sample preparation devices are directly coupled to MS instrumentation, is certainly a growing branch where micro- and nano-extraction approaches excel. Some of the most relevant methods recently developed in this field include the use of micro-solid phase extraction (SPE)[102,110,311], slug-flow microextraction (SFME)[82], single drop microextraction (SDME)[105], liquid phase microextraction (LPME)[106,108], membrane extraction[107,218], and polymer monolith microextraction (PMME)[310]. Solid Phase Microextraction (SPME), a world-wide recognized green sample preparation technique for GC[118] and LC[2,123,131,148,271,312,313] applications, certainly was not left behind[314]. As a matter of fact, the direct coupling of SPME fibres to MS instrumentation is not new, and it has been explored for almost two decades[81,180,210] (*i.e.* earlier than most popular AMS methods were reported[52,53]). Currently, diverse geometrical configurations of SPME have been coupled to mass spectrometry for a broad

range of applications including food, environmental and bioanalytical[81,136,267]. Surprisingly, to the best of our knowledge, few have exploited the genuine potential of the most known configuration of SPME: the fibre[182]. In this study, we present the direct coupling of biocompatible-SPME (Bio-SPME) fibres[152,182,242,313,315] to mass spectrometry via nano-ESI emitters as an useful tool for screening and quantitative analysis of small molecules present in samples of bioanalytical relevance. Major advantage of matrix compatible SPME devices that no matrix components, which can potentially block the nanospray opening, are introduced to the emitter.

3.2.2 Experimental Section

Mass Spectrometry

Experiments were performed with the use of a triple quadrupole mass spectrometer TSQ Vantage (Thermo Scientific, San Jose, USA). Nano-electrospray emitters: Econotip (Econo10, 1.0/0.58 OD/ID, mm), coated Glasstip (1.0/0.58, OD/ID, mm; 1 and 2 μm tip), were obtained from New Objective Inc. (Woburn, MA, USA). An in-house ionization source was built at the machine and electronic shop of the University of Waterloo (see Figure 3.1) to accurately position nano-ESI emitters in front of the mass spectrometer.

Reagents and materials

The following compounds were selected as model analytes to evaluate BioSPME-nanoESI: cocaine, diazepam, salbutamol, codeine, oxycodone, methadone, amitriptyline and imatinib. Deuterated analogues of each analyte were used for correction of intra- and inter-experiment variability.

Table 3.1 Target analytes, manufacturer, and SRM transitions monitored for each model compound in positive ionization mode.

Compound	Manufacturer	Matrix	Log P	Protein binding [%]	Parent [m/z]	Fragment [m/z]	Collision Energy	S-Lens
Diazepam	Cerilliant [1]	PBS	2.91	98	285.050	193.113	32	102
Diazepam-d_5	Cerilliant	PBS	-	-	290.075	198.179	33	113
Cocaine	Cerilliant	PBS	3.08	5	304.122	182.139	19	90
Cocaine-d_3	Cerilliant	PBS	-	-	307.140	185.190	20	91
Methadone	Cerilliant	Urine	4.20	90	310.189	265.281	14	82
Methadone-d_3	Cerilliant	Urine	-	-	313.199	268.304	15	77
Codeine	Cerilliant	Urine	1.20	-	300.136	152.146	63	124
Codeine-d_3	Cerilliant	Urine	-	-	303.139	152.135	64	118
Salbutamol	Cerilliant	Urine	0.01	-	240.146	148.179	17	70
Salbutamol-d_3	Cerilliant	Urine	-	-	243.144	151.170	19	67
Oxycodone	Cerilliant	Urine	1.67	45	316.121	241.215	29	100
Oxycodone-d_3	Cerilliant	Urine	-	-	319.140	244.246	28	100
Amitriptyline	Sigma-Aldrich [2]	Blood	4.92	≥ 90	278.148	233.461	16	86
Amitriptyline-d_6	TRC [3]	Blood	-		284.140	233.473	19	82
Imatinib	Sigma-Aldrich	Blood	2.48	95	494.180	394.790	26	123
Imatinib-d_3	TRC	Blood	-	-	497.204	394.785	28	128

1. Cerilliant (Round Rock, TX, USA), 2. Sigma-Aldrich (Sigma-Aldrich (Oakville, ON, Canada), 3.TRC, Toronto Research Chemicals (Toronto, ON, Canada); Log P, logarithm of its partition coefficient between n-octanol and water. Dwell time was 100 ms for all the analytes with a total spraying time of 45 seconds per replicate at 1.3 kV and 3 mm from the MS ion-transfer capillary. All the experiments were performed using a Thermo TSQ Vantage (Thermo Scientific, San Jose, USA). Protein binding information was obtained from www.drugbank.ca

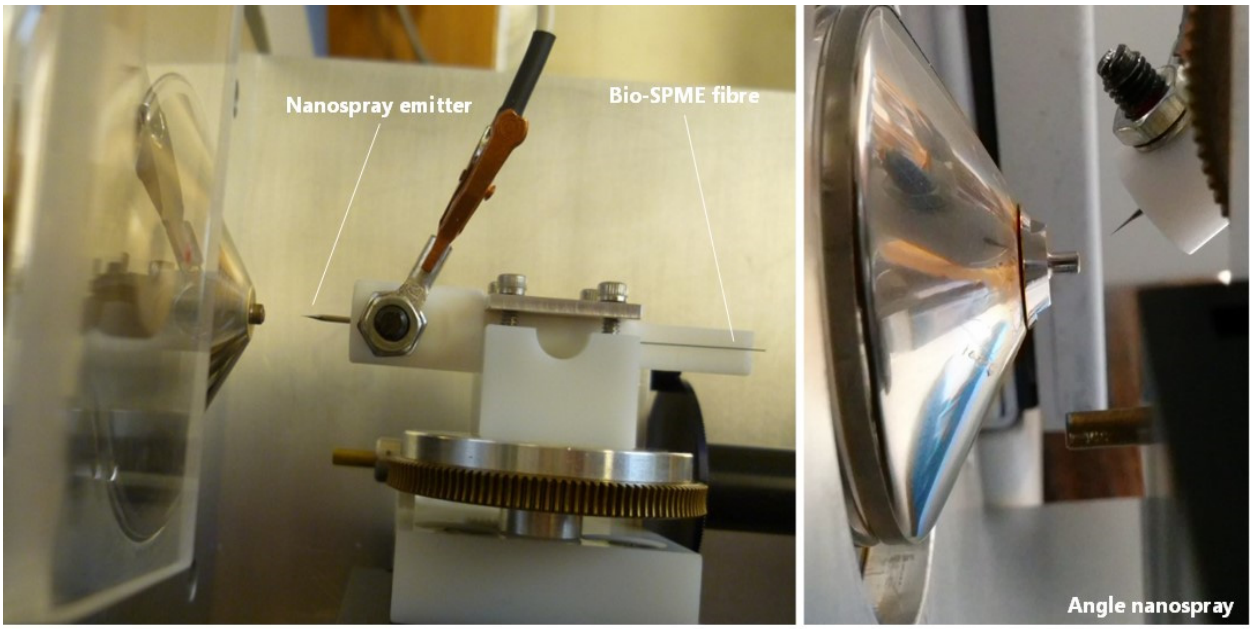

Figure 3.1 In-house ionization source for Bio-SPME-nano-spray. The 3D-moving stage (Newport Corporation, Irvine, CA) not only adjust the position with a precision of 0.02 mm in each dimension (25 mm moving path), but also tunes the spraying tip at different angles on the Z dimension (± 0.01º per moving mark). In order to ensure optimum ion transmission, the nano-spray emitter was position at 3 mm from the ion-transfer capillary.

Further details about compounds suppliers, properties, and SRM transitions are provided on Table 3.1. LC-MS grade solvents (acetonitrile, methanol, and water) used in all the experiments were purchased from Fischer Scientific. Biocompatible SPME mixed mode probes (*i.e.* C_{18}-SCX particles, 45 µm thickness, 15 mm coating length) were kindly provided by Supelco (Bellefonte, PA, USA). The phosphate-buffered saline solution (PBS) (pH 7.4) was prepared according to the procedure listed in Section 2.2.2. Pooled whole blood from healthy donors in potassium (K2) ethylenediaminetetraacetic acid (EDTA) was purchased from Bioreclamation IVT (Baltimore, MA, U.S.A.). Urine samples were collected from two healthy volunteers (one female and one male). Collection of urine from healthy volunteers for this particular study was under the approval of the Office of Research Ethical Board of University of Waterloo).

3.2.3 Results and Discussion

Rediscovering SPME-MS: Bio-SPME-nano-ESI, a new era

The direct interface of SPME fibres with MS analyzers has been investigated since the late 1990s[180,316]. For this purpose, different strategies have been followed by several groups around the world. Some of the most relevant approaches involve placing the fibre at the electron impact region of the MS[87], thermally desorbing the fibres prior to Inductively Coupled Plasma-MS (ICP-MS) analysis[165], desorbing the fibres in a solvent with high affinity for the analytes of interest preceding atmospheric pressure ionization (API), either by ESI[181]or APCI[172], ablating the analytes from the fibre surface with a laser either at vacuum[317] or at atmospheric pressure[175–177], and interfacing the fibres with AMS instrumentation, for instance DESI[179,203] or DART[206,207]. Although initial attempts to couple SPME fibres to MS using small desorption volumes were performed more than 10 years ago ($V_{des} \leq 10$ µL, via nano-ESI[210] or substrate spray[316]), recent endeavors have been focused on either developing new SPME-fibre based substrates[136,211,216] or desorbing SPME fibres on a large desorption/ionization chamber ($V_{des} \geq 100$ µL)[182]. For example, in a recent work reported by Ahmad *et al.*[182] an innovative approach to couple SPME to MS was proposed; however, given that the volume of the desorption chamber was greater than the optimum, the enrichment factor provided by Bio-SPME was not fully exploited. With the aim of taking advantage of the pre-concentration offered by SPME, this work continues the approach initially proposed by Walles *et al.*[210] In essence, it is intended to demonstrate that by using Bio-SPME fibres together with small desorption volumes[117,211] (*i.e.* $V_{des} \leq 4$ µL, on nano-ESI emitters[82,111,318,319]) remarkably low detection limits and satisfactory figure of merit can be attained with exceedingly short sample preparation times[136,267]. We used a home-made desorption/ionization that comprised a holder, in which a ball-end clamping screw enabled

straightforward connection of high voltage (HV) to the nano-ESI emitter, and ready and fast replacement of emitters between experiments (Figure 3.1). We used commercial emiters in order to make it easier for others to reproduce our method; we do, however, expect to improve performance using custom-made parts instead in the near future. Custom-made coatings[113,242] and emitters[111,319] enabling to tune the protocols for given applications even further are also expected to be used in near future.

The analytical workflow consisted of four main steps: extraction/pre-concentration, rinsing, desorption, and ionization (Figure 3.2). First, a preconditioned Bio-SPME fibre was inserted in a vial containing the sample matrix (*e.g.* V_{ext} ~ 10–1500 µL), and quick extraction/enrichment of the analytes was performed by agitating the sample at high speed (agitation at 3200 rpm, t≤ 2 min). Then, the fibre was rinsed in a vial containing LC/MS grade water (t ≤ 15 s) in order to remove matrix components that could potentially adhere to the coating surface. Subsequently, the fibre was introduced into an emitter prefilled with desorption solution. Lastly, and after allowing some time for the analytes to be desorbed in the selected solvent, a high electrical field between the emitter and the mass spectrometer was applied, and analytes were ionized via electrospray mechanisms[52,82,318].

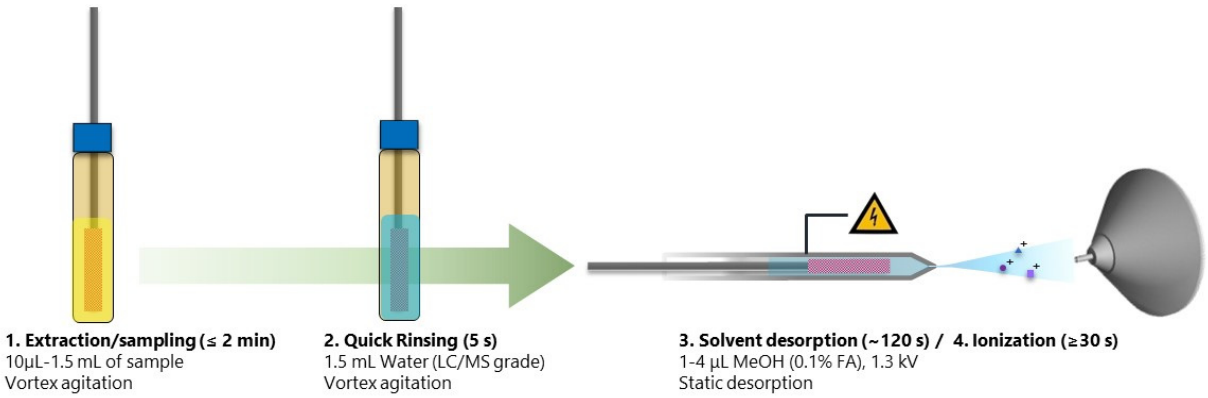

Figure 3.2 Experimental set up for Bio-SPME extraction from complex matrices and desorption–ionization using nano-ESI-MS/MS.

When optimizing SPME-nano-ESI desorption/ionization conditions, several factors should be taken into consideration. Some of the most important parameters include a) the chemistry of the target molecule, and its affinity for the coating and for the desorption solution; b) analyte desorption kinetics into a particular solvent; c) the geometrical characteristics of the emitter and its ionization efficiency[136,318,319]; and d) position relative to the mass spectrometer entrance. In regards to the SPME extraction phase, mixed mode was selected for this study due to its better performance when extracting polar analytes compared to C18. Emitter selection was performed according to its internal volume (*i.e.* small as possible to enhance the enrichment factor[115], but large enough that fiber could freely move in and out the emitter without being damaged), spray-current stability, intra- and inter-experiment reproducibility, and its cost. Among the studied emitters described in the experimental section, Econo10 provided the best compromise in terms of inter-analysis reproducibility/stability (Table 3.2) and price (*i.e.* ~ 6 US dollars per analysis).

Table 3.2 Inter-emitter reproducibility of commercial emitter commercialized by New Objective suitable for Bio-SPME-nano-ESI experiments. RSD, Relative Standard Deviation (n=3).

Compound	RSD [%] n=3		
	Econo10	BG75-2	BG75-4
Cocaine	6.1	5.8	6.1
Cocaine-d3	6.0	5.5	6.0
Ratio	0.2	0.4	0.2
Diazepam	3.3	12.7	3.3
Diazepam-d5	2.0	11.3	2.0
Ratio	2.0	1.5	2.0

All the experiments herein reported were performed using the aforementioned glass coated emitters (1.0/0.58 mm, OD/ID) filled with few microliters of acidified methanol (*i.e.* 0.1% formic acid) unless otherwise stated. Desorption volumes were set according to the length of the coating, always ensuring that the whole coating was completely immersed into the solvent (*e.g.* 4 µL for 15 mm fibres and 1 µL for 4 mm fibres). Considering the small tip size of these emitters (1 ± 0.5

μm), and aiming to prevent clogging and unstable spray-current, desorption solvent was filtered and degassed with helium prior to analysis.

Here, it is important to highlight that a slightly dry Bio-SPME fiber, for example, due to an excessive delay time preceding its introduction into the emitter, could be *per se* a significant source of bubbles that can distort the Taylor-cone formation and electrospray ionization[320]. Based on our experience, if the delay-time between the fibre rinsing step and its insertion into the emitter is longer than 30 seconds, bubble generation inside the emitter (Figure 3.3) can be anticipated as a result of microscopic dry spots/pores on the coating. This could be related to the low intrinsic wettability of the C_{18}-SCX particles used to manufacture mixed-mode Bio-SPME fibres[123]. Consequently, for all the experiments described in this manuscript, stable electrospray was guaranteed by using short fibre transition times from the rinsing vial to the nano-ESI emitter (t_{trans} ≤ 15 s). Indeed, when the affinity of the coating for the target analyte is high (*i.e.* large fibre constant, K_{fs}[2,115,118]), as well as the analyte hydrophobicity[113,131,152], fibres can be transported to the emitter inside the rinsing vessel with minimal/negligible analyte losses, thus preventing bubble

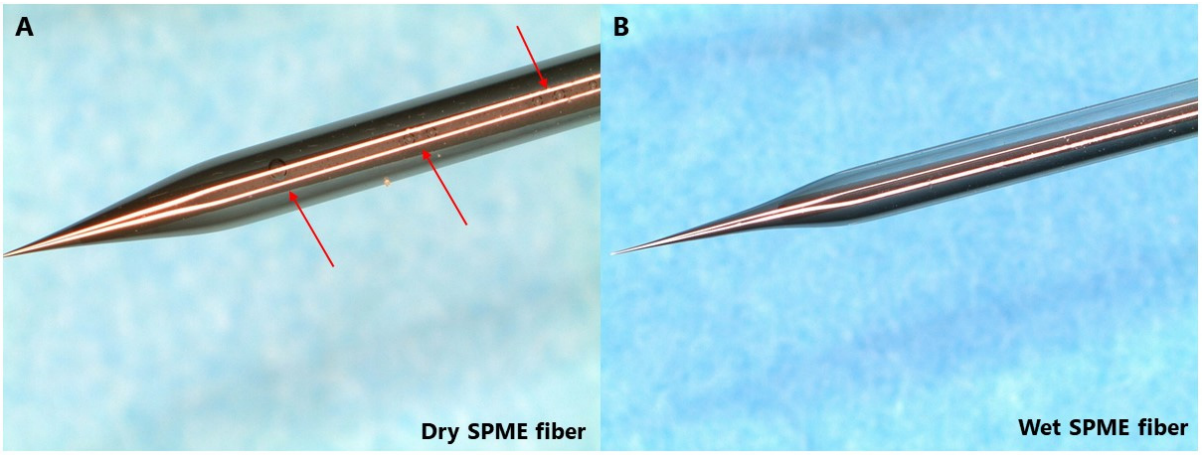

Figure 3.3 15 mm dry SPME fibre (**A**) versus wet SPME fibre (**B**) inserted into a nano-ESI emitter filled with 4 μL of methanol. Bubbles are indicated using red arrows.

formation due to dry surface. An alternate solution to this problem would be the use of coatings with better water-wettability[123], such as HLB (m-divinylbenzene and n-vinylpyrrolidone copolymer), and/or substrates with lower thermal conductivity, so the evaporation rate of the water is slower and the coating particles remain wet for longer times[321].

Given the slow flow rates inherent in nano-ESI (*i.e.* 20-80 nL min^{-1}; depending on solvent composition, voltage applied, and emitter tip architecture[322,323]), 4 µL of desorption solvent is enough to perform at least four instrumental replicates per fibre from a single emitter (Figure 3.4). Desorption time ($t_{des} \leq 5$ min) was selected in such a way that the monitored ion signal was reproducible between consecutive replicates (*i.e.* RSD ≤ 15 %, n =4, calculated using the area under the curve for each repetition as shown in Figure 3.4 and Table 3.3). In essence, a steady signal among experimental replicates means that the partitioning equilibrium between the fibre coating and the desorption solvent was reached and, consequently, it could be assumed that the amount of analyte in the desorption solvent is not statistically changing over the time. However, this does not necessarily mean that total desorption of the analyte extracted has been achieved. The amount of analyte desorbed from the fibre is certainly dependent on the strength and, in this particular approach, on the volume of the desorption solvent [117]. Undeniably, fibre desorption is the bottleneck step in the entire analytical process herein proposed. Given that the desorption time is selected based on the desorption kinetics of the analyte[117] and this depends, in addition to the affinity of the analyte for the coating, on the coating thickness and the agitation/thermal conditions in the desorption vessel, our current efforts are focused on the development of thinner fibre coatings (*e.g.* thickness ≤ 10 µm; mono or dual-layer coatings[115,267]), a heated desorption chamber (*i.e.* to decrease K_{fs}[117] and increase the diffusion coefficient of the analytes[324]), and a fibre vibration system, such the desorption step keeps pace with the entire analytical workflow.

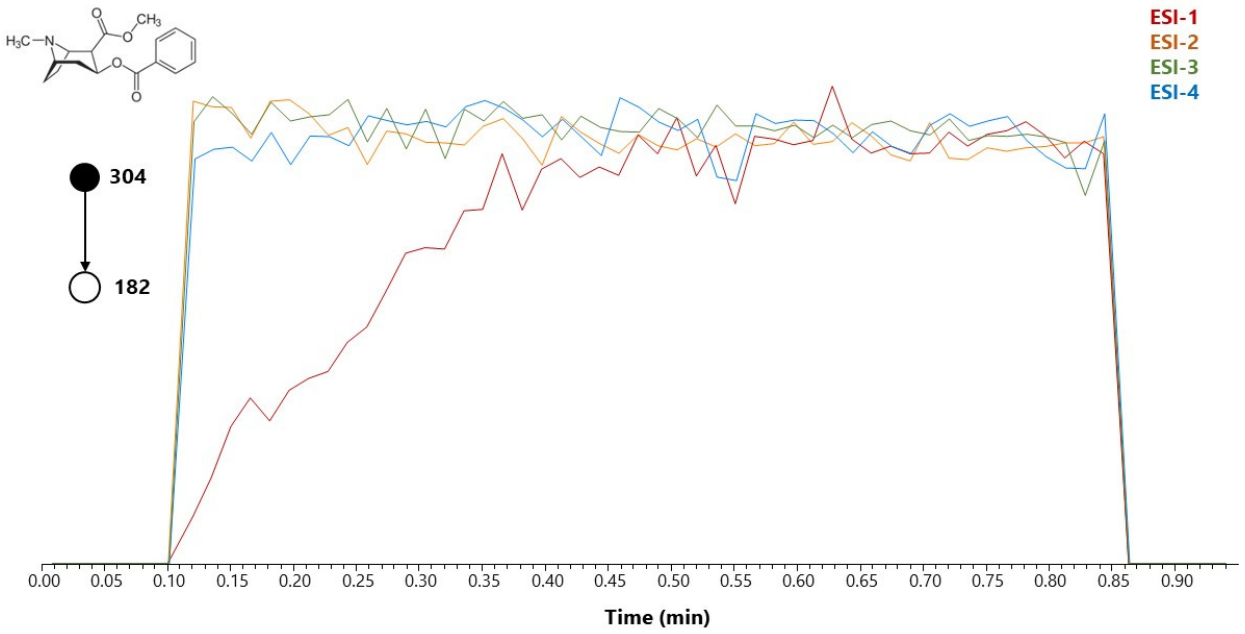

Figure 3.4 Cocaine ion-chronograms obtained using the same nano-ESI emitter (n=4). Signals correspond to 1 min extraction from 1.5 mL of PBS spiked with 75 ng mL^{-1} of the analyte. Extractions were performed using a 15 mm BioSPME mix mode fibre. The desorption volume was 4 µL and the desorption time was 5 minutes. Spraying voltage was 1.3 kV with an acquisition time of 0.9 min.

Table 3.3 Experimental replicate using a single nano-ESI emitter. (n=4). Signals correspond to 1 min extraction from 1.5 mL of PBS spiked with 75 ng mL^{-1} of the analyte. Extractions were performed using a 15 mm Bio-SPME mix mode fibre. The desorption volume was 4 µL and the desorption time was 5 minutes. Spraying voltage was 1.3 kV with an acquisition time of 0.9 min.

Compound	Replicate [area counts, au]			Average	SD	RSD [%]
	2	3	4			
Diazepam	25026413	27691475	29623102	27446997	2308076	8.4
Diazepam-d5	4120991	4486829	4756566	4454795	318996	7.2
Ratio	6.1	6.2	6.2	6.2	0.08	1.3
Cocaine	177866820	175990773	173211262	175689618	2342344	1.3
Cocaine-d3	31055950	29983291	29548704	30195982	775806	2.6
Ratio	5.7	5.9	5.9	5.8	0.08	1.4

Bio-SPME-nano-ESI-MS/MS: an unexploited tool

Not long ago, sample-preparation techniques directly coupled to MS were were avoided, because they were considered laborious[58]. In addition, for some microextraction techniques such SPME, the quantitation capabilities and throughput of the analysis was questioned due to the low analyte

recoveries and the long extraction times needed to achieve practical detection limits[136]. In contrast to what is normally believed, recent developments have demonstrated that different geometries of SPME not only allow quantitative analysis from complex matrices at trace levels (*i.e.* pg mL^{-1} levels), but also in short periods of time ($t_{ext} \leq 1$ min)[136,267]. Up to date, the quantitation potential of Bio-SPME fibres has not been exploited to its maximum[113,182,242], considering that during the desorption (inherent of SPME–LC methods), analytes are significantly diluted and non-efficiently ionized[117]. Herein, as a proof of concept, we demonstrated that Bio-SPME-nano-ESI can reach limits of quantitation (LOQs) of 34 and 100 pg mL^{-1} upon 1 min extraction from 1500 µL of PBS spiked with cocaine and diazepam, respectively (calibration functions were constructed on the basis of the signal ratio of the analyte and its isotopologue (A/Is) in three independent experiments; Figure 3.5). Furthermore, exceptional linearity in the range of 50 pg mL^{-1} up to 1 µg mL^{-1}, and outstanding accuracy (*i.e.* 87-98 %) at three different levels (*i.e.* 0.3, 7.5 and 200 ng mL^{-1}) was attained (see Table 3.4).

Certainly, higher concentration levels are not a limitation for Bio-SPME fibres. Thus, in cases where the affinity of the coating for the analytes is high, and analytes are present at concentrations larger than 100 ppb, shorter extraction times (≤ 1 min) could be used. Although the best combination of desorption/ionization conditions for SPME-nano-ESI (*e.g.* strength of desorption solution, emitter size, and fibre thickness) were not investigated in this study, the results obtained were rewarding[113,118,182]. Certainly, design of experiments (DOE)[311] would aid not only to improve current results, but also to decrease total analysis time.

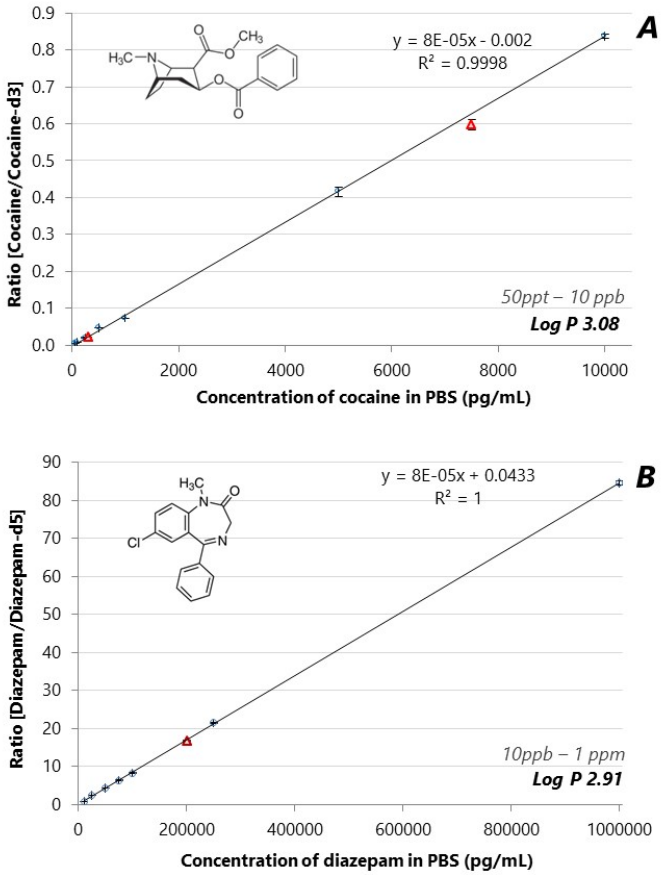

Figure 3.5 A. Quantitative analysis of PBS spiked with cocaine (50 pg ml^{-1} to 10 ng mL^{-1}) and its isotopologue [D_3] cocaine (12 ng mL^{-1}). **B.** Quantitative analysis of PBS spiked with diazepam (10 ng ml^{-1} to 1 µg mL^{-1}) and its isotopologue [D_5] diazepam (12 ng mL^{-1}). Bars represent the standard deviation of analyses for three replicates with independent fibres and nano-ESI emitters. Red triangles represent the accuracy levels evaluated for both compounds.

Table 3.4 Figures of merit, concomitant analysis of diazepam and cocaine in PBS.

Compound	Accuracy concentration Level (%)			LOD [pg/mL]	LOQ [pg/mL]
	300 [pg/mL]	7.5 [ng/mL]	200 [ng/mL]		
Diazepam	91 ± 3.9	97 ± 0.1	98 ± 0.9	34	102
Cocaine	92 ± 2.3	97 ± 1.3	87 ± 2.9	11	34

Analysis of controlled substances in urine samples

Due to the non-invasive sample collection and the typically large sample volumes available, urine is the most traditional matrix employed when monitoring abuse of illicit drugs[325] or doping in sport[326]. Since the amount of parent drug excreted in urine is typically low (*e.g.* parent drug could

be metabolized by the liver), analytical methods capable of providing sensitive analysis in the sub-ng per millilitre levels are needed[110,148]. Recently, Boyacı *et al.*[148] and Reyes-Garcés *et al.*[123,131] demonstrated that different geometrical formats of SPME were capable of meeting the Minimum Required Performance Levels (MRPL) set by the World Anti-Doping Agency (WADA) for the analysis of prohibited substances in urine. As a proof-ofconcept, we present the application of Bio-SPME fibres coupled to nanoESI-MS/MS for the determination of salbutamol, codeine, oxycodone, and methadone in urine. As can be seen in Figure 3.6 and Table 3.5, 1 minute extraction from 700 µL of urine was sufficient to achieve LOQs ranging between 100 and 500 pg mL^{-1}. As a matter of fact, LOQ values not only were below the MRPL levels (*i.e.* 50-100 ng mL^{-1})[148,325], but also rewarding correlation coefficients (>0.999) were observed for all the probes in the range evaluated (*i.e.* 100 pg mL^{-1} up to 500 ng mL^{-1}; see Figure 3.6). Since SPME derives its sensitivity and selectivity from the physicochemical/geometrical characteristics of the coating used, current research is directed towards the development of thinner coatings with greater affinity for the target analytes (*e.g.* HLB[131]), aiming to provide lower limits of detection without compromising total analysis time[136]. Although it was not evaluated in this manuscript, we foresee that SPME-nanoESI in combination with tandem-mass/high-resolving-power instruments could be used for the simultaneous screening of multiple controlled substances in a single analysis[123,131,136,148,267].

Table 3.5 Figures of merit, concomitant analysis of salbutamol, codeine, methadone, and oxycodone in pooled urine.

Compound	Accuracy concentration Level (%)		LOD [ng/mL]	LOQ [ng/mL]
	2.5 [ng/mL]	75 [ng/mL]		
Salbutamol	-	90 ± 1.4	1.1	3.3
Codeine	-	89 ± 1.2	2.1	6.4
Methadone	90 ± 1.8	89 ± 1.4	0.1	0.2
Oxycodone	-	90 ± 0.4	1.4	4.1

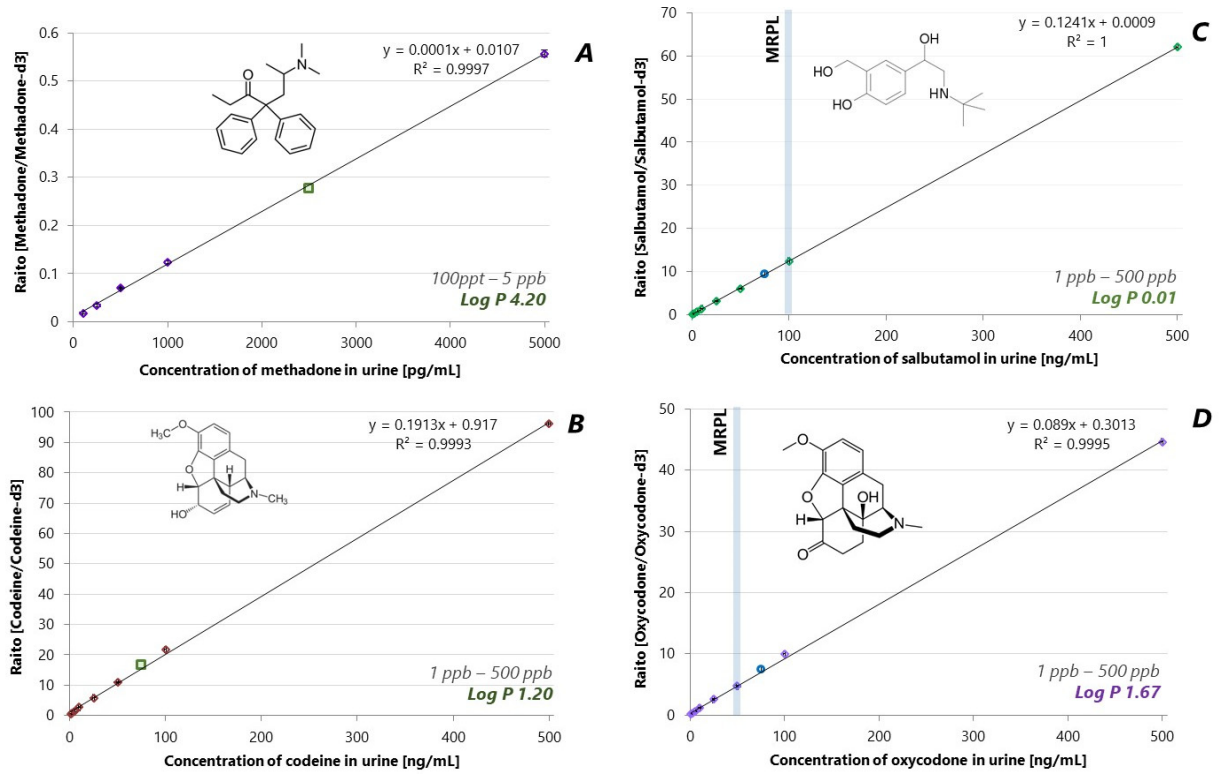

Figure 3.6 A. Quantitative analysis of urine spiked with methadone (100 pg ml^{-1} to 5 ng mL^{-1}) and its isotopologue [D_3] methadone (10 ng mL^{-1}). **B.** Quantitative analysis of urine spiked with codeine (1 ng ml^{-1} to 500 ng mL^{-1}) and its isotopologue [D_3] codeine (12 ng mL^{-1}). **C.** Quantitative analysis of urine spiked with salbutamol (1 ng ml^{-1} to 500 ng mL^{-1}) and its isotopologue [D_3] salbutamol (10 ng mL^{-1}). **B.** Quantitative analysis of urine spiked with oxycodone (1 ng ml^{-1} to 500 ng mL^{-1}) and its isotopologue [D_3] oxycodone (12 ng mL^{-1}). Bars represent the standard deviation of analyses for three replicates with independent fibres and nano-ESI emitters. Green squares and blue circles represent the accuracy levels evaluated for both compounds. MRPL, Minimum Required Performance Level.

Bio-SPME-nanoESI as a tool for Therapeutic Drug Monitoring (TDM)

In the era of personalised medicine, the development of bioanalytical methods that are capable of rapidly quantifying systemic concentrations of drugs with low therapeutic index or narrow therapeutic range, is crucial. Indeed, such methods should provide not only equal or better performance than the existing approaches (*e.g.* immunoassays and liquid chromatography-MS) in terms of accuracy and linear dynamic range, but also lower cost per sample and simpler operation. In order to fulfil these objectives, techniques such SFME[82] and SPE-Paper Spay (SPE-PS)[102] have

recently arisen as exciting alternatives for point-of-care TDM of diverse analytes in blood and plasma with minimal sample consumption and reasonable sample preparation. Although SPME has also been used in TDM applications[113,327], depending on the physicochemical properties of the analyte and its affinity for the extracting particles[113], relatively long sample preparation times (*i.e.* $t_{ext} \geq 10$ min) and moderately large sample volumes ($V_s \geq 1$ mL) were needed to achieve quantitative results via LC-MS/MS[131]. In view of this, Bio-SPME-nano-ESI is herein introduced as a simpler and faster approach for the quantitation of target analytes in whole blood samples. Given that SPME extracts via free concentration[113], it is expected that analytes largely bound to plasma proteins would provide extremely low extraction recoveries (*i.e.* worst-case scenario for SPME)[131]. Thus, aiming to evaluate the method under "extreme" conditions, two probes with protein biding larger than 90%, amitriptyline and imatinib[328], were selected. Unlike SFME and SPE-PS, when using Bio-SPME fibres, neither sample dilution[82] nor sample drying[102] is required. However, in comparison with the analysis of urine or PBS, additional rinsing steps[123] are needed to remove clusters of macromolecules that lingered to the coating surface during the extraction process that could potentially clog the nano-ESI emitter. Thus, for blood analysis the analytical process is the following: first, a pre-conditioned fibre (*i.e.* methanol/water, 1:1) is rinsed 10 seconds in LC-MS water prior to sampling in order to minimize attachment of proteins and cells on the coating/wire surface[123]. Then, extraction is performed by immersing the fibre 2 min in the vial containing the sample, and subsequently the fibre is rinsed for 5 seconds on a new vial containing LC-MS grade water. Afterwards, the fibre coating is carefully cleaned with a Kimwipe tissue and rinsed for another 5 seconds on a new vessel containing LC-MS grade water.

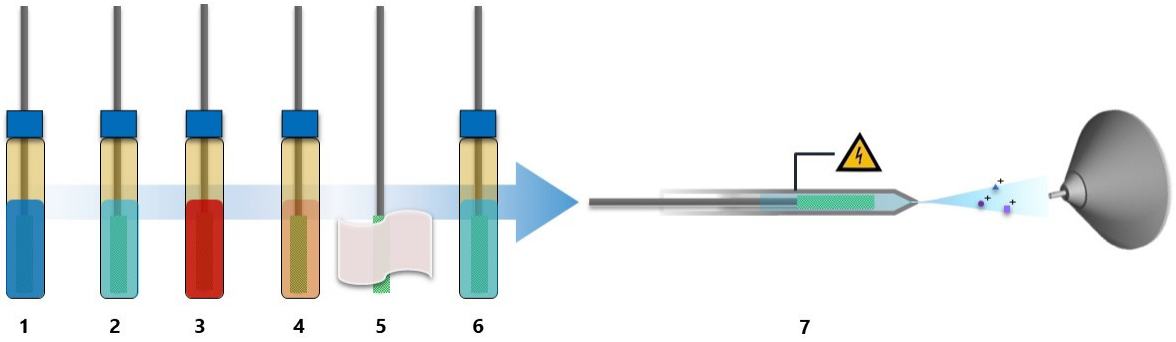

Figure 3.7 Experimental set up for Bio-SPME extraction from whole blood and desorption–ionization using nano-ESI-MS/MS. The analytical process can be summarized in 7 steps. **1.** Fibre pre-conditioning; **2.** Fibre rinsing in water to remove excess of methanol that might enhance protein/cell precipitation (10s); **3.** Extraction from whole blood (2 min); **4.** Fibre rinsing in water to remove cells and proteins attached to coating surface (5s); **5.** Fibre cleaning with a piece of Kim wipe tissue (5s); **6.** Additional rising step to remove small particles that might remained attached to the surface (5s); **7.** Desorption/ionization step using acidified methanol (0.1% FA).

Finally, the wet fibre is inserted on the nano-ESI emitter for desorption/ionization (Figure 3.7 summarizes the modified analytical procedure). It is important to stress that given the small tip size of the emitter used for the urine and PBS analysis (*i.e.* ~ 1± 0.5 μm), clogging might happen while performing instrumental replicates (*i.e.* multiple ionizations from the same emitter). Therefore, whole blood experiments were performed using emitters with a slightly larger tip size (*i.e.* ~ 2 ± 1 μm; BG-10-58-2-AP-20). When using these emitters plugging was never observed. Certainly, nano-ESI devices with larger emitter tip (*e.g.* 4-20 μm) would provide more robust analysis, especially when considering these for unattended high-throughput applications[37]. Even though the technique has not yet been optimized, we here have already demonstrated that limits of detection (LODs) in the sub-nanogram per millilitre range were achieved for amitriptyline and imatinib when performing 2 minutes extraction from 300 μL of whole blood (see Table 3.6). In addition, great accuracy (*i.e.* 91-93% at 100 ng mL^{-1}, Figure 3.8) and linearity were attained for both probes in the range assessed.

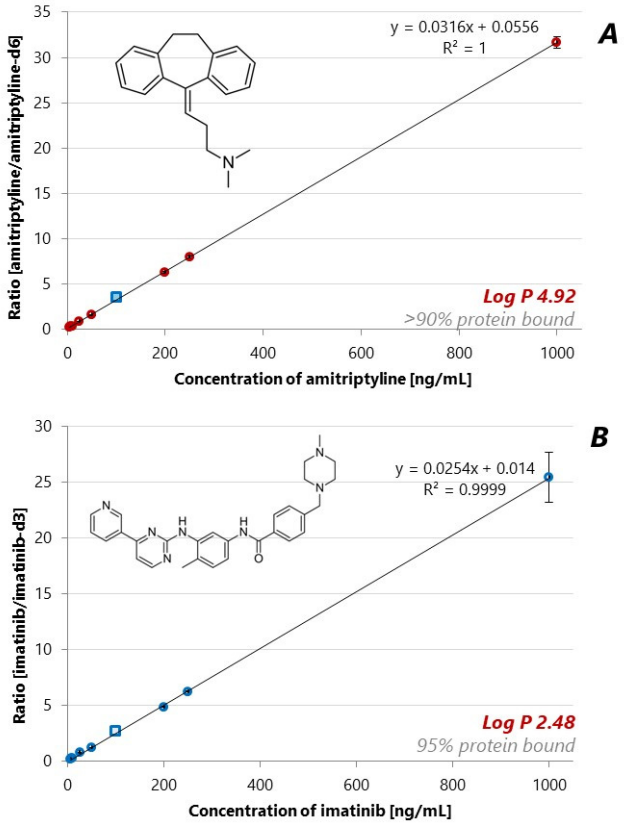

Figure 3.8 A. Quantitative analysis of whole blood spiked with amitriptyline (100 pg ml^{-1} to 5 ng mL^{-1}) and its isotopologue [D_6] amitriptyline (10 ng mL^{-1}). **B.** Quantitative analysis of whole blood spiked with imatinib (1 ng ml^{-1} to 50 ng mL^{-1}) and its isotopologue [D_3] imatinib (12 ng mL^{-1}). Bars represent the standard deviation of analyses for three replicates with independent fibres and nano-ESI emitters. Blue squares represent the accuracy levels evaluated for both compounds.

Table 3.6 Figures of merit, concomitant analysis of amitriptyline and imatinib in whole human blood.

Compound	Accuracy (%) 100 [ng/mL]	LOD [ng/mL]	LOQ [ng/mL]
Amitriptyline	110 ± 1.8	1.6	4.9
Imatinib	107 ± 1.0	2.3	7.0

Hence, due to speed of the analysis, the suitability of performing extraction/enrichment on-site, and the simplicity of the method, BioSPME could be used as an ideal tool for the fast correlation of drugs to its therapeutic efficacy or toxicity while treating a patient[152,313,315].

Towards targeted analysis in small blood volumes

Until now, the use of SPME fibers for quantitative analysis of target analytes in limited sample volumes ($V_s \leq 50$ µL) of biofluids, such as blood, has remained overall unexplored. Challenges involving getting reliable quantitative data at practical concentration ranges while keeping a simple sampling/sample preparation protocol have hindered the implementation of SPME in such cases. Chiefly, analytes with high protein binding coefficients typically provide extremely low recoveries by SPME; therefore, it is difficult to achieve useful quantitation limits by LC-MS/MS, unless long extraction times or larger coating surface areas are used[2,113]. Further, classical SPME fibers, with a coating length of 10-15 mm, are too long to be entirely in contact with small sample volumes unless a miniature vessel, such as a glass capillary or vial conical insert, is used for such purpose[329]. Finally, there are currently no appropriate protocols in place that ensure both total contact between fiber coating and sample[329], and efficient desorption/transmission of analytes into the mass spectrometer. To the best of our knowledge, the use of SPME fibers for the quantitation of target analytes in volumes below 10 µL has only been described in work conducted by Zhu and collaborators[329].

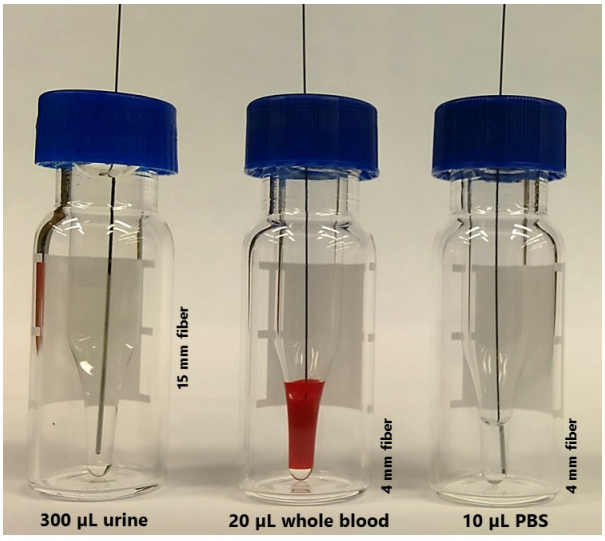

Figure 3.9 Small sample volume analysis using 15 and 4 mm mix-mode Bio-SPME fibers.

In order to challenge the new Bio-SPME-nano-ESI platform, it was used for the quantitative analysis in volumes ranging between 10 and 1500 µL. As shown in Figure 3.9, to ensure that the entire fiber remained immersed in the sample, glass vials with a fused-in conical insert and fibers with a coating length of 4 mm were used. At the outset, we wanted to demonstrate that independently of sample volume, the ratio of analyte to internal standard extracted by the fiber remained constant and that good signal was attained. As can be seen in Figure 3.10, 1-minute extractions from PBS spiked with cocaine and diazepam at 25 ng mL^{-1} yielded non-statistical differences among the five volumes evaluated (i.e. 10, 50, 100, 300, and 1500 µL). Therefore, based on these results, we proceeded to perform similar experiment in 20 µL of whole blood spiked with amitriptyline at concentrations of clinical relevance (*i.e.* 5-250 ng mL^{-1}). As can be seen in Figure 3.11, notable linearity, great accuracy, and good signal were obtained in the concentration range evaluated.

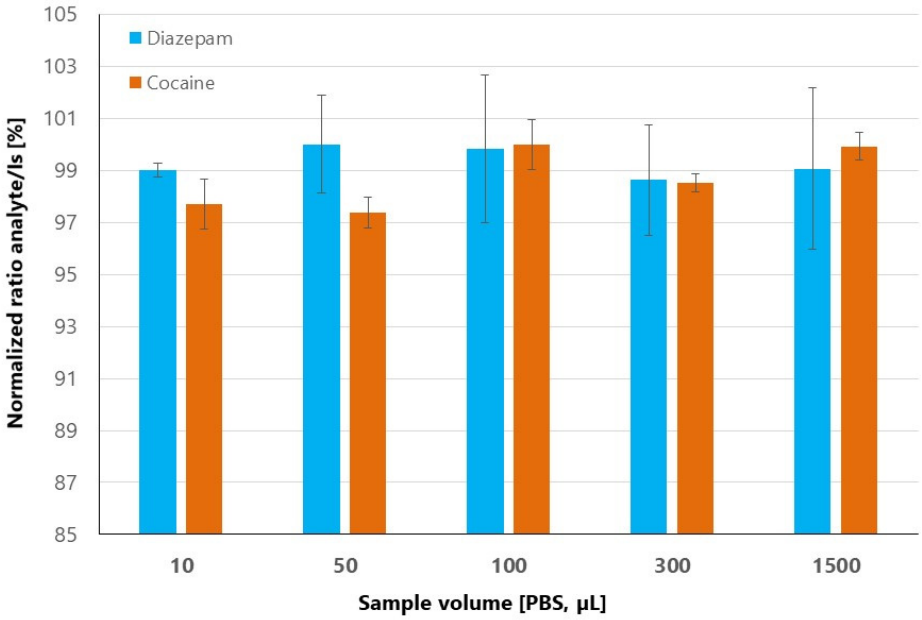

Figure 3.10 Comparison of analyte-to-internal standard ratios for cocaine and diazepam spiked at 20 ng mL^{-1} in five different volumes of PBS. Results were normalized for easier visualization. Internal standards were spiked at 10 ng mL^{-1}. Bars represent the standard deviation of analyses for three replicates with independent fibers and nano-ESI emitters.

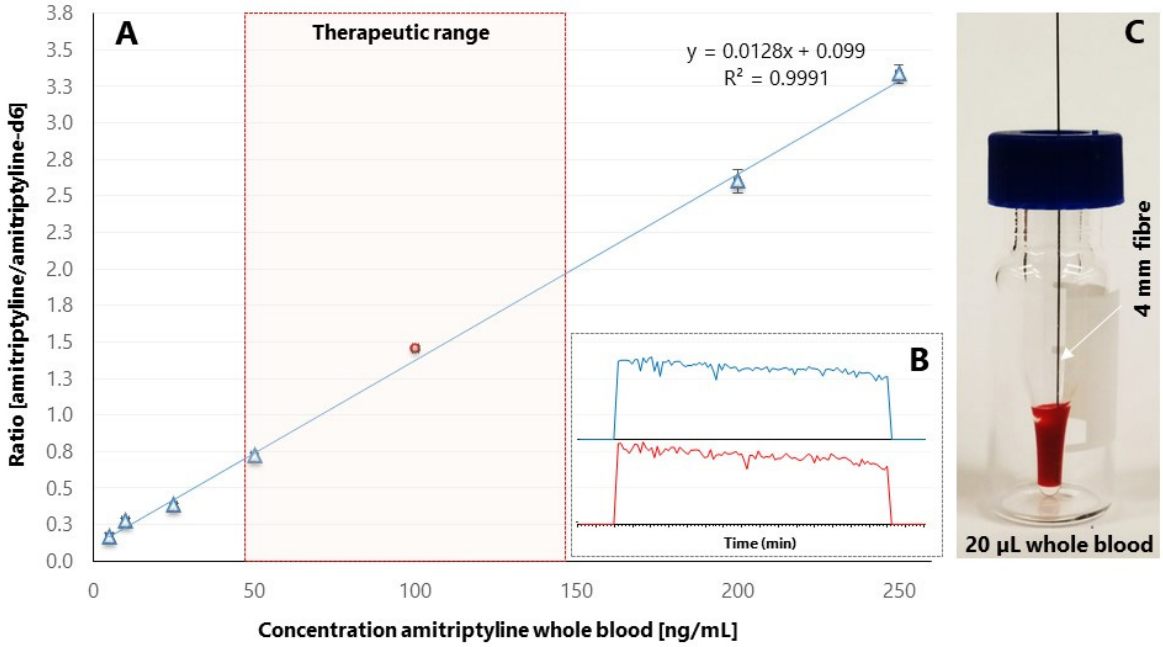

Figure 3.11 A. Quantitative analysis of whole blood spiked with amitriptyline (5 ng ml^{-1} to 250 ng mL^{-1}) and its isotopologue [D_6] amitriptyline (100 ng mL^{-1}). Sample volume is 20 µL with 2 min extraction/enrichment using 4 mm mix-mode Bio-SPME. Bars represent the standard deviation of analyses for three replicates with independent fibers and nano-ESI emitters. **B.** Ion chronogram of amitriptyline (top) and [D_6] amitriptyline (bottom) for an acquisition time of 45 s. **C.** SPME sampling from 20 µL of whole human blood using a 300 µL glass insert vial.

Although this application is just a proof-of-concept demonstrating the quantitation capabilities of Bio-SPME-nano-ESI, we anticipate its use in forensic and clinical applications where only minimal sample volumes are available. Currently, our group is working on the development of miniature devices that allow for the analysis of sample volumes below 10 µL (*i.e.* single cells[211,330], small pieces of tissue[263], and biofluids) without sacrificing analysis time or the extraction capabilities of SPME. Unlike other sampling devices coupled to nano-ESI[331,332], mini-SPME devices truly collect analytes of interest based on their affinity towards the extraction phase, while minimizing or removing potential interferences that might cause suppression/enhancement.

3.2.4 Summary

In this section, the Bio-SPME-nano-ESI platform was shown to rapidly and accurately determine total concentrations of target compounds in complex matrices. Furthermore, the suitability of these biocompatible probes to extract, identify, and quantify analytes present in small sample volumes was demonstrated for the first time. Through the selection of appropriate experimental conditions, the entire analytical process was completed in less than 7 min per sample with outstanding figures of merit. In addition to the abovementioned advantages, the Bio-SPME approach has a built-in clean-up step, which allows for the incidence of capillary plugging to be substantially reduced. In light of the results herein presented, we foresee the combination of SPME with nano-ESI as a rapid diagnosis tool for *in vivo* and *in situ* analyses of endogenous and exogenous substances in biological fluids[242] and tissue[152] samples. Certainly, the direct coupling of SPME-nanoESI to miniature mass spectrometers (MMS)[65,333] should bring a new dimension to what we know until now as on-site analysis in clinical, environmental, and forensic applications. Indeed, SPME-nanoESI and MMS, in combination with robotic platforms[334] is projected to be an ideal analytical tool for non-assisted time-resolved mass spectrometry applications[335] such monitoring at remote locations. Furthermore, in near future, Bio-SPME fibres in combination with Ion Mobility Spectrometry will be a key combination towards the analysis of compounds otherwise difficult to resolve by MS without LC[336].

Section 3.3 Fast quantitation of target analytes in small volumes of complex samples by matrix-compatible Solid Phase Microextraction devices

3.3.1 Introduction

The development of new analytical technologies capable of providing high quantitation performance while delivering simplified and fast analysis of small amounts of biological samples (*i.e.* V ≤ 10 μL) can undoubtedly impact the precision and efficiency of biological investigations in drug-development and point-of-care (POC) diagnosis[8,82,173,318]. Facilitated by the improved sensitivity and specificity provided by modern mass spectrometry (MS) instrumentation, the replacement of traditional complex laboratory procedures with integrated miniaturized methods has become a growing trend in POC diagnosis[65]. In recent years, advances in direct sample to mass spectrometry techniques such as paper-spray ionization, probe electrospray ionization, and touch spray[48,65,82,332] have allowed for the application of these methods towards the quantitative analysis of small volumes of biofluids. However, the sensitivity and precision typically achieved in the laboratory through adequate sample preparation prior to the MS quantification is traded off. Therefore, techniques capable of isolating and enriching target analytes from complex matrices with minimal processing time and adequate sample clean-up are highly desirable for applications that require direct introduction to MS [8,82,136,149,173].

As a concept, SPME embraces solventless microextraction technologies with different geometrical configurations that efficiently integrate sampling and sample clean-up, while also allowing for enrichment of the molar fraction of a given analyte in a single step[113]. Given the multiple advantages of this technique, including its feasibility to be coupled to different analytical instruments, SPME has been widely used for analysis of complex matrices such as biofluids,

tissues, and food samples[2,113,149,173,304]. The main objective of this report is to introduce a new SPME-device that allow for the analysis of samples characterized by a small volume and/or size. The proposed device consists of a polypyrrole (PPy) coated stainless steel micro-tip (Figure 3.12) which is capable of extracting the analytes by immersing the mini-fibre into few micro-litter of matrix. The micro-tip can be conveniently interfaced with MS instrumentation via chromatography, or directly interfaced via nano-electrospray-ionization (nano-ESI) for qualitative or quantitative determinations.

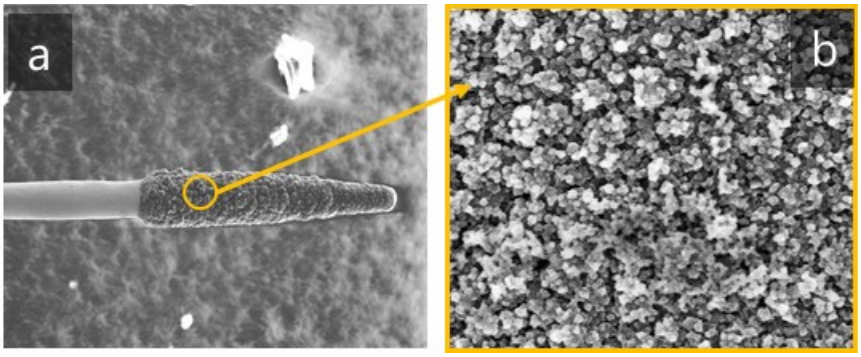

Figure 3.12 (**a**) SEM image of a 150 µm (100x) tip coated with a 5 µm layer of PPy; (**b**). SEM image of nano-structured PPy (5000x). Devices were made according to a protocol developed by Piri-Moghadam *et al*.[151]

3.3.2 Experimental section

Materials and reagents

The following compounds were selected as model analytes to evaluate PPy-tips: cocaine, diazepam, salbutamol, oxycodone, methadone and amitriptyline. Deuterated analogues of each analyte were used for correction of intra- and inter-experiment variability. Further details about compounds suppliers, properties, and SRM transitions are provided on Table 3.1. The phosphate-buffered saline solution (PBS) (pH 7.4) was prepared according to the procedure listed on Section 2.2.2. Pooled whole blood and pooled plasma from healthy donors in potassium (K2) ethylenediaminetetraacetic acid (EDTA) was purchased from Bioreclamation IVT (Baltimore,

MA, U.S.A.). Urine samples were collected from six healthy volunteers (three female and three male). Collection of urine from healthy volunteers for this particular study was under the approval of the Office of Research Ethical Board of University of Waterloo).

Mass Spectrometry

Experiments were performed with the use of a triple quadrupole mass spectrometer TSQ Vantage (Thermo Scientific, San Jose, USA). Nano-electrospray emitters: Econotip (Econo10, 1.0/0.58 OD/ID, mm) were obtained from New Objective Inc. (Woburn, MA, USA). An in-house ionization source was built at the machine and electronic shop of the University of Waterloo (see Figure 3.1) to accurately position nano-ESI emitters in front of the mass spectrometer.

Sample preparation

The established analytical workflow consisted of four main steps: extraction/pre-concentration, rinsing, desorption, and ionization. First, a preconditioned probe was inserted in a vial containing the sample matrix ($V_{ext} \sim 5$ μL), and quick extraction/enrichment of the analytes was performed ($t \leq 2$ min, static conditions). Next, the probe was rinsed in a vial containing LC/MS grade water ($t \leq 5$ s) to remove matrix components that could potentially adhere to the coating surface. Subsequently, the probe was introduced into a conical vial containing the desorption solution. Lastly, and after allowing some time for the analytes to be desorbed in the selected solvent, the solution was transferred to the nano-ESI emitter using a small syringe. Then, a high electrical field between the emitter (1.3 kV, positive mode; 2.5 kV, negative mode) and the mass spectrometer was applied, and analytes were ionized via electrospray mechanisms as shown in Figure 3.13.

Figure 3.13 Experimental set-up for PPy probe extraction from complex matrices and desorption–ionization using nanoESI-MS/MS or nanoESI-HRMS.

3.3.3 Results and discussion

The use of miniaturized SPME probes not only facilitates the analysis of samples characterized by limited availability of sample (*e.g.* single cells[211]), but can also diminish potential damage caused during *in vivo* sampling[149]. Although the use of small metal probes for the analysis of single cells and tissue has already been reported[211,332], due to the low sorption capacity and non-specific affinity of the metal surface (poor inter-device reproducibility), their application is limited to the determination of compounds present at high concentrations (*e.g.* phospholipids). In applications that require analysis of tissue or biofluids, device biocompatibility, defined not only as the capability to extract analytes of interest without significantly disturbing the system, but also in the sense of not co-extracting cell substructures and macromolecules (*e.g.* organelles, proteins, lipoproteins) needs to be examined with extreme care[2,304]. If the chosen materials are insufficiently biocompatible with the matrix under study, significant matrix effects are likely to occur, leading to poor or non-quantitation capabilities.[304] Accordingly, the determination of compounds at trace levels in small volumes and single cells requires biocompatible polymeric coatings with high affinity toward the compounds of interest. PPy, a biocompatible[2] material frequently used in biosensors[337] and biomedical engineering[338], is a well-known SPME extracting media for *in vivo* and *in vitro* bioanalysis[113]. However, the fabrication of miniature coated sampling devices has not

been extensively explored to date due to the multiple challenges associated with the fabrication of such devices (*e.g.* mechanical stability and thickness of the coating).[113] In this work, acupuncture needles with a diameter of 120 μm were electrochemically etched to a tip with a diameter size of approximately 5 μm. Then, PPy was electrochemically coated onto the surface according to a protocol developed by Piri-Moghadam *et al.*[151] Aiming to have a better understanding of the quantitation power afforded by the PPy tips, direct coupling to MS instrumentation was performed via nano-ESI emitters (Figure 3.13)[173]. Quantitative determinations of therapeutics and drugs of abuse in urine, plasma, and blood were carried out by performing static extractions from 5 µL of sample for 2 minutes. Subsequently, tips were rinsed for 3 seconds in water and then desorbed in 2 µL of solvent for 2 minutes. Limits of quantitation (LOQs) in the low-ng/mL range were obtained for all target analytes, with a total analysis time of less than 5 minutes in urine (see Figure 3.14 and Table 3.7). Likewise, rewarding results were obtained for oxycodone, salbutamol and methadone when analyzing plasma samples spiked with these target analytes (see Figure 3.15 and Table 3.8).

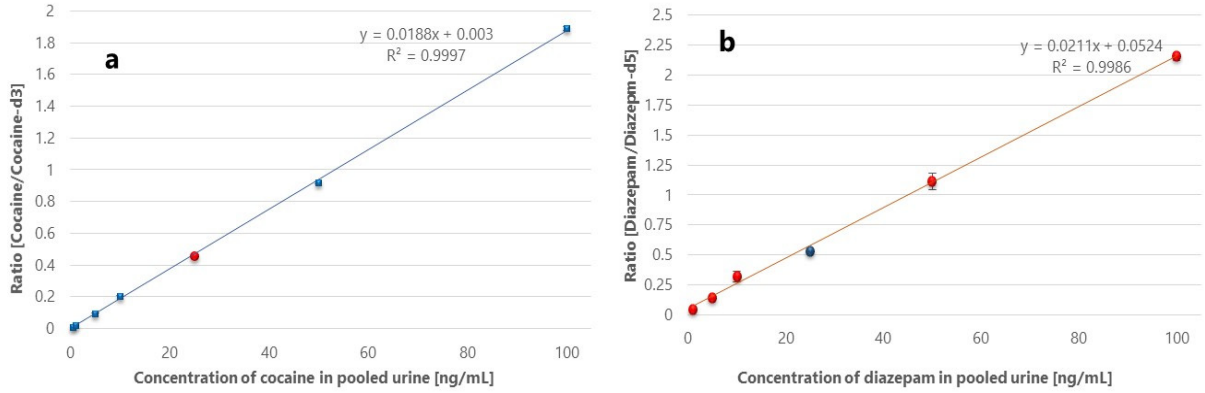

Figure 3.14 A. Quantitative analysis of urine spiked with cocaine (0.1 ng mL^{-1} to 100 ng mL^{-1}) and its isotopologue [D_3] cocaine (50 ng mL^{-1}). **B.** Quantitative analysis of PBS spiked with diazepam (1 ng mL^{-1} to 100 ng mL^{-1}) and its isotopologue [D_5] diazepam (50 ng mL^{-1}). Bars represent the standard deviation of analyses for three replicates with independent PPY-tips and nanoESI emitters. Dots in different colors represent accuracy validation points.

Table 3.7 Quantitative analysis of urine spiked with diazepam and cocaine. Accuracy and reproducibility obtained for mini-tips and nanoESI emitters (n=3).

	RSD [%]	
Concentration [ng mL^{-1}]	**Diazepam**	**Cocaine**
0.1	-	1.5
0.5	-	0.6
1	24.1	1.6
5	1.0	0.0
10	14.0	2.3
50	6.1	1.2
100	1.4	0.8
	Accuracy [%]	
2.5	-	82
25	82	88

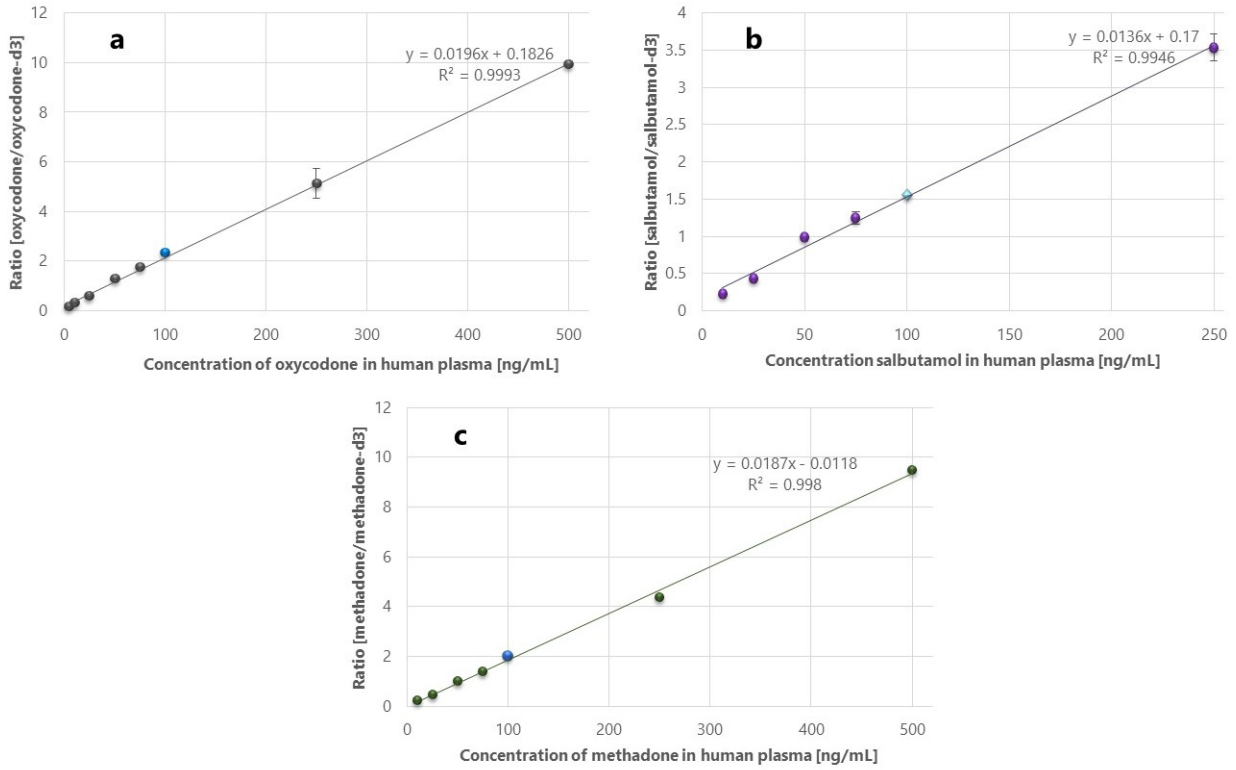

Figure 3.15 Quantitative analysis of plasma spiked with oxycodone (**a)**, salbutamol (**b**) and methadone (**c**), (1 ng ml^{-1} to 100 ng mL^{-1}) and their isotopologue. Bars represent the standard deviation of analyses for three replicates with independent PPY tips and nano-ESI emitters. Dots in different colors represent accuracy validation points at 100 ng mL^{-1}.

As expected, due to its small coated surface area, mini-tips cannot provide the same quantitation capabilities as the commercial coatings (see Figure 3.16 and Table 3.9) for the analysis of

amitriptyline in whole blood (LOQ ~ 25 ng mL^{-1}). Yet, the results are acceptable given the sample size and the therapeutic range of this drug (*i.e.* 50-150 ng mL^{-1}).

Table 3.8 Quantitative analysis of plasma spiked with salbutamol, methadone, and oxycodone. Accuracy and reproducibility obtained for SPME-tips and nano-ESI emitters (n=3).

	RSD [%]		
Concentration [ng mL^{-}	**Salbutamol**	**Methadone**	**Oxycodone**
5	-	-	3.8
10	3.3	5.5	10.8
25	3.7	5.0	4.3
50	1.2	1.6	5.8
75	7.1	1.1	1.7
250	5.1	0.1	1.0
500	2.1	1.0	6.1
		Accuracy [%]	
100	102	107	111

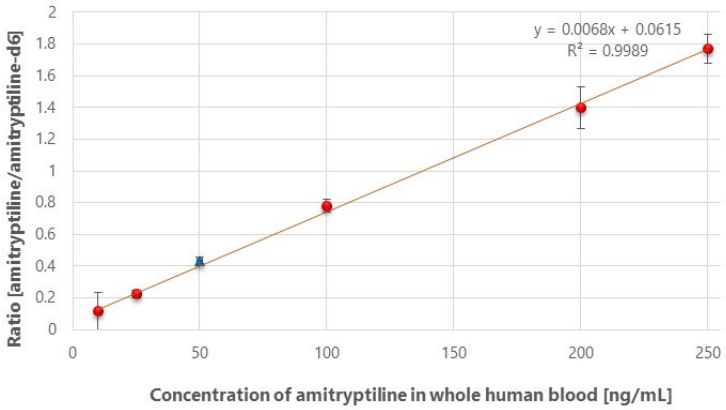

Figure 3.16 Quantitative analysis of whole blood spiked with amitriptyline (5 ng ml^{-1} to 250 ng mL^{-1}) and its isotopologue [D_6] amitriptyline (100 ng mL^{-1}). Bars represent the standard deviation of analyses for three replicates with independent PPY tips and nano-ESI emitters.

Table 3.9 Quantitative analysis of blood spiked with amitriptyline. Accuracy and reproducibility obtained for Coated Blade Spray when performing spot analysis (n=3). LOD, 5 ng/mL.

	RSD [%]
Concentration [ng mL^{-}	**Amitriptyline**
5*	2.0
10	1.3
25	0.6
50	0.4
200	2.1
250	2.0
	Accuracy
100	117

3.3.4 Summary

SPME acts as a chemical biopsy tool by enriching small molecules carrying chemical information about the investigated system without removing tissue, thus providing a much cleaner extraction. Currently, our group is working on the development of versatile biocompatible coatings (*e.g.* HLB-based) for miniature devices that allow for the extraction of a broader range of compounds, with potential for a variety of applications in fields such as plant biology and oncology, where small objects need to be scrutinized. Likewise, the application of SPME tips for *in-vivo* tissue "imaging" is expected in the near future. Essentially, imaging should be understood as the capability of these miniature devices to monitor metabolite or drug concentration changes in specific regions of the tissue with minimum invasiveness (*i.e.* spatial resolution information). If the object is sufficiently large, the damage caused by insertion can be considered negligible or sufficiently minimized, allowing for multiple samplings to be subsequently performed to investigate changes in the system as a function of time or applied stimuli. We foresee the SPME-tip device as a tool capable of complementing microdialysis and novel ambient ionization approaches suitable for the surgery room[61,199].

Section 3.4 Open Port Probe Sampling Interface for the Direct Coupling of Biocompatible Solid-Phase Microextraction to Atmospheric Pressure Ionization Mass Spectrometry

3.4.1 Introduction

Within the last twenty years, different strategies aimed at direct and efficient coupling of Solid Phase Microextraction (SPME) devices to mass spectrometry (MS) instrumentation have been explored for analysis of a broad range of matrices with high relevance in clinical, forensic, environmental, and food analysis.[80,136,151,173,183,184,192,210] To date, most reported direct couplings of SPME fibers are based on work performed either by Górecki *et al* [183,184] ("classical" thermal desorption used in gas-chromatography,[164,174]) or by Chen *et al.*[339] (solvent desorption, suitable for thermally labile compounds). In the latter, the desorption of extracted/enriched analytes occurs by placing the fiber on a desorption chamber (*e.g.* port, valve, or syringe),[114,172,180–182] filled with a solvent with high affinity for the analytes of interest, prior to the atmospheric pressure ionization event (either electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI)). Aiming to take full advantage of the molar fraction enrichment offered by SPME,[151] two of our recently reported endeavours applied the methodology initially proposed by Walles *et al.,*[210] where nano-electrospray ionization (nano-ESI) is used in combination with biocompatible-SPME (Bio-SPME) fibers/tips for fast quantitation of target analytes extracted from biofluids.[151,173] Although the combination of SPME with nano-ESI is fundamentally "ideal" for analysis of known/unknown substances from complex matrices, in that it yields high ionization efficiency[319] with minimal solvent consumption and long spray events that allow for numerous MS and MS^n experiments,[5,318] drawbacks of this coupling should also be considered in the development of further analytical applications. First, poorly wettable coatings, such as C18-coated fibers,[113,304] have been found to

be capable of generating bubbles inside the nano-ESI emitter (*i.e.* 'ambient-air' collected inside the particles is released when immersed onto the desorption solvent), which may possibly distort the Taylor-cone formation and, consequently, the electrospray ionization process. Second, the high cost per analysis (*i.e.* due to the non-reusability of the emitters), as well as the difficulties associated with automatization of the process are additional factors that could thwart the high-throughput implementation of SPME-nano-ESI. Aiming to solve the aforementioned concerns, this manuscript introduces the open port probe (OPP) sampling interface[36] as a novel, robust, sensitive, and ready-to-use interface for the direct coupling of Bio-SPME fibers to mass spectrometry. As a proof-of-concept, SPME-OPP is herein reported for the first time for determination of controlled substances in relevant clinical assays for urine: treatment of opioid-dependence (*i.e.* buprenorphine), pain management (*i.e.* fentanyl), and doping control (*i.e.* clenbuterol). Given that chromatography can be circumvented by using SPME-OPP, MRM^3 or DMS[336,340,341] were implemented to enhance compound selectivity while keeping with the speed and simplicity of traditional MS/MS analysis.

3.4.2 Experimental section

Materials and Supplies The following compounds were selected as model analytes to evaluate Bio-SPME-OPP: clenbuterol, fentanyl, and buprenorphine. Deuterated analogues of each analyte were used for correction of intra- and inter-experiment variability. Further details regarding compound suppliers, properties, multiple reaction monitoring (MRM), MRM^3 transitions, and DMS parameter settings are provided in Table 3.10 and 3.11. All LC-MS grade solvents (acetonitrile, methanol, and water) used in experiments purchased from Fisher Scientific (Bartlesville, OK, USA).

Table 3.10 Manufacturers, LogP, mass spectrometry, and DMS conditions monitored for each analyte.

Compound	Manufacturer	Log P[b]	Parent [m/z]	Fragment [m/z]	DP [V]	EP [V]	CE [V]	CXP[V]	SV [V]	CV [V]
Buprenorphine	Cerilliant[a]	4.53	468.3	396.2	120	10	55	10	NA	NA
Buprenorphine-d_4	Cerilliant[a]	-	472.3	400.2	120	10	55	10	NA	NA
Fentanyl	Cerilliant[a]	3.82	337.3	188.2	120	10	32	10	NA	NA
Fentanyl-d_5	Cerilliant[a]	-	342.2	188.2	120	10	32	10	NA	NA
Clenbuterol	Cerilliant[a]	2.33	277.1	203.1	60	10	24	10	4000	13.0
Clenbuterol-d_9	Cerilliant[a]	-	286.1	204.1	60	10	24	10	4000	13.0

Table 3.11 MRM^3 conditions for clenbuterol analysis.

Compound	MRM^3 transition	CE [V]	DP [V]	EP[V]
Clenbuterol	277.1-203.1-168.1	24	100	10
Clenbuterol – d_9	286.1-204.1-169.1	24	100	10

[a] Cerilliant (Round Rock, TX, USA); [b] Log P, logarithm of its partition coefficient between n-octanol and water (data taken from www.drugbank.ca). Dwell time was 50 ms for all analytes. DP: Declustering potential, EP: Entrance potential, CE: Collision energy, CXP: Collision cell exit potential. All experiments were performed using an SCIEX QTRAP® 6500+ MS/MS system with Ion Drive™ source and Electrospray Ionization (ESI) probe (SCIEX, Concord, Canada).

Biocompatible SPME mixed-mode probes (*i.e.* C_{18}-SCX particles, 45 μm thickness, 4 mm coating length) were kindly provided by Supelco (Bellefonte, PA, USA). Although non-biocompatible SPME devices could also be interfaced to OPP, the present work exclusively focuses on bioanalytical applications where the use of biocompatible devices is essential.[173] Urine samples were collected from six healthy volunteers (three female and three male). Collection of urine from healthy volunteers for this particular study was under the approval of the Office of Research Ethical Board of University of Waterloo.

DMS-MS system

A DMS system (SelexION+™, SCIEX, Concord, ON) was mounted in the atmospheric region between the sampling orifice of the QTRAP 6500+ (SCIEX) system and its electrospray ionization (ESI) source. The ESI probe was maintained at a voltage of 5500 V. A constant gas flow in the DMS cell was achieved by curtain gas flow (N_2; 30 psi, 7.1 L min^{-1}) and the primary stage vacuum pumping of the MS system. The temperature of the transport gas in the DMS cell was maintained at ~100 °C (DMS heater setting of 150 °C). For the experiments conducted in this study, the separation voltage (SV) was set at 4000 V, and the compensation voltage (CV) was set at 13.0 V for optimal transmission of clenbuterol and clenbuterol-D9.

Open-Port-Probe (OPP) sampling interface

As shown on Figure 3.17, the OPP sampling interface used in our experiments is similar to the one reported by Van Berkel and collaborators.[36,45] It is composed of a vertically aligned co-axial tube arrangement that enables solvent delivery to the sampling end through the tubing annulus (304 stainless steel, 1.75 mm i.d. × 3.18 mm o.d. × ~9 cm long; Grainger, Lake Forest, IL, USA), and

aspiration down the center tube (capillary tube; 254 µm i.d. x 361 µm o.d. × ~25 cm long; Upchurch Scientific, Oak Harbor, WA, USA) into the Ion Drive™ source driven by the nebulizer gas.[36] Each tube was secured within a PEEK Tee (Upchurch Scientific, Oak Harbor, WA, USA) so that solvent could be delivered by a solvent pump (200 Series; Perkin Elmer, Santa Clara, CA, USA). The aspiration force through the inner tube of the sampling probe was controlled by the flow rate of the nebulizing gas (nitrogen) into the ion source (90 psi), and the rate of the flow-in solvent was adjusted to achieve a dome-shaped sampling surface to maximize the contact area with SPME coatings (200 µL/min). The standard ESI electrode (100 µm i.d.) was replaced by one of equivalent length but with 150 µm i.d. to increase the accessible self-aspiration flow rate range of the system[36]. Positive ion mode ESI was used with ion source nitrogen gas settings GS1 = 90, GS2 = 70; curtain gas = 25; heated nebulizer temperature = 350 °C; and electrospray voltage = 5500 V.

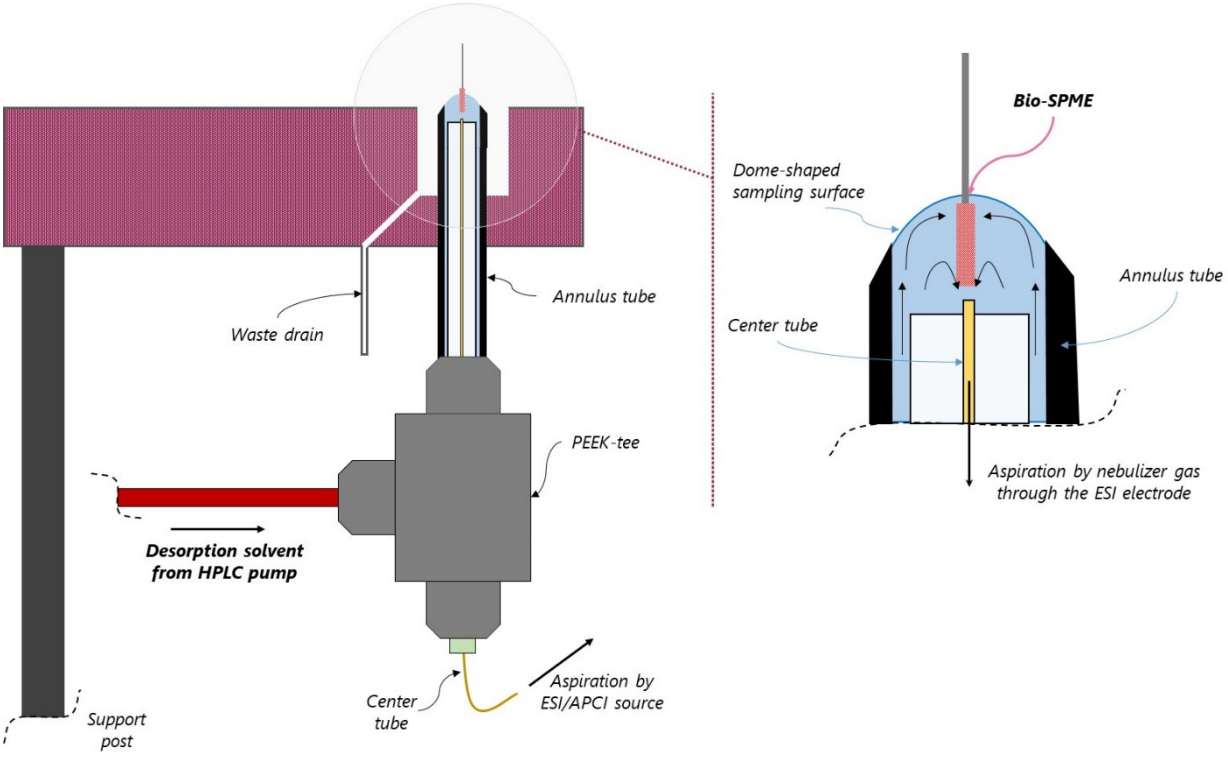

Figure 3.17 Schematic of the Open-Port-Probe interface used for desorption-ionization of Bio-SPME fibers. All experiments were performed using an SCIEX QTRAP® 6500+ MS/MS system with Ion Drive™ source and Electrospray Ionization (ESI) probe (SCIEX, Concord, Canada).

SPME extraction and desorption/ionization

The analytical workflow for SPME-OPP consisted of three simple steps: extraction/pre-concentration, rinsing, and desorption/ionization (Figure 3.18). First, the coating of the Bio-SPME fiber was preconditioned on a methanol-water (50:50) solution for about thirty minutes prior to analysis as described elsewhere[123]. Then, the Bio-SPME fiber was inserted in a vial containing the urine sample (V_{ext} ~ 300 µL), and quick extraction/enrichment of the analytes was performed by agitating the sample at high speed (agitation at 1500 rpm, t≤ 5 min). Next, the fiber was rinsed in a vial containing LC/MS grade water (t ≤ 10 s) to remove matrix components that could potentially adhere to the coating surface. Finally, analyte desorption was achieved by placing the SPME fiber for 5 seconds into the sampling dome of the OPP, such that it touched the continuous flowing stream. Thus, all the analytes extracted on the SPME fiber were desorbed and moved simultaneously from the open-port section of the probe (Figure 1) to the electrospray needle, where they were ionized via ESI mechanism. However, it is important to clarify that the desorption rate of each compound extracted on the fiber would mostly depend on its affinity for both the desorption solvent and the fiber coating. As shown in Figure 3.18, typical full width at half maximum **(**FWHM) occurs within 6 seconds with minimal peak broadening, so a new injection can be theoretically performed every 10-15 seconds. It should be noted that incomplete desorption of analytes was observed under the conditions used in this manuscript (*i.e.* ~ 80% eluted in 5 s). However, this was not a limitation in obtaining quantitative results, as the calibration functions were constructed on the basis of the signal ratio of the analyte and its isotopologue (A/Is) for twelve concertation levels in three independent replicates covering, in this way, the range between 0.1 and 100 ng mL^{-1}.

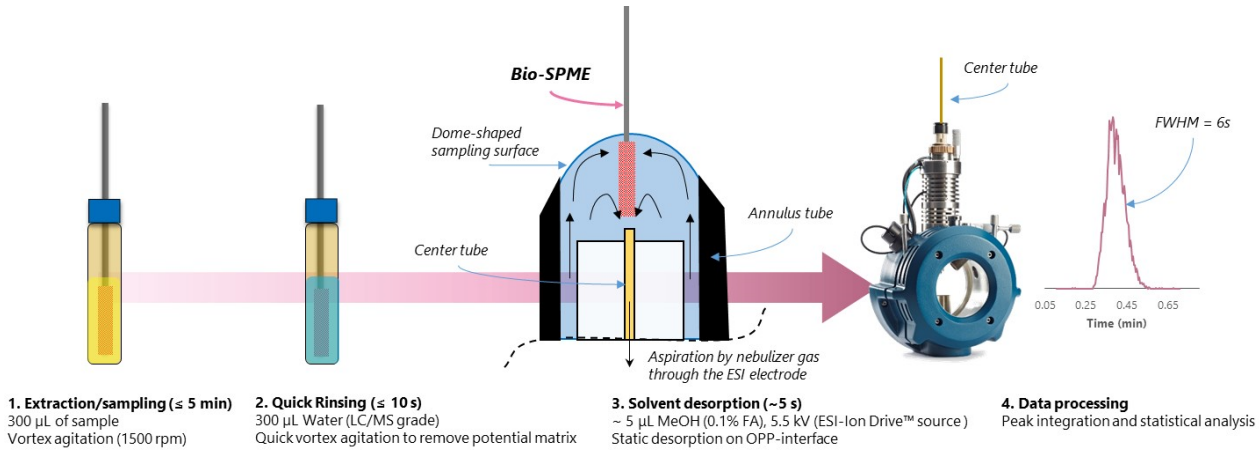

Figure 3.18 Experimental set-up for Bio-SPME extraction from complex matrices and desorption–ionization via OPP.

To determine the accuracy of the method, three validation points were evaluated. Although SPME fibers were used as consumables in this study (single use), it is worth mentioning that Bio-SPME fibers can be reused by implementing a cleaning step after the desorption/ionization cycle (*i.e.* mixture of methanol, isopropanol, and acetonitrile; 50:25:25). According to previous studies by Reyes-Garcés et al.[304], Souza-Silva et al.[143], and Musteata et al.[342], a Bio-SPME device can be reused at least 20 times. However, the reusability of the Bio-SPME fibre would depend on multiple factors (*e.g.* matrix of interest, effectiveness of the cleaning process, and efficiency of the desorption conditions). Therefore, in applications where fibres might be reused, the cleaning step should be carefully optimized according to both the matrix, the chemistry of the coating and its affinity towards the target compound.[148]

3.4.3 Results and discussion

SPME-OPP: a simple, fast, and sensitive coupling

Certainly, the greatest advantage of the OPP interface, particularly in comparison to other SPME direct couplings to MS,[173,174,182] is that it requires no modifications to the conventional ionization

source setup employed by most analytical labs, allowing the switch between LC-MS and OPP-MS to be achieved in just a few seconds. Indeed, the OPP herein described can be readily built by any skilled user, using mostly commercially available parts.[36] In addition, the OPP interface can be easily attached to the ESI/APCI source, and no expertise, other than that posed by an average LC/MS user, is required to operate it. Although a conventional LC pump was used in this study for solvent delivery, due to the low back pressure of the system (<30 psi), low-cost/back pressure pumps can also be implemented to reduce cost and avoid potential problems associated with high-pressure systems (e.g. leaking, expensive parts). Unlike nano-ESI emitters,[173,211,212] the OPP interface can be used for a long period of time with negligible inter-analysis carry-over owing to its continuous flowing principle.[36] In addition, the OPP interface is suitable for multi-SPME-fiber automation[161] and provides minimal risk of ionization interruption upon insertion of dry-fibers since it operates with large-size electrodes (*i.e.* 100-150 μm i.d. for ESI/APCI *versus* 1-2 μm i.d. for nano-ESI emitter; then, accounting for bubble formation). Building upon previous research,[36] the current study seeks to demonstrate that a noteworthy idea (direct-sample-introduction to MS by OPP) can be remarkably improved by using a sample preparation technology (*i.e.* Bio-SPME fibers) capable of isolating/enriching target analytes from complex matrices (*e.g.* biofluids, tissues, food samples) with minimal processing time (*i.e.* less than 2 minutes for the entire analytical process), and adequate sample clean-up (*i.e.* minimizing matrix effects and instrument contamination for long-term operation). Succinctly, the SPME-OPP coupling merges two attractive technologies to provide limits of quantitation at the low-part-per-billion level, or even part-per-trillion (depending on the analyte affinity for the coating and its ionization efficiency), in substantially short periods of time. Unlike the Open Probe device developed by Amirav's research group[30] for rapid coupling to electron ionization mass spectrometry, the OPP sampling interface

described in this study was designed for ESI or APCI. Although a triple quadrupole/linear ion trap was used in this study, the OPP system can also be coupled to any other mass analyzers such as single quadrupole, ion-traps, or time-of-flight as recently demonstrated by Van Berkel and collaborators[343]

Determination of controlled substances in urine samples

When monitoring controlled substances, such as in applications related to doping in sports, pain management, or abuse of illicit drugs, urine is most often selected as a matrix due to the non-invasive nature of its sample collection, and the large sample volumes available.[304] However, given the complexity of this matrix, as well as the low amount of parent drug excreted, analytical technologies capable of providing quantitative results in the sub-nanogram per milliliter range are highly desired. Recently, Reyes-Garcés et al.[131,304] and Boyacı et al.[148] demonstrated that different geometrical formats of SPME devices are capable of meeting the Minimum Required Performance Levels (MRPL) set by the World Anti-Doping Agency (WADA) for the analysis of multiple prohibited substances in urine by LC-MS. Although SPME-OPP coupling has yet to be perfected, still requiring parameters to be optimized (*e.g.* ideal fiber-coating thickness, strength of desorption solution, and shape of the open-port that allows for the smallest possible desorption volume), herein we demonstrated that limits of detection (LODs) in the sub-nanogram per millilitre range were achieved for fentanyl and buprenorphine upon performance of 2 min extractions from 300 µL of urine (Table 3.12, Figure 3.19). In addition, great accuracy (*i.e.* 93-108% at 3, 40, and 80 ng mL^{-1}, Figure 3.20) and linearity were attained for both analytes in the assessed range ($R^2 \geq 0.9987$). As can be seen in Table 3.12, our limits of quantitation (LOQ) are certainly below the MRPL values set for fentanyl and buprenorphine in urine (2 and 5 ng mL^{-1}, respectively[148]), which

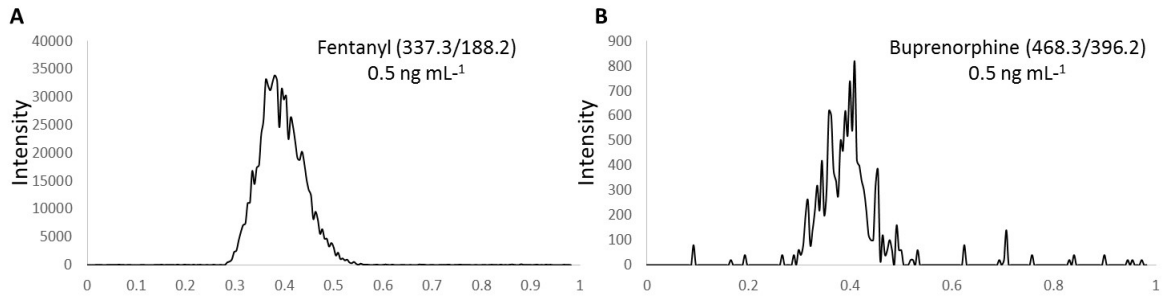

Figure 3.19 Fentanyl (**A**) and buprenorphine (**B**) ion-chronograms signals corresponding to a 5 min extraction at 1500 rpm from 300 µL of pooled urine spiked with 0.05 and 0.5 ng mL^{-1} of fentanyl and buprenorphine, respectively. Extractions were performed using a 4 mm Bio-SPME mix mode fiber.

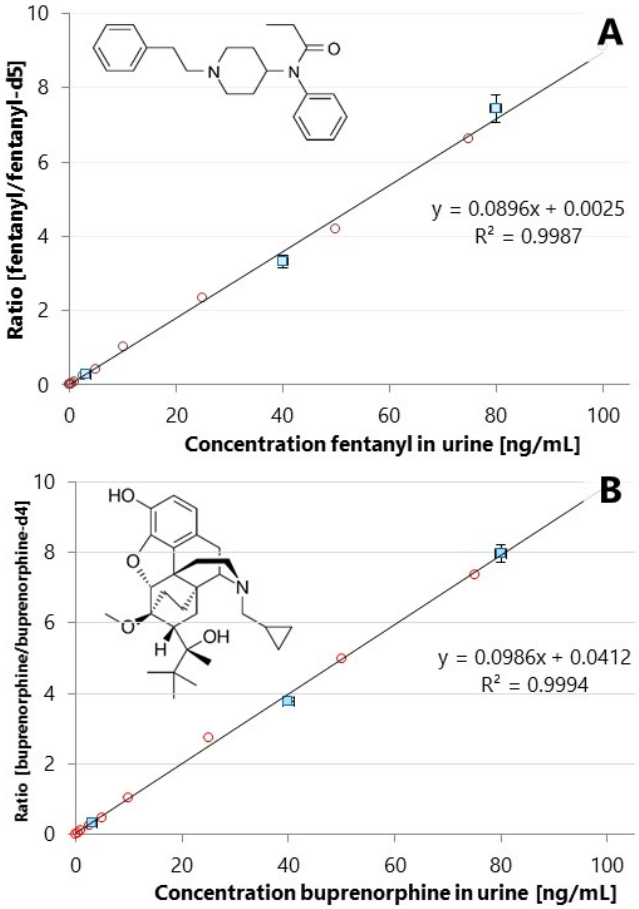

Figure 3.20 A. Quantitative analysis of urine spiked with fentanyl (50 pg ml^{-1} to 100 ng mL^{-1}) and its isotopologue [D5] fentanyl (10 ng mL^{-1}). **B.** Quantitative analysis of urine spiked with buprenorphine (500 pg ml^{-1} to 100 ng mL^{-1}) and its isotopologue [D4] buprenorphine (10 ng mL^{-1}). Blue squares represent the obtained accuracy levels (3, 40, and 80 ng mL^{-1}, respectively). Bars represent the standard deviation of analyses for three replicates with independent fibers.

Table 3.12 Figures of merit for the concomitant analysis of fentanyl and buprenorphine in urine by Bio-SPME-OPP in MRM mode.

Compound	Concentration accuracy (, ± SD)			LOD [ng/mL]	LOQ [ng/mL]
	3 [ng/mL]	40 [ng/mL]	80 [ng/mL]		
Fentanyl	3.2 ± 0.2	37.2 ± 1.4	83.1 ± 0.2	0.05	0.25
Buprenorphine	2.8 ± 0.1	37.9 ± 0.2	80.4 ± 0.3	0.25	0.50

proves the suitability of SPME-OPP-MS/MS for the quantitative determination of controlled substances in human urine.

Unlike fentanyl and buprenorphine, the quantitation of clenbuterol in urine is not a trivial task.[304] Indeed, in order to reach the required LOQ (0.2 ng mL^{-1}) for this compound, our group demonstrated that chromatographic separation in combination with tandem mass spectrometry are needed for the isolation of matrix interferences co-extracted by the SPME coating.[304] Although different direct sample-to-MS methods have reported the most abundant transitions (*m/z* 277→259 or 277→203) for the characterization/quantitation of clenbuterol in complex matrices,[111,257] our experience has shown that these transitions may lead to false positive results and untrustworthy LOQs (see Figure 3.21). Thus, aware of the extra challenges provided by the lack of a separation step, MRM^3 (*m/z* 277→259→168) was employed to overcome the lack of specificity encountered when exclusively performing MS/MS.[34,344,345] As shown in Figure 3.22, we demonstrated that Bio-SPME-OPP-MRM^3 can reach a LOQ value of 100 pg mL^{-1} upon performance of a 5 min extraction from 300 µL of urine spiked with clenbuterol. Furthermore, exceptional linearity in the range of 100 pg mL^{-1} up to 100 ng mL^{-1} and good accuracy (*i.e.* 82-96 %) at three different levels (*i.e.* 0.25, 2.5 and 75 ng mL^{-1}) were attained (see Table 3.13). Certainly, higher concentration levels are not a limitation. In cases where the affinity of the coating for the analyte is high and target compounds are present at concentrations larger than 100 ng mL^{-1}, shorter extraction times could be employed.

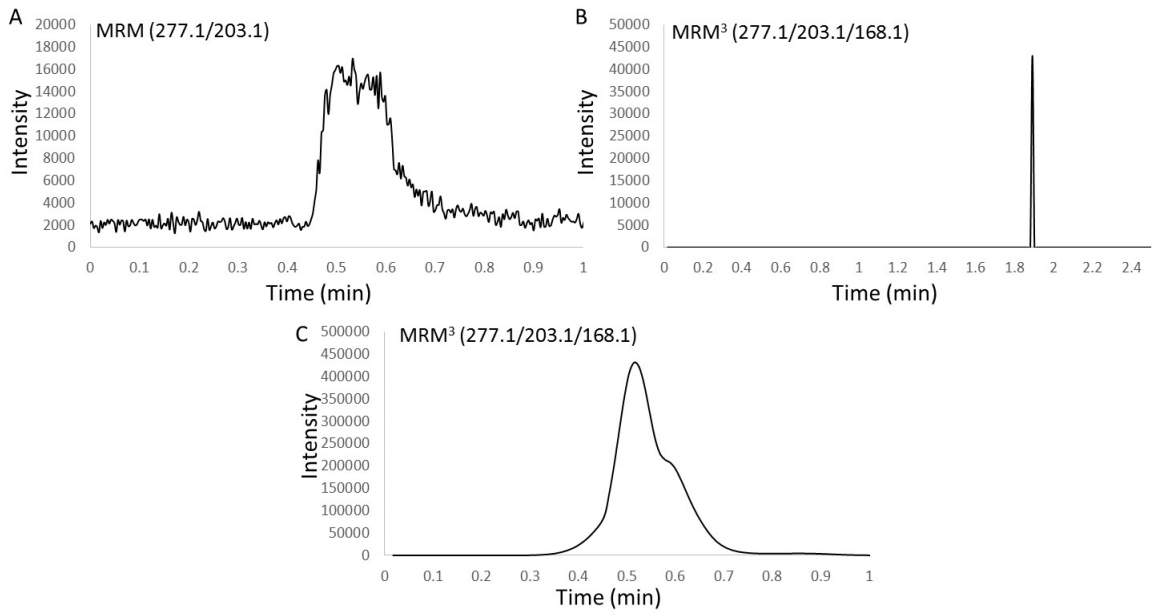

Figure 3.21 Ion-chronograms for clenbuterol obtained using (**A**) MRM (277.1-203.1), (**B**) MRM^3 (277.1-203.1-168.1) for a double-blank of urine (no standard or internal standard spiked) and (**C**) MRM^3 (277.1-203.1-168.1) for 0.2 ng/mL clenbuterol spiked in urine. Extraction conditions were 5 min extraction time and 1500 rpm from 300 µL of pooled urine, using a 4 mm Bio-SPME mix mode fiber.

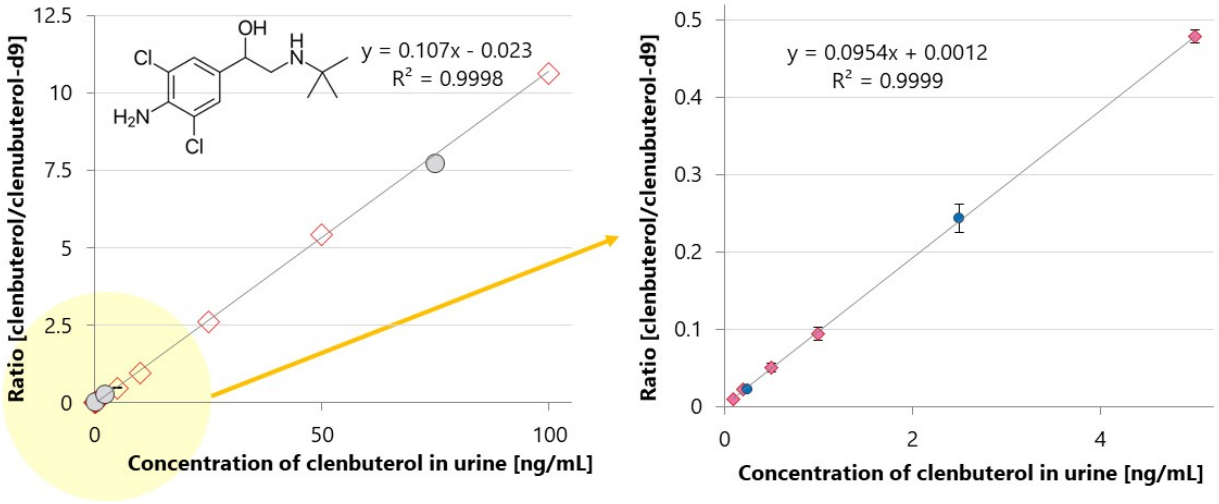

Figure 3.22 Quantitative analysis of urine spiked with clenbuterol (100 pg ml^{-1} to 100 ng mL^{-1}) and its isotopologue [D9] clenbuterol (10 ng mL^{-1}). Analyses were performed using SPME-OPP-MRM^3 (*m/z* 277→259→168). Blue circles represent the obtained accuracy levels (0.25, 2.5 and 75 ng mL^{-1}, respectively). Bars represent the standard deviation of analyses for three replicates with independent fibers.

Table 3.13 Figures of merit for the analysis of clenbuterol in urine by Bio-SPME–OPP-MRM^3

Compound	Concentration accuracy (± SD)			LOD [ng/mL]	LOQ [ng/mL]
	0.25 [ng/mL]	2.5 [ng/mL]	75 [ng/mL]		
Clenbuterol	0.3 ± 0.1	2.5 ± 0.2	73.1 ± 0.8	0.03	0.1

It is worth emphasizing that extraction on Bio-SPME fibers can also be performed under static conditions (*e.g.* during *in vivo* tissue analysis[113]), or at lower speeds of agitation. High-speed agitation was selected in this study with the aim to achieve the required LOQs with minimal total analysis time.[136,151]

In addition to MRM^3, it was demonstrated that DMS in combination with MS/MS could also be used for the quantitation of clenbuterol in urine via SPME-OPP. By tuning the adequate compensation voltage (CoV), transmission of the clenbuterol ions through the DMS cell can be achieved while co-extracted interferences are deflected.[341] As shown in Figures 3.23 and 3.24, no interference signals were detected and acceptable signal-to-noise ratio at the lowest quality control point tested (S/N ~ 4.8 for 0.25 ng mL^{-1}) was attained when using DMS.

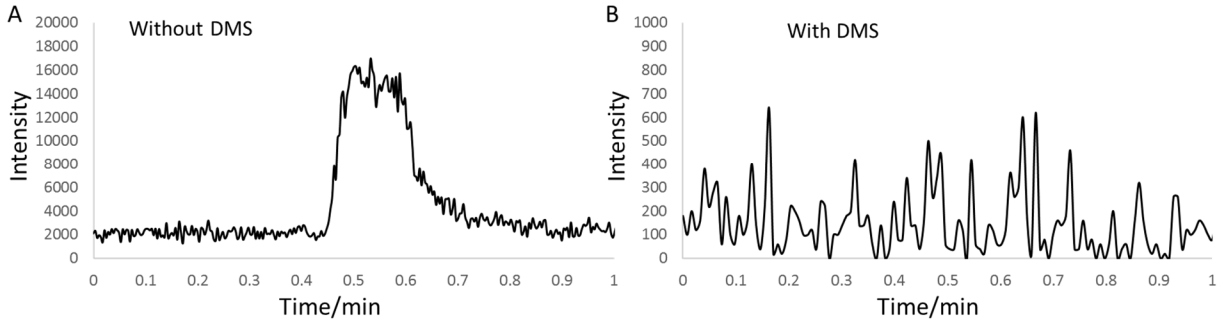

Figure 3.23 Ion-chronograms obtained for clenbuterol obtained using (**A**) only MRM (277.1-203.1) and (**B**) DMS and MRM (277.1-203.1) for a double-blank of urine (no-internal standard spiked). Extractions conditions were 5 min extraction time at 1500 rpm from 300 µL of pooled urine, using a 4 mm Bio-SPME mix mode fiber.

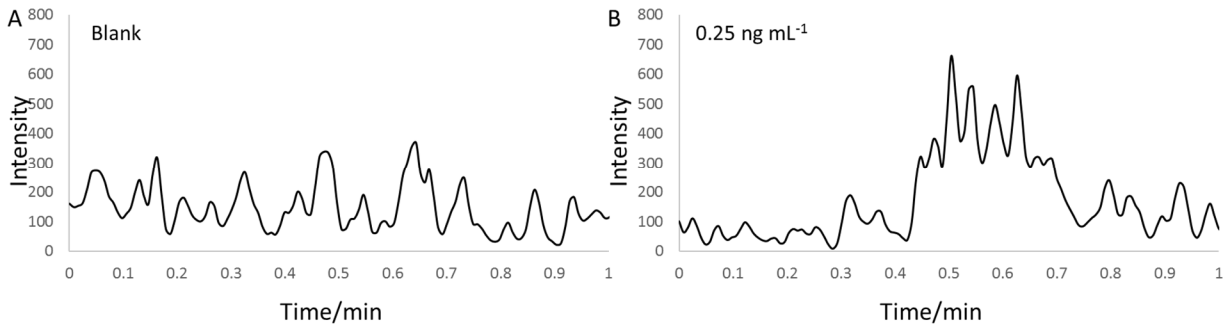

Figure 3.24 Ion-chronograms obtained using Bio-SPME-OPP and DMS-MS/MS. Extraction were performed from pooled urine blank (**A**) and pooled urine spiked with 0.25 ng mL^{-1} of clenbuterol (**B**). Extractions conditions were 5 min extraction time at 1500 rpm from 300 µL of pooled urine, using 4 mm Bio-SPME mix mode fiber.

Application of SPME-OPP towards analysis of tissue samples

Aiming to have a better understanding of the suitability of SPME-OPP for the study of tissue samples, extractions from agarose gel (2%) and homogenized brain tissue were performed. Both matrices were spiked with fluoxetine in concentrations ranging between 10 and 1000 ng mL^{-1}. Extractions were performed for 8 minutes using 4 mm mix-mode fibres, followed by a quick rinse in LC-MS water and quick wipe with a paper tissue. Right after sampling, fibres were stored at -80˚C until desorbed on the OPP interface. As can be seen in Figures 3.25 and 3.26, rewarding preliminary results were attained for the quantitation of fluoxetine. Future work will include determining the suitability of this approach to perform *in vivo* studies and validate a suitable calibration approach for such examinations. For instance, given the great sensitivity provided by SPME-OPP, one could consider the sampling rate approach, which allows performing calibration while within the linear region of the absorption profile[128]. The sampling-rate approach, validated under both laboratory and *in situ* environment, implies that the mass transfer rate remains constant throughout the duration of sampling, and that there is a simple relationship between the concentration of the target analyte in the sample matrix and the extracted amount of analyte[2,113].

Hence, in such scenario, the internal standard would need to be preloaded on the coating as to correct for potential variations during the extraction or ionization steps[346].

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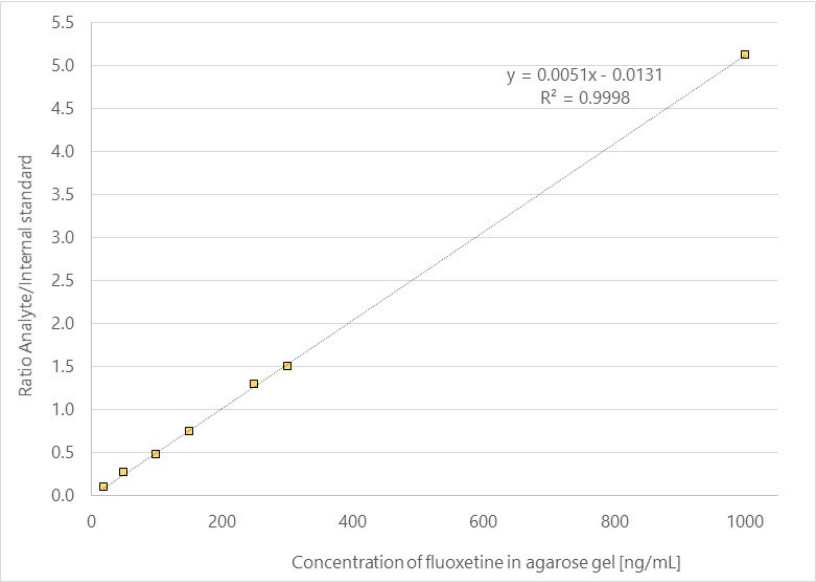

Figure 3.25 Quantitative analysis of agarose gel spiked with fluoxetine (10 ng ml^{-1} to 1000 ng mL^{-1}) and its isotopologue [D6] fluoxetine (200 ng mL^{-1}).

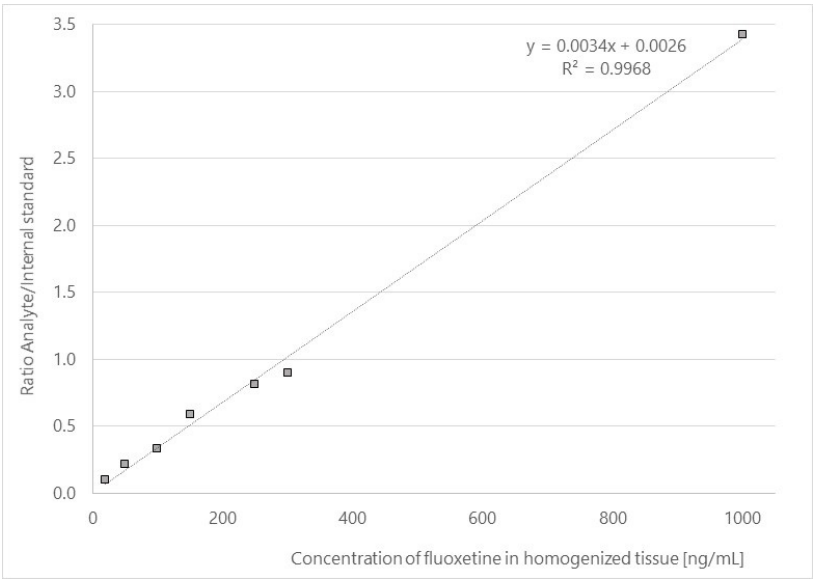

Figure 3.26 Quantitative analysis of homogenized brain tissue spiked with fluoxetine (20 ng ml^{-1} to 1000 ng mL^{-1}) and its isotopologue [D6] fluoxetine (250 ng mL^{-1}).

Similar to the work presented in Section 2.4, SPME-OPP can also be used for profiling studies of complex matrices. As a proof-of-concept, 30 minute extractions were performed from tissue samples from different animals purchased on a local market (*i.e.* salmon, beef, pork and chicken). After extraction, the fibres were rinsed in water, clean with a Kim-wipe and subsequently inserted on the interface. These studies were carried out on a SCIEX TripleTOF® 5600+ system located at SCIEX facilities. As can be seen in Figure 3.27 and 3.28, characteristic profiles of each kind and good separation between different tissues was attained on the PCA plot. Undeniably, these results are only a taste of multiple applications that can be carried on with this system. Certainly, by using SPME-OPP-HRMS with real-time recognition software, such the LiveID recently launched by Waters, the proposed profiling methodology has great potential to be used in food fraud investigations, microbe classification, and for rapid classification of tissue[5,61].

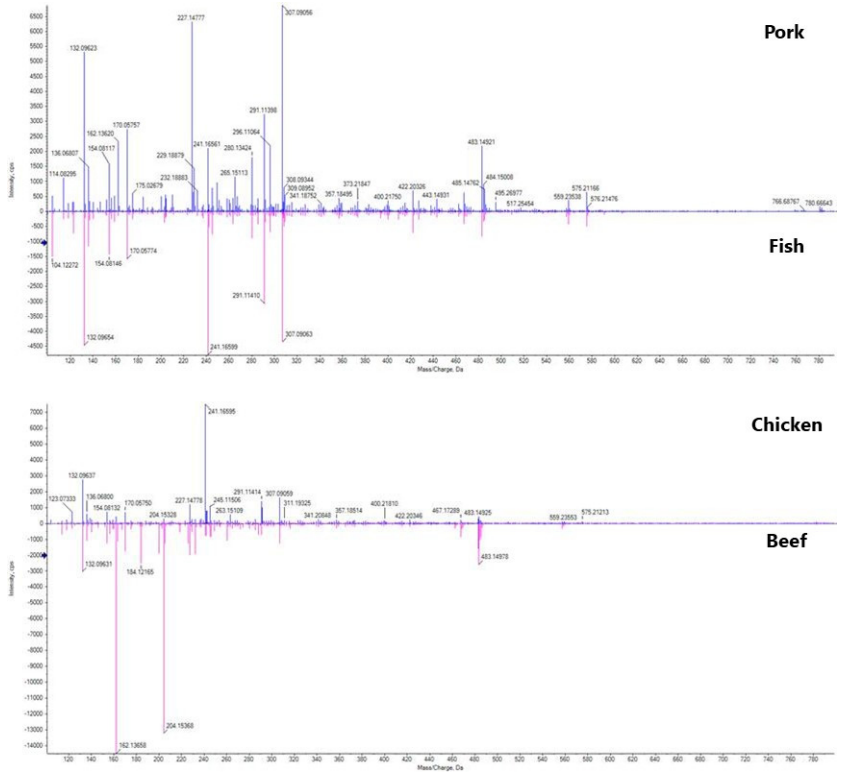

Figure 3.27 Mass spectra profile obtained after 30 min extraction from a piece of tissue of pork, salmon, chicken and beef.

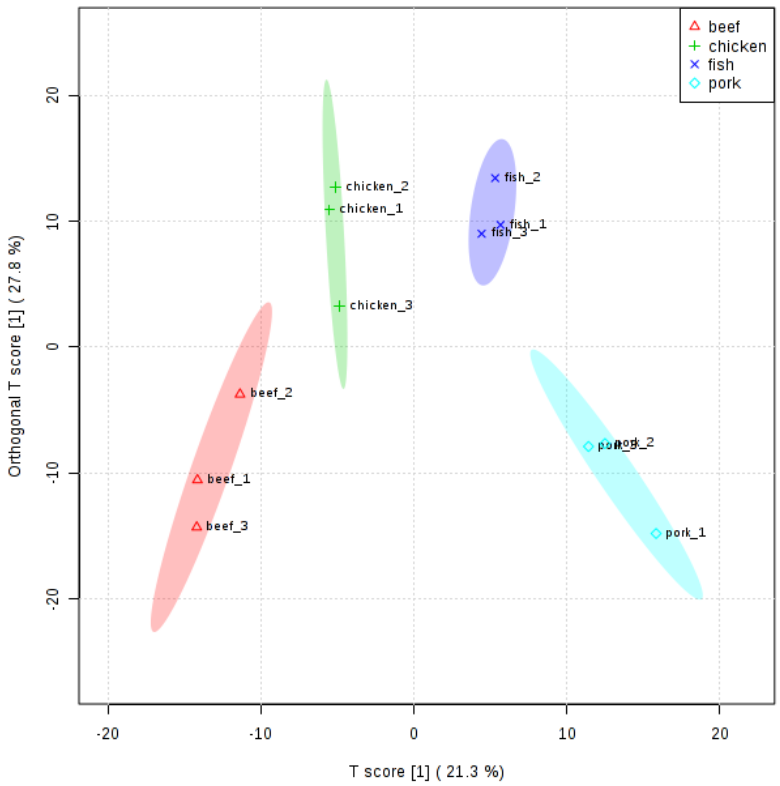

Figure 3.28 2 Three-dimensional PCA plot for identification of meat samples from different species (*i.e.* beef, pork, fish and salmon) using Bio-SPME-OPP-HRMS.

3.4.4 Summary

Unquestionably, application of Bio-SPME-OPP has tremendous potential in bioanalytical laboratories for fast determination of therapeutic drugs and prohibited-substances in complex matrices. Its suitability is owed to the multiple advantages it affords,[8,233,326] such as the simplicity of the coupling, its aptness for high-throughput analysis (*i.e.* less than 20 s per sample when running 96-samples at once), high sensitivity (*i.e.* sub-ng mL^{-1}), and moderate cost per analysis (*i.e.* reusability of the source and price of each SPME device).[131,304] Moreover, by using in-line technologies, such MRM^3 or DMS, enough selectivity enhancement can be achieved, thus making SPME-OPP a much faster alternative to classical LC-MS/MS based approaches. Certainly, the complete quantitation capabilities of SPME-OPP have yet to be discovered. Although this work

has been strictly focused on Bio-SPME fibers provided by the commercial vendor, fundamental work on SPME[155] has shown that lower LOQ values can be accomplished either by using SPME devices with a larger coated surface area (*e.g.* blades, mesh, or membranes [146,151,192]), increasing extraction time, enhancing the affinity of the coating for the analyte, or consuming larger sample volumes. Consequently, our future work will focus not only on achieving better figures of merit by employing novel SPME geometries amenable to OPP, but also in exploiting the versatility of analytes and matrices that can be analyzed by having ESI/APCI capabilities and DMS integrated into a single source. We foresee the combination of SPME-OPP with robotic platforms for non-assisted time-resolved mass spectrometry applications,[334] as well its implementation for fast determination of exogenous/endogenous compounds extracted *in vivo* at the surgery room.[5]

Section 3.5 Fast quantitation of opioid isomers in human plasma by differential mobility spectrometry/mass spectrometry via SPME/open-port probe sampling interface

3.5.1 Introduction

Simple, robust and high-throughput sample preparation workflows capable of yielding reliable quantitative analysis results are highly desired for the determination of pain-management substances and drugs of abuse in biological fluids[233]. Although dilute-and-shoot procedures have been evaluated for this purpose[21,22], most assays still require a sample clean-up step (e.g. solid-phase extraction or liquid-liquid extraction) for removal of biological matrix components that could cause contamination of the chromatographic column or ionization suppression[41]. Solid-phase microextraction (SPME), a green technology that combines sampling, sample clean-up, and analyte enrichment into a single step, has been widely used for high-throughput sample preparation with minimum handling[173], including in-vivo sampling, and on-site monitoring of several substances in different environmental, clinical and food applications.[113,228] Although SPME devices are capable of performing fast sample extraction from biological matrix, chromatographic separation (i.e. GC or HPLC) still is the platform of choice for the analysis of drugs of abuse, because the specificity may suffer from the loss of a chromatographic dimension (e.g. interferences can be observed when analyzing isomeric species with common fragment ions such as codeine and hydrocodone).[233,347] Therefore, the chromatography process has become the bottleneck to further improve the analysis throughput.[348]

Differential mobility spectrometry (DMS) is a gas-phase ion separation technology based on the mobility difference of target ions under high- and low-electrical fields,[341,349] and it has been proven to be a powerful tool to improve the selectivity of LC-MS analysis and remove endogenous

chemical interferences from samples to yield significant improvements in S/N and limits of detection.[350] In this study, a novel DMS device is introduced for the in-line separation and quantitation of isomeric opioids with shared fragment ions (*i.e.* codeine and hydrocodone, Figure 3.29) without requiring chromatographic separation. An open-port probe (OPP) sampling interface[36,343] was used to couple the high-throughput sample preparation based on SPME and the fast analysis based on DMS/MS. Employment of the OPP system allows merging the steps of analyte desorption and elution into the instrument by simply placing the SPME device in the open-end of the system for several seconds, without employment of high-pressure parts or injection valves. Good sensitivity, accuracy, and precision were demonstrated for the targeted analytes, with total analysis time per sample ranging between 10-15 seconds when performed by high-throughput means.

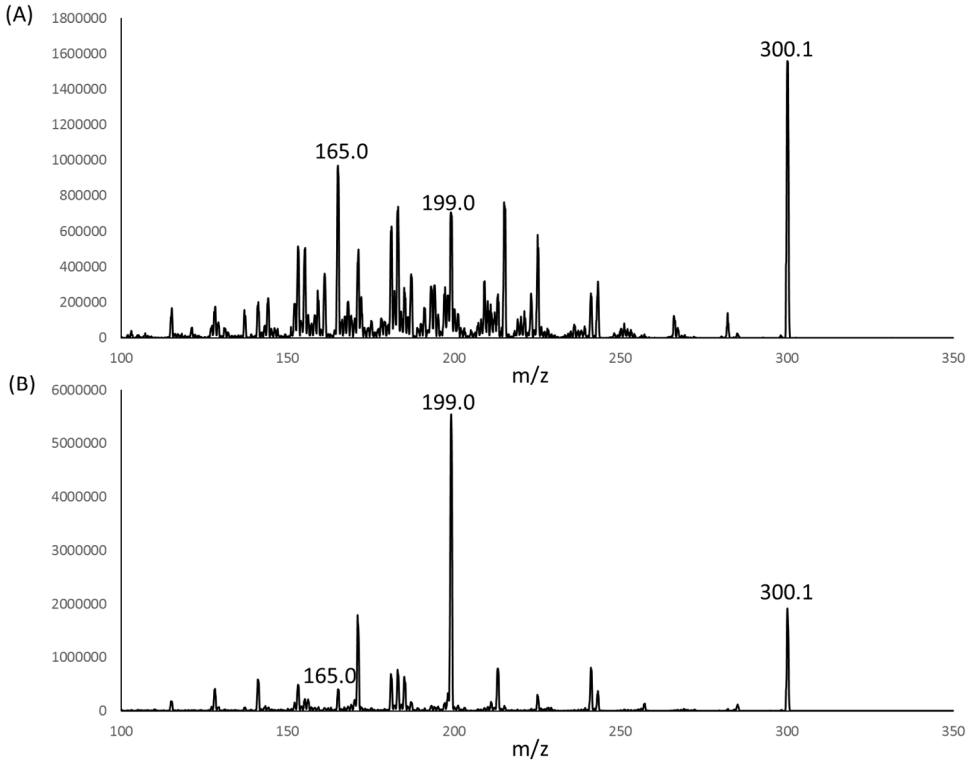

Figure 3.29 Product ions (MRM) of codeine (A) and hydrocodone (B) with 45 eV collision energy.

3.5.2 Experimental section

Reagents and supplies

Codeine, hydrocodone and their deuterated analogues were purchased from Cerilliant (Round Rock, TX, USA), and used without further purification. HPLC grade acetonitrile and methanol were bought from Caledon Laboratory Chemicals (Georgetown, ON, Canada). Distilled deionized water (18 MΩ) was produced in-house using a Millipore (Billerica, MA, USA) Integral 10 water purification system. Plasma samples were purchased from Bioreclamation IVT (Baltimore, MD, USA). Target analytes and internal standards (deuterated analogues) were spiked in the plasma (v/v) the day before extraction. Bio-SPME mixed mode probes (*i.e.* C_{18}-SCX particles, 45 µm thickness, 15 mm coating length) were kindly provided by Millipore-Sigma (Supelco; Bellefonte, PA, USA).

Open-port probe sampling interface

The OPP sampling interface used in this work was constructed based on the design introduced by Van Berkel and co-workers[36], and the device is described in Figure 3.17. This OPP sampling interface uses a vertically aligned, co-axial tube arrangement enabling solvent delivery to the sampling end (open-port) through the tubing annulus (304 stainless steel, 1.75 mm i.d. and 3.18 mm o.d., Grainger, Lake Forest, IL, USA), and aspiration down the centre tube (PEEK, 255 µm i.d. and 510 µm o.d., IDEX, Lake Forest, IL, USA) into the ion source driven by the nebulizer gas (fixed at 90 psi) through a 635 µm i.d. nozzle, and the ESI electrode i.d. was 150 µm. The rate of the flow-in desorption solvent (methanol) was adjusted (Perkin Elmer 200 LC pump, Waltham, MA, USA) to achieve a dome-shaped sampling surface to maximize the contact area with SPME coatings (fixed at 200 µL min^{-1}).

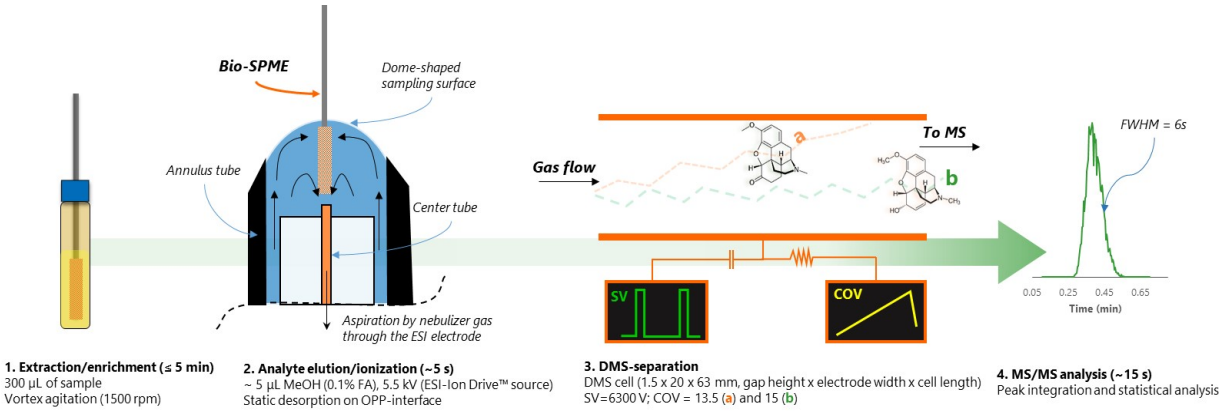

Figure 3.30 Workflow for Bio-SPME-OPP-DMS-MS/MS analysis of controlled substances from complex matrices.

Sample preparation with SPME and OPP sampling

The workflow of SPME based sample preparation consists of several steps including fibre conditioning, analyte extraction, fibre rinsing, analyte desorption and analyte elution into the mass spectrometer. By using OPP as the MS interface for SPME, the two latter steps are combined (see Figure 3.30), allowing for better analysis throughput and enhanced sensitivity (i.e. minimal analyte dilution). In addition, since the step of solution transfer is eliminated, chances of additional sample loss are avoided. In this study, a preconditioned SPME fibre was first inserted in a vial containing plasma sample (250 µL) for 5 min with vortex agitation (1500 rpm). Next, the fibre with extracted target analytes was rapidly rinsed in a vessel containing 0.3 mL water for 5 seconds to remove matrix components that could have potentially adhered to the coating surface. For the above two steps, multiple samples were processed simultaneously aiming to increase the analysis speed and reduce sample-to-sample variations. Finally, the SPME fibre was placed into the sampling port containing dome-shaped continuous-flow desorption solvent (i.e. methanol) for 5 seconds, and the desorbed analytes were carried to the ESI electrode for ionization.

Table 3.14 Mass spectrometry parameters used to monitor each analyte in the CoV ramp test.

Compound	Parent	Product	DP [V]	CE [V]	EP [V]	CXP [V]
Codeine	300.1	165.0	200	50	10	15
Hydrocodone	300.1	199.0	200	40	10	15
Norcodeine	286.1	268.1	180	40	10	11
Morphine	286.1	268.1	180	40	10	11
Hydromorphone	286.1	268.1	180	40	10	11

Table 3.15 DMS-MS parameters used to monitor codeine and hydrocodone in the SPME-OPP-DMS-MS experiments.

Compound	Parent [m/z]	Product [m/z]	SV	CV	DR
Codeine	300.1	165.0	6300	14.5	0
Hydrocodone	300.1	199.0	6300	16.5	0

DMS-MS system

A research grade DMS system (dimension of the DMS cell was 1.5 x 20 x 63 mm, gap height x electrode width x cell length) developed by SCIEX was mounted in the atmospheric region between the Turbo V^{TM} ion source (ESI) and the QTRAP® 5500 system's (SCIEX, Concord ON, Canada) sampling orifice. The ESI probe was maintained at a voltage of 5500 V. A constant gas flow in the DMS cell was achieved by the curtain gas flow (N_2; 20 psi, 5.5 L min^{-1}) and the primary stage vacuum pumping of the MS system. Nitrogen was also used as the throttle gas. The temperature of the transport gas was set at 100 °C. The analysis with conventional DMS cell was performed on a QTRAP® 6500+ system equipped with SelexION+^{TM} technology. The fundamental behaviour of DMS and the asymmetrical SV waveform has been thoroughly described in previous studies[341]. For the experiments conducted in this study, the optimal compensation voltage (CoV) for the ion transmission was determined first by scanning the CoV from 5 V to 20 V in 0.15-V increments with the separation voltage (SV) set at 6300 V (p-p). Analytes were dissolved in solution (methanol) to a concentration of 100 ng mL^{-1}, and infused into

the ESI source at a rate of 10 μL min^{-1}. During every CoV step, multiple-reaction monitoring (MRM) signals for each analyte (Table 3.14) were recorded, yielding an ionogram. The optimal CoV for the transmission of each analyte through the DMS device was determined and used for the SPME-OPP sampling experiments as shown in Table 3.15 (fixed SV and CoV settings for each individual ions without scanning process).

3.5.3 Results and discussion

Isomeric separation with DMS

DMS is a technology that can be used to separate gas-phase ions prior to analysis by MS.[341,351] In a DMS cell, a high frequency asymmetric waveform (SV), varying between high- and low-electric field regimes is applied across the ion transport channel between the two planner electrodes, perpendicular to the direction of the ion transport flow. The different mobilities exhibited under high- and low-electric field results in ions acquiring a "zigzag" trajectory between the electrodes. For successful transmission through the DMS cell and sampling into MS, a DC CoV can be applied to steer ions back on axis. This technology has been successfully used for both chemical noise elimination and isomeric ion separation (e.g. stereoisomers, structural isomers, and tautomers)[341]. Several approaches have been introduced aiming to enhance the resolving power of DMS, by either increasing the CoV difference (ΔCoV) or reducing the peak width in volts at half height (FWHM) in a CoV ramp scan. The modified transport gas has been widely used to improve the resolving power of DMS by adding a polar solvent (modifier) to the curtain gas[352]. Therefore, the analyte ions cluster with modifier molecules during the low-field portion of the waveform and undergo de-clustering during the high-field half-cycle, thus amplifying their differential mobilities with increased ΔCoV (*i.e.* better separation). Alcohols (e.g. methanol, isopropanol) are most frequently

used as modifiers, but their high proton affinity may induce a charge stripping from the analyte, which leads to a lower sensitivity.[353] The reduction of FWHM of a CoV peak is another approach for resolving power enhancement that is typically achieved by extending the ions' residence time within the DMS cell by reducing transport gas flow rate with the throttle gas.[354] It is important to highlight that although better separation can be attained, long in-cell ion residence time also directly leads to significant ion losses. In certain applications, chemical modifier and a throttle gas can be used simultaneously to further improve DMS resolving power.[355]

In this study, an alternative approach to enhance resolving power was developed by modifying the dimensions of the DMS cell from 1 mm x 10 mm x 30 mm (gap height x electrode width x cell length) to 1.5 mm x 20 mm x 63 mm. The enlarged volume of the DMS cell extends the residence time, which reduces the FWHM of studied analytes from 3.0 V to 0.8 V (Figure 3.31) without application of a throttle gas. Similar FWHM could be reached with the original cell (commercial cell with smaller volume) using 34 psi of throttle gas. However, due to the small ΔCoV attained with the commercial cell (0.8 V), even when using a throttle gas of 34 psi, it was not possible to detect codeine and hydrocodone individually at their optimum sensitivity (CoV peak apex). As an alternative, the CoV can be enlarged by increasing the gap height of the DMS cell. In the presented study, owing to the larger gap between the two electrodes, CoV as well as ΔCoV were increased to 1.5x for the same field strength. Thus, the combined benefits of both reduced FHWM and increased ΔCoV enabled the complete separation of these analytes (Figure 3.32). As can be seen in Figure 3.33, the interference between codeine and hydrocodone analysis was observed to be less than one percent. Although adequate separation for these isomers was accomplished by only modifying the geometry of the DMS cell, a chemical modifier and/or throttle gas can also be implemented for more challenging applications.

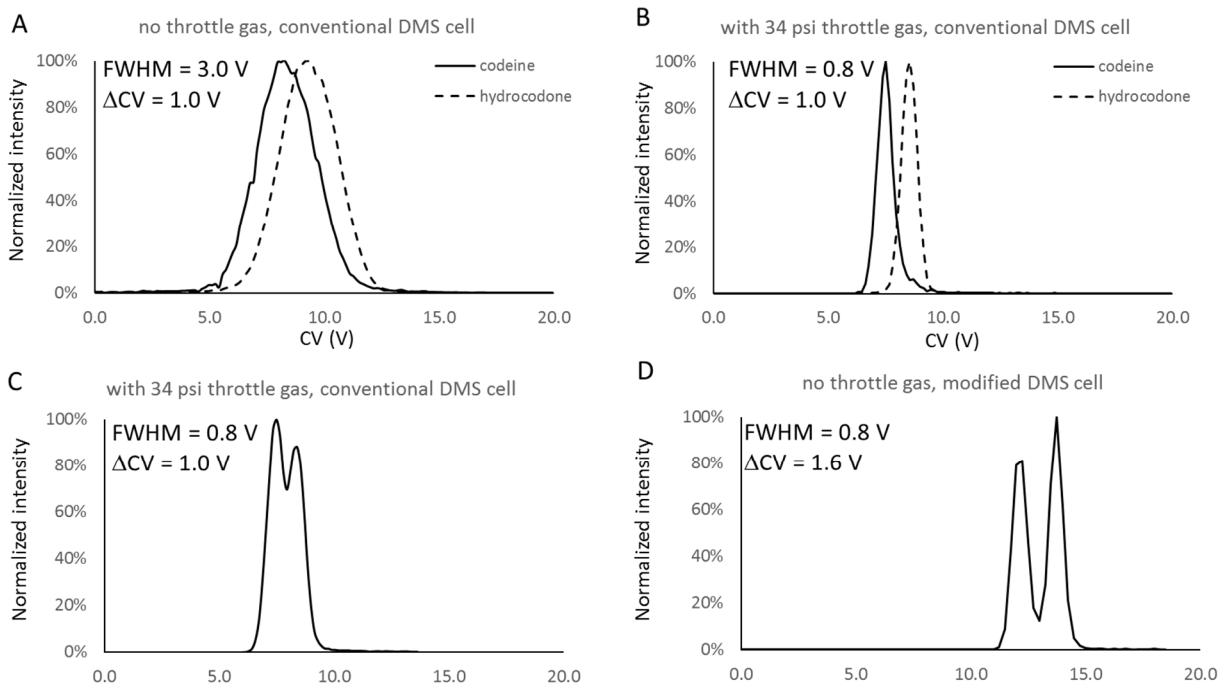

Figure 3.31 A. Overlapping ionograms generated from infusion of codeine or hydrocodone individually with the conventional DMS cell (1 x 10 x 30 mm) without applying throttle gas. SV was set at 4000 V. **B.** Overlaid ionograms generated from infusion of codeine or hydrocodone individually with the conventional DMS cell (1 x 10 x 30 mm) with 34 psi throttle gas applied. SV was set at 4000 V. **C**. Ionograms generated from infusion of a mixture of codeine and hydrocodone with the same condition of B. SV was set at 4000 V. **D**. Ionograms generated from infusion of a mixture of codeine and hydrocodone with the modified DMS cell (1.5 x 20 x 63 mm) without applying throttle gas. SV was set at 6000 V (same field strength with A, B, C).

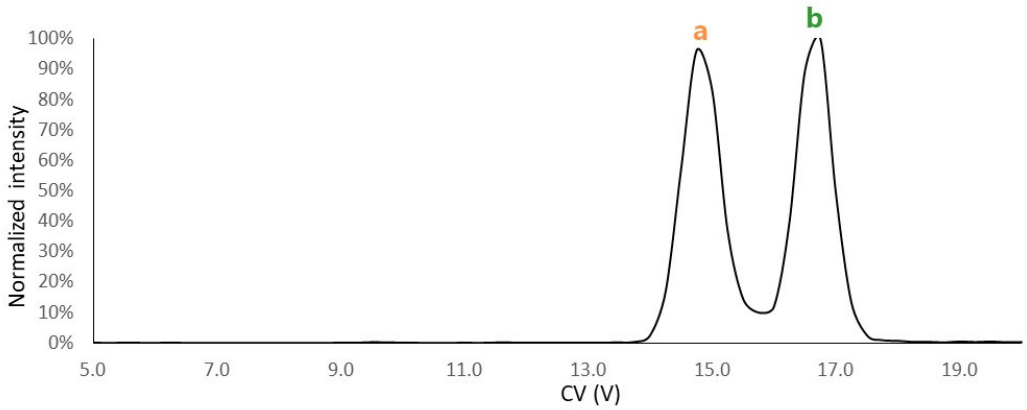

Figure 3.32 Ionogram generated from infusion of a methanolic solution containing a mix of codeine (a) and hydrocodone (b). The SV was set as 6300 V.

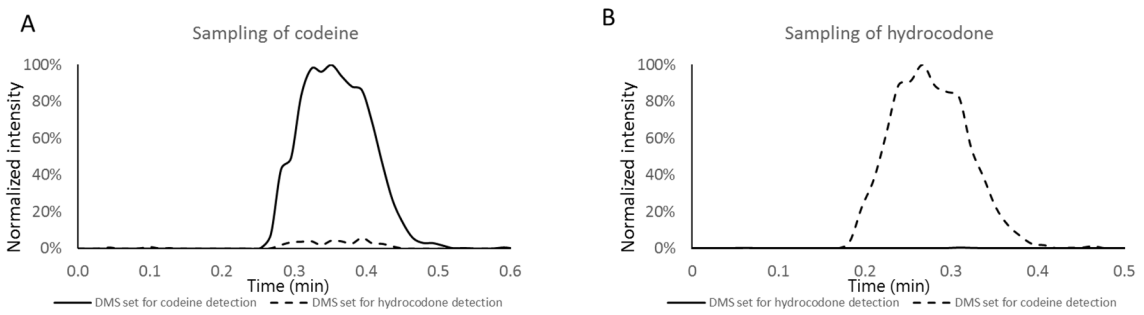

Figure 3.33 A. Ionograms of analytes extracted by an SPME fiber from human plasma spiked with 480 ng mL^{-1} codeine, with the optimal settings for the detection of codeine and hydrocodone, respectively. **B**. Ionograms of analytes extracted by an SPME fiber from human plasma spiked with 480 ng mL^{-1} hydrocodone, with the optimal settings for the detection of codeine and hydrocodone, respectively.

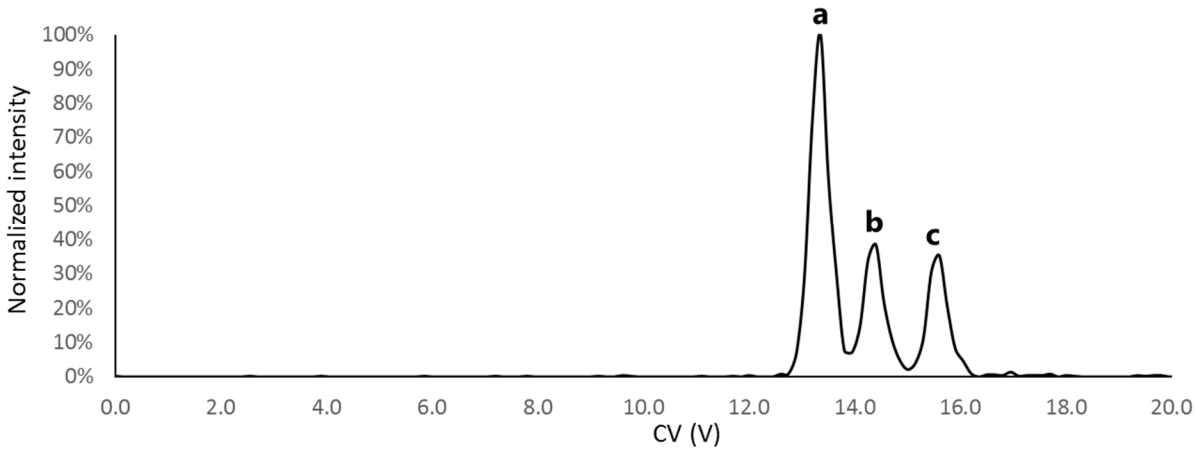

Figure 3.34 Ionogram generated from the infusion of a methanolic solution containing mixture of norcodeine (**a**), morphine (**b**) and hydromorphone (**c**). SV was set at 6300 V with psi throttle gas applied.

As an example, Figure 3.34 presents the separation of other opioid isomers (*i.e.* norcodeine, morphine, and hydromorphone) with shared fragment ions on the modified DMS cell with a 10 psi throttle gas.

Isomeric separation with DMS

The simultaneous quantification of codeine and hydrocodone from human plasma was chosen as the example assay to demonstrate the potential of the introduced SPME-OPP-DMS-MS platform

for the fast analysis of pain management drugs from biological matrix. As shown in Figure 3.35, our results met the analytical requirements set by the World Anti-Doping Agency (WADA). A limit of quantification (LOQ) of 1 ng mL^{-1} and linear dynamic range between 1 and 500 ng mL^{-1}, were demonstrated with good accuracy (85-106%) and linearity ($R^2 > 0.99$) (Table 3.16). Given that SPME devices can be arrange in a high-throughput assembly (*e.g.* 96 samples at the same time),[131] and there is no need for additional separation step other than that offered by DMS, the analysis can be as fast as 10-15 seconds per sample.

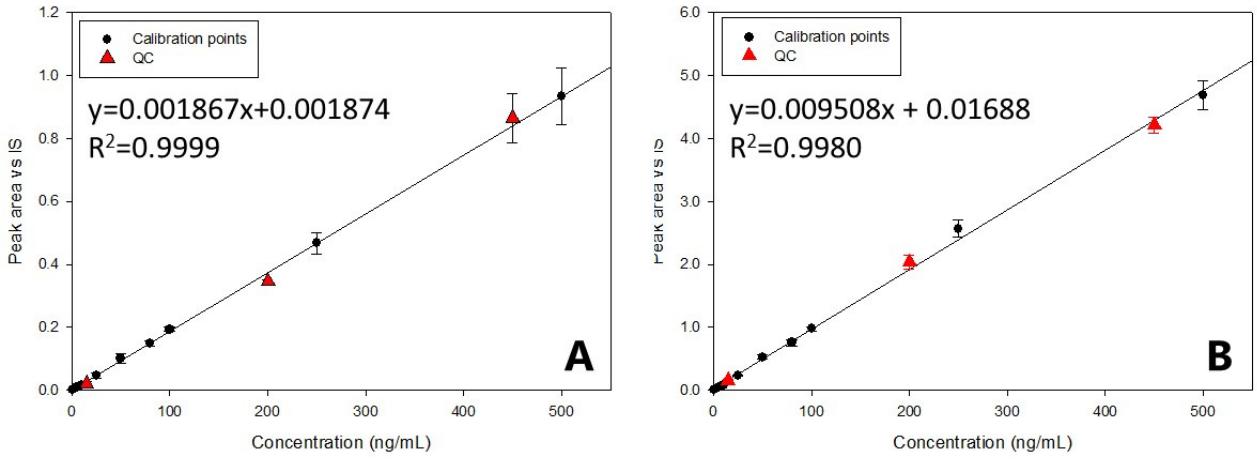

Figure 3.35 A. Quantitative analysis of plasma spiked with codeine (1 ng ml^{-1} to 500 ng mL^{-1}) and its isotopologue d3-codeine (50 ng mL^{-1}). **B**. Quantitative analysis of plasma spiked with hydrocodone (1 ng ml^{-1} to 500 ng mL^{-1}) and its isotopologue d3-hydrocodone (50 ng mL^{-1}). Red triangles represent the accuracy QC levels (30, 200, and 450 ng mL^{-1}, respectively).

Table 3.16 Figures of merit for the analysis of codeine and hydrocodone in plasma by SPME-OPP-DMS-MS analysis.

Compound	Concentration accuracy (ng/mL, ± SD)			LOD [ng/mL]	LOQ [ng/mL]
	15 [ng/mL]	200 [ng/mL]	450 [ng/mL]		
Codeine	12.7 ± 2.1	184.6 ± 9.8	476.0 ± 13.7	0.2	1.0
Hydrocodone	15.8 ± 2.7	212.7 ± 11.3	440.7 ± 12.7	0.5	1.0

3.5.4 Summary

A simple, robust and high-throughput technology based on SPME and DMS-MS was introduced for quantitative analysis of drugs of abuse and pain-management drugs in biofluids. Following extraction, SPME devices with isolated and enriched target analytes were directly coupled to the MS system via an OPP sampling interface, without further requirement of injection valves or high pressure parts. A modified DMS cell, with significantly improved resolving power, successfully distinguished isomeric species with shared fragment ions such as codeine and hydrocodone, without the assistance of lengthy LC gradients. The results presented here demonstrate the great potential of coupling SPME to DMS-MS in targeted analysis where regular SPME-MS, without the assistance of chromatographic separation, might fail to provide a true picture of the sample scrutinized. Similarly, this platform could be used for the application of SPME-OPP-DMS-MS towards lipids and metabolites profiling in the future.[5,29,356]

Chapter 4 Development of Coated Blade Spray and its application towards the analysis of complex matrices

4.1 Preamble

Chapter 4 consists of 5 sections that correspond to four manuscripts published in Angewandte Chemie (2), Analytical Chemistry, and Journal of Pharmaceutical and Biomedical Analysis, two submitted manuscripts and one manuscript in preparation. Most of the data, tables, and text presented within this chapter have already been incorporated in the aforementioned manuscripts, the details of which are listed below. Section 4.2 describes the development of Coated Blade Spray and its first implementation towards the quantitation of xenobiotics in PBS, urine, and plasma samples. Likewise, this section presents preliminary information regarding the fundamental operation of CBS. Section 4.3 describes the first application of CBS towards the determination of amitriptyline in droplets of blood. Further, the following chapter presents the development of a novel strategy that allows for the quantitation of multiple compounds at low part-per-billion levels in plasma and blood droplets. Section 4.4 presents the implementation of these technologies towards the ultra-fast quantitation of voriconazole in plasma samples, with achieved MS analysis times of less than 5 seconds per sample. Section 4.5 presents the development of a novel device that allows for the simultaneous performance of 96-CBS extractions, resultantly reducing total analysis time per sample to under 1 minute. Finally, section 4.6 introduces the application of HT-CBS towards the analysis of immunosuppressive drugs in blood samples. This method introduces the first instance where extraction with an SPME device is carried out from a matrix modified with organic solvent, a practice which was implemented with aims to release analytes heavily bound to red-blood cells, such as sirolimus and everolimus.

Section 4.2 include the following manuscripts:

1. Gómez-Ríos, G.A, Pawliszyn, J. *Development of coated blade spray ionization mass spectrometry for the quantitation of target analytes present in complex matrices*, Angew. Chem. 2014, 53, 14503-14507, DOI: 10.1002/anie.201407057

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I participated at all stages of the manuscript preparation process: the planning of experiments, manufacturing of devices, method development, sample preparation and analyte extraction procedures, data processing, manuscript writing and submission, as well as in manuscript replies to reviewers.

2. Piri-Moghadam, H.*, Ahmadi, F.*, Gómez-Ríos, G.A.*, Boyacı, E., Reyes-Garcés, N., Aghakhani, A., Bojko, B., Pawliszyn, J. *Fast Quantitation of Target Analytes in Small Volumes of Complex Samples by Matrix-Compatible Solid-Phase Microextraction Devices*, Angew. Chem. 2016, 128, 7636-7640, DOI: 10.1002/ange.201601476. *Equal contribution

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I participated at all stages of the manuscript preparation process: the planning of experiments, manufacturing of the ionization source, manufacturing of CBS devices, method development for CBS-MS/MS and HRMS, sample preparation and analyte extraction procedures, data processing, manuscript writing and submission, as well as in manuscript replies to reviewers. Dr. Ezel Boyacı and Nathaly Reyes-Garcés participated in the manufacturing of CBS devices used in the

manuscript, and in the performance of selected experiments for direct coupling. Other authors, as listed in Chapter 3, did not participate in the CBS experiments mentioned in this chapter.

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Section 4.3 includes the following manuscript:

3. Tascon, M.*, Gómez-Ríos, G.A*, Reyes-Garcés, N., Boyacı, E., Poole, J., Pawliszyn, J. *Ultra-fast quantitation of voriconazole in human plasma by coated blade spray mass spectrometry.* J Pharmaceut. Biomed., 2017, 144, 106-111, DOI: 10.1016/j.jpba.2017.03.009. *Equal contribution

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I participated at all stages of the manuscript preparation process: the planning of experiments, manufacturing of CBS devices, method development for determination of voriconazole, sample preparation and analyte extraction procedures, data processing, manuscript writing and submission, as well as in manuscript replies to reviewers. Dr. Marcos Tascon participated in the planning of experiments, in sample preparation procedures, and manuscript writing and

submission. Dr. Ezel Boyacı, Justen Poole, and Nathaly Reyes-Garcés participated in the manufacturing of CBS devices used in the manuscript.

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Section 4.4 includes the following manuscript:

4. Tascon, M.*, Gómez-Ríos, G.A*, Reyes-Garcés, N., Boyacı, E., Poole, J., Pawliszyn, J. *High-Throughput Screening and Quantitation of Target Compounds in Biofluids by Coated Blade Spray-Mass Spectrometry.* Anal. Chem., 2017, 89 (16), 8421-8428, DOI: 10.1021/acs.analchem.7b01877. *Equal contribution

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I participated at all stages of the manuscript preparation process: the planning of experiments, manufacturing of the high-throughput holder, method development for CBS, in sample preparation and analyte extraction procedures, data processing, manuscript writing and submission, as well as in manuscript replies to reviewers. Dr. Marcos Tascon participated in the planning of experiments, sample preparation procedures, and manuscript writing and submission. Dr. Ezel Boyacı, Justen Poole and Nathaly Reyes-Garcés participated in the manufacturing of CBS devices used in the manuscript.

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Section 4.5 includes the following manuscript:

5. Gómez-Ríos, G.A*, Tascon, M.*, Reyes-Garcés, N., Boyacı, E., Poole, J., Pawliszyn, J. *Rapid determination of immunosuppressive drug concentrations in whole blood by Coated Blade Spray-Tandem Mass Spectrometry (CBS-MS/MS).* (manuscript submitted), DOI: (pending). *Equal contribution

I participated at all stages of the manuscript preparation process: the planning of experiments, method development for immunosuppressive drugs, in sample preparation and analyte extraction procedures, data processing, manuscript writing and submission, as well as in manuscript replies to reviewers. Dr. Marcos Tascon participated in the planning of experiments, in the sample preparation procedure, and in the writing and submission of this manuscript. Nathaly Reyes-Garcés participated in the preparation of the CBS devices, as well as in the planning and execution of selected experiments. Dr. Ezel Boyacı and Justen Poole participated in the manufacturing of CBS devices used in the manuscript.

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Section 4.6 include the following manuscripts:

6. Gómez-Ríos, G.A*, Tascon, M.*, Reyes-Garcés, N., Boyacı, E., Poole, J., Pawliszyn, J. *Quantitative analysis of biofluid spots by coated blade spray mass spectrometry, a new approach to rapid screening.* (manuscript submitted), DOI: (pending). *Equal contribution

I participated at all stages of the manuscript preparation process: the planning of experiments, method development for spot analysis, sample preparation and analyte extraction procedures, data processing, manuscript writing and submission, as well as in manuscript replies to reviewers. Dr. Marcos Tascon participated in the planning of experiments, in the sample preparation procedure, and in the submission of the manuscript. Dr. Ezel Boyacı, Justen Poole, and Nathaly Reyes-Garcés participated in the manufacturing of CBS devices used in the manuscript.

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7. Gómez-Ríos, G.A*, Tascon, M.*, Reyes-Garcés, N., Aquaro, V., Rickert, D., Kasperkiewicz, A., Pawliszyn, J. *Lab-on-a-blade.* (manuscript in preparation) *Equal contribution

I participated at all stages of the manuscript preparation process: the planning of experiments, method development for analysis of ultra-small volumes, sample preparation and analyte extraction procedures, data processing, manuscript writing and submission, as well as in manuscript replies to reviewers. Dr. Marcos Tascon participated in the planning of experiments, in the sample preparation procedures, and in the submission of the manuscript. Nathaly Reyes-

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Section 4.2 Development of coated blade spray ionization mass spectrometry for the quantitation of target analytes present in complex matrices

4.2.1 Introduction

The rapid development of ambient ionization techniques during the beginning of the twenty-first century[46,57,58,191]has allowed for the introduction of new solid phase microextraction (SPME) applications. As recently reviewed[216], different geometries of SPME have been coupled to direct analysis in real-time (DART), desorption electrospray ionization (DESI), surface enhanced laser desorption ionization (SELDI), and matrix-assisted laser desorption ionization (MALDI) in a broad range of applications[104,144,175–177,179,194,210]. The present study reports the development of a novel SPME configuration that allows its use, without further modifications, as an ambient ionization method for mass spectrometry.

4.2.2 Experimental section

Mass Spectrometry

Experiments were carried out either on a TSQ Vantage™ or an Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA). To guarantee the accurate position of the blades in front of the mass spectrometer during all experiments, an in-house ionization source was built at the University of Waterloo (Figure 4.1 and 4.2). The 3D-moving stage (Newport Corporation, Irvine, CA) not only adjusts the position with a precision of 0.02 mm in each dimension (25 mm moving path), but also tunes the spraying tip at different angles on the Z dimension (± 0.01º per moving mark). In order to ensure optimum ion transmission, the position of the blade-tip should not be offset more than 5 mm in all the directions from the centre of the ion-transfer capillary.

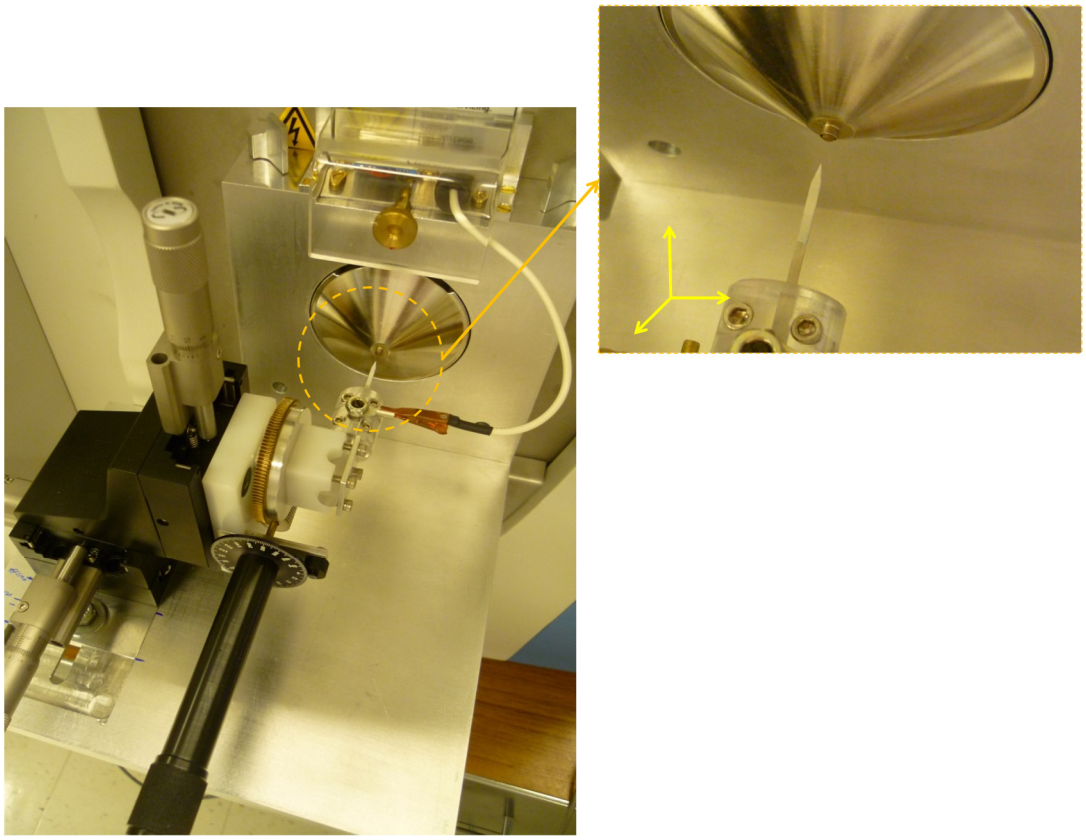

Figure 4.1 In-house ionization source for blade spray technology. The 3D-moving stage (Newport Corporation, Irvine, CA) not only adjust the position with a precision of 0.02 mm in each dimension (25 mm moving path), but also tunes the spraying tip at different angles on the Z dimension (± 0.01º per moving mark). In order to ensure optimum ion transmission, the position of the blade-tip should not be offset more than 2 mm in all directions from the centre of the ion-transfer capillary.

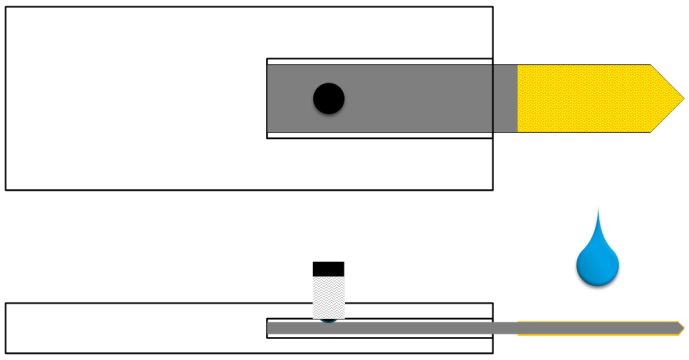

Figure 4.2 Schematic of the spring loading system for easy replacement and accurate positioning of blade spray devices

Blades were made by hand at the machine shop of the University of Waterloo. Coated blades were prepared in house by spraying a C_{18}-polyacrylonitrile (C18-PAN) solution according to a protocol developed in our laboratory.

Biological samples

A phosphate-buffered saline solution (PBS) (pH 7.4) was prepared according to the protocol described in Section 2.2.2. Pooled human plasma and whole blood from healthy donors in potassium (K_2) ethylenediaminetetraacetic acid (EDTA) were purchased from Lampire Biological Laboratories (Pipersville, PA, USA). Urine samples were collected from two healthy volunteers. Collection of urine from healthy volunteers for this particular study was under the approval of the Office of Research Ethical Board of University of Waterloo.

Materials and supplies

The following compounds were selected as model analytes to evaluate CBS: cocaine and diazepam. Deuterated analogues of each analyte were used for correction of intra- and inter-experiment variability. Further details about compounds properties and SRM transitions are provided on Table 2.1. LC-MS grade solvents (acetonitrile, methanol, and water) used in all the experiments were purchased from Fischer Scientific. Calibration curves of each analyte were prepared on each matrix between 0.01 and 100 ng mL^{-1}.

4.2.3 Results and discussion

Coated blade spray (CBS) was conceived as an ideal compromise between sample preparation and direct coupling to mass spectrometry. In essence, the device comprises a stainless steel sheet

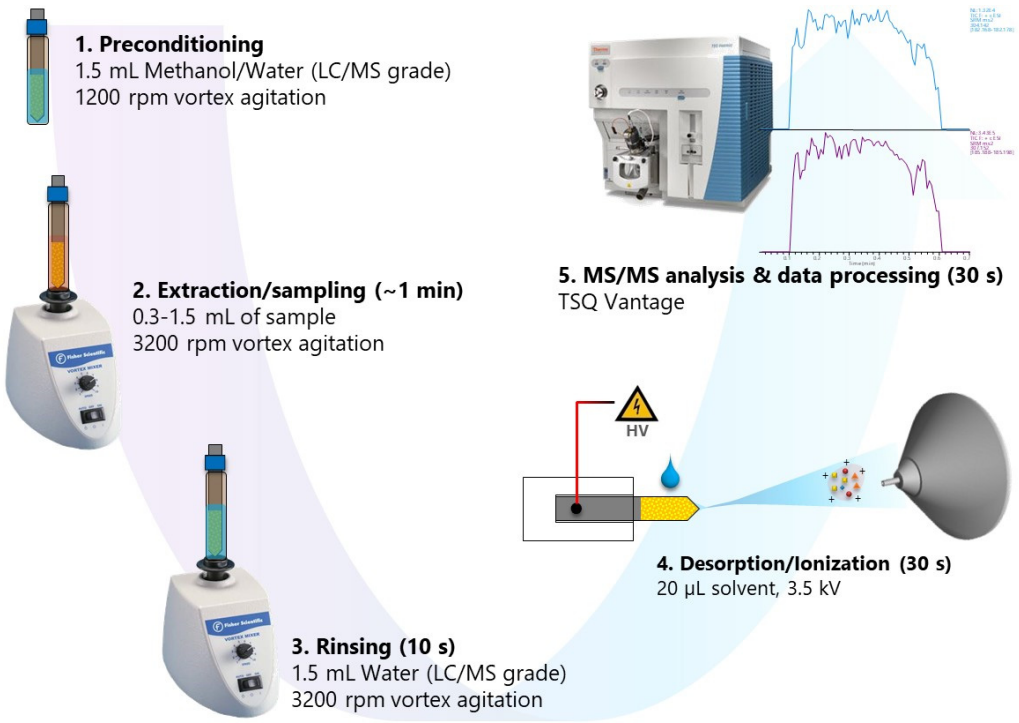

Figure 4.3 Experimental set up for coated blade-spray extraction and desorption/ ionization.

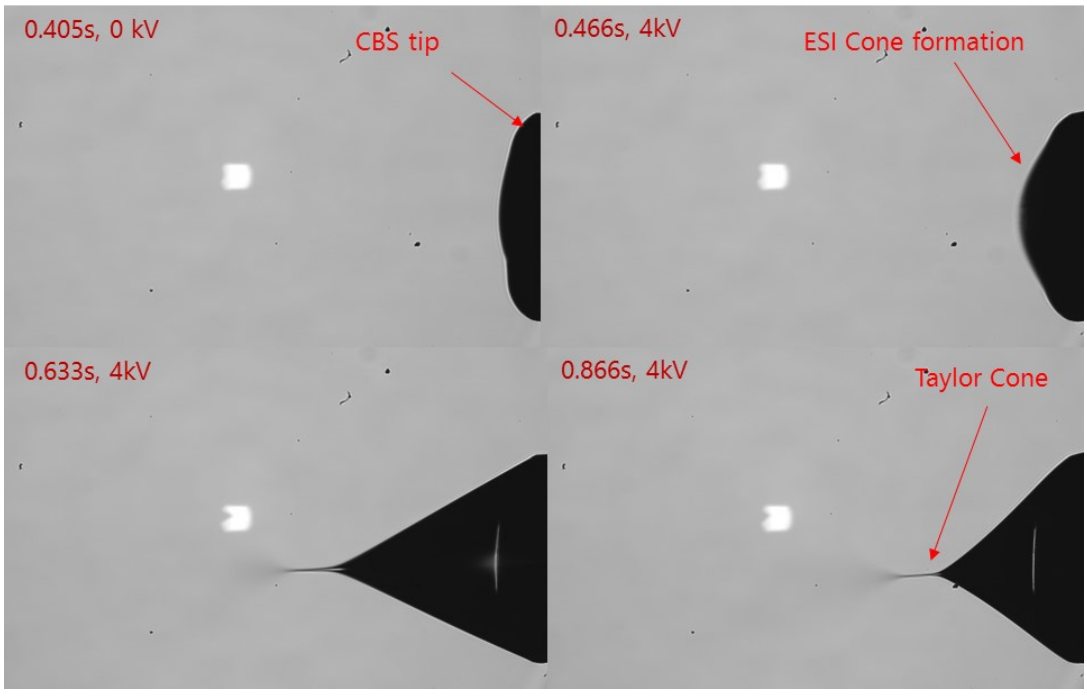

Figure 4.4 Photograhps taken at the tip of a CBS while starting the ESI event. A voltage of 4 kV was applied on a blade wetted with 20 µL of methanol.

(Ø ≤ 200μm) cut as a "*gladius sword*" and coated with a biocompatible polymer (C_{18}-PAN). As a sample preparation method, the SPME coating (Ø ≤ 80μm) simultaneously isolates and enriches the analytes present in the matrix without removing matrix itself[150]. In addition, since the coating is matrix compatible and tuned towards the analytes of interest and the extraction occurs via free form, the technique allows for clean-up of undesirable artefacts that might provide ion suppression or enhancement typically observed placing the matrix directly in front of mass spectrometer[149,150,357]. As an ambient ionization technique, the coated blade acts as a solid-substrate ESI source[46,58]; ions of the extracted/pre-concentrated analytes are generated by applying a high electric field to a blade pre-wetted with a small volume (≤ 20 μL) of desorption solution (see Figure 4.4). As summarized on Figure 4.3, the analytical process consists of three simple steps: first, a pre-conditioned blade is inserted in a vial containing the sample matrix (300-1500 μL) and quick extraction/enrichment is performed by agitating the sample at high speed (vortex agitation at 3200 rpm, t ≤ 1 min); then, the blade is rapidly rinsed in a vial containing water (t ≤ 10s), aiming to remove matrix components adhered to the coating surface; finally, the blade is installed on the blade-holder, in which a spring-loading based system allows straightforward connection of high voltage (HV) to the blade and its easy and fast replacement between experiments (Figure 4.2). Similar to other substrate-spray methodologies, the duration and intensity of the analyte signal is affected by the amount extracted, the volume and chemical features of desorption solvent, the wetting time (time preceding application of HV), and the spraying voltage[49,90,95,225,358,359]. In fact, given that coated blade-spray is a merger between sample preparation and ambient ionization, the selection of adequate desorption/ionization conditions depends on the following: a. the chemistry of the analyte and its affinity for the desorption solvent; b. kinetics of the analyte partitioning between coating layer and the solvent drop (elution efficiency

of the analytes), and c. spray efficiency of the solvent at the tip of the blade[191,358]. Optimization of such parameters using experimental design resulted in 17.5 µL of methanol, 37.5 s wetting time, and 3.5 kV as the most favourable conditions for analysis of diazepam (DZP). However, it is worth emphasizing that these values are valid only for the compound studied in the present work, and during the development of a new blade-spray analytical method, all these parameters must be thoroughly addressed by the analyst.

In view of the fact that CBS and paper spray (PS) have similarities in the design of the devices (differences and resemblances between PS and CBS were summarized on ESI, Table 4.1)[191], an analogy in the ionization mechanism is plausible[25].There are three key resemblances among the techniques. First, the design of the spraying tip, which consists of a macroscopically sharp point with an optimum tip angled at less than 90º. Second, the desorption solution transports the analytes towards the tip once a high potential is applied between the device and the mass spectrometer inlet. Third, the electrical field created among the blade and the MS inlet induces a charge that accumulates at the vertex of the device, causing ionization, as previously described for electrospray[225,358]. However, provided that CBS is made of conductive materials such as stainless steel rather than a porous non-conductive substrate as paper, it could be assumed that a steady electric field is produced at the CBS tip. As a result of the stable electric field gradient between the metal tip and the MS inlet is generated and a more reproducible and efficient ion formation during the spray is expected. Consequently, method sensitivity with CBS is presumed to be improved not only by the enrichment factor provided by SPME, but also by the enhancement in ionization. It is important to highlight that similar to PS[358] corona discharge can also occur in CBS when electrical potentials above 4.5 kV are applied to the metal blade (particularly when the solvent has been depleted). Although the mechanisms governing CBS ionization have not been

Table 4.1 Comparison of CBS to other direct-to-MS technologies.

Features	Paper Spray (PS)	Coated Blade Spray (CBS)	online-SPE-MS
Substrate	Paper	Stainless Steel	-
Ionization mechanism	ESI	ESI	ESI/APCI
Solvent assistance	Yes	Yes	Yes
Extraction mechanism	No	SPME	SPE
Sample clean-up	Limited	Yes	Yes
Sample normalization	No	Yes	Yes
Sample preparation	Limited	Yes	Yes
Extraction selectivity	No	Yes	Yes
Suitable for complex matrices	Yes	Yes	Yes
Small sample volumes (≤ 50 µL)	Yes	Yes	Yes
Typical sample volumes (50 - 1000 µL)	No	Yes	Yes
Large sample volumes (≥ 1000 µL)	No	Yes	No
Direct tissue analysis	Yes	Yes	No
in-vivo analysis	No	Yes	No
on-site analysis (portable)	Yes	Yes	No
Linear dynamic range	low ppb-ppm	low ppt-ppm	low ppt-ppm
Device robustness	Low	High	High
MS analysis time	60-120s	1-120s	10-15s
Total analysis time	2-120 min[a]	0.5-90 min[b]	1 min ≥[c]
Cost per analysis	Low	Moderate	High
Reusability	Disposable	Disposable	Reusable
Desorption/ionization automation	Yes	Yes	Yes
Sample preparation automation	No	Yes	Yes
Workflow simplicity	Yes	Yes	Yes
Compatibility with all MS vendors	Yes	Yes	No[d]
Quantitation of multiple analytes (*via*	Yes	Yes	Limited
Green analytical method	Yes	Yes	No
Surface sampling (rubbing)	Yes	No	No
Ambient ionization under small voltages	Yes	No	No

[a] Depending upon drying of the sample on the substrate
[b] Sample preparation time can be extended until equilibrium or exhaustive extraction is attained
[c] Depending upon complexity of the matrix
[d] Technology is exclusively commercialized by a particular MS manufacturer

thoroughly explored yet by the authors of this manuscript, the protocol for the analysis of complex mixtures is simple and rapid. Surely, a better understanding of the fundamentals involved in the coated blade spray mass spectrometry should allow for improvements in the performance of the technique, as well as widening the scope of its applications[58,198,358].

Since its conception, ambient ionization methods have been developed to circumvent several steps in the analytical process[57]. Indeed, sample preparation approaches coupled to MS have been erroneously visualized by ambient mass spectrometrists as unnecessary, byzantine, and tedious. Conversely to what is normally believed[57], SPME extraction/enrichment can be performed in a short period of time, and the LOD of the method is generally constrained by the instrumental capabilities rather than by the intrinsic features of the coating[149,228]. For instance, 15 seconds are enough to extract a quantifiable amount of analyte at the low ppb level by a traditional LC/MS method (Figure 2.6). Direct coupling of SPME to MS can easily surpass these detection limits, since the desorption/dilution step inherent in most SPME-LC methods is removed from the analytical procedure. In addition, since extractions are carried-out at pre-equilibrium[115], the amount of analyte collected is controlled by the convection conditions (*i.e.* boundary layer), the extraction time, and the surface area of the extracting phase. Hence, under an identical sampling setting, CBS can exceed the sensitivity achieved by other SPME geometries owing to its high surface area[124]. By merely increasing the interaction time between the coating and the sample matrix from 15 seconds to 1 minute, lower LODs can be achieved. As can be seen in Figure 4.5, LOQs as low as 1 ppt were estimated by performing 1 minute extraction from 1.5 mL of phosphate buffered saline (PBS) spiked with cocaine (calibration functions were constructed based on the signal ratio of the analyte and its isotopologue [A/I_S]), obtained with three independent CBS) . Furthermore, the linear dynamic range of the method, evaluated up to 1 ppm, showed astounding

linearity. Beyond any doubt, high concentration levels are not a limitation for SPME[115]. Indeed, in cases where the affinity of the coating for the analytes is high and analytes are present at concentrations superior to 50 ppb, shorter extraction times (≤ 30 s) can be performed.

The remarkable features of blade-spray technology, in comparison to other ambient mass spectrometry devices, include its reusability and intra/inter-device reproducibility[144,231]. Extractions performed with three independent blades (n=12) from 1.5 mL of a PBS solution containing 10 ppb of DZP showed intra/inter-blade relative standard deviations (RSD) lower than 1.8% (Table 4.2). Certainly, producing coatings having reproducible characteristics resulted in excellent reproducibility and repeatability. Besides, since the extraction phase normalized the sample matrix placed in front of mass spectrometer, by extracting only small molecules (*i.e.* analytes of interest) in amounts corresponding to their free concentration in the sample, this ensure minimized matrix effects and therefore reproducible response of the instrument.

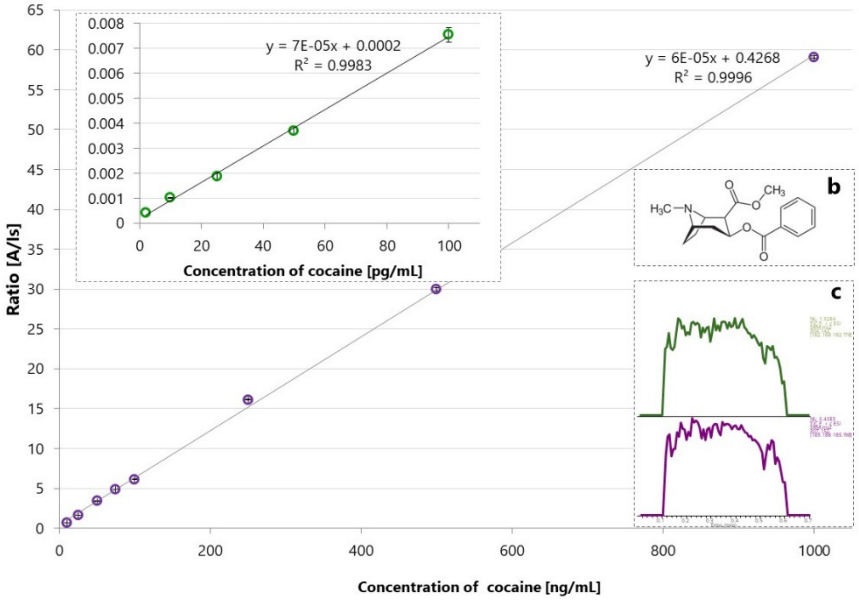

Figure 4.5 a. Quantitative analysis of PBS spiked with cocaine (2 pg mL^{-1} to 1 µg mL^{-1}) and its isotopologue [D_3] cocaine (14.5 ng mL^{-1}). Bars represent the standard deviation of analyses for three replicates with independent blades. a. Insert plot shows low-concentration range; **b**. Molecular structure of cocaine; **c**. Ion chronogram of cocaine and [D_3] cocaine for 30 seconds acquisition.

Table 4.2 Inter- and intra-blade reproducibility (n=12). Results are reported as ratio of analyte (diazepam) versus internal standard isotopologue [D_5] diazepam. 1 min extractions were performed using vortex agitator set-up at maximum speed (3200 rpm). Extraction from 1.5 mL of PBS spiked with10 ng $mL^{-1}$of each substance. Analyses were performed using Thermo TSQ on SRM mode. Blade spray conditions: 17.5 µL methanol, 3.5 kV, and 37 s wetting time. SD, standard deviation. RSD, relative standard deviation.

Repetition	Blade 1	Blade 2	Blade 3	
1	0.99	1.01	1.00	
2	0.98	1.00	1.00	
3	1.02	0.97	0.99	
4	0.98	0.98	0.99	**Summary**
Average	0.99	0.99	1.00	0.99
SD	0.02	0.02	0.01	0.01
RSD	1.8	1.7	0.7	1.3

In addition, carry-over was negligible by implementing a cleaning step once the extraction/desorption-ionization cycle was completed. A mixture of methanol (MeOH, 50%), isopropanol (IPA, 25%), and acetonitrile (ACN, 25%) was found on preliminary experiments to be an ideal solution to get rid of most residual analytes from preceding extractions[148]. It is worth emphasizing that the cleaning step should be optimized according to the chemistry of the coating and its affinity towards the analyte of interest. In cases in which large variability exists in sample concentration among samples (*e.g.* low ppt to high ppb or even ppm levels), blades should be constrained to single use. Otherwise, a small amount of analyte (few pg) could potentially linger on the blade after the desorption/cleaning cycle, yielding possible false positives. As a solution, while working with compounds with high affinity towards the coating[133] that are present in the sample at concentrations higher than 50 ppb, shorter extraction times (≤ 30 s) are recommended to diminish the amount of analyte enriched; this precaution would guarantee a complete removal of the non-sprayed analytes during the cleaning step. In addition, by using thin coatings, efficient equilibration of the analytes can be achieved (faster extractions), together with more effective desorption/ionizations. Thus, blades coated with a consistent and reproducible thinner layer of sorbent are under development in our laboratory (see Section 4.3).

In contrast to PS, another exceptional characteristic of CBS is the mechanical strength provided by the use of a stiff substrate in the creation of the blades. As a result, deformation/damage of the device does not occur, regardless of the sample dimensions. Thus, CBS could be used to perform extractions from small volumes (*e.g.* few microliters, as extractive blood spot) up to large volumes (*e.g.* hundreds of litres, as on-site monitoring of a watercourse/lake). Additionally, analogous to thin film microextraction (TFME)[231], CBS can be implanted into tissue for *in vivo* monitoring of endogenous and exogenous substances. Indeed, the geometry of the device for *in vivo* applications might not be limited to a flat surface. For instance, a cylindrical coated device (*i.e.* metal pin with Ø ≤ 150µm) could provide mildly-intrusive access inside the tissue[346], otherwise non-accessible by modern methods (*e.g.* DESI), with minimal damage. Succinctly, contrary to most ambient ionization techniques, SPME-based spray approaches can be used for *in situ, in vivo, ex vivo* and on-site applications independently of the sample characteristics (*e.g.* volume, structure, complexity, and viscosity) with truly minimal sample preparation.

An exclusive characteristic of blade spray is its capability to perform reproducible and independent desorption/ionization from each side of the blade (Figure 4.6). Hence, analysis in duplicate of each sample from a single extraction is feasible when the blade is coated with the same extraction phase on both sides. Indeed, multiple attempts towards the development of a comprehensive coating for SPME have been investigated[357]. The universal approach for complete coverage, solid phase extraction (SPE), consists of combining two or more extraction phases with different affinities for the analytes, *e.g.* coatings that provide hydrophobic and hydrophilic interactions. However, by using this procedure, a compromise has to be often made in regards to the chemistry of both coatings, and generally an intermediate phase is obtained. The device herein presented can be coated with a different polymeric phase on each side; thus, covering two different ranges of

polarities and consequently providing a genuinely comprehensive analysis in a single experiment[357]. Considering the above mentioned, CBS appears to be a practical tool for metabolomics studies; however, further research regarding its reproducibility and quantitation capabilities without using internal standard (I_s) is still required prior to its application for untargeted analysis in complex matrices. As recently reported by our group[267], once the SPME geometrical characteristics that affect the desorption/ionization of the analytes are controlled, reproducible quantitation without I_s is feasible. Therefore, our ongoing research is primarily focused on optimizing the geometry of CBS and coating characteristics to provide run-to-run and batch-to-batch reproducible results even when an I_s is not used for signal correction. Considering that CBS, as a SPME device, reduces matrix effects and provides matrix normalization (*i.e.*

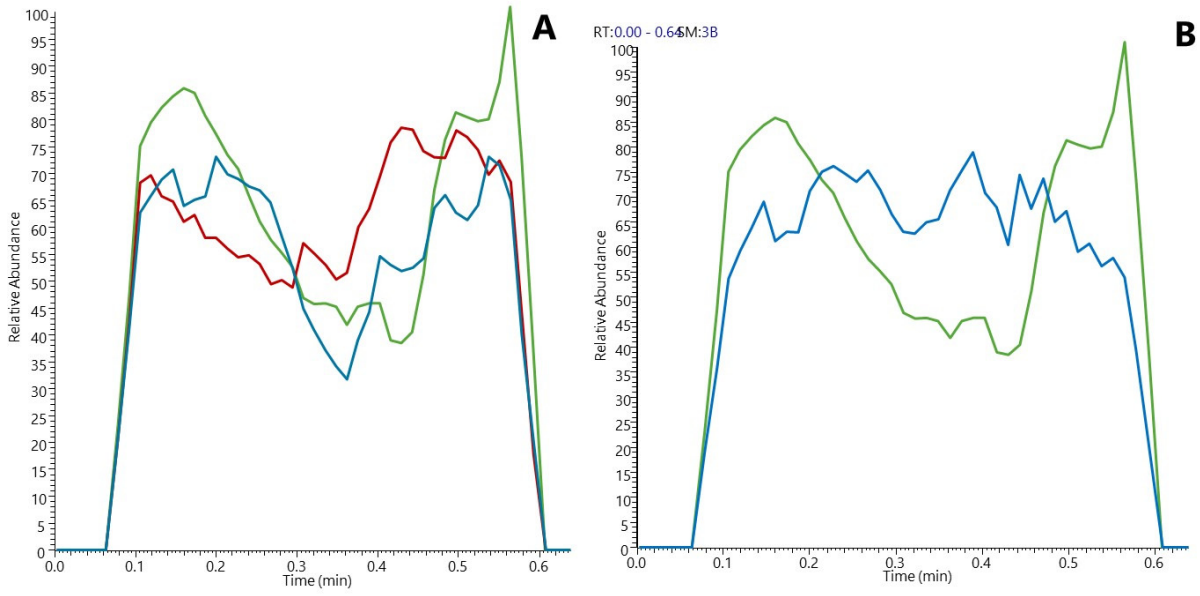

Figure 4.6 A. Ion chronograms of three desorptions/ionizations of cocaine from the same side of the blade (blade spray conditions: 15 µL methanol, 4 kV, and 45s wetting time). 1 min extractions were performed using a vortex agitator set-up at maximum speed (3200 rpm). **B.** Ion chronograms of cocaine obtained from the desorption/ionization of both sides of the blade. Extractions from 1.5 mL of PBS spiked with 100 ng $mL^{-1}$of cocaine using a single blade. Analyses were performed using Thermo Exactive Orbitrap on full scan mode.

analytes are extracted/pre-concentrated in a well-defined non-interfering matrix which is the coated sorbent), precise geometrical conditions (*e.g.* thin homogenous layer of particles[267]) will facilitate its application as a quantitative tool in metabolomics research. Given the simplicity and speed of analysis with CBS, the technique can be said to be an ideal device for the screening of pharmaceutical drugs or illicit compounds in biological samples. Blade spray was used for the quantification of cocaine and diazepam in urine and plasma. As can be seen in Figure 4.7, exceptional linearity was achieved in both matrices.

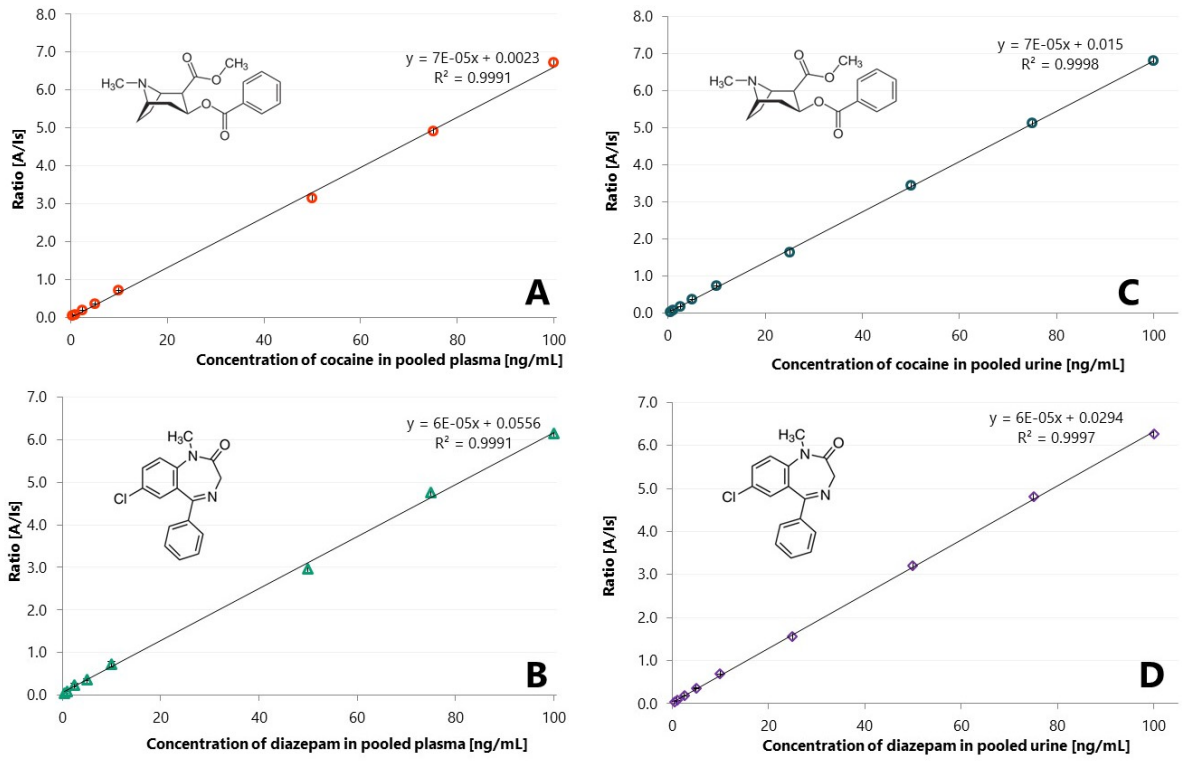

Figure 4.7 A. Quantitative analysis of plasma spiked with cocaine (500 pg mL^{-1} to 100 ng mL^{-1}) and its isotopomer [D_3] cocaine (14.5 ng mL^{-1}). **B.** Quantitative analysis of plasma spiked with diazepam (500 pg mL^{-1} to 100 ng mL^{-1}) and its isotopomer [D_5] diazepam (16 ng mL^{-1}). **C.** Quantitative analysis of urine spiked with cocaine (500 pg mL^{-1} to 100 ng mL^{-1}) and its isotopomer [D_3] cocaine (14.5 ng mL^{-1}). **D.** Quantitative analysis of urine spiked with diazepam (500 pg mL^{-1} to 100 ng mL^{-1}) and its isotopomer [D_5] diazepam (16 ng mL^{-1}).

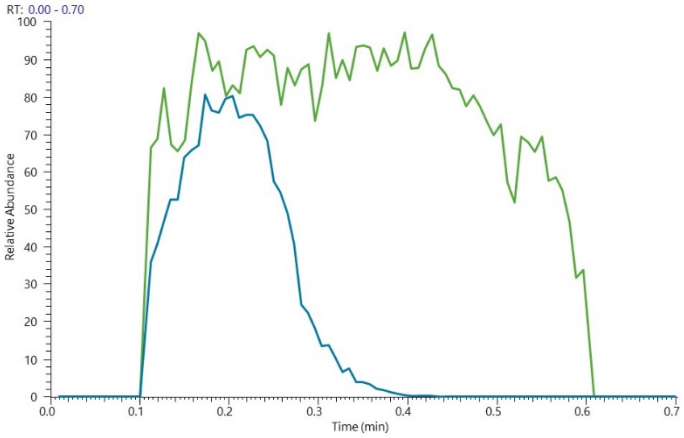

Figure 4.8 Ion chronograms of a blade sprayed after 10s rinsing step in water (green) *versus* blade sprayed without rinsing (blue). 1 min extraction from 1.5 mL of PBS spiked with500 pg $mL^{-1}$of cocaine using a single blade. Analyses were performed using a Thermo TSQ on SRM mode.

Similar to the results obtained in PBS, LOQs of 0.5 and 2 pg mL^{-1} were estimated for cocaine in plasma and urine, respectively. As expected, in a comparison made with nanopure water spiked with target analytes (Figure 4.8), it was found that the rinsing step was critical in the diminishment of ionization suppression from salts (*e.g.* from urine/PBS) and attachment of biomolecules to the coating surface (*e.g.* from plasma). In summary, by using a microextraction device to extract/transfer the analytes from the sample matrix to the MS system, matrix effects for analytes with low binding are significantly minimized, and detection limits are similar independently of the matrix (*e.g.* cocaine, 5 % protein binding)[131]. Indubitably, sample clean-up provided by CBS is convenient not only for quantitation purposes, but also to extend the operative time of the mass spectrometer by minimizing instrument maintenance and providing steady instrumental sensitivity. Unlike cocaine, LOD/LOQ for DZP in plasma were estimated in 15 and 50 ppt, respectively. Certainly, the quantification limit is higher in comparison to urine and PBS (LOQ ~ 5 ppt). However, it is worth emphasizing that DZP is 98% bound to plasma proteins and, as an SPME device, CBS equilibrates only with the free-portion of analyte in the sample[2]. Total analysis time (extraction from a complex matrix without pre-treatment, rinsing, desorption/ionization, peak

integration, and quantitation of total concentration) is less than 2 minutes per sample when performing manual operation of the blades.

Nowadays, multiple efforts are directed towards the development of powerful LC-MS/MS or GC-MS/MS methods that allow for the analysis of controlled substances in complex matrices. Given the complexity of the samples, such procedures entail cumbersome and extensive sample preparation steps. Consequently, approaches that allow fast, quantitative, and direct analysis are highly demanded. As a proof of concept, blade spray was used to screen 21 compounds controlled by the World Anti-Doping Agency (WADA) and the United Nations Office of Drugs and Crime (UNODC)[131,360]. Selected reaction monitoring (SRM) mode was used to uniquely identify each substance (see Table 2.1). Although desorption/ionization conditions were not optimized for each analyte, all substances were detected at 20 ppb, and 14 of these compounds provided hypothetical LODs lower than 50 ppt (*e.g.* clenbuterol, 6-acetylcodeine, and toremifene). As blade spray derives its selectivity and sensitivity from the chemical and physical properties of the coating used, current research is directed towards coatings with higher affinity for the target compounds in order to reach lower limits of detection[131]. Undeniably, the capability of CBS to simultaneously screen multiple substances of interest in a single analysis, without sacrificing sensitivity or increasing the analysis time, is an outstanding characteristic of this technique. Thus, the application of CBS for the concomitant monitoring of numerous pesticides in food commodities, personal care products in wastewater, or doping substances in high-performance competitions is foreseen in the near future (as discussed in Section 4.4)[81].

Fundamentally speaking, CBS is capable of quantifying even more compounds in a single analysis because the electrospray event can be extended by using a continuous solvent supply. As a proof-of-concept, a glass capillary was connected to a syringe pump which was continuously supplying

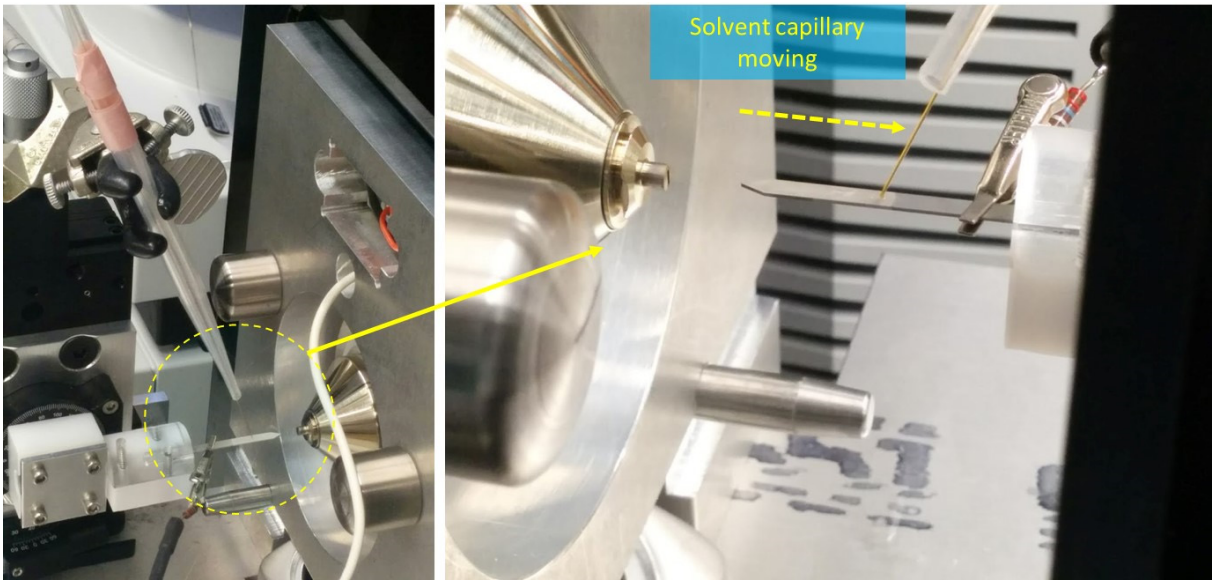

Figure 4.9 Experimental set-up used for segmented CBS desorption. Glass capillary was moved by hand from the tip of the blade towards the back of the coating while high-voltage was on.

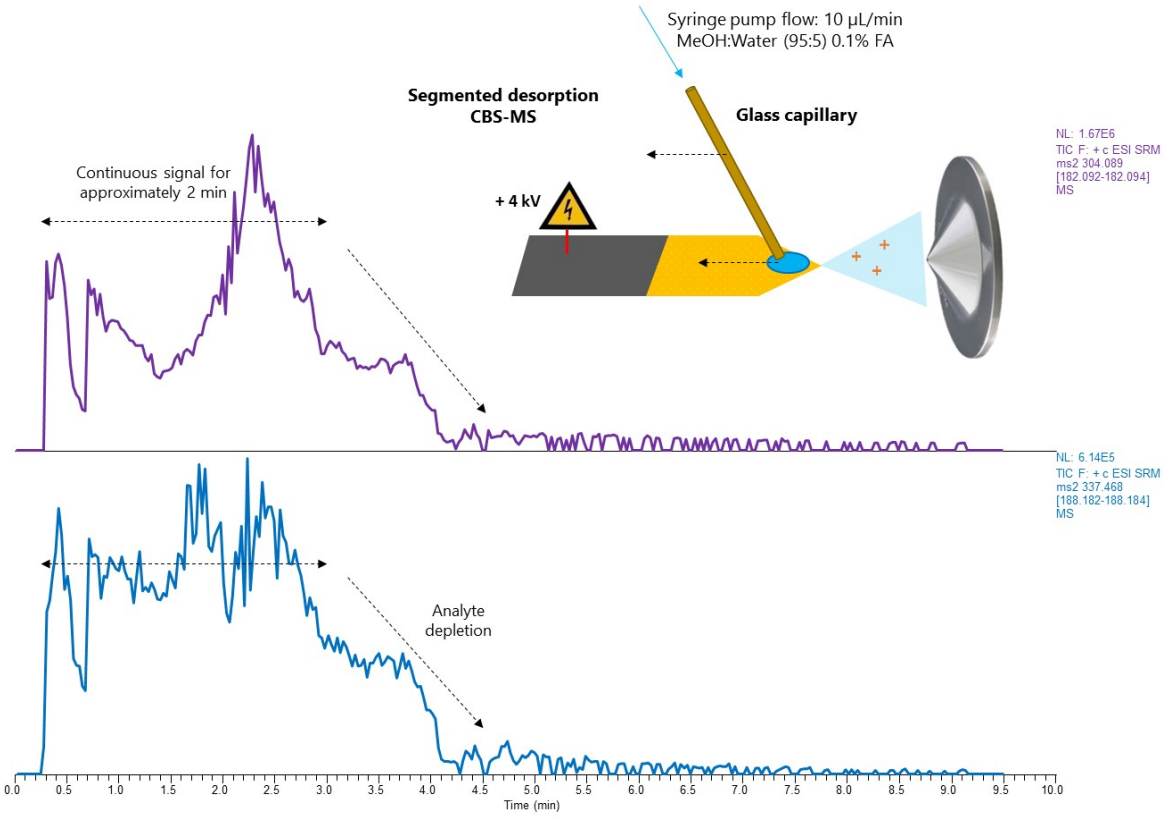

Figure 4.10 Ion chronograms for cocaine (top) and fentanyl (bottom) obtained from a CBS that extracted for 10 min from 300 µL of PBS spiked at 1 ng mL^{-1} with the aforementioned compounds.

a flow of 10 µL/min of desorption solvent (see Figure 4.9). Unlike the previous experiments, where a finite amount of desorption solvent was added with a pipette, the capillary was moved by hand from the tip of the blade towards the back. Thus, by performing a segmented desorption, rather than a "complete" desorption, a continuous signal was attained. As can be seen in Figure 4.10, after approximately 2.5 minutes, signal drops as a result of analyte depletion. Certainly, increase and decrease on the ion signal are a consequence of either: inaccurate position of the capillary on the blade, imprecise speed while moving the capillary, or irregular distance from the capillary to the coating area. Although preliminary, these results showed the potential of CBS of performing long spray events which is critical when hundreds of compounds need to be monitored[243]. Our efforts are currently focused on developing an interface that allows having a steady analyte signal for no less than 2 minutes.

Towards small sample volumes

SPME Micro tips, discussed in Chapter 3, can effectively sample small volumes of selected parts of a living object. If the object is sufficiently large, the damage caused by insertion can be considered negligible or sufficiently minimized, allowing for multiple samplings to be subsequently performed to investigate changes in the system as a function of time or applied stimuli. However, if the sample is so small as to be substantially disturbed or consumed during extraction, or if the living object is so small as to not withstand sampling without loss of life, repetition of the sampling procedure is not possible. For such cases, exhaustive extraction on a coated larger flat surface might be a more attractive approach. To pursue this line of inquiry, CBS[136] was implemented for the first time as a novel strategy for the analysis of small volumes of biofluids[136]. Unlike applications involving the tips, no additional instrumentation (*i.e.* LC or nano-

ESI emitter) or sample containers (*e.g.* vial) are needed to perform CBS analyses. As summarized in Figure 4.11, the analytical process consists of three simple steps: first, a drop of sample (≤ 10 µL) is placed on the coated blade for 5 minutes (static conditions); then, the blade is rapidly rinsed in a vial containing water (t ≤ 10s); and finally, the blade is installed in front of the MS system for analysis, as described in the experimental section[136]. As a sample preparation method, the coating (thickness ≤ 10µm) simultaneously isolates and enriches the analytes present in the sample. Indeed, given the high affinity of HLB[304] for a broad range of compounds, multiple rinsing steps can be performed to wash off matrix components potentially adhered to the surface with minimal to no loss of analytes.

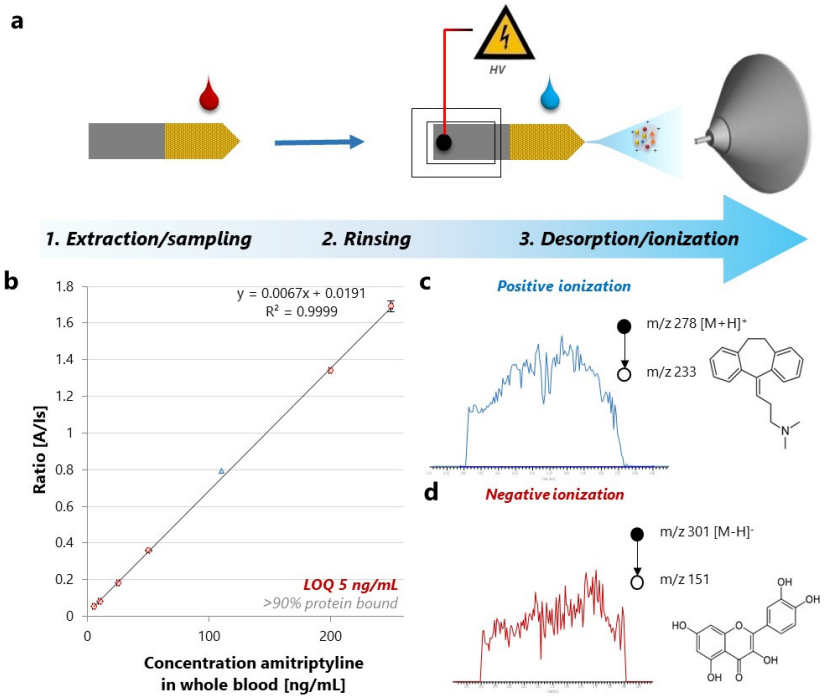

Figure 4.11 a. Experimental set up for analysis of small volumes with coated blade spray. **b.** Quantitative analysis of whole blood spiked with amitriptyline (5 ng ml^{-1} to 250 ng mL^{-1}) and its isotopologue [D_6] amitriptyline (100 ng mL^{-1}). 5 µL of sample were spotted on the blade, and 5 min static extraction/enrichment conducted using HLB-PAN coated blades. Bars represent the standard deviation of analyses for three replicates with independent blades. **c.** Ion chronogram of amitriptyline; acquisition time of 30 s in positive mode SRM (278 → 233 m/z); **d.** Ion chronogram of quercetin; acquisition time of 30 s in negative mode; SRM (301 → 251 m/z).

Our results demonstrated that not only was good reproducibility attained (RSD ≤ 2%, calculated based on the signal ratio of the analyte and its isotopologue [A/I_s]), but also noteworthy improvement of the LOQ for amitriptyline was achieved in comparison to that obtained with use of the tips (LOQ ~ 5 ng mL^{-1} *versus* 25 ng mL^{-1} attained with the tips). Essentially, the extraction efficiency of SPME techniques can be enhanced by increasing the affinity of the coating for the analytes (chemistry of the extraction phase) and/or by increasing the volume or active surface area of the extraction phase. Certainly, the coated area of the blade where the droplet is placed significantly surpasses the total area of the SPME-tip. Therefore, in cases where both the affinity of the analyte for the coating and the extraction recovery are high (*e.g.* low matrix-binding), CBS should conceptually be considered as an open-bed SPE device, rather than as an SPME device. Finally, as a proof-of-concept, CBS was used for the qualitative determination of quercetin in 5 µL of homogenized onions (Figure 4.11). Negative ionization was used for identification of the analyte collected by the blade (301 → 251 m/z) after 5 minutes of contact. The obtained results evidence the great potential of CBS for finger-printing and population studies of microorganisms such as cells, fungi, and bacteria. Hence, instead of inserting a mini-probe into a cell, single/multiple cells can be placed on top of the coated surface, and then burst to release their cytoplasmatic content. After allowing some time for extraction, the remaining cell components can then be washed, enabling subsequent determinations to be performed either by high-resolution (*i.e.* metabolite profiling) or tandem mass spectrometry (*i.e.* target-analysis).

4.2.4 Summary

Other major future applications of CBS involve *in situ* applications within the medical field. The ability to deliver a rapid prognostic metric of clinical condition is certainly important in the critical-

care setting (emergency and surgery units)[152]. Thus, molecular diagnostic and prognostic instruments, which are able to provide doctors with fast and reliable results, are highly desired in such facilities for personalized diagnosis and treatment of patients. Although CBS reusability is undoubtedly beneficial in fields where high-throughput is needed and hundreds of samples need be processed on a daily basis, blade spray is also envisioned as a splendid single use device for trace analysis. For this reason, our group is currently working on the development of a cost-effective disposable device. By coupling disposable blade spray (dCBS) to portable/easily-deployable mass spectrometers[361], fast, reliable and "real-time" measurements will be provided on site. Thus, due to their speed, sensitivity, selectivity, and linear dynamic range, dBS-MS/MS will be an avantgarde tool not only for point-of-care therapeutic drug monitoring, but also in diverse forensic, food, medical and environmental applications. Indeed, the many advantages of using CBS and other SPME-MS configurations will certainly encourage analytical scientists around the world to choose these swifter sample preparation approaches prior to direct introduction to MS analysis over traditional, more time-consuming methods.

Section 4.3 Ultra-fast quantitation of voriconazole in human plasma by coated blade spray mass spectrometry

4.3.1 Introduction

Voriconazole is an antifungal drug from the family of the triazoles. As a derivative of fluconazole, voriconazole possesses a fluoropyrimidine group rather than a triazole moiety in addition to an alpha methylation on the tertiary carbon [362]. In contrast to the first generation of triazole antifungals, voriconazole has a broader spectrum of action[363,364] and has been demonstrated as effective against fungal infections caused by *Aspergillus*[365] and *Fusarium* species[364]. The mechanism of action consists of the inhibition of cytochrome P450 enzyme lanosterol 14-alpha-demethylase, which prevents the conversion of lanosterol to ergosterol, an essential component of cell membranes[366]. Although the effectiveness of this antifungal drug has been widely proven, toxicity risks and a narrow therapeutic concentration window have been reported. Indeed, plasma concentrations above 6 μg/mL have been associated with clinical events such as visual impairment or photopsia, abnormal hepatic function, and higher bilirubin levels[367,368]. On the other hand, in cases where voriconazole plasma concentrations were below 2 μg/mL, a great proportion of patients suffered a significant progression in their infections[369]. In addition, high pharmacokinetic variability of this drug due to erratic bioavailability and variation in drug metabolism has also been documented[370]. Consequently, the development of an accurate, reliable, fast, and cost-effective analysis of voriconazole in plasma is highly desirable.

Most of the methods for voriconazole determination to date involve the use of liquid chromatography (LC) with either ultra-violet (UV)[362] or mass spectrometry (MS)[371–374] detection. In addition, methods employing gas chromatography-MS (GC-MS)[375] and capillary

electrophoresis (CE)[376] have also been reported. Within the LC-MS applications, the fastest LC separation was attained in 3 minutes[377] and the sample volume consumption was in the range between 20 µL[371] and 200 µL[377] of plasma. However, when the sample preparation step is considered in the total analysis time, none of the published methodologies can be completed in less than 15 minutes considering that even in the simplest approaches protein precipitation and centrifugation steps are required in order to have an extract suitable for liquid chromatography[373]. Recent developments in bioanalytical applications using solid phase microextraction (SPME) have demonstrated that this technology is an attractive alternative to conventional methods due to its simplicity, sensitivity and speed of analysis[136]. In SPME, a device coated with a polymeric material is used to extract/enrich analytes from a given sample prior to instrumental analysis. By using novel ultra-thin biocompatible coatings, faster and efficient extractions of small molecules from biofluids can be easily attained[113,119,151]. Despite the well-known advantages of using LC, emerging technologies such ambient mass spectrometry[46,58,94,95,378] and the direct coupling of SPME to very sensitive and selective mass spectrometers have shown outstanding results when aiming to decrease the total analysis time[173]. Among the recently developed SPME-MS technologies, coated blade spray (CBS)[136], SPME-TM-direct analysis in real time (SPME-DART)[267], Bio-SPME-nano-ESI[173], Bio-SPME-OPP[36] and SPME-DBDI[379] excel by the limits of detection achieved. Succinctly, CBS was developed as an ideal compromise between sample preparation and direct coupling to mass spectrometry and it behaves as an SPME device for extraction and as a solid-substrate ESI source when performing ionization. When comparing CBS to other SPME-MS technologies, the former offers additional advantages such as simplicity of the set-up (see Figure 4.12), negligible MS carry over (*i.e.* no-cross talking among experiments),

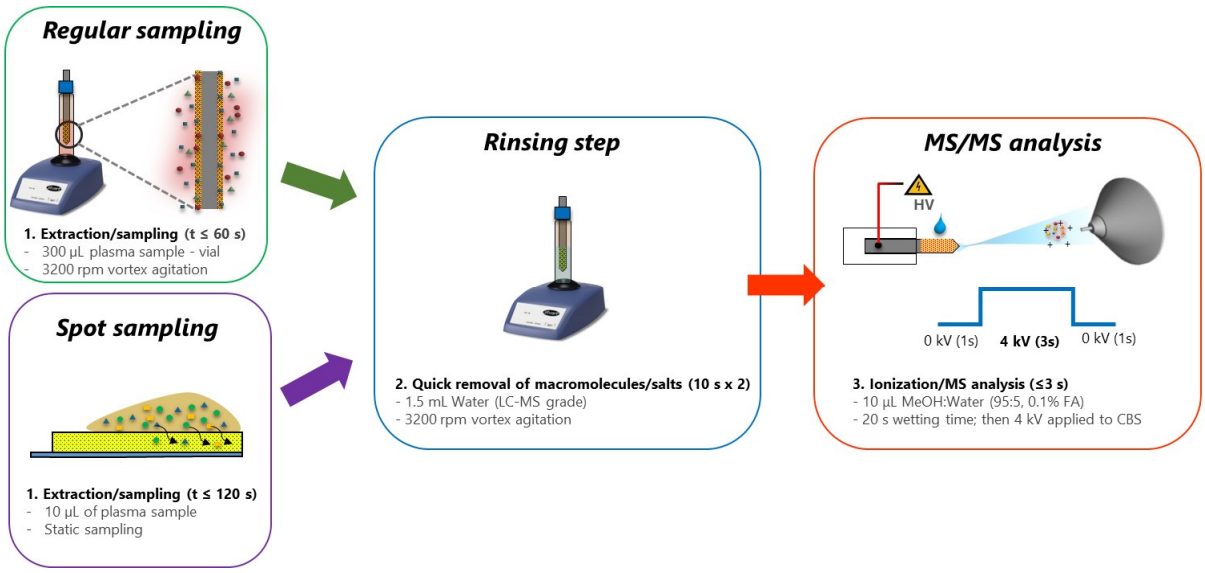

Figure 4.12 Schematic of the experimental set-ups for extraction/enrichment of voriconazole from plasma and desorption–ionization via Coated Blade Spray-Mass Spectrometry.

minimal solvent use (≤ 20 µL), and no-need of expensive parts for the construction of the ionization source[136]. In addition, CBS is the device with the largest coated surface area available for extraction when compare to the other SPME-devices used for direct coupling to MS; therefore, low or even sub parts per billion levels can be easily achieved when rapid extractions (time ≤ 2 min) are performed from small sample volumes (≤ 300 µL).

In this work, we propose the ultra-fast determination of voriconazole in human plasma by two different CBS-based approaches. In the first strategy, voriconazole is extracted on the CBS device by fully immersion of the coated blade in 300 µL of human plasma during 1 minute under vortex agitation. In the second approach, analytes are enriched on the CBS by placing a 10 µL droplet of plasma for 2 minutes on the coated area. After the completion of the extraction process in both cases, a quick rinsing step is performed in order to remove matrix components potentially adhered to the surface. Once complete, analytes were directly desorbed/ionized from the CBS device and analyzed by mass spectrometry without the need of additional sample preparation or

chromatography. Both methodologies were fully validated in terms of linearity, LOD, LOQ, repeatability, reproducibility, relative matrix effects and accuracy employing 5 different lots of plasma and 4 different patients.

4.3.2 Experimental section

Mass Spectrometry

All the experiments were performed using a TSQ Quantiva (Thermo Scientific, San Jose, California, USA). Data was processed using Trace Finder version 3.0 (Thermo Scientific, San Jose, California, USA). A home-made coated blade spray interface was built at University of Waterloo, and a thorough description of the operation of this system can be found elsewhere[136].

Materials and supplies

Methanol (MeOH), acetonitrile (ACN), isopropanol (IPA) and water were all LC-MS grade and purchased from Fisher Scientific. Formic acid was purchased from Sigma Aldrich (Saint Louis, USA). Voriconazole and the deuterated analogue voriconazole-d_3 were obtained from Sigma Aldrich (Milwaukee, USA) and Cerilliant (Saint Louis, USA), respectively. Individual stock standard solutions of voriconazole at a concentration of 5000 $\mu g \cdot mL^{-1}$ were prepared in methanol and stored at -80 °C. Human plasma (with K_2-EDTA as anticoagulant) from different patients was purchased from Bioreclamation IVT (Westbury, New York, USA). All the plasma samples were spiked and stored overnight at 4 °C prior to use to reach drug-protein binding equilibrium. HLB particles (~ 5 µm particle diameter) were kindly provided by Waters Corporation (Wilmslow, UK). Stainless steel blades were purchased from Shimifrez Inc. (Concord, Ontario, Canada). Blades (15

mm length) were coated with HLB-polyacrylonitrile (HLB-PAN) slurry according to a protocol developed in our laboratory (see Section 4.4.2).

Sample preparation

All the CBS devices were cleaned after manufacturing for 30 minutes using a 40:40:20 (MeOH/ACN/IPA, v/v/v) solution and then conditioned for 30 minutes with a 50:50 (MeOH/water, v/v) solution prior to extraction process. It is important to point-out that, regardless of the application, CBS can be dried prior to the extraction step. The first of two methodologies to be described consisted of the extraction/enrichment of voriconazole from 300 µL of human plasma for 1 minute using vortex agitation (3200 rpm), followed by two independent rinsing steps in 300µL of water (under agitation, $t \leq 5s$). The second approach involved the spotting of a 10 µL droplet of plasma onto the coated surface of the CBS device. After 2 minutes of extraction, two rinsing steps of 5 seconds using water were performed. A schematic of both procedures is presented in Figure 4.12. After extraction, the CBS devices were positioned in the home-made interface and 10 µL of a 95:5 MeOH/water v/v 0.1% formic acid solution were added on the coated area to desorb the analytes ($t \leq 20$ s). Subsequently, a voltage of 5.5 kV was established between the CBS and the MS entrance to electrospray the target analytes from the tip of the blade. All the analysis were carried out exclusively in positive ionization mode. Optimum collision energy and RF-lenses conditions were tuned for each compound by direct infusion of methanolic standards. MS/MS transitions, optimum collision energy (CE), and RF-lenses voltage were 350.18→281.05, 16.5 V, and 58 V for voriconazole, respectively; and 353.18→284.12, 16.5 V and 59 V for voriconazole-d_3, respectively. The total MS/MS event was 3 seconds with a dwell time of 100 ms for each compound. Xcalibur software (Thermo Scientific, San Jose, California, USA, version 4.0) was employed for data acquisition. Calibration curves were prepared in the range between 0.1

µg/mL and 50 µg/mL. Extractions from each calibration point were performed using three independent replicates. The linear regression was plotted as the area of the analyte divided by the area of the internal standard as a function of the concentration (unweighted linear least squares). Voriconazole-d_3 was spiked in plasma samples as an internal standard at a concentration of 5 µg/mL. Both approaches were validated in terms of LOD, LOQ, linearity, repeatability, reproducibility, accuracy and relative matrix effects. The latter was evaluated by following the criterion proposed by Matuszewski, B. K. which consists in the comparison of the RSD associated to the slopes of five calibration curves from five different lots of biofluids [131,380].

4.3.3 Results and discussion

Aiming to develop an ultra-fast analysis, while monitoring two MS/MS transitions (*i.e.* compound of interest and its internal standard), the electrospray event was set to 3 seconds using a dwell time of 100 ms for each compound. Thus, a minimum of 15 scans per compound is obtained without significantly increasing the total analysis time. Ion chronograms related to the selective reaction monitoring (SRM) of voriconazole at different concentration levels, including the plasma blank, are presented in Figure 4.13. As can be seen, the full width at half maximum (FWHM) of each pulse is exactly three seconds and the instrumental signal increases with an increase in the plasma concentration of voriconazole.

In order to optimize the enrichment step, extraction time profiles were constructed at the extraction conditions of both CBS approaches: extraction with forced convection (~ 3200 rpm) and spot sampling. As can be seen in Figure 3A, equilibrium between the coating and the plasma sample is not reached even with an extraction time of 300 seconds for high speed extraction. Taking into account that the optimization of the extraction time was performed with the lowest point of the

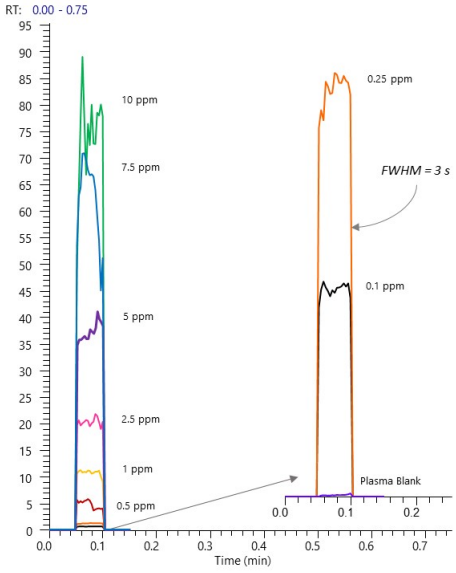

Figure 4.13 Ion chronograms related to the selective reaction monitoring (SRM) of voriconazole (350.18→281.05) at different concentration levels, including the plasma blank.

calibration curve (*i.e.* 0.1 µg/mL) and, consequently, all the extraction times fulfilled the LOQ required (*i.e.* 1 µg/mL), the optimum extraction time for the first CBS approach was selected based on the lowest relative standard deviation (RSD, %) attained with the raw data (non-corrected by internal standard) at the shortest extraction time. Thus, the best compromise between speed and reproducibility was obtained with 60 seconds of extraction where the RSD for the raw data was below 15% and the signal-to-noise ratio (S/N) was about 1000. On the contrary, for spot analysis (10 µL of plasma), given the high surface-to-volume ratio, a plateau on the amount extracted was reached after 120 seconds of extraction (see Figure 4.14B) [151]. Therefore, this time point was chosen for spot analysis, as it is the extraction time at which equilibrium was achieved and that provides the lowest RSD (~ 3%) and a S/N of 500. It is worth to emphasize that in spot analysis long extraction times can lead to drying of the biofluid droplet on the coating surface. This undesired effect can promote the co-extraction of analytes that could cause matrix effects (*e.g.* ion suppression) as well as could induce protein precipitation on the coating surface.

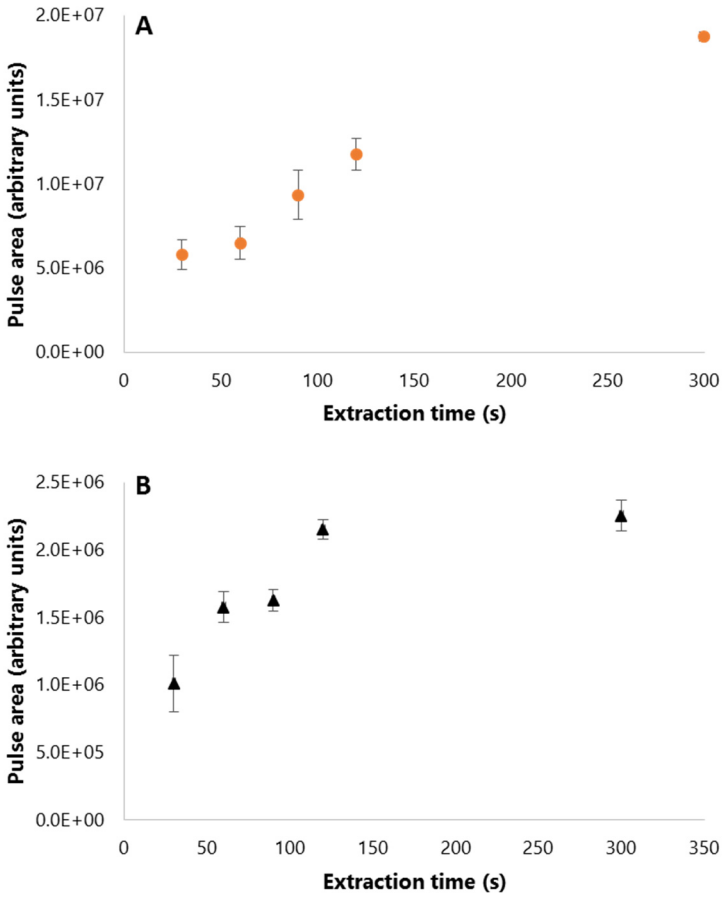

Figure 4.14 Extraction time profile of A) regular sample volume approach and, B) droplet approach. Concentration of voriconazole in plasma 0.1 $\mu g \cdot mL^{-1}$.

Thus, to avoid a significant decrease on the instrumental sensitivity and a detriment to the experimental reproducibility extraction times shorter than 5 min are recommended for SPME spot sampling. Once both extraction times were optimized, full calibration curves in plasma samples were constructed to qualify the most relevant figures of merit of each approach. As can be seen in Table 4.3, LOD and LOQ of 0.003 and 0.1 µg/mL, respectively, were obtained for the regular sample volume strategy (~ 300 µL). It is worth highlighting that despite the short extraction time ($t \leq 60$ s) the LOQ is at least ten times lower than the required concentration range (*i.e.* 1-6 µg/mL). The correlation coefficient (R^2), repeatability, and reproducibility were acceptable with values of

Table 4.3 Figures of Merit for Plasma Analysis of voriconazole using HLB CBS-MS/MS.

Methodology	LOD ($\mu g.mL^{-1}$)	LOQ* ($\mu g.mL^{-1}$)	Linearity ($\mu g.mL^{-1}$)	Linear equation	Correlation coefficient (R^2)	Repeatability RSD, % (n=3)	Reproducibility RSD, % (n=3)
Regular volumes	0.003	0.1	0.1-50	y=0.1877 x - 0.0889	0.990	0.3-2.3	6.2-7.7
Spot volumes	0.006	0.1	0.1-50	y=0.1824 x - 0.0009	0.999	0.5-0.9	4.5-7.0

*LOQ was the lowest calibration point tested

Table 4.4 Relative matrix effects for five different lots of plasma (n=3).

Methodology	Relative matrix effects												
	Lot A		Lot B		Lot C		Lot D		Lot E		Average	SD	RSD, %
	slope	RSD, %	slope	RSD, %	slope	RSD, %	slope	RSD, %	slope	RSD, %			
Regular volumes	0.194	1.4	0.200	1.7	0.218	2.7	0.215	0.8	0.178	0.9	0.20	0.02	8.1
Spot volumes	0.191	0.8	0.196	1.4	0.213	2.5	0.206	0.6	0.181	1.0	0.20	0.01	6.3

Table 4.5 Accuracy and relative standard deviation values obtained for four patient plasma samples spiked at five different concentration levels (n=3).

Methodology	Concentration ($\mu g \cdot mL^{-1}$)	Patient 1		Patient 2		Patient 3		Patient 4	
		54/180[a]		85/137[a]		675/189[a]		569/197[a]	
		56/C/M[b]		29/B/M[b]		40/B/M[b]		52/H/F[b]	
		Accuracy	%RS	Accuracy	%RSD	Accuracy	%RSD	Accuracy	%RSD
Regular volume analysis	**0.5**	120.6	0.8	115.6	5.5	118.2	4.8	115.7	3.9
	1	93.6	0.4	102.5	1.5	100	0.8	111.5	2.5
	2.5	91.8	1.4	90.6	0.5	101.9	1.0	104.3	2.0
	5	97.4	1.5	90.5	15.9	96.4	1.9	110.8	0.4
	10	103.3	2.4	106.7	2.4	116.7	1.9	114.5	1.0
Spot volume analysis	**0.5**	117.2	0.9	122.3	8.3	107	3.9	112.8	3.5
	1	93.7	0.6	101	1.1	97.9	0.3	111.8	1.8
	2.5	95.4	0.7	90.6	3.3	100	1.3	103.6	1.4
	5	102.8	0.9	101.8	1.9	97	1.0	110.5	0.8
	10	104.5	0.5	107.4	0.4	116.6	0.7	112.4	0.3

[a] Triglycerides/cholesterol levels, respectively (mg/dL); [b] Age/Race/Gender
Gender: C=Caucasian; B=Black; H=Hispanic – Gender: M=Male; F=Female

0.9904, 0.3-2.3% and 6.2-7.7%, respectively. In the case of the spot sampling, although the LOD was two time higher (0.006 μg/mL) and LOQ were the same as those obtained with the fast convection approach, the figures of merit were slightly improved. Certainly, lower experimental error values can be explained due to the fact that the extraction on the spot sampling is performed under virtually exhaustive extraction conditions while the extractions in regular sample volumes were performed under non-equilibrium conditions and without automation [131].

Aiming to evaluate relative matrix effects, the slopes of the calibration curves of voriconazole prepared in five different lots of plasma were compared. As can be seen in Table 4.4, acceptable relative matrix effect were found for both methods, with slightly lower inter-slope errors attained for the spot approach in accordance with the already discussed results. With the objective of studying the accuracy of the proposed methodologies, five validation points within the therapeutic range of voriconazole (*i.e.* 0.5, 1, 2.5, 5, and 10 μg/mL) were prepared in plasma samples from four different patients and analyzed by CBS-MS/MS (n=3; each level). Thus, plasma samples from patients with different medical background (e.g. diverse triglycerides/cholesterol levels, race, gender, and age) were selected in order to investigate possible lack of accuracy at such conditions. As can be seen in Table 4.5, the accuracy values were in the ranges of 90.5 - 120.6% and 90.6 - 122.3 % for regular volumes and spot approach, respectively. It is important to point out that of the twenty accuracy experiments performed, only two of them showed accuracy results slightly higher than 120% at the lowest validation point (*i.e.* 0.5 μg/mL). Therefore, it can be concluded that the accuracy attained was satisfactory regardless of the cholesterol levels and the methodology used.

The total time of analysis in the case of regular sample volumes, when performing the experiments one-by-one, is in the order of 120 seconds per sample. It comprises 60 seconds of extraction, 20 seconds of rinsing, 20 seconds of desorption and 3 seconds of mass spectrometry analysis. However, if both processes are automatized (*i.e.* extraction in 96-well system and desorption/ionization) [129,131,133], the total analysis time can be reduced to just 25 seconds per sample [95]. Although our spot sampling approach cannot be as fast as the aforementioned technology, diminutive sample volumes can be analyzed with a total analysis time of only 180 seconds per sample. Hence, when comparing the proposed CBS-based approaches against other analytical strategies used for the determination of voriconazole in plasma samples (most of them addressed by means of LC), the former can unquestionably outperform the total analysis time (~ 10 minutes) [371,377,381] attained by those technologies.

4.3.4 Summary

In this work, two methodologies for the ultra-fast quantitation of voriconazole in human plasma were developed. Both approaches have all the advantages of SPME such as analyte enrichment and sample clean-up, as well as the speed and simplicity of direct coupling to mass spectrometry offered by CBS. Although the current set-up permits the quantitation of voriconazole down to 0.1 μg/mL in 120 seconds, in the near future fully automated systems will reduce the total analysis time to only 25 seconds per sample. Certainly, the regular volume approach would prove extremely convenient for the quantitation of this antifungal drug in routine clinical analysis, while the droplet approach is more appropriate to perform *in vitro* pharmacokinetic studies in small animals, as it allows for great temporal resolution since the total procedure do not last more than 180 seconds and the sample employed can be as small as 10 μL. In summary, our results demonstrated that

CBS-MS not only has a remarkable potential for the fast determination of therapeutic drugs in biofluids, but also eliminates the analytical burden of the classical sample preparation (*e.g.* tedious procedures) and liquid chromatography approaches (*e.g.* usage of complex fluidics and large solvent volumes).

Section 4.4 High-Throughput Screening and Quantitation of Target Compounds in Biofluids by Coated Blade Spray-Mass Spectrometry

4.4.1 Introduction

High-throughput analysis of complex samples via mass spectrometry (MS) is becoming increasingly relevant across a broad range of fields where large sets of compounds must be identified and quantitated[382]. Although high-throughput analysis has many applications, the most relevant include screening for contaminants in environmental samples[383] and pesticides in food matrices[384], as well as the determination of drug panels in biofluids[25]. Ideally, screenings should not only be fast, sensitive, and capable of being automated, but they must also provide acceptable precision and accuracy. Thus far, most of the analytical technologies developed for quickly screening target compounds in biofluids via MS require at least one sample preparation step prior to a chromatographic separation process[360,385,386]. Unavoidably, the sum of all these stages typically results in analytical workflows that cost more and take longer than is ideal. Moreover, some of these methods involve substantial labor and a significant amount of consumables (e.g. solvents, sorbents, salts, cartridges)[387]. Thus, it is unsurprising that MS-based screening technologies are currently shifting towards faster and cheaper methodologies. One example is ambient mass spectrometry (AMS)[52,388], which is a group of technologies that promote ion generation from untreated samples under atmospheric conditions. AMS requires no sample preparation or separation steps (no-analyte enrichment or matrix alteration), which makes it applicable for biofluid analysis, for example, by spiking/spotting a portion of the sample on a solid substrate, which is then interfaced with the mass spectrometer to generate ionic species via electrospray ionization (e.g. Paper Spray (PS)[49,83,90]). Indeed, multiple PS can be arranged in an

autosampler in order to perform high-throughput determinations[83,95]. Likewise, biofluid can be spotted on a substrate (e.g. mesh or glass slide) that is then automatically placed between a jet of charge species and the mass spectrometer (*e.g.* via desorption electrospray ionization (DESI)[344,389], or via direct analysis in real time (DART))[209]. Furthermore, automated analysis can be conducted by using a continuous flowing liquid to sample a surface containing a dried sample (e.g. dried blood spot) such as liquid extraction surface analysis (LESA)[24], or via a liquid microjunction surface sampling probe (LMJ-SSP)[390]). Despite AMS's simplicity, it can fail to provide adequate sensitivity when trying to quantify concentrations of target compounds at low or sub parts-per-billion (ppb) in biofluids[101,214]. In addition, the lack of sample preparation not only leads to significant ion suppression, but it also makes AMS-based technologies prone to requiring instrument maintenance more frequently. Aiming to enhance the performance attained by PS in terms of sensitivity, some applications have recently reported the use of coated PS, yet increasing the cost and decreasing the simplicity of the technology[214,217]. Moreover, while several AMS procedures have notably claimed analysis times in the range of a few seconds per sample, these times refer only to the instrumental period and do not take into account the sample pretreatment step (*e.g.* drying time of the biofluid spot on the paper[214,391]). Therefore, there is a great need for proficient sample preparation approaches that can deliver high-throughput MS analysis; as such approaches will enable rapid screening without compromising the method sensitivity or the mass spectrometer's integrity. In response to these issues, technologies that efficiently combine on-line solid-phase extraction (SPE) and MS[102,392]—such the RapidFire (RF)[29,40,41] by Agilent Technologies, or the TurboFlow by Thermo Fisher Scientific[33,38]—have been developed as potential solutions. For instance, the entire SPE-MS process in the RF is automated, and the instrumental analysis time range is between 10 and 15 seconds per sample[40]. Since these

technologies inherently preconcentrate the analytes of interest, quantitative screening at low ppb concentrations in complex matrices is feasible[41]. In spite of these systems' swiftness and simplicity, pure sample matrices cannot be directly injected on them, and at least one or two sample pre-treament steps are required in order to prevent damaging either the fluidics or the SPE cartridges. For example, in the case of urine, a diluting step is sufficient to introduce the sample into the MS system. However, matrices containing high protein concentration require special handling to prevent SPE clogging (e.g. protein precipitation and centrifugation). Moreover, unmanned instruments and multiple high-pressure pumps tend to contain intricate electromechanical systems that may require frequent maintenance, which can contribute to higher operational costs. The above-mentioned factors and challenges clearly demonstrate the ongoing need for the development of novel technologies that can address them. Solid-Phase Microextraction (SPME) is one such potential solution. SPME embraces solventless microextraction technologies with different geometrical configurations that combine sampling, analyte extraction, sample clean-up, and compound enrichment in a single step[228], and it has been proven to be effective for the anaysis of complex biological matrices on multiple occasions[393]. Furthermore, the direct coupling of SPME to MS has recently been demonstrated to be an extremely powerful tool in bioanalytical determinations. Combining these technologies improves the limits of quantitation, accelerates analysis throughput, and diminishes potential matrix effects when compared to direct sample introduction coupled to MS[136,151,173,178,192,220]. Coated blade spray (CBS) is an SPME-based device that can be directly interfaced with MS instruments. Essentially, CBS is a stainless steel sheet (350 µm, Ø) with a swordlike geometry (Figures 4.15 and 4.16) that is coated with polymeric adsorbent particles. However, unlike other SPME devices, it can also be

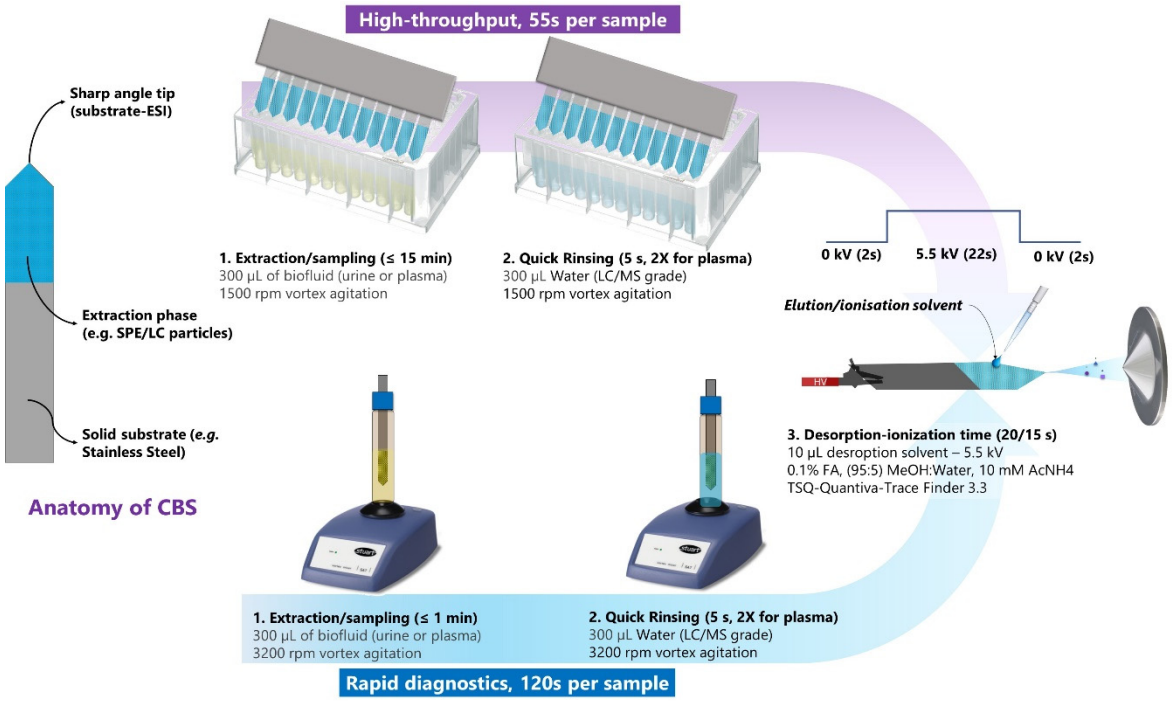

Figure 4.15 Experimental set-up for CBS-MS analysis of complex matrices, rapid diagnostics, and high-throughput configuration.

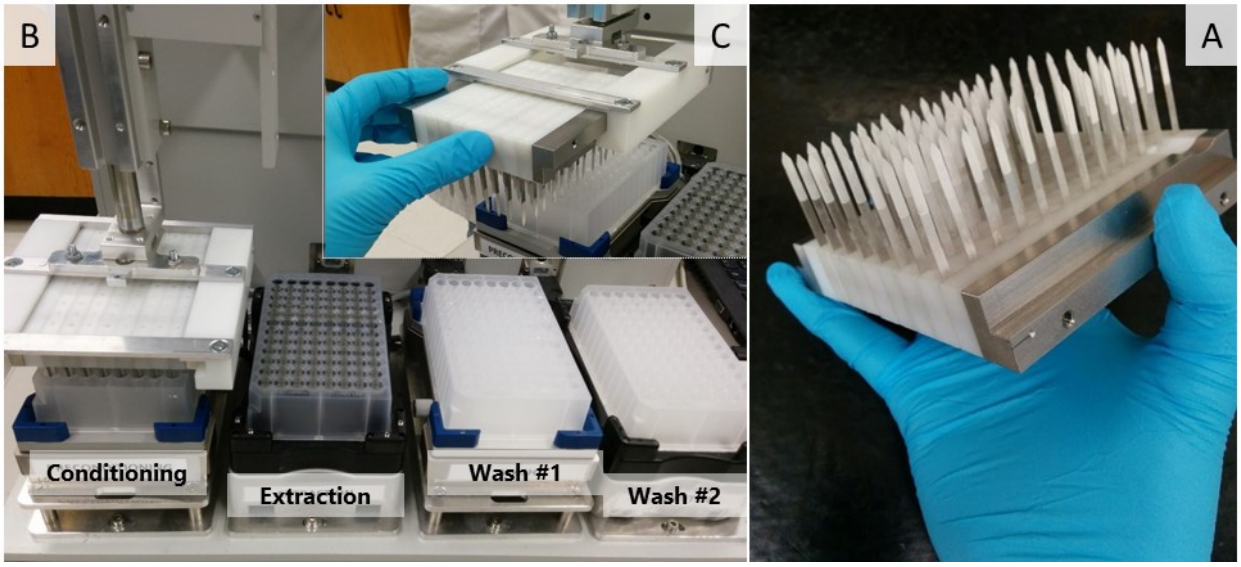

Figure 4.16 (A) CBS-brush with 8 rows of 12 blades each (96 blades total); **(B)** Concept 96-autosampler[25,131] ; and **(C)** Easy installation mechanism of the CBS-brush on the autosampler arm.

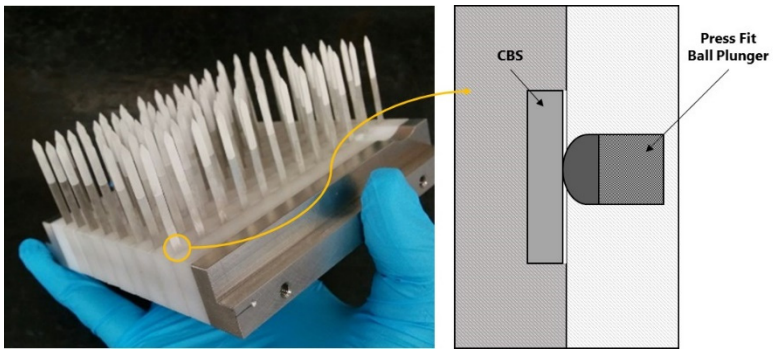

Figure 4.17 In house CBS-96 holder. Picture depicts the system for simple CBS installation/removal based on a press fit ball plunger.

used as a solid-substrate ESI source[136,220]. In this study, we assess CBS's suitability for use in high-throughput screening and in the quantiation of multiple compounds. Essentially, we perform 96 extractions simultaneously, which allows us to not only decrease the total analysis time per sample, but also to enhance the method's precision, accuracy, and LOQs. For this purpose, we selected a panel of compounds, including controlled substances, pain-management drugs, and therapeutic medications (*i.e.* mixture of anabolics, β-2 agonists, diuretics, stimulants, narcotics and β-blockers)[25,304]. In order to ensure that this assessment was as chemically comprehensive as possible, the list of proof-of-concept substances comprises a broad range of molecular weights, moieties, protein binding, and polarities. Furthermore, we tested our system in common bioanalytical matrices, such plasma and urine. Unlike online SPE-MS, no additional sample pretreatment is needed, and the entire analytical process is completed in less than 1 min.

4.4.2 Experimental section

Materials and supplies

Formic acid was purchased from Sigma-Aldrich (Saint Louis, USA), and LC-MS-grade methanol (MeOH), acetonitrile (ACN), isopropanol (IPA) and water were purchased from Fisher Scientific.

The following compounds were selected as model analytes for evaluating high-throughput-CBS (HT-CBS): methamphetamine, methamphetamine-d_5, carbamazepine, carbamazepine-d_{10}, propranolol, propranolol-d_7, clenbuterol, clebuterol-d_9, diazepam, diazepam-d_5, codeine, codeine-d_3, cocaine, cocaine-d_3, sertraline, sertraline-d_3, citalopram, citalopram-d_6, fentanyl, fentanyl-d_5, buprenorphine, buprenorphine-d_4, morphine, morphine-d_6, methadone, methadone-d_3, oxycodone, lorazepam, bisoprolol and stanozolol were acquired from Cerilliant Corporation (Round Rock, TX, USA). As noted, deuterated analogues of most analytes were used to correct for intra- and inter-experiment variability. In cases where no deuterated analogues were available as to correct for differences during extraction/ionization, the analyte and the internal standard paired were denoted in Table S1 with a superscript lowercase letter. For further details regarding compound properties, and selected reaction monitoring (SRM) transitions, see Table 4.6. Individual stock standard solutions were prepared in methanol at a concentration of 1000 $\mu g \cdot mL^{-1}$ and stored at -80 ºC. The phosphate-buffered saline solution (PBS) (pH 7.4) was prepared according to the procedure outlined in the Section 2.2. Human plasma (with K2-EDTA as anticoagulant) that had been pooled from different batches was purchased from Bioreclamation IVT (Westbury, New York, USA). All the plasma samples were spiked and stored overnight at 4 ºC in order to achieve the drug-protein binding equilibrium. Urine samples were collected from 10 healthy donors (five female and five male). The urine collection process for this particular study was conducted with the approval of the University of Waterloo's Office of Research Ethical Board.

CBS manufacturing

Stainless steel blades, which were purchased from Shimifrez Inc. (Concord, Ontario, Canada), were then coated using a slurry of hydrophilic lipophilic balance particles and polyacrylonitrile

Table 4.6 Target analytes and internal standards, polarities (LogP), minimum required performance levels (MRPL), and SRM transitions monitored for each model compound in positive ionization mode.

Compound	Log P	MRPL	Precursor	Product	Collision Energy	RF-Lens
Methamphetamine [a]	2.07	100	150.373	91.040	20	30
Oxycodone [a]	1.07	50	316.098	241.054	27	71
Metamphetamine-d_5 (IS) [a]			154.970	92.040	20	30
Carbamazepine [b]	2.45	-	237.304	194.097	19	57
Lorazepam [b]	2.39	50-240	321.054	274.889	21	72
Carbamazepine-d_{10} (IS) [b]			247.106	204.146	21	65
Propranolol [c]	3.48	100	260.070	116.111	18	62
Bisoprolol [c]	1.87	100	326.035	116.200	17	66
Propanolol-d_7 (IS) [c]			267.137	116.111	19	61
Clenbuterol	2.94	0.2	276.971	202.995	16	60
Clenbuterol-d_9 (IS)			286.092	204.015	17	48
Diazepam	2.82	5	284.995	193.054	32	82
Diazepam-d_5 (IS)			290.090	198.111	32	86
Morphine [d]	0.89	50	286.049	152.060	55	79
Salbutamol [d]	0.44	100	240.071	148.071	18	41
Morphine-d_6 (IS) [d]			292.076	152.060	55	86
Codeine	1.19	2	300.385	165.054	39	78
Codeine-d_3 (IS)			303.122	165.071	41	82
Cocaine	1.97	100	304.089	182.093	18	60
Cocaine-d_3 (IS)			307.055	185.111	20	50
Sertraline [e]	5.06	≤300	306.356	159.000	26	51
Stanozolol [e]	4.33	2	329.192	81.037	43	116
Sertraline-d_3 (IS) [e]			309.030	158.929	31	106
Methadone	3.93	50	310.048	265.007	15	57
Methadone-d_3 (IS)			313.272	268.166	15	57
Citalopram	3.5	≤300	325.094	109.071	28	73
Citalopram-d_6 (IS)			331.119	109.071	28	92
Fentanyl	4.12	1	337.468	188.183	22	70
Fentanyl-d_5 (IS)			342.261	188.111	24	72
Buprenorphine	4.63	5	468.250	396.111	38	119
Buprenorphine-d_4 (IS)			472.336	400.093	39	147

(HLB-PAN) according to procedure described below. HLB particles were kindly provided by Waters Corporation. The coating length and thickness was 15 mm and 10 μm, respectively. The dipping solution is prepared by mixing 5 g of polyacrylonitrile (PAN) solution and 72.5 mL of dimethyl formamide (DMF) on a container. This mixture is heated at 90 °C for 1 hour. After

cooling down, 6.3g of the solution are mixed with 0.65g of HLB particles (5 µm) on a 20 mL vial overnight prior to start the coating procedure. Preceding to any coating application, CBS should be etched with hydrochloric acid (HCl, 37 %) for 15 minutes. After etching the devices must be thoroughly clean with water and acetone and they should be kept on a desiccator with nitrogen flow prior to execute the coating process. Ideally, the coating solution should be continuously stirred during the coating procedure. For coating, the desired coated area (e.g. 15 mm) is immersed onto the slurry solution for approximately 10 seconds and subsequently remove and cure at 125 °C for 1 min. For the preparation of CBS used in this manuscript a single dipping step was followed.

Bioanalytical protocol

The workflow for the biofluid analysis consisted of four simple steps: extraction/preconcentration, coating rinsing, analyte desorption, and subsequent ionization. In the case of non-automated processes, extraction is performed by inserting the CBS into a vial containing 300 µL of biofluid and agitating it for 1 minute (3200 rpm, vortex agitation). Once this step has been completed, the blades are then placed into a vial containing water and agitated under vortex conditions twice ($t \leq$ 5s each) in order to remove matrix components that are physically attached to the coating surface. After this washing step, the blades are manually installed on the interface for MS analysis. As shown in Figure 4.17, automated CBS extractions were carried out using a Concept-96 system (Professional Analytical Systems (PAS) Technology, Magdala, Germany). This robotic sample preparation unit has been described in detail elsewhere[131,304]. Unlike the manual protocol, automated experiments were performed using 96-well plates (volume of 500 µL). A holder capable of arranging up to 96-CBS devices was constructed at the University of Waterloo's machine shop

(Figure 4.16). As can be seen in Figure 4.17, each blade can be manually positioned in a groove where a press-fit ball plunger fixes the blade via a clicking mechanism. An additional feature of the 96-CBS holder is that it can be easily installed/removed from the automatic arm by means of a rail-like mechanism. In contrast to manual experiments, the Concept-96 system cannot achieve much higher agitation speeds, with a maximum speed of 1500 rpm (orbital agitation); therefore, longer extraction times were selected in order to guarantee the best quantitation capabilities.

Mass Spectrometry analysis

All the experiments described in this manuscript were carried out using a TSQ Quantiva mass spectrometer (Thermo Fisher Scientific, San Jose, California, USA), and data processing was performed using Trace Finder 3.3 (Thermo Fisher Scientific, San Jose, California, USA). To guarantee that the blades were accurately positioned in front of the mass spectrometer during all experiments, an in-house ionization source was built at the University of Waterloo (see Figure 4.18). As a matter of fact, aiming to improve the spray stability, which might have been affected by the air conditioner flows on the laboratory, a plastic enclosure was built. Figure 4.19 portray the results obtained with and without the enclosure. Undeniably, by enclosing the CBS interface lower signal variability can be attained. Hence, the aforementioned device was used otherwise stated. Once the CBS had been installed on the interface, 10 µL of a 95:5 MeOH/water v/v 0.1% formic acid solution was applied to the coated area in order to desorb the analytes ($t \leq 20$ s). After analyte elution on the desorption solution, a voltage of 5.5 kV was established between the CBS and the MS entrance to generate electrospray from the tip of the blade (see Figure 4.4).

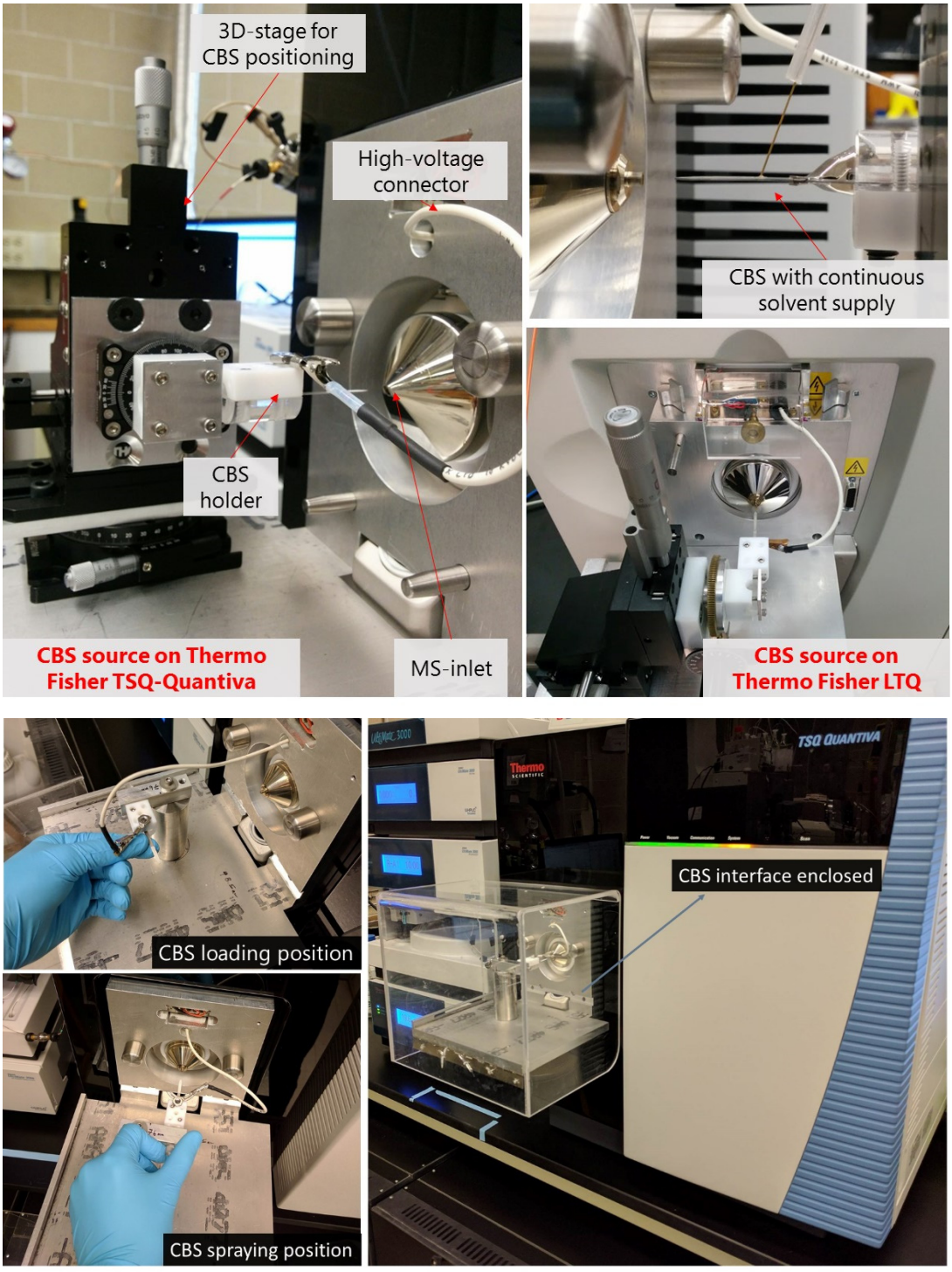

Figure 4.18 CBS-MS interfaces used for the direct coupling of CBS to Thermo Fisher TSQ-Quantiva and Thermo Fisher LTQ with multiple degrees of freedom (top). CBS-MS interface with enclosed system and one degree of freedom for easy CBS loading-spraying (bottom).

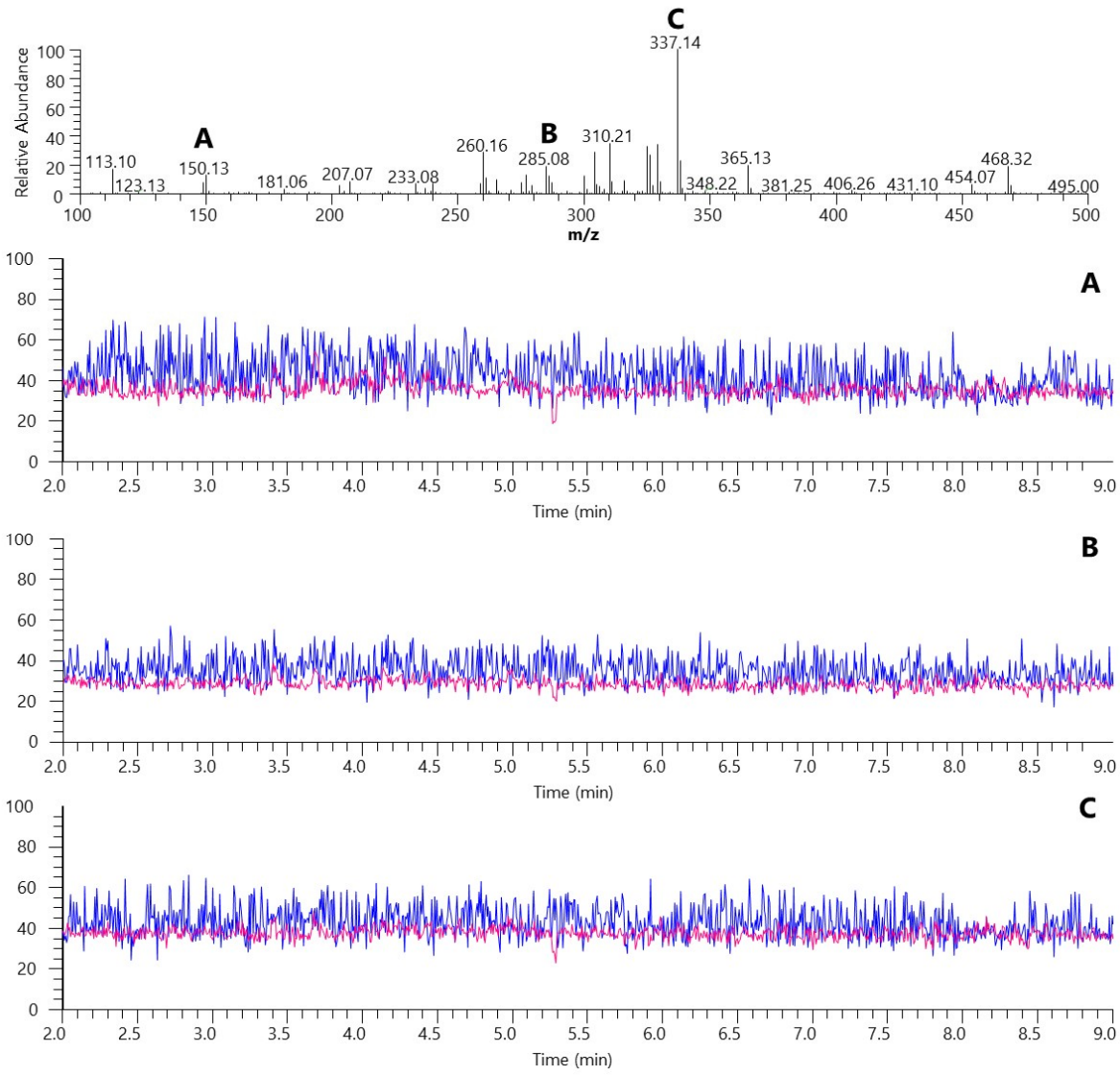

Figure 4.19 Ion chronograms of (**A)** methamphetamine (m/z 150), (**B**) diazepam (m/z 285) and (**C**) fentanyl (m/z 337) obtained by direct infusion of a 1 µg mL^{-1} solution of the compounds listed on Table 4.16. In this experiment, CBS was used as an ESI source with a set-up similar to the one presented in Figure 4.9. A spray voltage of 5.5 kV was applied. Pink and blue lines represent the signal obtained with and without the enclosure system displayed on Figure 4.18, respectively.

All analyses were carried out in positive ionization mode. Optimum collision energy and RF-lens conditions were tuned for each compound via the direct infusion of methanolic standards. MS/MS transitions, optimum collision energy (CE), and RF-lens voltages for each analyte can be found in Table 4.6.

Characterization

The methodologies were characterized with respect to linearity, precision, accuracy, and LOQ. Calibration functions were constructed on the basis of the signal ratio of the analyte and its isotopologue (A/Is) for 12 concertation levels in three independent replicates from 0.05 $ng \cdot mL^{-1}$ to 100 $ng \cdot mL^{-1}$. Furthermore, three different concentrations levels (3, 40, and 80 $ng \cdot mL^{-1}$) were analyzed in order to assess precision and accuracy. LOQs were calculated as the lowest calibration point with precision values lower than 20%.

4.4.3 Results and discussion

CBS-MS: a novel technology for concomitant and rapid screening

CBS has numerous advantages over most SPME-MS couplings, such as: low solvent consumption per analysis ($\leq 15\mu L$)[182]; no fluidics requirements (no pumps, valves or tubes are used)[178]; no gas or heating requirements[174,192]; no need for a desorption chamber[114,173,182]; no need of a pumping mechanism during the sample preparation process[114,197,394]; and no need for a sampling vessel when analyzing small sample volumes[114,173,182]. In addition, CBS can be coated with any extractive material, thus offering broader analyte coverage than other devices[114,197,211,212] Furthermore, as presented in this manuscript, CBS is capable of quantifying multiple compounds in a single analysis because the area under the curve used for quantitation purposes lacks the shape of a Gaussian peak with a limited peak width[114,164,167,174,178], and the electrospray event can be extended by using a continuous solvent supply. Perhaps the greatest advantage of CBS is that the blade acts as both the extraction device and the ionization source. Given all its features, CBS demonstrates to be a good alternative for sample preparation and direct-to-MS technologies[395]. In order to use the full potential of CBS important variables such coating type and speed of extraction should be

carefully optimized. For instance, if a reduction of the extraction time is aimed, fast convection conditions are required. Thus, either vortex agitation or high-speed orbital agitation (*i.e.* 1500-3200 rpm) are strongly recommended (see Figure 4.15). Furthermore, based on previous work done in our group[131], HLB coated blades were selected with the objective of providing a wide and balance analyte coverage (*i.e.* extraction of compounds with octanol-water partition coefficient ranging between 0.44 to 5.06, see Table 4.6).

Non-automated analysis

In this study, two workflows (manual and automated) suitable for completely different bioanalytical scenarios have been proposed. The non-automated methodology, which consists of performing analyte extraction by hand (Figure 4.15), is chiefly intended for point-of-care therapeutic drug monitoring or any application where the rapid assessment of an analyte concentration needs to be performed[8,9]. Certainly, it is quite useful when single or a small number of analyses are required, as this keeps the total analysis time for the extraction and quantitation under 2 minutes per sample. As can be seen in Table 4.7, LOQs below 0.5 ng/mL were attained for the majority of the analytes when performing 1 min extractions from 300 µL of PBS. Moreover, good accuracy (82.3 to 116.2 %) and precision (≤ 8%) were attained at the three tested quality control (QC) levels (3, 40 and 80 ng/mL).

Urine analysis Since urine is widely available and uninvasive to collect, it is the most suitable matrix for performing multi-residue analysis of prohibited substances[233]. However, urine is also one of the most challenging biological matrices; as such, it was unsurprising that urine's LOQ median (3.75 ng/mL) was 10 and 7 times higher than in PBS and plasma, respectively (see Table

4.8). Although the minimum required performance levels (MRPLs) set by the World Antidoping Agency (WADA) were not achieved for all the controlled substances (e.g. clenbuterol), all drugs of abuse were quantified at the cut-offs set by the most common currently used screening assays (*e.g.* 5 ng/mL is the lowest cut-off level in urine; *i.e.* fentanyl)[396]. Likewise, good accuracy (83-110%), and great precision (≤ 10%) were achieved at the three QC levels tested for all the studied probes (3, 40 and 80 ng/mL).

Plasma analysis Plasma samples are a great alternative to complement urine analysis in prohibited substances determinations[360]. In fact, plasma, or serum, has been the preferred matrix over whole blood samples and, as a result, most clinical reference data is based on either plasma or serum concentrations[304,347]. However, plasma samples pose their own set of challenges. For instance, since SPME-based technologies extract via free concentration, substances characterized by high protein binding are expected to exhibit higher limits of quantification[397]. Furthermore, due to plasma's intrinsic characteristics (e.g. viscosity due to protein content), rinsing steps are critical for removing clusters of macromolecules/salts that might be lingering on the coating surface during the extraction process, which can potentially cause ion suppression or instrument contamination. As expected, LOQs in plasma are equal to or considerably better than in urine; however, compounds that are heavily bound to proteins stand as an exception to this trend (e.g. diazepam or buprenorphine, see Table 4.9). Nonetheless, our results are in perfect agreement with the quantitation requirements for both drugs of abuse in blood samples and the therapeutic cut-off levels of most medications[396]. In addition to the quantitation capabilities, the characterization of our experiments showed good accuracy (80 to 120 %), precision (≤ 8%), and linearity for all study probes at the three tested QC levels (3, 40, and 80 ng/mL).

Table 4.7 linear regression slopes, intercepts, and correlation coefficients (R^2). Limits of quantitation (LOQ), accuracy (%), and precision (percentage relative standard deviation, RSD %) values for three different concentration levels. All values were calculated based on the protocol in the experimental section for the non-automated approach and PBS as a sample.

Compound name	Slope	Intercept	R^2	LOQ ($ng \cdot mL^{-1}$)	Accuracy (n=3), %			Precision (n=3), RSD %		
					3 ($ng \cdot mL^{-1}$)	40 ($ng \cdot mL^{-1}$)	80 ($ng \cdot mL^{-1}$)	3 ($ng \cdot mL^{-1}$)	40 ($ng \cdot mL^{-1}$)	80 ($ng \cdot mL^{-1}$)
Methamphetamine	0.546	0.509	0.9995	0.5	95.7	101.2	87.8	19.3	9.1	3.6
Carbamazepine	0.018	0.026	0.9952	0.5	109.9	105.8	93.9	17.9	3.9	4.8
Propranolol	0.151	0.041	0.9968	0.25	116.2	107.1	100.5	4.4	4.9	3
Clenbuterol	0.121	0.008	0.9971	0.25	100.3	101.5	94.9	1.5	2.3	1.7
Diazepam	0.009	0.001`	0.9967	0.1	104.7	106.7	97	2.3	4.5	0.7
Codeine	0.084	0.002	0.9980	0.5	113.7	105.5	99.4	4.9	1	1.5
Cocaine	0.093	-0.009	0.9977	0.25	107.4	101.3	96.5	5.4	1.7	4.2
Sertraline	0.063	0.022	0.9962	0.25	92.1	101.8	96.4	2.9	8.3	2.6
Citalopram	0.095	-0.024	0.9979	0.25	112	104.4	98.7	1.3	5.4	0.9
Fentanyl	0.098	0.009	0.9980	0.5	110.6	106.4	97.1	1.2	3.8	0.7
Buprenorphine	0.012	0.003	0.9982	2.5	88.4	106	101.6	9.2	3	0.9
Morphine	0.076	0.017	0.9996	1	105.2	97.4	103.8	4.8	12.7	13.3
Methadone	0.105	-0.005	0.9948	0.25	102.9	107	99.2	6.7	1.4	3.5
Salbutamol	0.263	0.370	0.9949	0.5	82.3	105.2	90	23.4	14	14.9
Oxycodone	0.072	0.032	0.9992	0.25	110.2	93.4	93.6	36.5	21.8	22
Lorazepam	0.004	0.004	0.9938	2.5	110.3	110.6	91.6	8.7	33.1	37.3
Bisoprolol	0.292	-0.178	0.9997	0.25	115	97	84.4	17.7	11.8	14.2
Stanozolol	0.052	0.103	0.9951	0.5	88.9	96.4	74.5	10.8	10.5	10.7

Table 4.8 linear regression slopes, intercepts, and correlation coefficients (R^2). Limits of quantitation (LOQ), accuracy (%), and precision (percentage relative standard deviation, RSD %) values for three different concentration levels. All values were calculated based on the protocol in the experimental section for the non-automated approach and plasma as a sample.

Compound	Slope	Intercept	R^2	LOQ ($ng \cdot mL^{-1}$)	Accuracy (n=3), %			Precision (n=3), RSD %		
					3 ($ng \cdot mL^{-1}$)	40 ($ng \cdot mL^{-1}$)	80 ($ng \cdot mL^{-1}$)	3 ($ng \cdot mL^{-1}$)	40 ($ng \cdot mL^{-1}$)	80 ($ng \cdot mL^{-1}$)
Methamphetamine	0.43	-0.18	0.9996	0.5	110.8	91.8	95.4	2.7	2.5	6
Carbamazepine	0.017	0.0019	0.9992	0.5	101.8	97.3	91.4	8.5	4.7	6.2
Propranolol	0.14	0.098	0.9991	0.5	88.7	81.7	78.8	8.5	13.2	10.9
Clenbuterol	0.11	-0.025	0.999	0.5	110.5	79.4	82.6	9.3	9.6	8.9
Diazepam	0.0077	0.045	0.9914	10	-	77.1	81.2	5.6	17.1	7.9
Codeine	0.078	0.074	0.9993	1	69.9	93.7	99.4	3.8	4.6	1.5
Cocaine	0.082	0.063	0.9992	0.5	87.1	84.1	85.2	4.9	9.3	10.6
Sertraline	0.06	0.19	0.9961	2.5	25.5	80.9	83	14.4	13.6	9.2
Citalopram	0.08	0.083	0.9952	0.25	79.7	87.6	84.9	8.1	12.4	1.9
Fentanyl	0.087	0.089	0.9983	0.25	81	83.8	77.8	12	3.3	6.4
Buprenorphine	0.012	0.15	0.9877	25	-	79.2	71.6	-	3.3	3.1
Morphine	0.11	12	0.9998	2.5	89.9	98.2	101.7	6.4	0.9	4.1
Methadone	0.078	0.19	0.9883	0.5	55.3	79.2	88.1	11.7	11.4	9.7
Salbutamol	0.13	0.19	0.981	0.5	54.3	89.5	104.5	4	7.1	4
Oxycodone	0.04	0.12	0.9984	1	100.8	88.8	101.2	1.8	21.2	9.8
Lorazepam	0.0012	0.011	0.9865	25	-	99.9	121.9	-	12.9	18.3
Bisoprolol	0.37	0.59	0.9983	0.25	55.7	97.7	103	15.9	3.3	13.9
Stanozolol	0.013	0.12	0.8342	25	-	56.8	85.4	-	1.8	22

Table 4.9 linear regression slopes, intercepts, and correlation coefficients (R^2). Limits of quantitation (LOQ), accuracy (%), and precision (percentage relative standard deviation, RSD %) values for three different concentration levels. All values were calculated based on the protocol in the experimental section for the non-automated approach and urine as a sample.

Compound	Slope	Intercept	R^2	LOQ ($ng \cdot mL^{-1}$)	Accuracy (n=3), %			Precision (n=3), %		
					3 ($ng \cdot mL^{-1}$)	40 ($ng \cdot mL^{-1}$)	80 ($ng \cdot mL^{-1}$)	3 ($ng \cdot mL^{-1}$)	40 ($ng \cdot mL^{-1}$)	80 ($ng \cdot mL^{-1}$)
Methamphetamine	0.42	0.44	0.9991	2.5	87	93	100	3.3	2.8	3.5
Carbamazepine	0.014	0.077	0.998	10	-	99	104	4.7	5.8	5.7
Propranolol	0.13	-0.077	0.9981	0.5	132	96	108	3.1	12.9	5.9
Clenbuterol [a]	0.12	0.065	0.9983	2.5	102	94	103	6.6	0.8	3.8
Diazepam	0.0092	0.0085	0.9996	2.5	93.8	95.8	99.9	8	1.3	1.9
Codeine	0.083	0.14	0.9986	10	-	94.9	99.9	-	1.3	3.4
Cocaine	0.086	0.028	0.9986	0.5	98.2	96.1	105.1	4.5	1.5	1.4
Sertraline	0.063	0.1	0.9978	5	-	92.9	97.6	4.5	5.8	0.9
Citalopram	0.061	0.0045	0.9964	1	107.8	91.8	98.6	6.7	15.7	8.4
Fentanyl	0.096	0.021	0.9991	0.25	102.4	94.3	104.7	5.4	2.3	1.2
Buprenorphine	0.014	0.1	0.9985	10	-	88.5	98.2	-	1.7	2.1
Morphine	0.086	0.13	0.9965	10	-	98.8	105.6	-	6.6	4.1
Methadone	0.09	0.062	0.9996	0.5	89.6	94.8	106.1	2.7	2.1	2.1
Salbutamol	0.14	0.23	0.9921	10	-	93.2	105.9	-	27.5	17.8
Oxycodone	0.017	-0.0075	0.9997	5	-	104.6	108.7	-	13.4	13
Lorazepam	0.0021	0.027	0.9992	25	-	90.4	83.7	-	10.5	28.9
Bisoprolol	0.18	0.0042	0.9995	0.25	109.4	91.1	94.3	3.8	3.3	7.6
Stanozolol	0.075	0.58	0.9943	10	-	85.5	101.9	-	3	9.7

[a]The required LOQ for clenbuterol was not achieved (0.2 $ng \cdot mL^{-1}$)

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High-throughput analysis

The automation of a given analytical method decreases operating costs, minimizes experimental error during measurement, and increases the analysis throughput[14,95]. For the past ten years, researchers have explored the use of SPME devices for high-throughput analysis by using robotic autosamplers that enable parallel analyte extraction in a 96-well plate format [25,129,131,133,157,162,304]. However, prior studies have generally introduced the sample extract into the MS system via gas or liquid chromatography[133]. To the best of our knowledge, this is the first application where, after the unmanned high-throughput sample preparation process, the SPME devices are directly coupled to the mass spectrometer. As shown in Figure S1, a novel holder was designed to facilitate rapid and independent CBS installation; thus, unlike the original blade system[25,131], our proposed prototype is electrically isolated and can be effortlessly uninstalled from the holder to performed MS analysis. Besides, unlike previous designs[133], the holder can be mounted on the Concept-96 autosampler in just a few seconds (see Figure 4.17). Moreover, since automation is able to process multiple samples simultaneously, it allows for an increase in extraction/enrichment time of the SPME process without dramatically increasing the total analysis time[25,393]. Consequently, an extraction time of 15 minutes (~9 s per sample) was selected with the goal of increasing the amount of analyte extracted and decreasing the LOQs previously attained by the manual procedure. As can be seen in Tables 4.10 and 4.11, our results demonstrate that HT-CBS not only decreased the median LOQ (3.5 and 1.5 fold decrease for urine and plasma, respectively), but it also improved the precision and accuracy attained at all the QC concentration levels (2.5% and 3% median precision for plasma and urine, respectively; 98.5% and 100.6% median accuracy for plasma and urine, respectively). Moreover, as shown in Figure 4.20, great linearity was attained for all compounds over the assessed range in both matrices, even when their deuterated analogues were

Table 4.10 Figures of merit for the high-throughput quantitation of multiple analytes in human plasma via CBS-MS/MS

Compound	Slope	Intercept	R^2	LOQ ($ng \cdot mL^{-1}$)	Accuracy (n=3), %			Precision (n=3), %		
					3 $ng \cdot mL^{-1}$	40 $ng \cdot mL^{-1}$	80 $ng \cdot mL^{-1}$	3 $ng \cdot mL^{-1}$	40 $ng \cdot mL^{-1}$	80 $ng \cdot mL^{-1}$
Methamphetamine	0.11	-0.0353	0.9964	1.0	106.9	90.4	98.3	0.4	0.2	0.8
Carbamazepine	0.07	-0.0003	0.9961	0.1	103.6	96.8	95.0	1.5	3.8	2.5
Propranolol	0.20	-0.064	0.9944	1.0	106.4	91.4	97.6	0.6	1.4	0.7
Clenbuterol	0.11	-0.013	0.9956	0.5	103.8	91.3	97.5	1.3	0.2	1.2
Diazepam	0.13	-0.032	0.9953	1.0	106.0	91.8	99.4	0.1	1.8	1.2
Codeine	0.09	-0.031	0.9955	1.0	103.3	90.8	98.0	0.5	0.3	1.4
Cocaine	0.09	-0.013	0.9961	0.5	104.7	92.3	96.5	0.8	0.6	0.2
Sertraline	0.06	-0.023	0.9950	1.0	105.6	89.7	98.5	4.2	0.6	1.7
Citalopram	0.10	-0.019	0.9967	0.5	104.4	90.3	98.9	0.8	1.0	0.4
Fentanyl	0.07	-0.014	0.9953	0.5	101.3	91.0	96.6	2.0	0.7	0.7
Buprenorphine	0.09	-0.029	0.9927	2.5	121.8	91.3	93.9	7.5	2.9	4.3
Morphine	0.07	0.081	0.9940	1.0	104.4	90.7	94.8	2.4	2.4	0.7
Methadone	0.01	-0.001	0.9959	0.5	103.3	91.4	99.4	0.5	0.5	0.5
Salbutamol	0.03	-0.002	0.9916	0.5	111.4	101.1	93.4	6.7	9.1	2.5
Oxycodone	0.17	-0.018	0.9957	0.5	104.2	92.6	100.3	3.3	4.1	2.3
Lorazepam	0.10	0.054	0.9822	1.0	104.1	97.8	108.6	9.2	14.3	3.4
Bisoprolol	0.10	-0.028	0.9973	0.1	106.3	93.5	101.5	2.7	6.2	2.3
Stanozolol	0.04	0.134	0.9741	10	-	84.6	95.4	-	6.0	8.2

Table 4.11 Figures of merit for the high-throughput quantitation of multiple analytes in human urine via CBS-MS/MS.

Compound	Slope	Intercept	R^2	LOQ ($ng \cdot mL^{-1}$)	Accuracy (n=3), %			Precision (n=3), %		
					3 $ng \cdot mL^{-1}$	40 $ng \cdot mL^{-1}$	80 $ng \cdot mL^{-1}$	3 $ng \cdot mL^{-1}$	40 $ng \cdot mL^{-1}$	80 $ng \cdot mL^{-1}$
Methamphetamine	0.12	0.009	0.9961	0.25	91.4	102.6	97.8	0.3	1.4	1.6
Carbamazepine	0.06	0.067	0.9943	2.5	85.4	111.4	95.2	4.9	0.5	4.9
Propranolol	0.21	-0.003	0.9965	0.25	89.5	104.7	98.9	0.2	1.1	0.6
Clenbuterol	0.11	0.038	0.9961	0.25	91.5	103.1	98.1	1.0	0.6	0.6
Diazepam	0.12	0.014	0.9942	0.5	95.2	103.6	101.1	1.3	0.4	1.9
Codeine	0.09	0.018	0.9932	1.0	94.3	101.3	96.0	1.5	0.3	0.9
Cocaine	0.09	-0.003	0.9953	0.25	93.5	103.3	98.3	0.7	1.0	0.1
Sertraline	0.06	-0.003	0.9904	2.5	115.6	105.9	97.7	1.4	2.9	1.5
Citalopram	0.06	0.0004	0.9929	0.5	90.3	111.5	96.4	1.7	10	3.8
Fentanyl	0.07	-0.003	0.9961	0.25	92.6	103.2	97.1	0.7	0.6	0.3
Buprenorphine	0.11	-0.008	0.9929	0.5	92.4	102.7	100.0	2.2	0.7	2.8
Morphine	0.06	0.173	0.9938	1.0	94.4	104.0	102.4	8.1	1.5	5.2
Methadone	0.01	-0.0003	0.9962	0.25	94.5	104.3	99.5	0.6	1.0	0.4
Salbutamol	0.04	0.014	0.9909	10	-	88.9	89.0	-	15.6	7.3
Oxycodone	0.13	0.098	0.9972	1.0	92	112.8	103.0	3.7	0.9	1.3
Lorazepam	0.08	0.443	0.9908	10	-	121.8	122.1	-	19.9	10
Bisoprolol	0.06	-0.007	0.9925	0.5	94.5	104.8	90.4	4.0	1.2	0.3
Stanozolol	0.10	0.175	0.9952	5	-	115.5	110.7	-	5.5	11

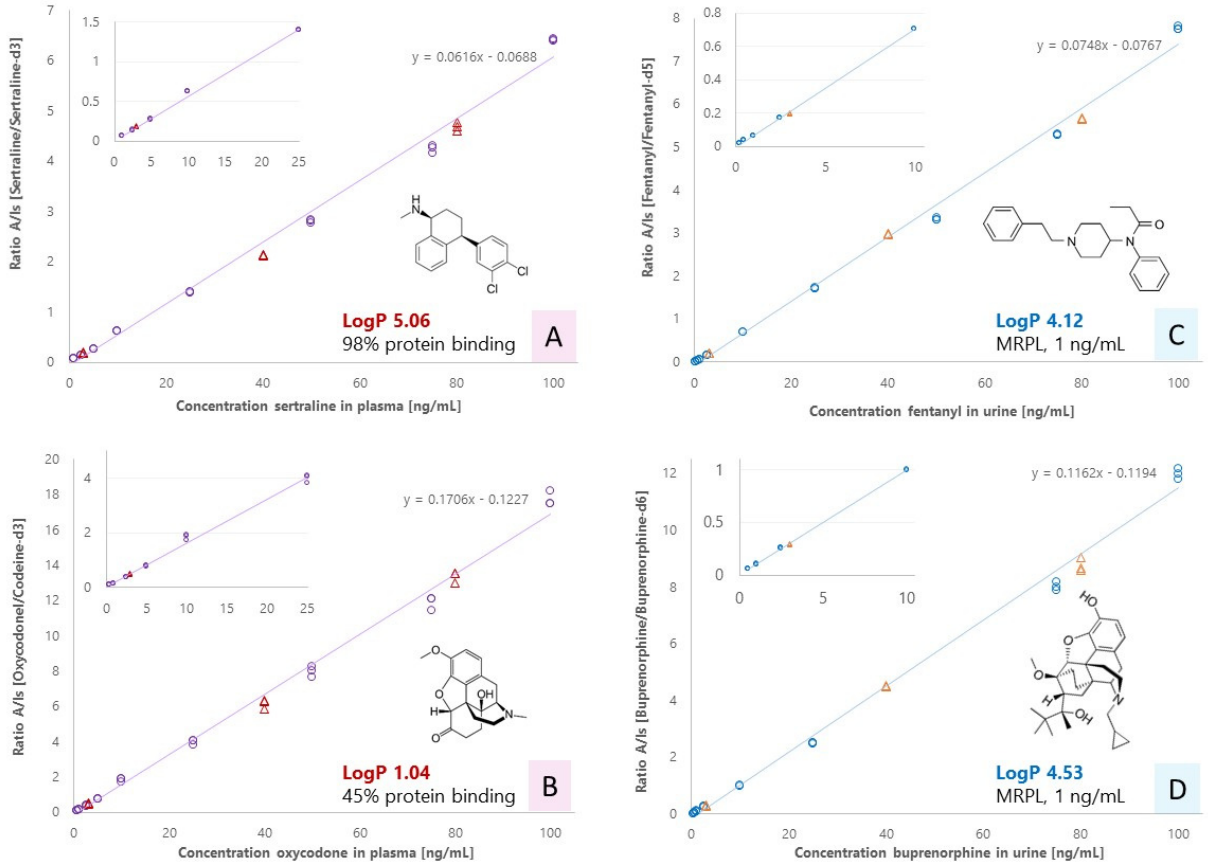

Figure 4.20 A. Quantitative analysis of plasma spiked with sertraline (0.5 to 100 ng mL−1) and its isotopologue [D3] sertraline (10 ng mL−1). B. Quantitative analysis of plasma spiked with oxycodone (0.5 ng ml−1 to 100 ng mL−1) and [D3] codeine as internal standard (10 ng mL−1). C. Quantitative analysis of urine spiked with fentanyl (0.25 to 100 ng mL−1) and its isotopologue [D5] fentanyl (10 ng mL−1). D. Quantitative analysis of urine spiked with buprenorphine (0.5 ng ml−1 to 100 ng mL−1) and its isotopologue [D6] buprenorphine (10ng mL−1). Three replicates with independent CBS devices were run for each calibration and accuracy point. The red and orange triangles represent the accuracy levels evaluated for all compounds on each matrix.

not used for correction (e.g. oxycodone). Furthermore, since CBS bypasses the chromatographic and desorption steps of the former SPME protocol[133], HT-CBS can offer the same or better quantitative results than those attained by Boyacı et al. for HT-SPME, but in 1/33 of the time (less than 55 s per sample)[25]. Given the number of compounds and internal standards screened in our method (*i.e.*30), a cycle time of 1.5 seconds is required when using a dwell time of 50 ms. Hence, if the desired target is 15 scans per compound, the fastest electrospray event possible will be 22.5s.

Thus, with a total sample preparation time of less than 10 s per sample, the MS time now becomes the bottleneck in our methodology. Undeniably, the speed of the analysis can be further enhanced by decreasing the dwell time at the expenses of the attainable LOQ. For instance, by decreasing the dwell time from 50 to 10 ms, one could shorten the MS analysis time to less than 5 s; at the same time, the LOQ might only increase by a factor of 2.2 times as the signal-to-noise ratio is proportional to the square root of the dwell time (S/N ~ $\sqrt{t}$). Theoretically, the LOQ attained via the HT-CBS methodology would allow for a decrease in dwell times because the WADA and clinical laboratory reference values were met below requirements[25,396]. However, this is just a conjecture, and full experimental validation will be indispensable in determining the LOQ for a given application that uses shorter dwell times.

Notwithstanding the good results attained, it is important to bear in mind that no technique is perfect, and the lack of a separation step (*e.g.* chromatography) may result in insufficient selectivity for some compounds when exclusively performing MS/MS analysis[34,178]. Interferences could be observed when the target analyte is co-extracted from the matrix with isomeric species that share the same fragmentation pattern. Certainly, this is an issue inherent of any direct-sample-to-MS approach and not only observed by SPME-MS. Although CBS is capable of performing ultra-fast extraction/enrichment from biofluids, it is important to highlight that the coatings developed up to date are not selective enough to discriminate between the compound of interest and a potential isobar or isomer. For instance, as shown in Table 4.10, the HT-CBS method could not meet the MRPL set for clenbuterol in urine (0.2 ng/mL)[25,233,345] as isobars that share the MS/MS transition set for clenbuterol (m/z 277→ 203) were co-extracted from urine[25,178,344]. Hence, the "blank" signal in urine samples is significantly higher than the one observed on PBS samples. Undeniably, such results may lead to false positives and untrustworthy LOQs.

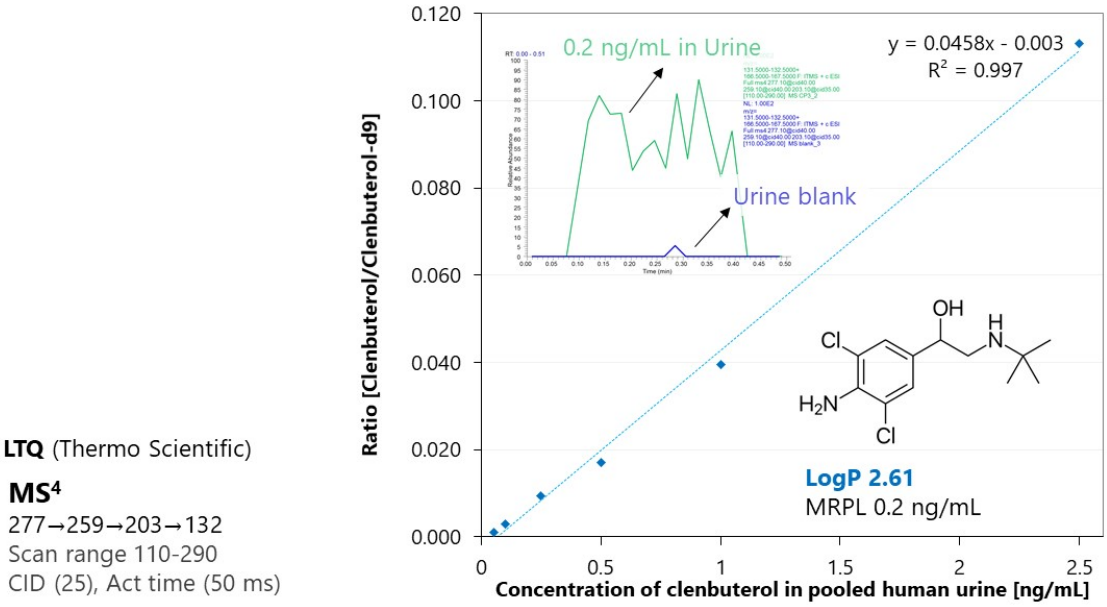

Figure 4.21 Quantitative analysis of urine spiked with clenbuterol (50 pg ml^{-1} to 2.5 ng mL^{-1}) and its isotopologue [D9] clenbuterol (10 ng mL^{-1}). Analyses were performed using CBS-MS^4 (*m/z* 277→259→203→132).

This lack of specificity can be overcome by using tandem MS in time[8,34,178]. As can be seen in Figure 4.21, we achieved an LOQ of 0.05 ng/mL by using MS^4 (277→259→203→132) on a linear trap quadrupole (LTQ). Certainly, other on-line technologies, such as ion-mobility based approaches, can also be used to enhance instrumental selectivity without compromising total analysis time[29,178,336,341].

4.4.4 Summary

Compared to other SPME-MS couplings, CBS offers additional features that allow it to be implemented in a broader range of applications[114,174,178,267]. For instance, CBS can hold longer acquisition times (t ≥ 15s), which allows it to monitor more MS events and, consequently, more compounds per analysis. Moreover, when compared to direct-sample-to-MS approaches[83], CBS offers efficient compound enrichment, better sensitivity, wider sample volume range, and

negligible contamination of the MS system. Furthermore, as with other SPME devices[313,393], CBS can be used as a means of transporting the "sample" from the sampling site to the laboratory, and it can be shipped by mail with no reasonable expectations of occupational exposure to the biofluid or any other potentially infectious materials[398]. In this work, we have demonstrated that CBS-MS/MS is a suitable platform for rapidly and accurately quantitating multiple target compounds in complex matrices. The methodology was characterized for 18 compounds of interest, including therapeutic medications, pain management drugs, and prohibited substances in plasma and urine samples[151]. Essentially, CBS-MS can be used either as a rapid diagnostic tool in point-of-care applications (*e.g.* single extraction on-site, 2 min per sample), or for high-throughput determinations via a robotic autosampler (96-well approach, 55s per sample). Indeed, the HT-CBS method met both the WADA[25] and clinical laboratory[396] minimum required performance levels for all the assessed substances. Thus, our current work is focused on the development of a CBS-autosampler that allows up to 96-samples to be processed simultaneously without supervision of each CBS's MS event. We hypothesize that a fully optimized system that includes all the sample prep and MS steps will enable us to achieve a 15 s sample-to-sample duty cycle. It must be highlighted that in this manuscript our goal was to demonstrate the quantitation capabilities of CBS towards a wide range of compounds with different physicochemical properties; though, when developing clinical applications, metabolites of each drug under study should also be considered and included on the determination of the parent drug total concentration. For instance, an enzymatic hydrolysis (*e.g.* using a β-glucuronidase) can be implemented on the workflow prior to the enrichment step[148]. Finally, we demonstrated that, when dealing with compounds with known interferences (*e.g.* clenbuterol), on-line technologies such tandem MS in time[34,65] can be used to enhance selectivity and reach the required limits of quantitation. Undeniably, the methodology

described in this paper is not limited to bioanalytical applications; indeed, it can be easily implemented for fast screening/quantitation in other analytical applications, such determining pesticide levels or emerging contaminants in environmental or food matrices[139,278].

Section 4.5 Rapid determination of immunosuppressive drug concentrations in whole blood by Coated Blade Spray-Tandem Mass Spectrometry (CBS-MS/MS)

4.5.1 Introduction

Immunosuppressive drugs (ISDs) are a class of medication that control or reduce the immune system's activity[399]. ISDs are regularly used to prevent the rejection of transplanted organs and tissues, as well as to treat autoimmune diseases or illnesses, such as psoriasis, lupus, and rheumatoid arthritis. Traditionally, ISD therapy requires drug concentrations to be closely monitored due to their narrow therapeutic range[27]. Essentially, an under-dose of ISDs can lead to graft (*i.e.* a piece of living tissue that is transplanted surgically) rejection/impairment and an over-dose can cause severe nephrotoxicity and/or overimmunosuppresion, which can subsequently result in an excessive risk of infection and malignancies[13]. Therefore, frequent Therapeutic Drug Monitoring (TDM) of ISDs in whole blood concentrations is essential for patients who have recently experienced organ transplantation[6,44,400]. Historically, immunoassays have been used to assess ISD concentrations in a patient's blood[27,399]. However, a report recently published by Seger *et al.* shows that approximately 50% of tacrolimus (TAC) and cyclosporine (CycA) determinations are currently performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) [13,401]. Furthermore, determinations for sirolimus (SIR) and everolimus (EVR), which are both mTOR (mechanistic target of rapamycin), are conducted using by LC-MS/MS in a range of 70 to 75%[400]. On average, approximately 60% of ISD determinations are performed via LC-MS/MS; this is largely due to immunoassay determinations' high level of risk for showing either matrix-induce method bias (*i.e.* susceptibility to false-positives caused by other matrix components)[13], or cross-reactivity-induced method bias (*e.g.* interaction of metabolites of the ISDs), which displays

higher concentrations than those reported by LC-MS/MS[13,401]. State-of-the-art MS instruments have allowed limits of quantitation (LOQ) as low as 0.1 ng mL^{-1} for TAC [402,403], 0.2 ng mL^{-1} for SIR/EVR[16,404], and 2 ng mL^{-1} for CycA[405] to be reached. Despite the outstanding quantitative and selective capabilities provided by MS systems, these high-tech analytical instruments require clean sample extracts in order to generate reproducible and reliable results for long periods of time. Hence, there is a need for adequate sample preparation approaches that guarantee the following parameters: a.) the satisfactory release of ISDs from the erythrocytes; b.) acceptable enrichment of the drugs on the extractive phase (*i.e.* solvent or particles); and c.) removal of potential matrix interferences. Most of the methods developed to date rely on protein precipitation (PP) followed by either liquid-liquid extraction (LLE) or solid-phase extraction (SPE). According to a survey conducted by Seger *et al.*[13], approximately 72% of the sample preparation work currently performed in clinical laboratories for ISD determinations is done manually by the analyst rather than by an automated liquid handling platform[13,27]; as such, this manual analytic procedure is both more tedious and prone to increased error in the determination[406]. Although the chromatographic step is not necessarily the bottleneck of the entire analytical process, the time required for each chromatographic run is between 2 and 4 minutes per sample[27]. Hence, the turnaround times can be anywhere from two hours up to multiple days, depending on the laboratory protocols[13]. Undeniably, there is a need for a method that can provide faster throughput—not only in the analysis stage, but also during the sample-preparation steps[8]. Methods that allow rapid interfacing with mass spectrometry[407]—for example, Paper Spray (PS)[94,408], Rapid-Fire (RF)[44], Turbo-Flow (TF)[409,410], and Laser Diode Thermal Desorption[411]—have been recently developed as alternatives for the rapid determination of immunosuppressants in whole blood[6]. Unfortunately, none of these technologies are capable of both sample preparation and the direct coupling with the instrument.

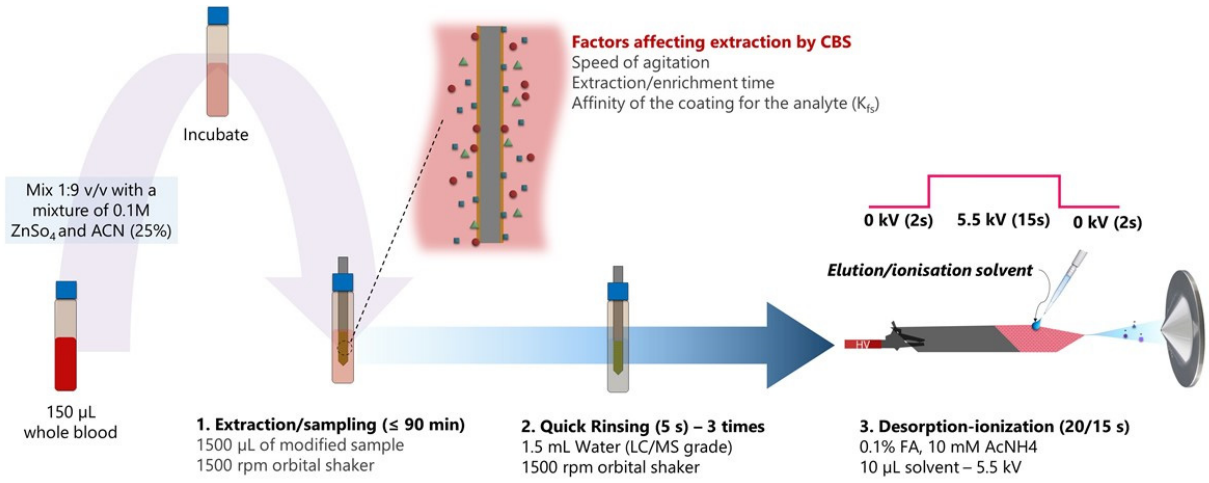

Figure 4.22 Experimental workflow for the determination of ISDs in whole blood via CBS-MS/MS

Coated Blade Spray (CBS) is a technology that efficiently integrates sample preparation and direct coupling to MS on a single device [395]. Essentially, CBS consists of a stainless-steel sheet carved in a sword-like fashion which is coated with a biocompatible extractive composite polymer (Figure 4.22). As a solid-phase microextraction (SPME) device, CBS simultaneously isolates and enriches analytes of interest present in the matrix without collecting the matrix itself [151]; as an ambient ionization device, CBS acts as a solid-substrate electrospray ionization (ESI) source [8,9]. Owing to its simplicity, CBS-MS/MS results suitable for fast (≤ 1 min per sample) and quantitative (low or sub-ng/mL) determinations of various substances have been previously demonstrated in urine, plasma, and whole blood samples[151,220,395]. In this study, we present CBS-MS/MS as a novel tool for the rapid and simultaneous determination of TAC, SIR, EVR, and CycA. By using a fully automated sample preparation procedure, we dramatically simplify the method and enable total analysis times as short as 3 minutes with turn-around times of less than 90 minutes.

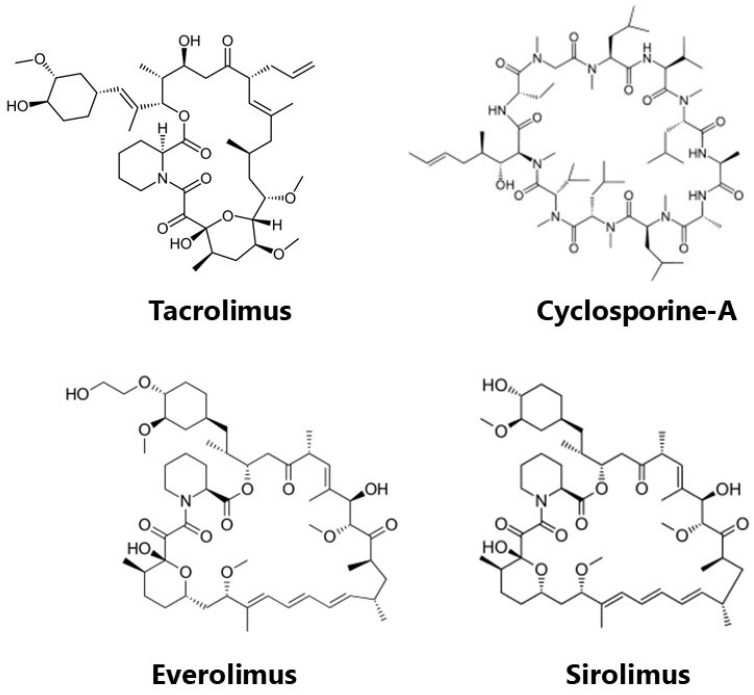

Figure 4.23 Chemical structure of the target ISD

4.5.2 Experimental section

Materials and supplies

LC-MS grade Methanol (MeOH), acetonitrile (ACN), isopropanol (IPA), and water were purchased from Fisher Scientific. Formic acid was purchased from Sigma–Aldrich (Saint Louis, USA). TAC, SIR, EVR, and CycA (see Figure 4.23) were obtained from Sigma–Aldrich (Milwaukee, USA). Deuterated analogs, namely tacrolimus-d2C1, sirolimus-d3, everolimus-d4, and cyclosporineA-d4 were purchased from TRC-Chemicals (Toronto, ON, Canada). Liquichek™ whole blood immunosuppressant quality control (4 levels QC) standards purchased from Bio-Rad (Mississauga, ON, Canada). Human whole blood (with K2-EDTA as the anticoagulant) from different patients was acquired from Bioreclamation IVT (Westbury, New York, USA). All blood samples were spiked and stored overnight at 4 °C prior to use in order to reach drug-protein binding equilibrium. HLB particles (~5 µm particle diameter) were kindly provided by Waters Corporation

(Wilmslow, UK). Stainless steel blades (15mm), which were purchased from Shimifrez Inc. (Concord, Ontario, Canada), were coated with HLB-polyacrylonitrile (HLB-PAN) slurry according to a proprietary protocol described in Section 4.4.

Table 4.12 Mass spectrometry parameters used to monitor each ISD.

Compound	Polarity	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens (V)
Tacrolimus	Positive	821.488	768.350	19.9	85
Tacrolimus-d2C1	Positive	824.522	771.481	20.4	85
Sirolimus	Positive	931.540	864.425	16.5	85
Sirolimus-d3	Positive	934.606	864.497	16.1	88
Everolimus	Positive	975.578	908.454	16.4	87
Everolimus-d4	Positive	979.609	912.528	15.5	88
Cyclosporine A	Positive	1219.80	1202.729	16.3	96
Cyclosporine A-d4	Positive	1223.87	1206.854	11.4	97

Mass Spectrometry

All the experiments reported herein were performed using a TSQ Quantiva mass spectrometer (Thermo Scientific, San Jose, CA, USA), and the acquired data was processed using Trace Finder version 3.0 (Thermo Scientific, San Jose, CA, USA). A custom-made coated blade spray interface was built at the University of Waterloo; a thorough description of the operation of this system can be found elsewhere[395]. Each drug was detected as a single charged ammonium adduct[400], with one selected reaction monitoring transition recorded for each analyte (dwell time ~ 50ms): *m/z* 821→768 for TAC; *m/z* 931→864 for SIR; *m/z* 975→908 for EVR; and *m/z* 1219→1202 for CycA. Further details regarding collision energy and RF-lens values are presented in Table 4.12.

Sample preparation

All of the CBS devices were cleaned for 30 min using a 40:40:20 (MeOH/ACN/IPA, v/v/v) solution before being conditioned for an additional 30 min with a 50:50 (MeOH/water, v/v)

solution prior to the extraction process. It is important to point out that, regardless of the application, CBS can be dry prior to the extraction step. The analytical workflow consisted of three stages (see Figure 4.22): a.) analyte enrichment; b.) coating cleaning, wherein nonspecific attachments of matrix components were quickly removed from the coated surface (10 s water-rinse); and c.) MS analysis, in which a minute droplet of elution/ionization solvent (10 µL of methanol:water 95:5, 0.1% FA, 10 mM AcNH4) was placed onto the coating[220,395]. Following analyte desorption (~20s), a high-voltage was applied (~5.5 kV) to the non-coated area of the blade in order to generate an electrospray from the CBS tip for 20s (see Figure 4.24)[395]. The extraction procedure was performed using a 1:9 mixture of 150 µL of EDTA-anticoagulated blood sample with a water-acetonitrile solution (Water:ACN, 85:15, 0.1M $ZnSO_4$); the objective of this procedure was to burst cells that were present in the matrix and to denature proteins that were bound to the target analytes[400], thereby increasing the free concentrations of ISDs[151]. Next, analyte enrichment was performed by immersing the CBS in the denatured sample for 90 minutes at 25 ºC. This process was done by working in batches (*i.e.* automated 96-samples; see Figure 4.25), producing an average analysis time of 90s, and an individual sample turnaround time of approximately 90 min. The MS analysis time was approximately 30s. Automated CBS extractions were carried out using a Concept-96 system (Professional Analytical Systems, PAS, Magdala, Germany). This robotic sample preparation unit has been described in detail elsewhere[131,304]. Matrix-match whole-blood calibration functions were constructed based on the signal ratio of the analyte and the isotope-labelled internal standard (A/Is) with seven calibrators in four independent experiments covering a range between 1 and 100 ng/mL for EVR/SIR/TAC and 10-1000 ng/mL for CycA (see Tables 4.13, 4.14 and 4.15). Linear regressions were plotted using weighted linear least squares (1/x). The quantitation of the ISDs was validated in terms of LOD, LOQ, linearity,

repeatability, reproducibility, accuracy, and relative matrix effect for whole blood containing different hematocrit levels.

Table 4.13 Calibration points for levels of tacrolimus, everolimus, and sirolimus.

Calibration points	**1**	**2**	**3**	**4**	**5**	**6**	**7**
ng/mL	1	2.5	5	10	15	25	50

Table 4.14 Calibration points for levels of Cyclosporine A.

Calibration points	**1**	**2**	**3**	**4**	**5**	**6**	**7***
ng/mL	10	25	50	100	150	250	500

Table 4.15 Liquid check quality control (QC) standards acquired from Bio-Rad.

Calibration points [ng·mL^{-1}]	**Level 1**	**Level 2**	**Level 3**	**Level 4**
Cyclosporine	55.6	179	324	699
Everolimus	N/A	3.14	6.78	17.6
Sirolimus	3.62	7.74	13.1	N/A
Tacrolimus	3.94	9.05	17.0	26.7

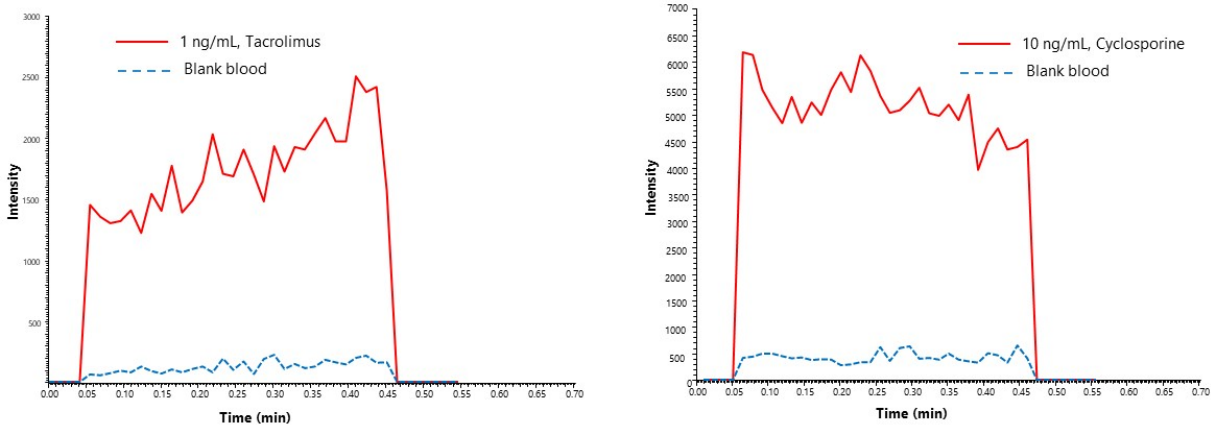

Figure 4.24 Ion chronograms for TAC and CycA in whole blood spiked at 1 and 10 ng mL^{-1} (red line), respectively, overlay with representative chronograms from blank blood (blue line).

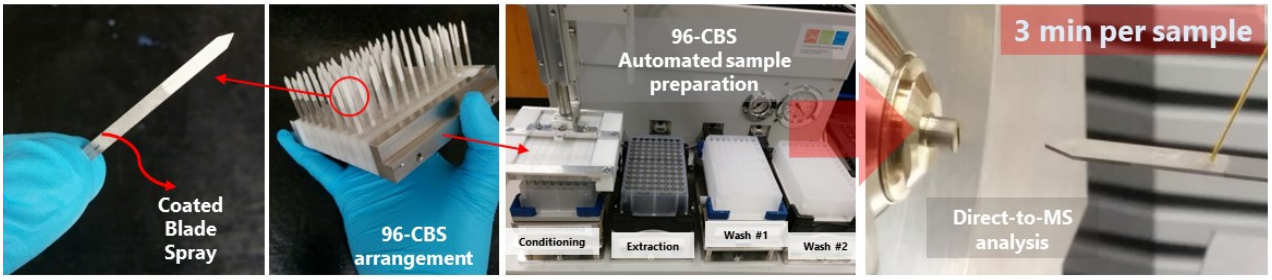

Figure 4.25 Automated CBS sample preparation set-up for ISDs analysis.

4.5.3 Results and discussion

The quantitative determination at low concentration levels (*e.g.* low part-per-billion) of drugs heavily bound (≥80%) to plasma proteins is a challenge for any sample preparation technology that extracts via-free concentration, like CBS[134]. This challenge is particularly great if the targeted drugs are not only bound to proteins, but are also partitioned into the red blood cells, as is the case with ISDs. As a consequence, the most commonly used methods for ISD analysis involve protein precipitation using a solution consisting of methanol and zinc sulfate ($ZnSO_4$), in order to break the cell walls and denature the proteins that bind the drugs. Once this has been done, sample clean-up using online-SPE and analyte separation/detection via LC-MS/MS analysis is conducted. Unsurprisingly, early attempts at quantifying ISDs in intact blood samples using protocols that had previously been designed for plasma and urine[220,395] were not successful. Given that SPME has traditionally been a solvent-free sample preparation technology, our next step consisted in conducting extraction using a water-modified matrix (mixture 1:1 of 100 µL blood and 100 µL of a 0.1M $ZnSO_4$ solution). However, as can be seen in Figure 4.26, we only attained acceptable results for CycA, while the LOQs for TAC and EVR were significantly above the therapeutic range (~10-25 ng mL^{-1}).

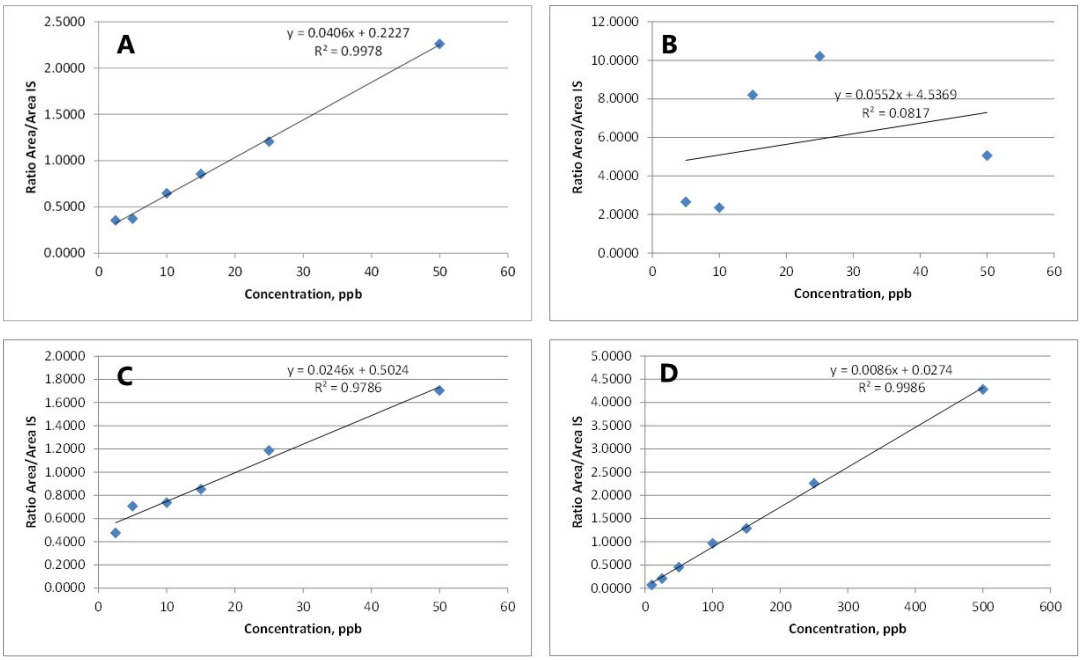

Figure 4.26 Quantitative determination of (A) TAC, (B) SIR, (C) EVR, and (D) CycA. Extractions were performed from 100 µL of whole human blood pre-mixed with 100 µL of a 0.1M ZnO4-solution. 20 min of extraction at 2000 rpm was followed by three rinsing steps in fresh water of 5s each.

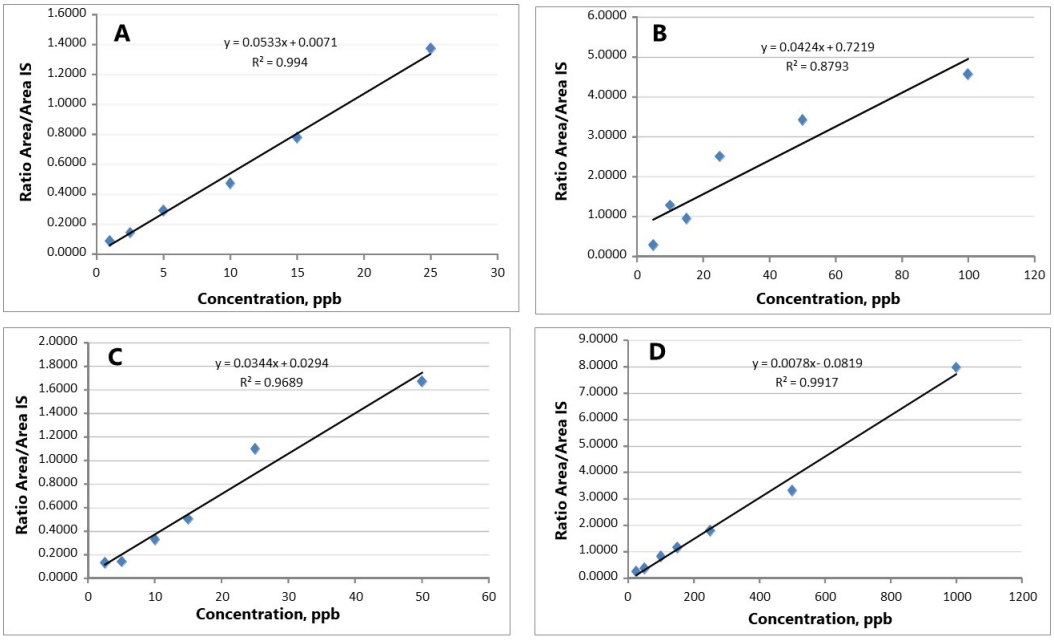

Figure 4.27 Quantitative determination of (A) TAC, (B) SIR, (C) EVR, and (D) CycA. Extractions were performed from 200 µL of whole human blood pre-mixed with 500 µL of a 0.1M ZnO4-solution and 500 µL of LC-MS water. 30 min extraction at 2000 rpm was followed by three rinsing steps in fresh water of 5s each.

Moreover, SIR, the analyte with the largest partitioning with erythrocytes (~95%), was barely detected using this protocol. Therefore, we increased the sample volume (*i.e.* 200 µL of blood) and the volume of "denaturing" mixture (500 µL 0.1M $ZnSO_4$ solution and 500 µL of water) in an attempt to increase the free-concentration of SIR. Although these conditions allowed us to improve the LOQ for TAC and EVR (~5-10 ng mL^{-1})—and to slightly improve the detectability for SIR (see Figure 4.27)—the results fell far short of the required values. As an alternative, we also evaluated extraction using whole blood samples that had been frozen (-80 °C) and thawed. Nonetheless, as shown in Figure 4.28, the results were only satisfactory for CycA. In order to determine if the HLB-coated blades were effective for the extraction of ISDs, extractions were performed using non-modified human plasma samples that had been spiked with four ISDs. As can be seen in Figure 4.29, the results revealed that HLB-CBS was capable of extracting ISDs from plasma with LOQs in the order of 1 ng mL^{-1} for SIR/ TAC, 5 ng mL^{-1} for EVR (~74% protein binding in plasma[400]), and 10 ng mL^{-1} for CycA. This results suggest that the poor extraction of ISDs in whole blood was undoubtedly linked to their partitioning into the erythrocytes (see Table 4.13). Thus, we were compelled to defy the traditional solventless philosophy of SPME[113] as a result of the complexity of the matrix-analyte interactions.

Essentially, this class of compounds can only be quantified via SPME-based technologies under the following conditions: the matrix consists of blood cells that are fully denatured; the analytes have been released into the solution phase; and the CBS devices are used as open-bed SPE substrates[151]. Basically, this approach entails adding a mixture of water, 0.1M $ZnSO_4$ solution, and organic solvent (*e.g.* methanol or acetonitrile, MeOH or ACN) to the biofluid in order to modify the matrix viscosity and the analyte-protein binding properties, and to denature the red blood cell walls.

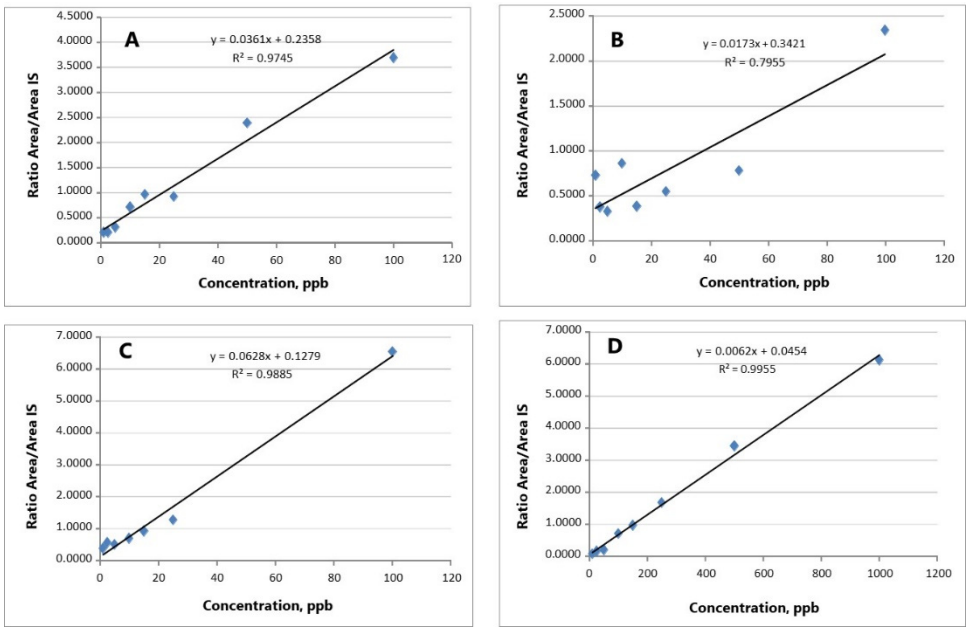

Figure 4.28 Quantitative determination of (**A**) TAC, (**B**) SIR, (**C**) EVR, and (**D**) CycA. Extractions were performed from 200 µL of whole human blood frozen (-80 °C, 1h) and thawed. 30 min extraction at 2000 rpm was followed by three rinsing steps in fresh water of 5s each.

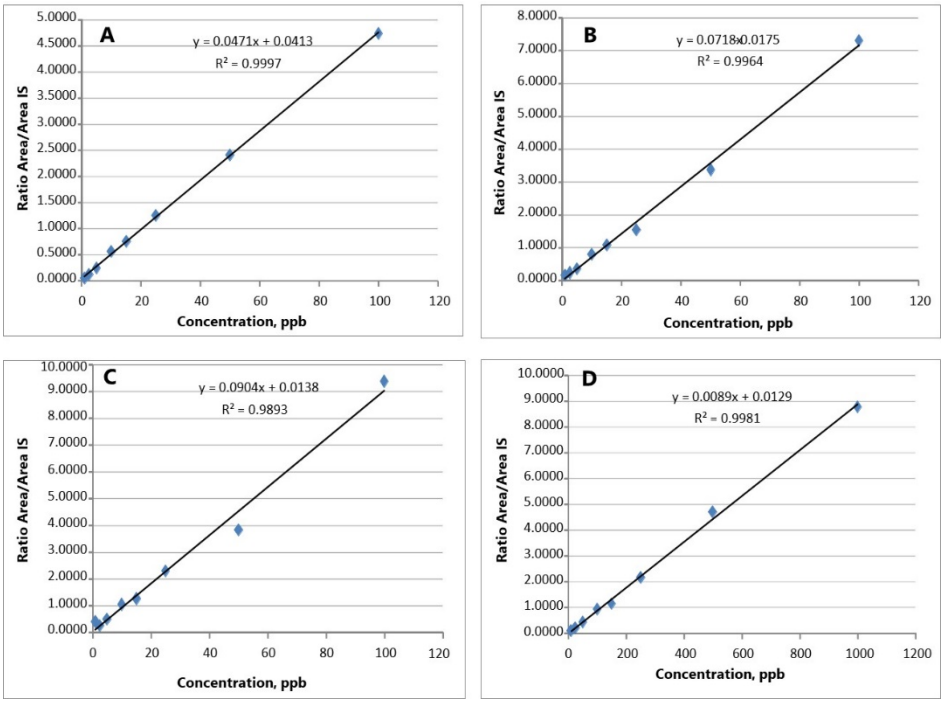

Figure 4.29 Quantitative determination of (**A**) TAC, (**B**) SIR, (**C**) EVR, and (**D**) CycA. Extractions were performed from 300 µL of human plasma. 30 min extraction at 2000 rpm was followed by three rinsing steps in fresh water of 5s each.

This approach results in a marked increase in the free concentration of the ISDs, which promotes their extraction onto the coating particles. Nevertheless, the addition of solvent to a matrix creates extra challenges for analyte extraction. For instance, if the amount of organic solvent is too large, analyte partition may be driven onto the solvent layer rather than onto the extractive particles due to a dramatic decrease in the analyte partition coefficient (K_{fs}). This will result in a notable reduction in the amount that is extracted. Likewise, an insufficient amount of organic solvent will lead to an unsatisfactory release of the analytes, and, consequently, inadequate LOQs. Our initial assessments were performed using diverse ratios of MeOH. As shown in Figure 4.30, a mixture containing 50% MeOH allowed for the highest recoveries for all ISDs. Based on these observations, and knowing that more than 50% organic solvent will dramatically affect the extraction recovery, we also studied ACN as a substitute for MeOH. Our results indicated that, when using ACN only 25% of organic solvent was sufficient to get the best recovery (see Figure 4.31). Furthermore, and more importantly, ACN-denaturing mixtures displayed better performance than MeOH-based mixtures (see Figure 4.31).

Hence, this ratio was selected as the ideal compromise for quantitating ISDs, and it was used to determine the best extraction time. Our results indicate that extraction times around of 100 minutes are necessary to collect a sufficient amount of ISDs to allow us to reaching the required LOQs (see Figure 4.33)[27]. Longer extraction times were not evaluated as this will increase the turn-around time beyond what traditional methods can attained. A further comparison under optimum extraction conditions showed that HLB coatings performed approximately two times better than C18 coatings for the enrichment of ISDs (see Figure 4.34). Additionally, we found that using a larger amount of blood is a viable alternative strategy for increasing the amount of analyte extracted from the matrix (see Figure 4.35).

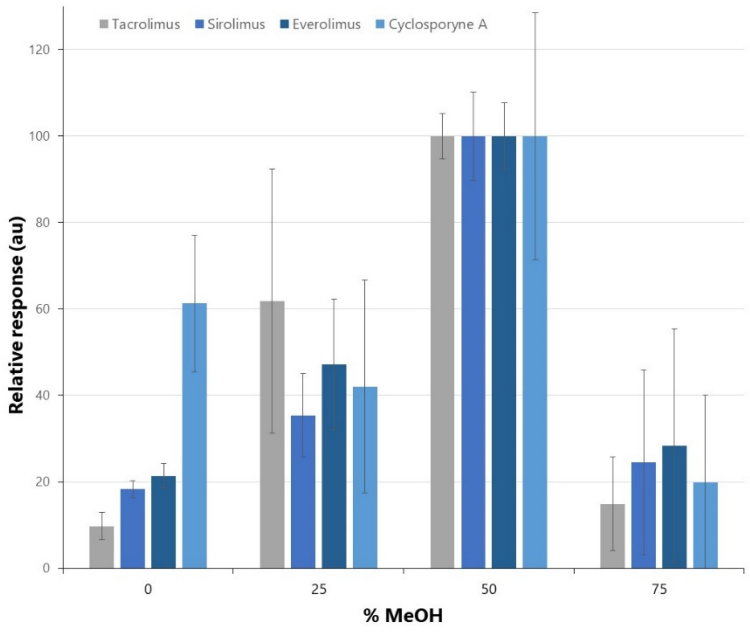

Figure 4.30 Optimization of organic content (MeOH, %) required to achieve the highest instrumental response. Extractions were performed from 100 µL of whole human blood spiked at 50 ng mL-1 with TAC/SIR/EVR and 500 ng mL-1 for CycA, and pre-mixed with a 0.1M ZnO4-solution and MeOH according to their respective ratios. 30 min extraction at 2000 rpm was followed by three rinsing steps in fresh water of 5s each.

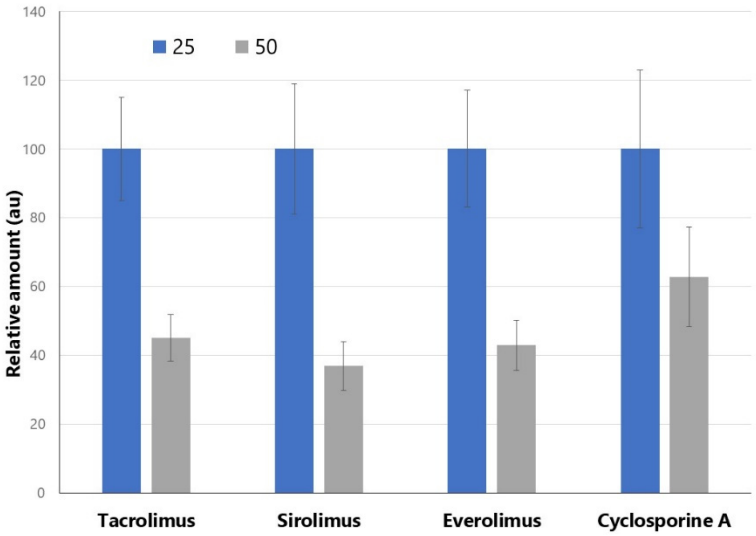

Figure 4.31 Optimization of organic content (ACN, %) required to achieve the highest instrumental response. Extractions were performed from 100 µL of whole human blood spiked at 50 ng mL^{-1} with TAC/SIR/EVR and 500 ng mL^{-1} for CycA, and pre-mixed with a 0.1M ZnO_4-solution and CAN according to their respective ratios. 30 min extraction at 2000 rpm was followed by three rinsing steps in fresh water of 5s each.

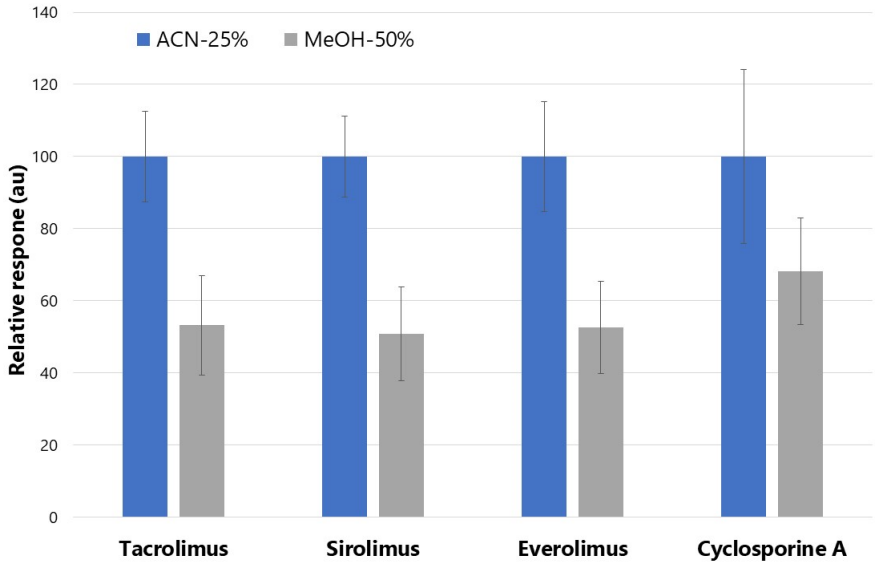

Figure 4.32 Comparison between two different organic solvent denaturing mixtures: 25% ACN versus 50% MeOH.

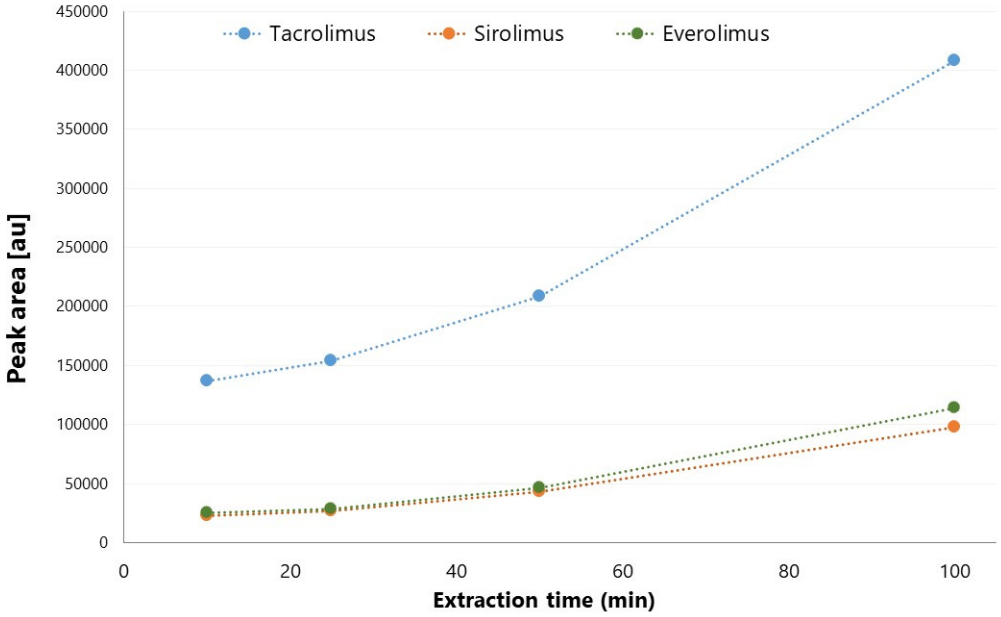

Figure 4.33 Extraction time profile for three of the ISDs (10, 25, 50, 100 min). Extractions were performed from whole human blood spiked at 50 ng mL^{-1} with TAC/SIR/EVR, and pre-mixed with a 0.1M ZnO_4-solution and ACN (25%) according to their respective ratios.

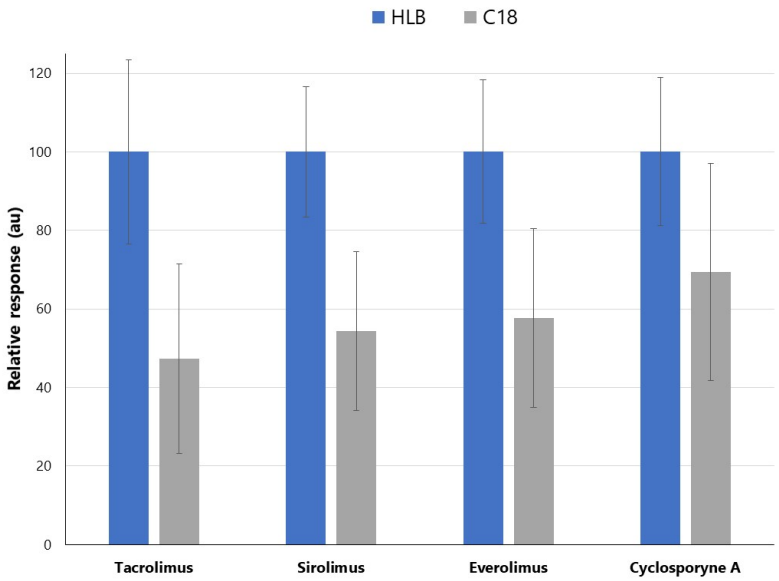

Figure 4.34 Comparison of two different coating chemistries for the extraction of ISDs from a modified blood-matrix. Extractions were performed from whole human blood spiked at 50 ng mL^{-1} with TAC/SIR/EVR and 500 ng mL^{-1} for CycA, and pre-mixed with a 0.1M $ZnSO_4$-solution and ACN (25%) according to their respective ratios. 90 min extraction at 2000 rpm was followed by three rinsing steps in fresh water of 5s each.

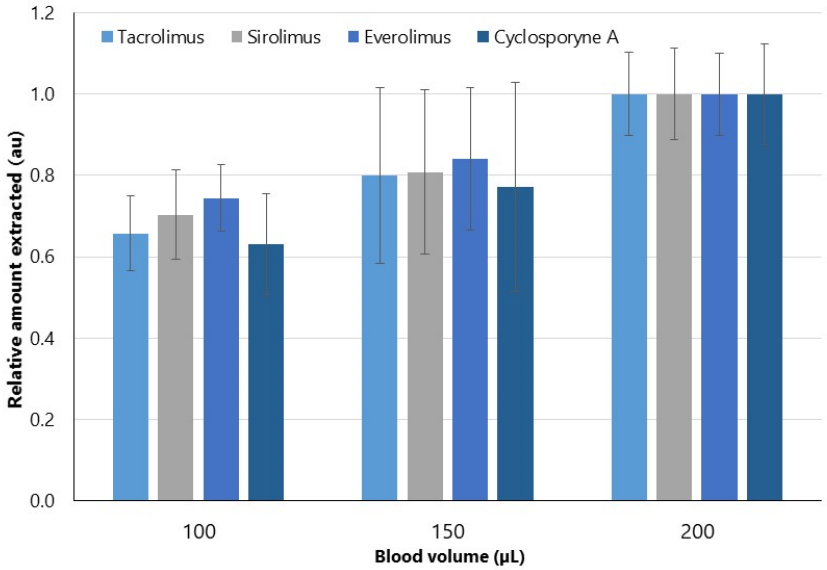

Figure 4.35 Comparison of three different blood sample volumes for the determination of ISDs from a modified blood-matrix. Extractions were performed from whole human blood spiked at 50 ng mL^{-1} with TAC/SIR/EVR and 500 ng mL^{-1} for CycA, and pre-mixed with a 0.1M $ZnSO_4$-solution and ACN (25%) according to their respective ratios. 90 min extraction at 2000 rpm was followed by three rinsing steps in fresh water of 5s each.

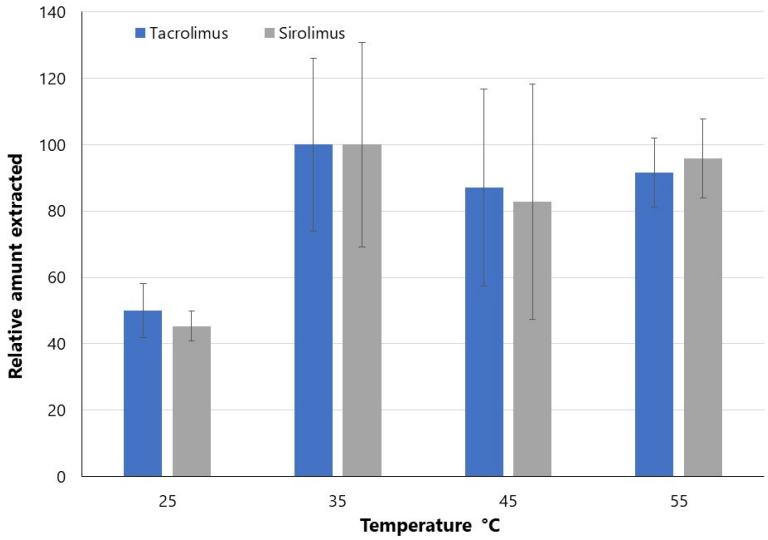

Figure 4.36 Comparison of four different extraction temperatures for the determination of ISDs from a modified blood-matrix. Extractions were performed from whole human blood spiked at 50 ng mL^{-1} with TAC/SIR/EVR and 500 ng mL^{-1} for CycA, and pre-mixed with a 0.1M $ZnSO_4$-solution and ACN (25%) according to their respective ratios. 90 min extraction at 2000 rpm was followed by three rinsing steps in fresh water of 5s each.

However, this option would be limited to the amount of sample available. Finally, we found that we were able to achieve a significant improvement in the extraction efficiency for all ISDs by increasing the extraction temperature (see Figure 4.36). Indeed, we observed that the amount of analyte extracted reached a maximum at 35°C. This behavior is mainly related to a compromise between the increasing of the diffusion coefficient of the target analytes and the reduction of the affinity of the coating for the ISDs as the temperature increases.

Based on all of the above-mentioned findings, we proceeded to evaluate the capabilities of CBS-MS/MS for the quantitation of ISDs in whole blood. Our final protocol consisted of performing extractions from 150 µL of blood mixed with 325 µL of ACN (25%) and 1025 µL of 0.1M $ZnSO_4$-solution. The extraction time used in this protocol was 90 min at 1500 rpm using a 96-well plate that had been heated to 35°C.

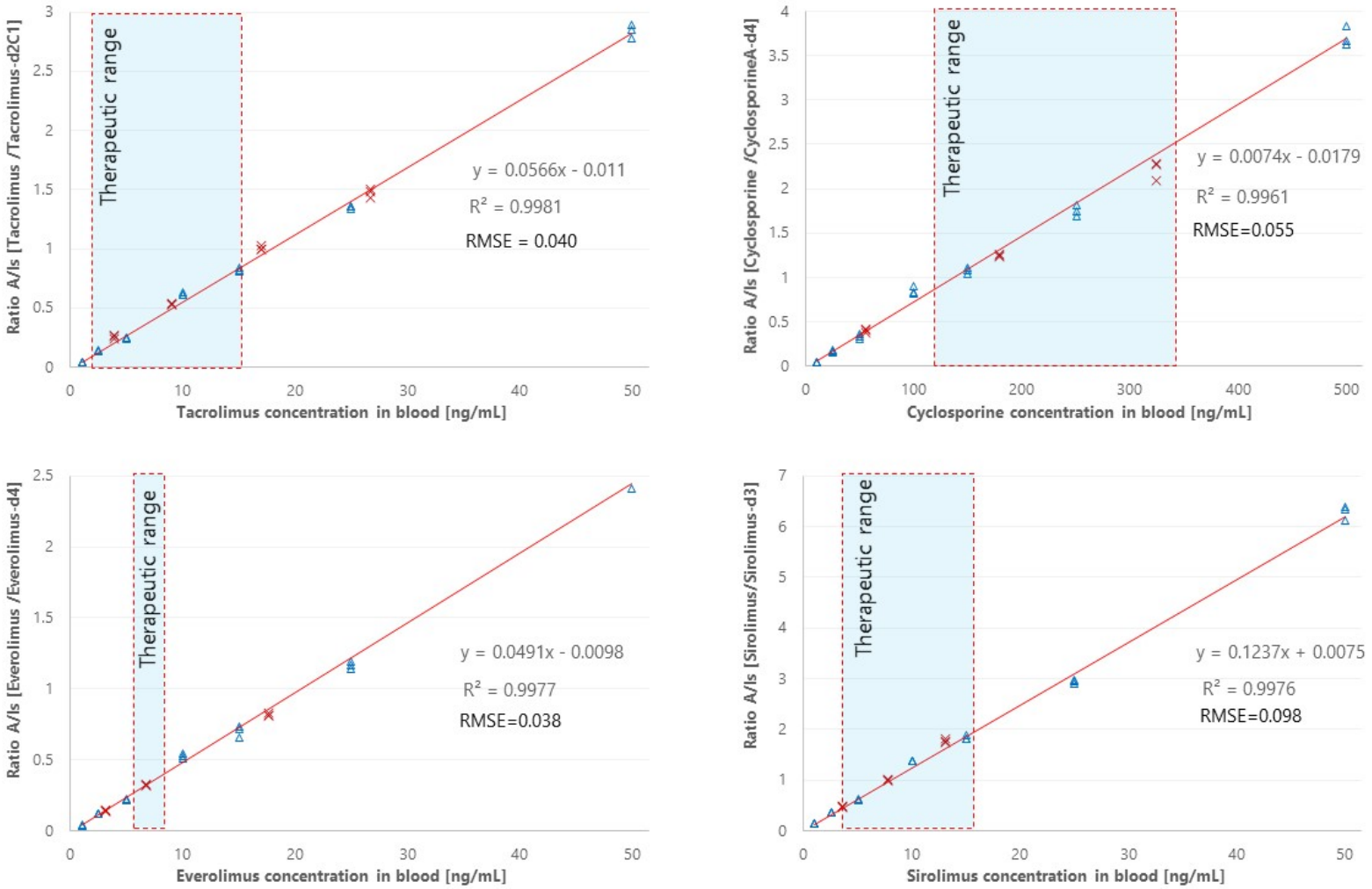

Figure 4.37 Linear regression curves for TAC, CycA, EVR and SIR in whole blood. Analyses were performed using CBS-MS/MS from modified blood matrix with ACN and zinc sulfate. Quantification was performed with the entire area under the curve for each analyte, normalized by IS, to obtain calibrations curves as shown (blue triangles). Red marks represent the Liquichek™ QC standards.

Table 4.16 Figures of merit for the determination of ISDs in whole blood. Extractions were performed from 150 µL of blood mixed with 325 µL of ACN (25%) and 1025 µL of 0.1M $ZnSO_4$-solution. Extraction time was 90 min at 1500 rpm using a 96-well plate heated at 35°C. RMS: root-mean-square error; LDR: linear dynamic range.

Compound	Distribution in Erythrocytes (%)	Typical Concentrations in ISD-TDM Samples [ng/mL][13,27]	LOD [ng/mL]	LOQ [ng/mL]	LDR [ng/mL]	RMSE
Cyclosporine	41-58	50−350	3	10	10-1000	0.055
Tacrolimus	~85	3−15	0.3	1	1-100	0.040
Sirolimus	~95	3−20	1	2.5	2.5-100	0.064
Everolimus	≥75	3−15	0.3	1	1-100	0.038

Table 4.17 Validation of protocol herein proposed using Liquichek® Bio-Rad standards (n=4)

Compound	QC-1	QC-2	QC-3	QC-4
Cyclosporine	101 ± 4.3	95 ± 1.1	93 ± 5.0	87 ± 2.1
Tacrolimus	119 ± 4.9	106 ± 1.3	106 ± 1.8	98 ± 2.8
Sirolimus	101 ± 2.8	103 ± 1.0	109 ± 1.9	-
Everolimus	-	96 ± 1.9	100 ± 0.4	96 ± 1.5

Table 4.18 Comparison of ISDs calibration curves obtained using blood with different hematocrit levels (n=4)

Hematocrit (%)	Linear least squares slope values			
	TAC	SIR	EVR	CycA
70	0.0226	0.0459	0.0228	0.0101
45	0.0219	0.0441	0.0210	0.0100
20	0.0205	0.0499	0.0220	0.0090
Average	0.0216	0.0466	0.0219	0.0097
RSD (%)	5	6	4	6

Figure 4.37 presents the linear calibration curve attained for each of the studied compounds. The LOQs achieved using this methodology were 10 ng mL^{-1} for CycA, 1 ng mL^{-1} for EVR/ TAC, and 2.5 ng mL^{-1} for SIR (see Table 4.16). Good linearity ($R^2 \geq 0.996$) was attained over the evaluated range for all ISDs. As can be seen in Figure 4.37, the coefficient of variation (CV) of the calibrators for all ISDs was in accordance with the assay precision levels prescribed by the International Association of Therapeutic Drug Monitoring and Clinical Toxicology Immunosuppressive Drug Scientific Committee (IATDMCT; ≤ 10%)[13]. Furthermore, this method had a median accuracy of 100.5% (see Table 4.17), which was evaluated using third-party-prepared calibrators (*i.e.* Liquichek™ calibrators by Bio-Rad, see Table 4.15). Finally, as presented in Table 4.18, no statistical differences (RSD ≤ 7 %) were observed in the calibration curve slopes of ISDs in blood with different hct values (20, 45, and 70 hematocrit %)[412]. The above-mentioned result can be primarily attributed to the strongly denatured media employed during the extraction step. In other words, due to the extreme conditions of the solution, a quantitative and reproducible amount of analyte is released and extracted in its free form regardless of the hct level. Although this manuscript strictly presents a proof-of-concept of CBS's suitability for ISD determination, a full validation of the technology using a certified LC-MS/MS method with real samples is presently being undertaken in our laboratory.

4.5.4 Summary

This study demonstrates that CBS-MS/MS is a suitable tool for routine ISD determinations in clinical laboratories. Moreover, CBS offers a workflow that is simpler than that of traditional methods by eliminating the need for chromatographic separation, while providing a clean extract that allows for long-term instrumental operation with minimal maintenance. Furthermore, as CBS

integrates all analytical steps in one device, it eradicates the risk of instrumental carry-over. Fully automated sample preparation simplifies the method and allows for total analysis times as short as 3 minutes with turn-around times of less than 90 minutes. LOQs of 10 ng mL^{-1} for CycA, 1 ng mL^{-1} for EVR/ TAC and 2.5 ng mL^{-1} for SIR, were obtained. Moreover, excellent linearity and more than acceptable CV (≤ 10%) for all the validation points evaluated within the therapeutic range. Finally, it was demonstrated that no statistical differences (RSD ≤ 7 %) in sensitivity were obtained when blood with different hct values was analyzed. However, it is worth noting that, beyond the analytical validation herein reported and, in order to use CBS-MS/MS in routine clinical practice for TDM measurements, some clinical performance parameter must be considered; for example, long-term stability, performance of the instrument/assay combination under typical routine circumstances (*e.g.* several samples a day), different medical conditions (e.g. co-medication, lipemia, jaundice, impaired renal function) or different physical blood storage approaches (e.g. hemolysis).[13] Further validation is required using samples from real patients, as this will allow for more detailed examination of potential in-source fragmentation-related bias (e.g. inadvertent dissociation of labile conjugated metabolites in the API region). The aims of our current efforts have been threefold: to decrease the sample volume required to perform analysis; to provide a fully-automated CBS-MS platform; and to assess tacrolimus (ERM-DA110a), which is the only certified reference material available in the market for ISD quantitation[399,401].

Section 4.6 Quantitative analysis of biofluid spots by coated blade spray mass spectrometry, a new approach to rapid screening

4.6.1 Introduction

Efficient, simple, and cost-effective methods that allow for quantitative analysis of small volumes of biofluids are critical for the advancement of personalized medicine and drug development. State-of-the-art mass spectrometry (MS) instrumentation in combination with innovative and easy-to-use microsampling technologies have facilitated the development of new analytical methodologies.[8,173] Applications such as new-born screening,[413,414] therapeutic drug monitoring,[173,347,415,416] and drug metabolism pharmacokinetics (DMPK)[417] have greatly benefitted from these advances. Such microsampling devices are consisted of, or contain, a piece of paper or polymeric absorbent in which a droplet of biofluid can be collected then dried. Following, devices can be either stored for further studies or sent to the laboratory for immediate analysis. Typically, the analytical workflow for determination of analytes of interest collected on these devices consists of multiple steps prior to quantitation via MS, including liquid extraction, extract clean-up, analyte elution (e.g. by using solid phase extraction, SPE), and chromatographic separation.[418] Aiming to increase the throughput of analysis, on-line technologies that combine all these steps have been developed and thoroughly assessed by other researchers.[419] Further, in the last ten years, scientists have developed multiple ground-breaking technologies allowing for the eradication of the sample-prep/separation stages from the analytical work-flow.[8] These technologies, allowing for direct MS analysis of dried biofluid spots (e.g. paper spray, PS), have gained remarkable popularity, being adapted in numerous applications.[8,49] However, sample preparation cannot be entirely overlooked; indeed, several unavoidable effects intrinsic to ionization and detection processes can resultantly

emerge from a lack of sufficient sample preparation, including ion suppression, poor sensitivity, and potential instrument contamination.[80,113] Aiming to solve these issues, a new chapter in this novel era of MS was written by merging micro-sample preparation technologies with ambient ionization MS approaches.[102,173,420] However, while such methods can certainly address the aforementioned effects, not all of them provide a pragmatic approach to analysis (*e.g.* complex operation, long analysis times, or expensive equipment/parts required per analysis). In this regard, there still exists a demand for a tool that not only improves limits of quantitation (LOQ) and minimizes matrix effects, but that can also offer high throughput compatibility for rapid diagnostics.[178] Coated Blade Spray (CBS), an SPME-based technology designed for enrichment of analytes of interest from complex sample matrices, can be directly coupled with MS instruments for rapid quantitative or qualitative analysis.[136] CBS can be described as a sword-like stainless steel sheet coated with polymeric adsorbent particles, which act as a solid-substrate ESI source (Figure 1).[46] Unlike classical microsampling devices, CBS functions by extracting/enriching analytes of interest from a given sample, rather than through a collection of dried sample spots.[151] Contrary to general assumptions,[83,91,98] CBS has been shown to handle a broad range of sample volumes (*i.e.* µL to L).[136,151,220] Herein, having accepted the challenge of employing CBS towards analysis of minimum amounts of sample (≤ 10 µL), we present a thorough validation of the quantitation capabilities of CBS for analysis of small volumes of biofluids.

4.6.2 Experimental section

Materials and reagents

Formic acid was purchased from Sigma-Aldrich (Saint Louis, USA), and LC-MS-grade methanol (MeOH), acetonitrile (ACN), isopropanol (IPA) and water were purchased from Fisher Scientific.

The following compounds were selected as model analytes for evaluating the quantitation capabilities of CBS in droplet analysis: methamphetamine, methamphetamine-d5, carbamazepine, carbamazepine-d10, propranolol, propranolol-d7, clenbuterol, clebuterol-d9, diazepam, diazepam-d5, codeine, codeine-d3, cocaine, cocaine-d3, sertraline, sertraline-d3, citalopram, citalopram-d6, fentanyl, fentanyl-d5, buprenorphine, buprenorphine-d4, morphine, morphine-d6, methadone, methadone-d3, oxycodone, lorazepam, bisoprolol and stanozolol were acquired from Cerilliant Corporation (Round Rock, TX, USA). As noted, deuterated analogues of most analytes were used to correct for intra- and inter-experiment variability. For further details regarding compound properties, and selected reaction monitoring (SRM) transitions, see Table 4.6. Individual stock standard solutions were prepared in methanol at a concentration of 1000 µg·mL-1 and stored at -80 ºC. Stainless steel blades, which were purchased from Shimifrez Inc. (Concord, Ontario, Canada), were then coated using a slurry of hydrophilic lipophilic balance particles and polyacrylonitrile (HLB-PAN) according to a protocol developed in our laboratory (see Section 4.4.2 for further details) with HLB particles kindly provided by Waters Corporation. The coating length and thickness was 15mm and 10µm, respectively.

Biological samples

A phosphate-buffered saline solution (PBS) (pH 7.4) was prepared by adding 8.0 g of sodium chloride, 0.2 g of potassium chloride, 0.2 g of potassium phosphate, and 1.44 g of sodium phosphate to 1 L of nanopure water. Human plasma (with potassium (K2) ethylenediaminetetraacetic acid (EDTA) as anticoagulant) had been pooled from different batches was purchased from Bioreclamation IVT (Baltimore, MA, U.S.A). Pooled whole blood from healthy donors in potassium K2-EDTA was purchased from Bioreclamation IVT (Baltimore, MA,

U.S.A.). All the plasma and blood samples were spiked and stored overnight at 4 ºC in order to achieve the drug-protein binding equilibrium. All the experiments and methods herein reported were done with the approval of the University of Waterloo's Office of Research Ethical Board.

Mass Spectrometry

All the experiments described in this manuscript were carried out using a TSQ Quantiva mass spectrometer (Thermo Fisher Scientific, San Jose, California, USA), and data processing was performed using Trace Finder 3.3 (Thermo Fisher Scientific, San Jose, California, USA). To guarantee that the blades were accurately positioned in front of the mass spectrometer during all experiments, an in-house ionization source was built at the University of Waterloo (see Figure 4.18). Once the CBS had been installed on the interface, 10 µL of a 95:5 MeOH/water v/v 0.1% formic acid solution was applied to the coated area in order to desorb the analytes ($t \leq 20$ s). After analyte elution on the desorption solution, a voltage of 4 kV was established between the CBS and the MS entrance to generate electrospray from the tip of the blade. All analyses were carried out in positive ionization mode. Optimum collision energy and RF-lens conditions were tuned for each compound via the direct infusion of methanolic standards. MS/MS transitions, optimum collision energy (CE), and RF-lens voltages for each analyte can be found in Table 4.6.

Analytical methodology

All the CBS devices were cleaned after manufacturing for 30 min using a 40:40:20 (MeOH/ACN/IPA, v/v/v) solution and then conditioned for 30 min with a 50:50 (MeOH/water, v/v) solution prior to extraction process. It is important to point-out that, regardless of the application, CBS can be dried prior to the extraction step. As illustrated in Figure 4.38, the

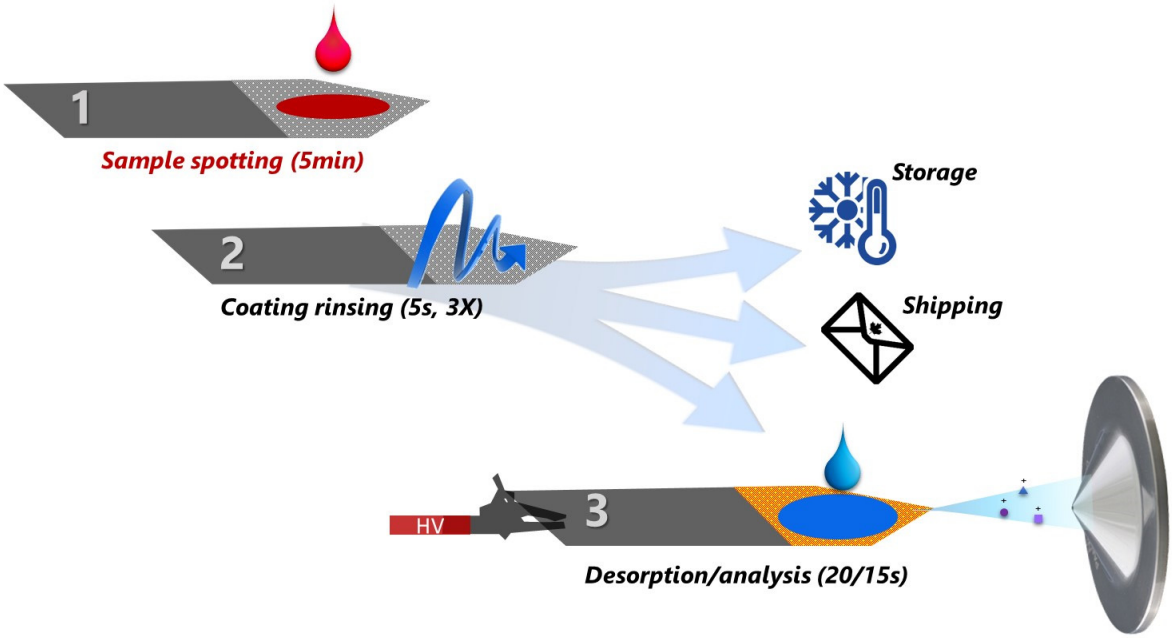

Figure 4.38 Experimental set-up for quantitative analysis of blood or plasma droplets via Coated Blade Spray-Mass Spectrometry (CBS-MS).

analytical workflow consists of three simple steps. First, 10 µL of biofluid is spotted onto the coated area of the blade, then left to interact with the extracting particles for 5 minutes. Next, the CBS is rapidly rinsed for 10 seconds with water, aiming to remove any potential matrix that could have adhered to the surface. Subsequently, the blade is placed in front of the MS system for analysis, and a droplet (10 µL) of elution/spraying solvent is added onto the coated area. Finally, after 20 seconds, ions of the extracted/pre-concentrated analytes are generated by applying a high electric field (+ 4kV) between the blade and the mass spectrometer.[136] All the methods herein reported were performed according to University of Waterloo safety guidelines and regulations.

Method validation

The methodologies were validated with respect to linearity, precision, accuracy, and LOQ. Calibration functions were constructed on the basis of the signal ratio of the analyte and its

isotopologue (A/Is) for 10 concertation levels in four independent replicates from 0.25 ng·mL^{-1} to 100 ng·mL^{-1}. Furthermore, three validation points at concentrations of 3, 40, and 80 ng·mL^{-1} were analyzed in order to assess precision and accuracy. LOQs were calculated as the lowest calibration point with precision values lower than 20%.

4.6.3 Results and discussion

An initial assessment of CBS as a tool for analysis of biofluid spots was performed by employing, as model, phosphate buffered saline (PBS) samples spiked with 17 compounds from different classes and comprising a broad range of molecular weights, functional groups, and polarities (Table 4.6), including controlled substances (e.g. clenbuterol), pain management drugs (e.g. buprenorphine), and drugs of abuse (e.g. fentanyl). As shown in Table 4.19, by using blades coated with hydrophilic–lipophilic balanced (HLB) particles, LOQs equal or lower than 1 ng·mL^{-1} and outstanding figures of merit were reached for all analytes under study (*i.e.,* SPME balance coverage). In view of these promising results, CBS was then employed towards analysis of analytes spiked on human plasma. As summarized in Table 4.20, LOQs for all compounds were, in almost all cases, below the minimum required performance levels (MRPL) set by the World Andi-Doping Agency (WADA), the cut-off established by the Substance Abuse and Mental Health Services Administration (SAMHSA), or the analytical quantitation limits established by certified clinical laboratories (i.e., LOQ ~ 1-5 ng·mL^{-1}). As shown in Table 4.21, similar results were obtained for analyses of blood samples spiked with the same target analytes (i.e., LOQ ~ 1-10 ng·mL^{-1}). Although LOQs were higher for plasma and blood spots, the validation of the methodology yielded reassuring results at all concentration levels (Tables 4.19-4.21).

Table 4.19 Figures of merit for determination of multiple substances in PBS spots via CBS-MS/MS. Red color denotes compounds that did not match the required MRPL levels, or showed poor accuracy/precision.

Compound	LOQ, ng·mL^{-1}	Accuracy, %			Precision, %		
		3 ng·mL^{-1}	40 ng·mL^{-1}	80 ng·mL^{-1}	3 ng·mL^{-1}	40 ng·mL^{-1}	80 ng·mL^{-1}
Methamphetamine	0.5	93	103	98.3	3	2.1	2.8
Carbamazepine	1	107.7	102.6	97.8	9.1	1.8	1.8
Propranolol	1	96.5	101.8	97.7	7.0	2.2	1.4
Clenbuterol	1	102.4	104.9	97.6	6.4	1.5	1.2
Diazepam	0.5	102.2	101.1	97.1	5.8	5	2.2
Codeine	0.5	104.8	103.9	96.1	20.8	19.6	5.6
Cocaine	0.5	94.1	108.1	97.8	0.9	9.9	2
Sertraline	0.5	95.1	102.3	98.5	5.4	4.5	1.5
Citalopram	1	97.9	106.4	97.3	3.4	5.7	2.3
Fentanyl	0.25	95.1	110.3	100.6	1.6	1.6	2
Buprenorphine	0.25	102.1	101.8	95.4	3.7	7.4	3
Morphine	1	108.3	102.7	96.6	9.3	1.9	2.2
Methadone	0.25	95.5	106.8	97.5	1.9	2.7	1.2
Salbutamol*	5	120.3	133.3	110.5	37.5	25.2	14.4
Oxycodone	1	109.1	105.4	103.5	2.8	7.8	4.7
Lorazepam	25	-	95.5	88.3	-	19	7.3
Bisoprolol	0.25	98.4	109.5	95.4	9.8	12.3	16.2

Table 4.20 Figures of merit for determination of multiple substances in plasma spots via CBS-MS/MS. Red color denotes compounds that did not match the required MRPL levels, or showed poor accuracy/precision.

Compound	LOQ, ng·mL^{-1}	Accuracy, %			Precision, %		
		3 ng·mL^{-1}	40 ng·mL^{-1}	80 ng·mL^{-1}	3 ng·mL^{-1}	40 ng·mL^{-1}	80 ng·mL^{-1}
Methamphetamine	2.5	108.5	98.7	100.9	4.3	2.7	3
Carbamazepine	1	103.1	101.4	106.8	8.7	1.5	4
Propranolol	2.5	110.4	97.6	106.7	1.5	3.4	3.4
Clenbuterol	2.5	113.7	97.2	104.6	7.7	1.6	2.3
Diazepam	2.5	113.5	97	102.9	5.9	1.2	2
Codeine	5	-	96.2	94.1	-	8.6	11
Cocaine	2.5	110.9	98.5	102.1	7.4	1.3	0.9
Sertraline	2.5	100.5	91.3	107.3	5.1	1.8	3.1
Citalopram	2.5	107.5	95.5	103.5	2.9	3.8	1.5
Fentanyl	2.5	107.7	98.2	104.5	4.5	0.6	2.9
Buprenorphine	2.5	92.7	102.9	101.4	15.2	7.5	7.9
Morphine	5	-	99.5	97.4	-	5.2	5.9
Methadone	5	-	96.9	102.8	-	1.8	0.8
Salbutamol*	2.5	111.9	106.5	104.7	8.6	7.2	15
Oxycodone	5	-	99.2	110.8	-	3.13	1.11
Lorazepam	-	-	-	-	-	-	-
Bisoprolol	1	96.1	97.6	98	6.8	1.7	5.2

Table 4.21 Figures of merit for determination of multiple substances in blood spots via CBS-MS/MS. Red color denotes compounds that did not match the required MRPL levels, or showed poor accuracy/precision.

Compound	LOQ, ng·mL^{-1}	Accuracy, %			Precision, %		
		3 ng·mL^{-1}	40 ng·mL^{-1}	80 ng·mL^{-1}	3 ng·mL^{-1}	40 ng·mL^{-1}	80 ng·mL^{-1}
Methamphetamine	10	-	88.6	123.8	-	4.1	1.3
Carbamazepine	1	94.8	89.8	115.5	4.2	0.5	1.2
Propranolol	5	-	90.5	95.2	-	1.2	1.8
Clenbuterol	1	105.7	93.8	112.6	12.3	2.3	1.1
Diazepam	2.5	113	94.3	111	13	3.9	1.2
Codeine	2.5	102.4	95.3	75.3	2.5	3.8	16
Cocaine	2.5	119.8	93.3	96.8	10.9	1	4
Sertraline	5	-	92.3	101.8	-	0.2	0.1
Citalopram	2.5	118.6	93.1	111.6	7.7	2.5	3.7
Fentanyl	2.5	112.7	91	110.9	3	1.1	4.4
Buprenorphine	1	96.1	87.8	100.6	4.2	6.0	13
Morphine	10	-	92.9	23.8*	-	1.9	22
Methadone	1	104.2	92.8	105.9	8.3	1.1	5.5
Salbutamol*	10	-	98.4	23.1*	-	1.6	11.4
Oxycodone	10	-	93	135.0*	-	1.6	17.7
Lorazepam	10	-	95	68.04	-	4.1	8.1
Bisoprolol	1	93.7	94.8	49.2*	22.8	6	4.1

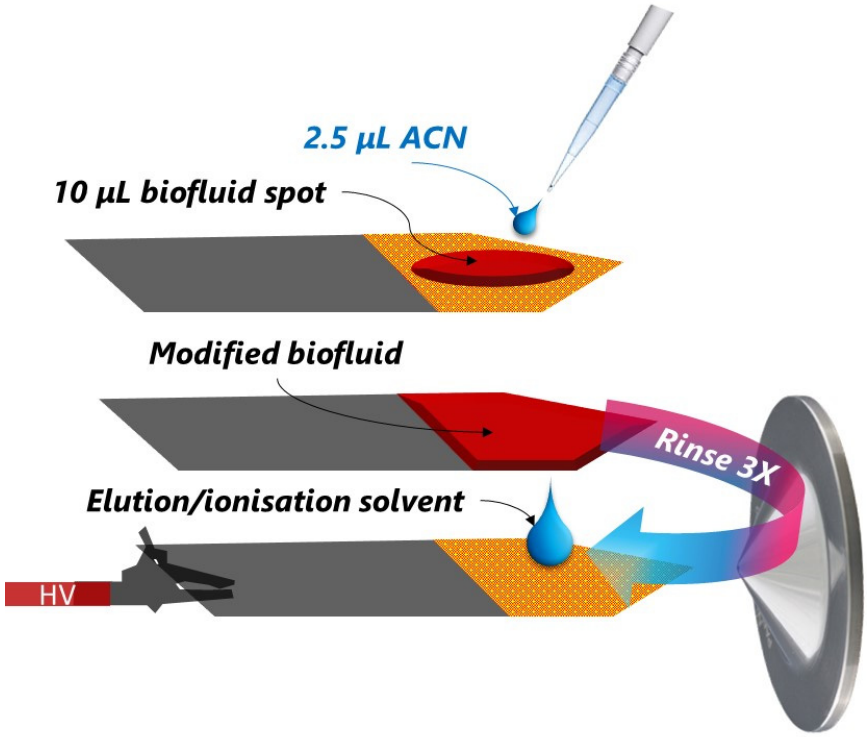

Figure 4.39 SPME-CAN methodology towards analysis of target compounds heavily bound to proteins and/or red blood cells via CBS.

Given that CBS, like any other SPME device, extracts via free concentration, analytes largely bound to plasma proteins or red blood cells are expected to provide lower extraction recoveries in comparison to those provided by PBS (*i.e.,* worst-case scenario for SPME-related technologies).[131] Aware of the intrinsic limitations of CBS, we decided to shift the paradigm and defy the solventless philosophy of SPME.[2] As portrayed in Figure 4.39, our idea entailed adding a minuscule amount of organic solvent (*i.e.* acetonitrile, ACN) to the biofluid spot so as to modify the matrix viscosity as well as the analyte-protein-binding properties, resultantly increasing the free concentration of the analytes under study, and thus facilitating their extraction onto the coating particles.[98] However, the addition of solvent onto an SPME surface holding a biofluid droplet brings up extra challenges for analyte quantitation. For instance, if the amount of organic solvent on the coating

is too large, analyte partition may be driven onto the solvent layer rather than onto the extractive particles, due to a dramatic decrease in the analyte partition coefficient (K_{fs}), and consequently, decrease the free concentration of the analyte. Likewise, if extraction/enrichment time is too long ($t \geq 5$min), precipitation of macromolecules (*i.e.,* proteins[347]), as well as blood skeletonization on the coating, may occur. Such events might lead to significant ionization suppression and potential instrument contamination. Thus, optimization of solvent volume and interaction time were critical steps in the development of this method. As shown in Figures 4.40 and 4.41, the highest response was attained when using 2.5 μL of ACN and 5 minutes of contact time. Unquestionably, these results evidence the relevance of the clean-up feature intrinsic of SPME-based devices such CBS, which are inbuilt so as to prevent the undesired attachment of potential contaminants/interferences. As can be seen in Figure 4.42 and Table 4.22, employment of this new method, named SPME-CAN, resulted in lower LOQs for all studied probes (i.e., 2-20 fold enhancement), without sacrificing total analysis time. It is important to point out that deuterated internal standards were not available for all analytes under investigation (salbutamol, oxycodone, bisoprolol, lorazepam). Nonetheless, the presented findings demonstrated that CBS-MS/MS was a suitable technique for quantitative analysis of all the studied compounds, even when the deuterated analogue of the target compound was not available (Figure 4.42). An additional feature of CBS for point-of-care applications is its ability to guarantee analyte stability on the coating prior to instrumental analysis. Our findings showed that the majority of the compounds were stable on the coating, even at room temperature, for up to 7 days (Figure 4.43). Certainly, further stability can be accomplished by storing the blades at low temperatures (-30/-80 ˚C).[2,313] While the stability of CBS has only been evaluated to 30 days at freezing conditions at this time, a long-term storage evaluation experiment (> 6 months) is currently under way in our laboratory.

Table 4.22 Figures of merit for determination of multiple substances in blood spots by SPME-CAN methodology via CBS-MS/MS.

Compound	Log P[a]	Protein binding (%)[b]	LOQ, Spot-alone ng/mL	LOQ, SPME-CAN ng/mL	Accuracy (n=4), %			Precision (n=4), %		
					3 ng/mL	40 ng/mL	80 ng/mL	3 ng/mL	40 ng/mL	80 ng/mL
Salbutamol	0.44	-	10	2.5	113.6	81.7	86.3	7.1	7.5	12.7
Morphine	0.99	30-40	10	2.5	118.6	86.9	87.2	6.5	6.3	3.6
Oxycodone	1.04	45	10	2.5	110.7	81.9	93.6	6.9	11.4	5.6
Codeine	1.20	7-25	2.5	0.5	94.2	80.8	93.7	6.5	4	3.2
Cocaine	1.97	-	2.5	0.25	92.9	86.4	96.1	3.5	2.4	1.2
Methamphetamine	2.23	-	10	0.5	90.1	84.3	95.7	2.9	2.1	1.6
Bisoprolol	2.30	30	1	0.5	95.8	87.7	95.6	4.2	3.1	7.7
Diazepam	2.63	98.5	2.5	0.5	95.5	80.5	93.4	3.7	4.5	2.3
Carbamazepine	2.77	76	1	0.25	113.2	90.7	95.3	1.7	1.8	2.9
Clenbuterol	2.94	-	1	0.5	95.9	83.3	96.4	2.8	1.7	0.9
Lorazepam	2.98	89-93	10	2.5	128.4	91.5	103.6	8.5	8.7	16.4
Propranolol	3.03	>90	5	0.5	92.0	83.2	92.6	1.8	1.9	1.9
Citalopram	3.58	80	2.5	0.5	95.7	85.3	94.6	4.5	2.5	2.4
Fentanyl	4.12	80-85	2.5	0.25	93.2	85.7	95.2	2.3	1.7	0.8
Methadone	4.14	85-90	1	0.25	96.2	92.8	93.5	2.6	1.5	0.9
Buprenorphine	4.53	96	1	0.5	98.4	85.8	97.8	7.2	8.3	4.1
Sertraline	5.06	98	5	0.25	96.5	87.1	95.1	2.4	8.5	3.3

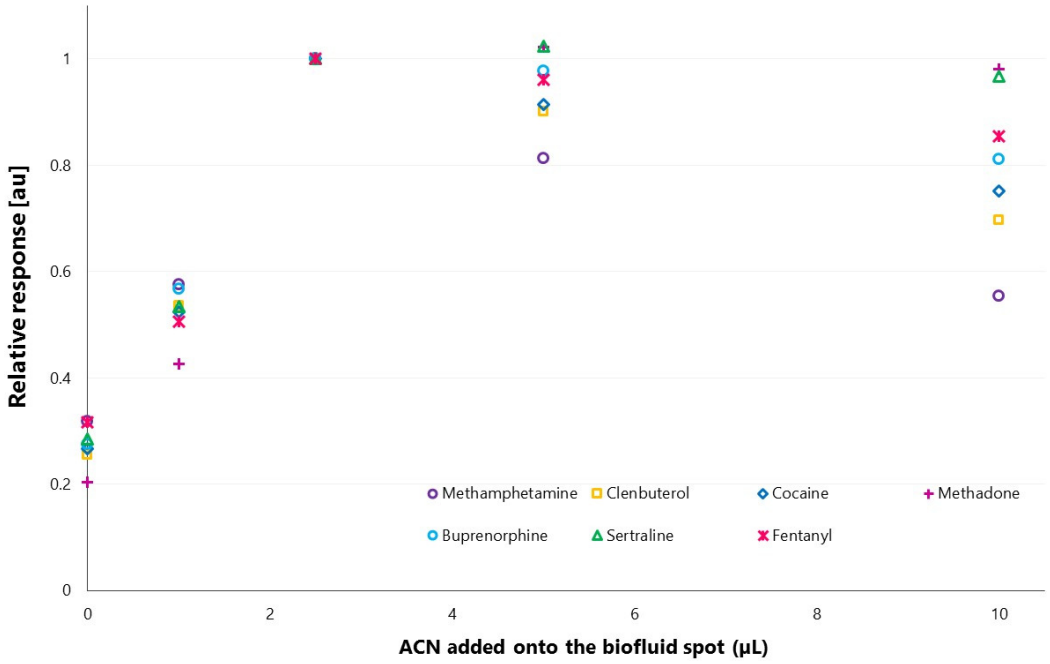

Figure 4.40 Optimization of the volume of ACN added to biofluid spot.

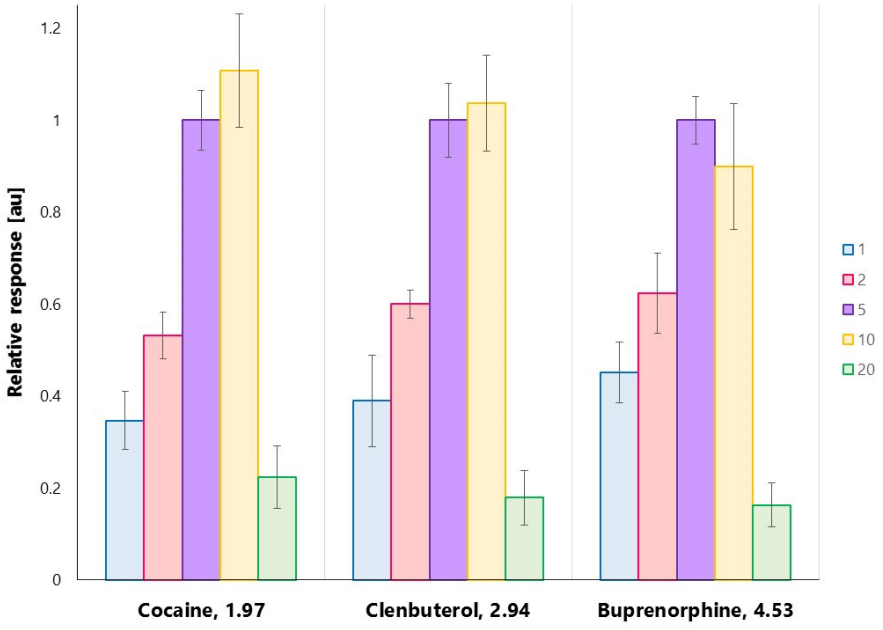

Figure 4.41 Optimization of interaction time (min) between modified spot and coated blade

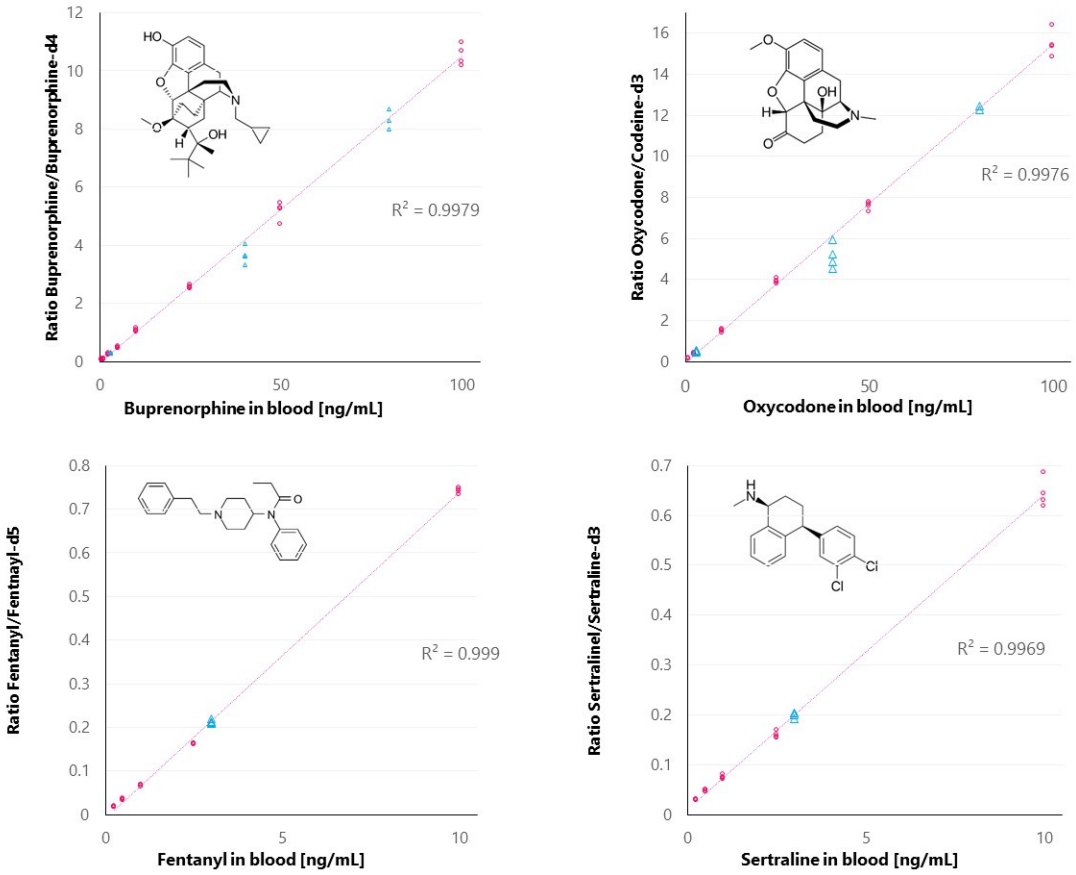

Figure 4.42 Quantitative analysis of whole blood spiked with buprenorphine (0.5-100 ng mL^{-1}), oxycodone (2.5-100 ng mL^{-1}), fentanyl (0.1 ng mL^{-1} to 10 ng mL^{-1}), and sertraline (0.25-10 ng mL^{-1}). Total sample volume was 10 µL and total analysis time ≤ 7 min via MS/MS.

Internal standard preloading and application to on-site sampling

All of the experiments described up to this point were achieved using blood spiked with both the analyte of interest and its internal standard. Given that in real case scenarios, such as drug pharmacokinetic studies in rats, the blood sample only contains the target drug (or its metabolites), and that adding an internal standard can be cumbersome due to the ultrasmall amount of analyte that need to be spiked, pre-loading an internal standard on the blade prior to spotting the real sample was evaluated. Essentially, the internal standard was preloaded on the blade by spotting 5 µL of 1 ppm solution of IS and letting it dry for a few minutes at room temperature.

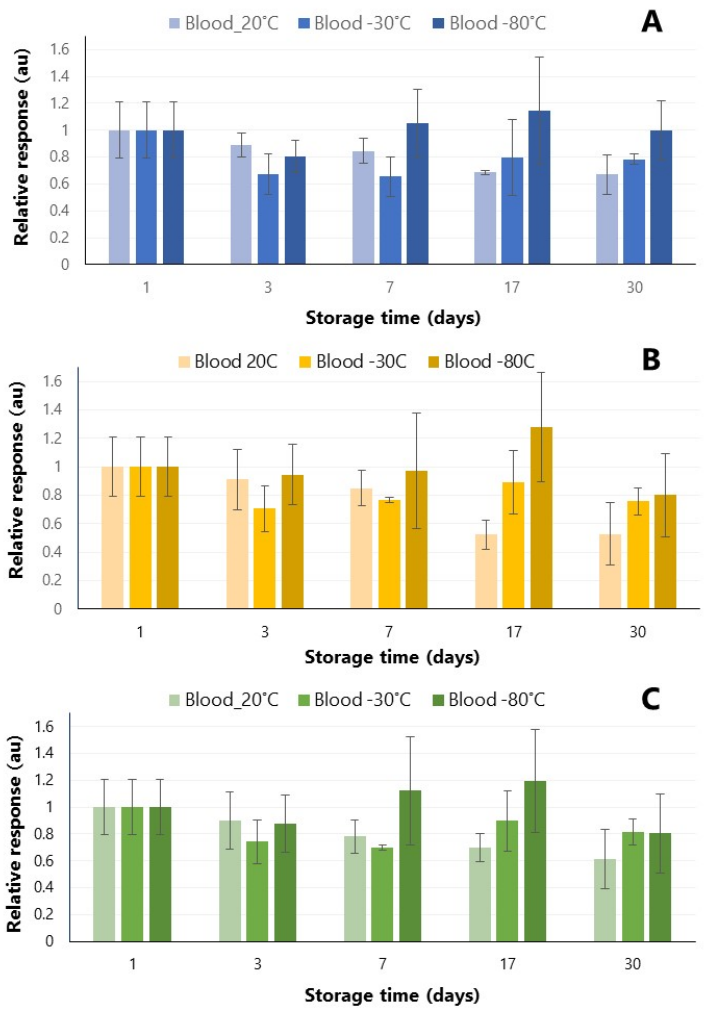

Figure 4.43 Storage stability of analytes extracted from blood spots on CBS devices for several days. A. Cocaine; B. Methamphetamine; C. Fentanyl

As can be seen in Figure 4.44, good linearity was attained when studying rat blood only spiked with fluoxetine. Based on this results, this methodology was implemented towards the quantitation of fluoxetine in rats that were administrated with such drug. The sampling took place at the laboratories of the Center for Addiction and Mental Health (CAMH) as part of our collaboration with Doctor Clement Hamani and his team on the implementation of SPME for brain studies. Basically, by using CB 300 K2EDTA microvette tubes (SARSTEDT, Germany), approximately 15 µL of blood was collected from the tail of the rats and 5 µL of such blood was placed into the coated area of a blade pre-loaded with internal standard.

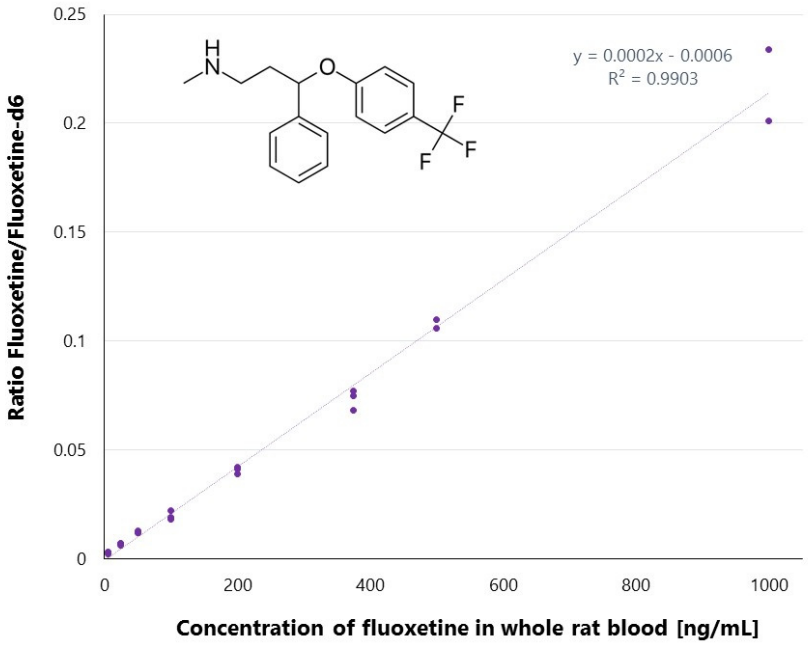

Figure 4.44 Quantitative analysis of whole blood spiked with fluoxetine (0.01-1000 ng mL^{-1}) and fluoxetine-d6 preloaded on the CBS.

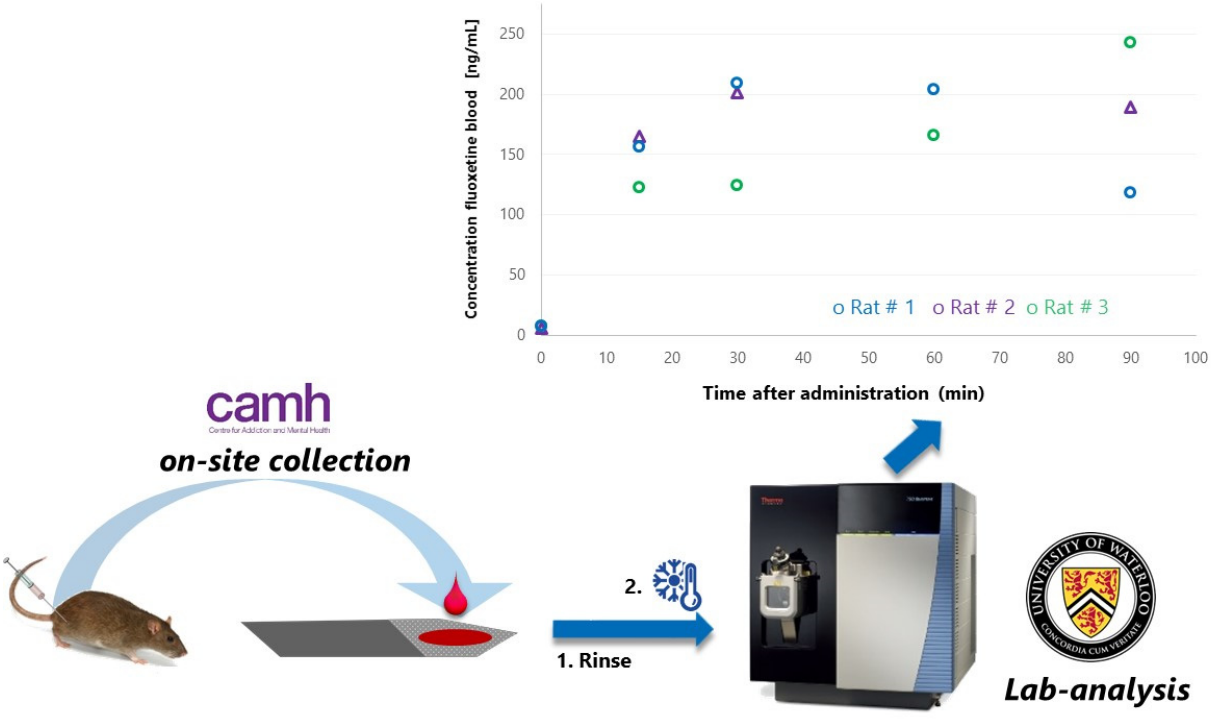

Figure 4.45 On-site monitoring of fluoxetine in whole rat blood (5 µL) using CBS preloaded with fluoxetine-d6. Sampling was performed at CAMH and instrumental analysis at UW.

As described through this section, the contact time between the spot and the coating was 5 minutes, followed by 2 rinsing steps in fresh water. After gently cleaning the coating with a Kim-wipe, the blades were stored on a vial and brought to the laboratory at University of Waterloo under cold chain (-80 °C). It is important to point-out that the extractions were performed using the original protocol developed for droplet analysis (*i.e.* no-solvent addition). Furthermore, it must be indicated that the blood samples used in this study were part of another study between our group and Dr. Hamani's group. Consequently, the sampling times were adjusted according to such study and I have no control over when the experiment starts or ends. Nevertheless, as can be seen in Figure 4.45, this methodology allowed us monitoring over the time the concentration of fluoxetine in real "patients" (rats). Currently, our group is focused on evaluating this methodology towards the determination of multiple targets from a single droplet of blood as described below.

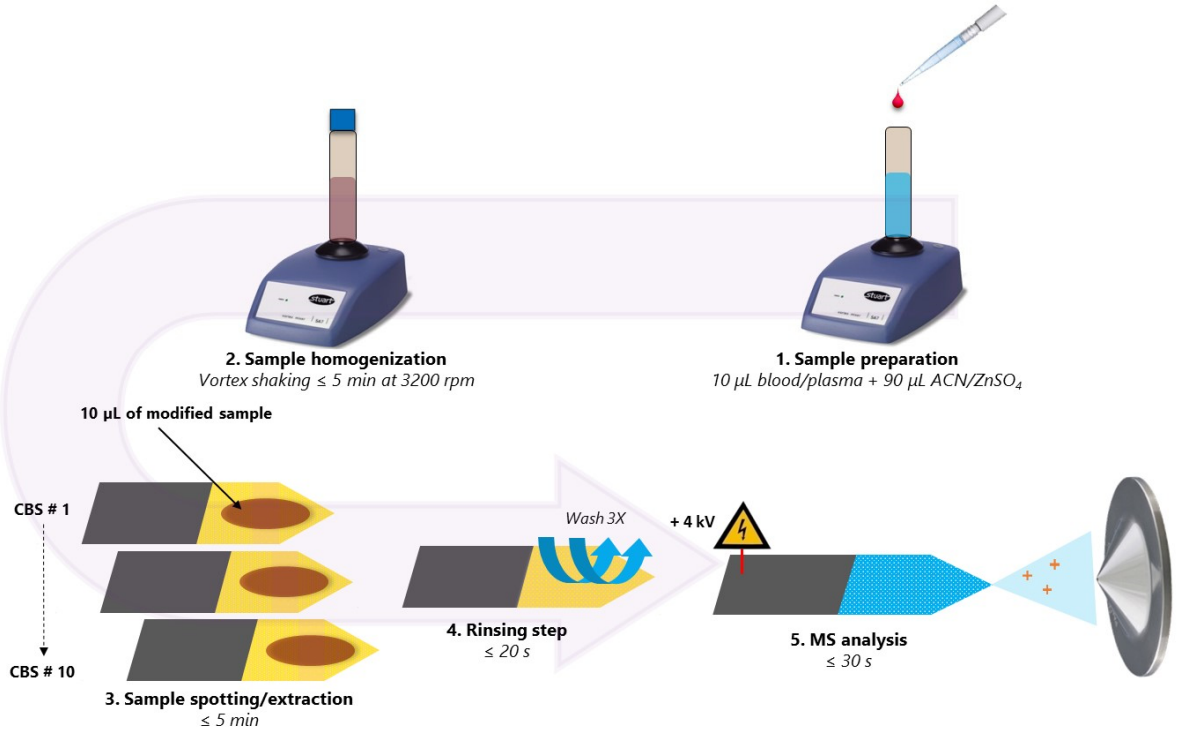

Figure 4.46 Lab-on-blade workflow for the analysis of multiple analytes with different CBS from a single droplet of biofluid.

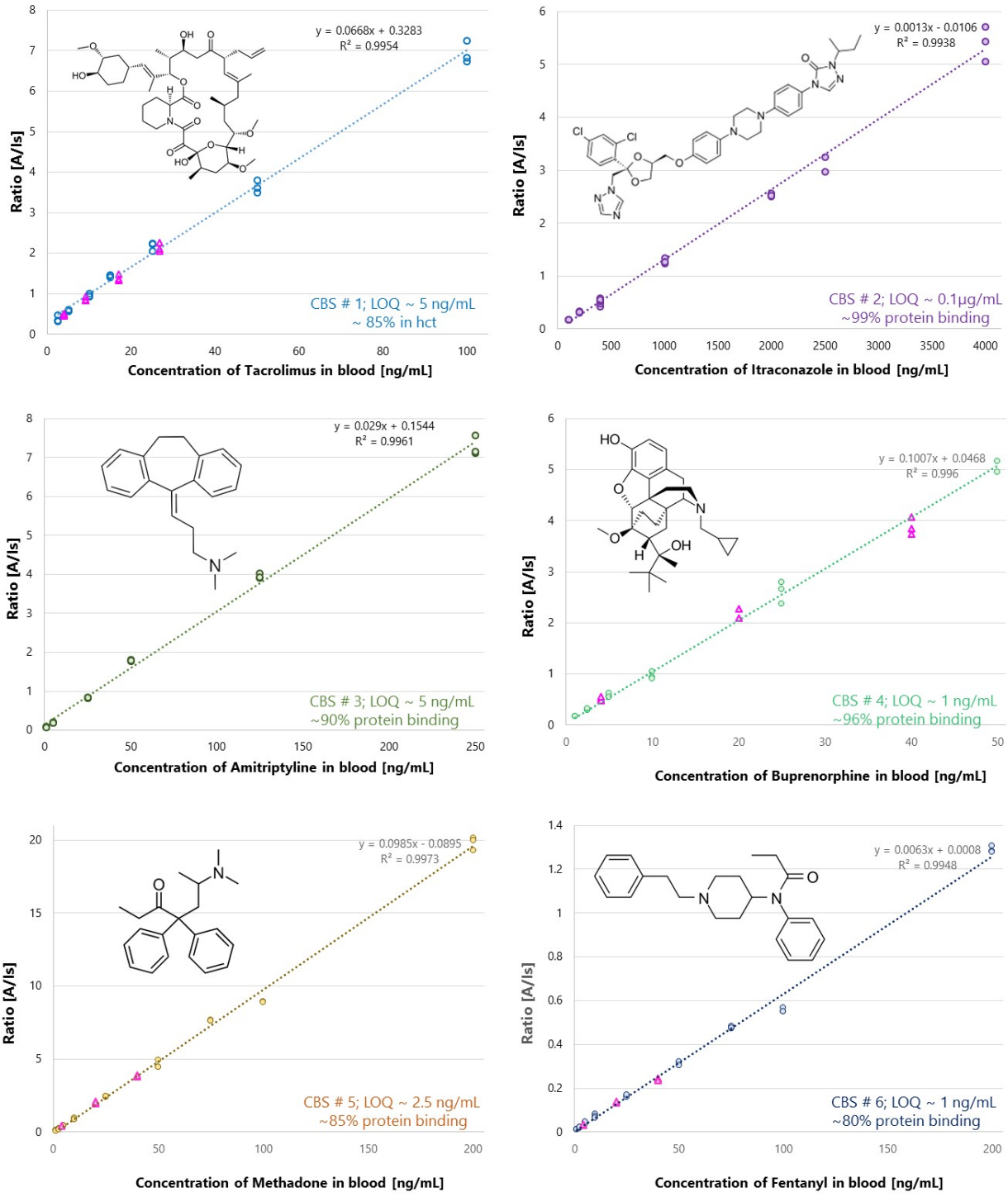

Figure 4.47 Quantitative determination of TAC, itraconazole, amitriptyline, buprenorphine, methadone and fentanyl in 10 µL whole human blood using Lab-on-a-blade protocol. Pink triangles indicate validation points plotted against the calibration curve. Each analysis was performed with an independent CBS with a total of three replicates (n=3) per calibrator or validation point. Each point corresponds to a single 10 µL sample.

Lab-on-a-blade

As a proof-of-concept, the methodology used for the analysis of immunosuppressants was implemented for the determination of multiple target compounds from a single droplet of biofluid. As shown in Figure 4.46, the procedure comprises mixing a droplet of blood (*i.e.* 10 µL) with 90 µL of the ACN/$ZnSO_4$ mixture disclosed in Section 4.5. After 5 minutes of homogenization under vortex agitation, 10 µL of the "modified sample" was applied on the coated area of the CBS. Finally, after 5 minutes of contact, the CBS devices were rinsed 3 times and placed in front of the MS for analysis. Unlike the workflows describe in the previous sections, at least 10 independent CBS experiments can be performed from a single droplet of blood. For instance, Figure 4.47 presents exemplary calibration curves for TAC, itraconazole, amitriptyline, buprenorphine, methadone and fentanyl attained with independent blades. Certainly, these results are just preliminary and further optimization is undergoing in our laboratory in order to attain lower limits of quantitation, particularly for TAC. Undeniably, the lab-on-a-blade (LOB) protocol appears as an attractive method for applications where not only the sample is limited but analytes with diverse analytical requirements (*e.g.* ionization or extraction conditions) need to be analyzed concomitantly and in a rapid fashion.

4.6.4 Summary

In summary, the potential of CBS for analysis of various compounds present in small volumes of biofluids was thoroughly validated. Unlike other SPME-MS approaches, no additional instrumentation is required for analysis, as the blade acts as both the extraction device and ionization-source.[174,178] Similar to dried blood spot (DBS) or PS methods, sample containers are not needed for sample collection, as the sample can be simply spotted onto the coated area of the

blade. Thus, after enriching the analytes on the coating, the CBS device can be shipped to the laboratory for immediate analysis, or simply stored under cold chain pending examination (Fig. 1).[391,398] Likewise, we foresee the application of CBS towards analysis of less invasive matrices such as urine or saliva,[123,421] as an extractive matrix spot.[200,422] In the same line, one could see the suitability of this technology for fingerprinting applications and population studies of microorganisms such as fungi and bacteria.[59,423,424] Certainly, the SPME-CAN methodology herein proposed can be easily implemented with other SPME substrates consisted of a flat geometry, such as SPME-Transmission Mode,[267] for spot analyses. As a future direction, our current work focuses on the development of a technology that allows for simple pre-loading of internal standards to facilitate quantitation of small sample volumes and a hassle-free sample manipulation approach.[93,425] Definitely, CBS-MS is not meant to solve all analytical problems, and combinations with on-line technologies such as differential mobility spectrometry[341] (or ion-mobility[336]) and multiple reaction monitoring with multistage fragmentation (MRM^3) may be necessary to quantify more challenging compounds (*e.g.* isobars with share fragment ions).[178] Nevertheless, the findings herein presented are quite encouraging towards the development of a cost-effective tool that can be easily implemented for on-site analysis and rapid diagnostics.[65,426,427]

5. Summary and future directions

5.1 Summary

This PhD dissertation introduced novel analytical technologies capable of delivering simplified and fast qualitative and quantitative analysis of complex matrices. These approaches, based on the direct coupling of SPME to MS, were shown to be rapid, simple to operate, deployable, cost-effective, and able to provide results close to real-time, while causing negligible instrument contamination, thus guaranteeing its reliable and long-term operation. In addition to providing biocompatibility and adequate sensitivity, all devices herein discussed can be arranged with automated systems to provide high-throughput determinations (*i.e.* $\geq$ 96 samples simultaneously). Furthermore, workflows suitable either for analysis of semi-solid samples, such as biological tissues, or liquid matrices, such biofluids, were developed for use of the presented technology. With respect to the analysis of aqueous matrices, the developed methodologies afford sampling of volumes that range from micro-droplet size ($V \leq 20$ µL) to hundreds of milliliters. Such advances are positioned to have an immediate impact on the speed, precision, and efficiency of biological investigations in drug development and point-of-care (POC) diagnosis. The strategies herein disclosed can be essentially applied to any field that requires robust, inexpensive, sensitive, and rapid workflows such as clinical, toxicological, doping, and forensic sciences.

Aiming to have a quick overview of the results/observations described in this dissertation, Table 5.1 presents a fair comparison of the SPME-MS technologies herein disclosed and those developed by other groups in recent years for rapid and reliable analysis of complex matrices. Succinctly, CBS[136] stands as one of the most comprehensive SPME-MS technologies introduced to date due to its well-defined benefits over other SPME-MS couplings.

Table 5.1 Comparison between SPME-MS technologies

Feature	SPME-MS technology					
	nanoESI	**OPP**	**CBS**	**DESI**	**DBDI**	**DART**
Ionization mechanism	ESI	ESI/CI	ESI	ESI	CI	CI
Desorption mechanism	Solvent	Solvent	Solvent	Solvent	Thermal	Thermal
MS front-end modification	Moderate	Minimal	Moderate	Significant	Significant	Significant
Reusability of the ionization source	No[a]	Yes	No[b]	Yes	Yes	Yes
Reusability of the SPME device	Yes	Yes	Yes	Yes	Yes[c]	Yes[c]
Cost per sample[d]	Significant	Moderate	Minimal	Moderate	Moderate	Moderate
Easiness to operate	Moderate	Simple	Simple	Complex	Simple	Simple
MS-total analysis time	≥ 5 min	≤ 20 s	≥ 2 min	-	≤ 20 s	≤ 20 s
Fastest MS acquisition	≤ 5s	15s	≤ 5s	-	15s	5-15s
Suitable for profiling applications	Yes	Yes	Yes	Yes	Yes	Yes
Suitable for in-vivo applications	Yes	Yes	Yes [f]	Yes	Yes	Yes
Suitable for mass spectrometry imaging	No	No	No	Yes	No	No
No need of a vessel for sampling	Yes[g]	Yes[g]	Yes	Yes[g]	Yes[g]	Yes[g]
Sensitivity attained	Moderate	High	Moderate	Low	High	Moderate
Requires fluidics[h]	No	Yes	No	Yes	Yes	Yes
Energy consumption (Watts)	Minimal	Minimal	Minimal	Minimal	Significant	Significant
Solvent consumption (μL/min)	Minimal	Significant	Minimal	Minimal	-	-
Gas consumption (mL/min)	-	Significant	-	Significant	Significant	Significant

a Single emitters are used for independent experiments to prevent carry-over
b Depending on the application, CBS devices could be potentially reused
c Depending on the desorption temperature
d Cost per sample is determined by devices used, solvent and/or gas consumption, energy requirements, among others
e MS/MS, MRM^3, HRMS from a single device
f Depending on the size of the blade (valid only for ultra-small CBS)
g Depending on the geometry of the SPME device, it must be flat, with sufficient surface area to handle a droplet
h Pumps, valves, syringes

Some of these benefits include: no gas or heating requirements for the desorption/ionization step;[174,267] low solvent consumption per analysis (≤ 15μL);[182] no fluidic requirements (*i.e.* pumps, valves, syringes or tubes);[178] no need for a desorption chamber;[114,173,182] no need for a sampling vessel when analyzing small sample volumes;[114,173,182] and no requisite of a pumping mechanism during the sample preparation process[114,197,394]. In addition, CBS can be coated with different extractive materials on each side, thus offering numberless experimental opportunities not afforded by other SPME interfaces.[114,197,211,212] For instance, CBS offers introduction of more one replicate at a time in the MS instrument, simply by independently spraying each side of the sampling device. Likewise, a derivatization reaction could be carried out on only one side of the blade, thus enhancing the selectivity and sensitivity of the method for a specific group of analytes, while the other size can be employed for an untargeted analysis of the system under investigation. Furthermore, in comparison to DBDI or OPP, CBS is capable of quantifying more compounds in a single analysis, since the area under the curve that is used for quantitation lacks the shape of a Gaussian peak, which is limited in width,[114,164,167,174,178] while the electrospray event can be extended with the use of a continuous solvent supply (*i.e.* analyte is introduced at a given spray rate). The extended electrospray event also allows for multiple MS experiments (*i.e.* MS/MS, MRM^3, DMS/IMS, HRMS) to be carried out with a single device. However, it is fair to say that technologies that introduce all extracted analytes into the mass spectrometer within a narrow window of time (*i.e.* peak or band) allow for higher sensitivity (*i.e.* higher signal to noise ratio, S/N). Two examples of such technologies include SPME-OPP[178] and SPME-DBDI.[174] Unfortunately, sensitivity is attained at the expense of the total number of compounds that can be analyzed, especially when using tandem mass spectrometry, as the number of analyzed compounds is dependent on the amount of transitions chosen per compound (*i.e.* single reaction monitoring,

SRM) and the dwell time selected for each transition. CBS is able to address this limitation by offering an additional feature that enables faster analysis times in comparison to DBDI or OPP. As shown in Chapter 4, spraying times as short as 3 seconds (or shorter) can be performed for small sets of analytes due to the good spray stability offered by CBS. Essentially, the total analysis time required for a given CBS application hinges on the total number of analytes being targeted for investigation. For example, for a small set of compounds (≤ 10), CBS can collect an adequate number of data points in 1-2 seconds when a dwell time of 25 ms is used. Indeed, it would be difficult for technologies that depend on fluidic systems to outperform CBS's speed of analysis while maintaining the low-cost per analysis. Unquestionably, the greatest advantage offered by CBS is that the blade acts as both the extraction device and the ionization source. In summary, among all the technologies herein discussed, CBS is perhaps the SPME-MS approach with the highest chance of shifting the paradigm of direct sample introduction to MS.

5.2 Future directions

Great advances with respect to the development and application of diverse SPME-MS technologies have been presented in this dissertation. However, due to the novelty of these technologies, there are several factors that require further investigation. For instance, the SPME-TM devices herein described were strictly focused on the use of stainless steel meshes. Although the performance of the stainless steel SPME-TM was outstanding for direct immersion analysis, employment of this technology for *in vivo* applications (*e.g.* road testing) would require the use of biocompatible molded/extruded polymers,[304] biocompatible 3D printed materials,[428] or other alternative substrates.[429] Furthermore, the SPME-TM device presented in this dissertation for direct immersion applications was manufactured by welding a mesh spot on a metal support blade.[267]

However, state-of-the-art manufacturing technologies, such as the one employed for the production of CBS (*i.e.* photochemical etching),[219] can be used for the preparation of individual self-supported meshes. Furthermore, high-throughput mesh arrangements, similar to the ones used for liquid chromatography,[131] can be constructed to enhance the manufacturing speed of these devices. In addition, employment of the 96-mesh on a single holder would allow for significant reductions in total analysis time per sample. Undeniably, further work on SPME-TM will also be directed towards the use of novel coating chemistries that utilize smaller particle sizes, and innovative mesh designs that allow for improved ion transmission.[229] Yet, the development of novel geometries and coating characteristics will necessitate further investigations of the desorption and ionization fundamentals related to such devices.[232] For instance, there is need for a deeper understanding of the physical and physicochemical parameters (*e.g.* proton affinity, henry's constant, thermal conductivity of the substrate, polymeric phase chemistry affinity, particle size, porosity) that govern instrumental sensitivity. Undeniably, the employment of modeling tools, such as COMSOL, would be quite useful in the optimization of such parameters.[135] Certainly, the availability of such information would enable the tuning of devices to favor the highest possible instrumental response.

With respect to SPME-nanoESI, the results herein reported showed that further work is needed in relation to the wettability and smoothness of the tested commercial coatings. Such developments would help prevent the formation of droplets inside emitters, which would in turn enable employment of longer electrospray events, further facilitating the employment of this technique towards diverse MS experiments.[173] In regards to the commercial applicability of this technique, the total cost of emitters needs to be reduced in order for employment of this technology to become feasible in commercial applications. To that extent, the implementation of high-throughput

analysis that utilizes commercially available automatized nanoESI systems might aid not only in enhancing the speed of analysis, but also contribute towards an overall decrease in the total cost per sample.[37,56] Although this work was chiefly focused on targeted studies, employment of nanoESI will fundamentally allow for the attainment of diverse information regarding the system under investigation. Therefore, it is anticipated that in a foreseeable future, combinations of SPME-nanoESI with MS/MS, HRMS, and IMS-MS will facilitate the attainment of maximum amounts of information gathered from a single sample/fiber/emitter.

Certainly, the SPME-OPP results herein presented are just a foreword for a technology with a bright future. As matter of fact, on-going work is focused on the optimization of desorption conditions (*e.g.* open chamber dimensions, evaluation of dynamic versus static desorption, effect of pump-flow and capillary inertness on band-broadening) and features of the SPME device (*e.g.* geometry, dimensions, coating thickness ad coating chemistry). As previously discussed in this work, owing to the inherent operational mechanism of the OPP (*i.e.* analytes are introduced into the MS system as a peak), this technology shows great potential of becoming one of the most sensitive SPME couplings available to date once all features listed above have been optimized. It is clear, at least for the author of this dissertation, that SPME-OPP has already found a niche in the implementation of SPME for rapid diagnosis at the surgery room, the clinical setting, or any application that requires the use of small SPME devices while guaranteeing adequate sensitivity (*e.g. in vivo* sampling from brain).[313]

Undeniably, CBS is a technology that has great potential to replace existing direct-to-MS technologies and on-line SPE-MS approaches for the analysis of complex matrices. Future work on CBS will be directed towards improving the selectivity of the method, either by implementing on-coating derivatization approaches,[286] through the use of smart materials as coatings (*e.g.*

molecular imprinted polymers or metal organic frameworks),[430] or by addition of non-chromatographic separations such as ion mobility or differential mobility prior to MS detection.[178] In addition, CBS is moving towards fully-automated and integrated sample preparation and MS events, allowing for total analysis times of less than 15s for biofluids. Novel methodologies for determination of drug metabolites will include an enzymatic hydrolysis step in the workflow prior to the enrichment step.[233] This new step will not dramatically affect the total analysis time, and will enhance the sensitivity of the method for the target drug. Furthermore, on-going experiments have shown the potential of CBS for quantitation of target analytes from smaller sample volumes ($\leq$ 5 µL). Moreover, by making the abovementioned enhancements, one can foresee the implementation of CBS in the analysis of more challenging analytes, such as testosterone and neurotransmitters. Much like other SPME-MS technologies, one can predict the immediate application of CBS as a tool for rapid profiling studies[70,285] that, when used in tandem with portable mass spectrometers, will enable rapid qualitative or semi-quantitative on-site analysis.[196] Certainly, current efforts are concentrated on the commercialization and implementation of CBS by other laboratories around the globe.

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Author: Hamed Piri-Moghadam,Fardin Ahmadi,German Augusto Gómez-Ríos,Ezel Boyacı,Nathaly Reyes-Garcés,Ali Aghakhani,Barbara Bojko,Janusz Pawliszyn

Publication: Angewandte Chemie

Publisher: John Wiley and Sons

Date: May 9, 2016

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Biocompatible Solid-Phase Microextraction Nanoelectrospray Ionization: An Unexploited Tool in Bioanalysis

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Anal. Chem., **2016**, *88* (2), pp 1259–1265
DOI: 10.1021/acs.analchem.5b03668
Publication Date (Web): December 9, 2015

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Abstract

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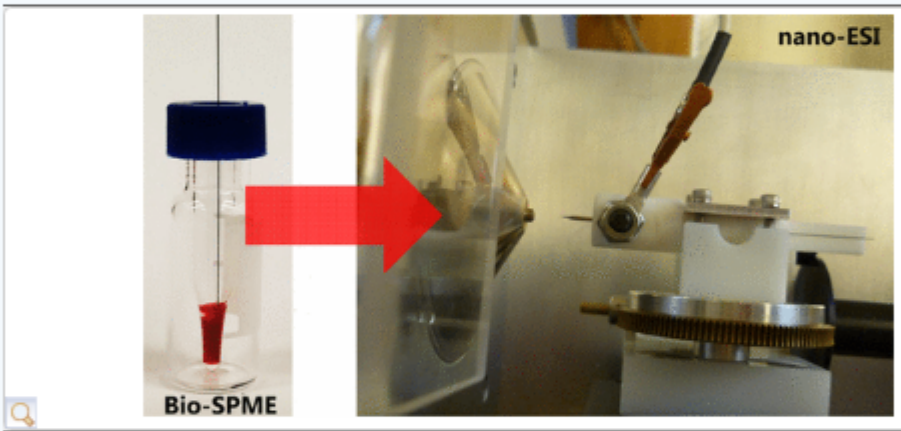

In recent years, different geometrical configurations of solid-phase microextraction (SPME) have been directly coupled to mass spectrometry, resulting in benefits such as diminishing matrix effects, improvement of detection limits, and considerable enhancement of analysis throughput. Although SPME fibers have been used for years, their potential for quantitative analysis when directly combined with mass spectrometry has not been explored to its full extent. In this study, we present the direct coupling of biocompatible SPME (Bio-SPME) fibers to mass spectrometry via nanoelectrospray ionization (nano-ESI) emitters as a powerful tool for fast quantitative analysis of target analytes in biofluids. Total sample preparation time does not exceed 2 min, and by selecting an appropriate fiber length and sample vessel, sample volumes ranging between 10 and 1500 μL can be used. Despite the short extraction time of the technique, limits of detection in the subnanogram per milliliter with good accuracy (≥90%) and linearity (R^2 > 0.999) were attained for all the studied probes in phosphate-buffered saline (PBS), urine, and whole blood. Given that Bio-SPME–nano-ESI efficiently integrates sampling with analyte extraction/enrichment, sample cleanup (including elimination of matrix effects in the form of particles), and ionization, our results demonstrated that it is an advantageous configuration for bioanalytical applications such as therapeutic drug monitoring, doping in sports, and pharmacological studies in various matrixes.

Solid phase microextraction (SPME)-transmission mode (TM) pushes down detection limits in direct analysis in real time (DART)

G. A. Gómez-Ríos and J. Pawliszyn, *Chem. Commun.*, 2014, **50**, 12937
DOI: 10.1039/C4CC05301J

DOI: 10.1039/C7AN00718C (Paper) *Analyst*, 2017, Advance Article

Towards on-site analysis of complex matrices by solid-phase microextraction-transmission mode coupled to a portable mass spectrometer *via* direct analysis in real time†

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Received 30th April 2017, Accepted 17th June 2017

First published on 20th June 2017

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