Development and application of SPME technologies for the rapid sampling and analysis of anthropogenic compounds in the environment

by

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Author’s Declaration

This thesis consists of material all of which I authored. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Due to the wide spread use of pharmaceuticals in both human and animal populations, the contamination of surface waters resulting from the outflow of waste water treatment facilities is of growing concern. Conventional methods used for the determination of these compounds often require extensive sample preparation in order to achieve appropriate limits of detection and quantitation. As a result, analytical methods which utilize these procedures are limited in their throughput capacity, while also generating large volumes of solvent waste. Coated blade spray (CBS) is a solid phase microextraction (SPME) technique which enables the direct to mass spectrometry analysis of extracted compounds with the application of limited organic solvent in order to desorb analyte and perform electrospray ionization. Demonstrated herein is the application of CBS for the concomitant MS/MS analysis of 12 pharmaceuticals in environmental waters which was able to demonstrate LODs for all compounds at concentrations of less than 50 ng/L while employing a 30 second analysis time. As select pharmaceuticals are susceptible to bioaccumulation, the analysis of fish tissue as a marker of environmental pollution is also an area of interest. For this reason a device was developed, the SPME needle, which is able to perform rapid tissue analysis without the need for a protective sheathing needle or house, simplifying the sampling process. The device was further validated to be reproducible and not effected by puncture through protective tissue. Finally, the device was incorporated into a projectile which was validated to enable rapid one handed SPME sampler administration in a matter of seconds in an on-site proof of concept.

Acknowledgments

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Finally, I would like to thank Muskies Canada for their discussion and input in their feedback pertaining to the recessed SPME device development. In addition, I would like to thank Peter Levick of Muskies Canada and Dan Weller of McMaster University, for their gracious help in obtaining the muskellunge samples.

Dedication

I would like to dedicate this thesis to my friends and family who have supported me throughout my undergraduate and graduate education.

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List of Abbreviations

Abbreviation	Meaning
AA	Ammonium acetate
ACN	Acetonitrile
ARA	Arachidonic acid
CBS	Coated blade spray
CI	Confidence interval
DART	Direct analysis in real time
DESI	Desorption electrospray ionization
DHA	Docosahexaenoic acid
DI	Direct immersion
DMF	Dimethylformamide
DVB	Divinylbenzene
EDX	Energy dispersive x-ray
EPA	Eicosapentaenoic acid
ESI	Electrospray ionization
EXT	External calibration
FA	Formic acid
GC	Gas chromatography
H_2O_2	peroxide
HLB	Hydrophilic lipophilic balanced
IMS	Ion mobility spectrometry
LAESI	Laser ablation electrospray ionization
LC	Liquid chromatography
LLE	Liquid liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
MS	Mass spectrometry
nitinol	Nickel titanium alloy
PAN	Polyacrylonitrile
PDMS	Polydimethylsiloxane
PS	Paper spray
PUFAs	Polyunsaturated fatty acids
SA	Standard addition calibration
SEM	Scanning electron microscope
SPE	Solid phase extraction
SPME	Solid phase microextraction
SRM	Select reaction monitoring
TM	Transmission mode
UV	Ultra-violet light
v%	Volume percent
WWTF	Waste water treatment facility

Chapter #1: Introduction

1.1 Pharmaceuticals in environmental water

The concentrations of pharmaceuticals in environmental waters is an issue of growing concern. As one would expect, the environmental concentration of pharmaceuticals in surface waters is highly, with these compounds being reported all over the world.[1–4] For example carbamazepine, a drug used to treat bipolar disorder and epilepsy, has been found at concentrations ranging from 0.2 ng/L to 0.75 µg/L in the Grand River (Ontario, Canada).[5,6] Due to the multiple classes of prescription and non-prescription pharmaceuticals currently used, the long-term environmental repercussions of the release of such compounds into the environment are not yet fully understood. It has however been observed that at trace concentrations of select pharmaceuticals mixture effects can amplify the biological response exhibited by non-target organisms.[7] In addition, the presence of a target pharmaceutical in a sample can hint at a much more complicated underlying picture as drug metabolites and/or transformation products may also be present.[8] As a result, analysis procedures which monitor particular pharmaceuticals along with their major metabolites are beginning to be reported and should become more prevalent as time progresses.[9,10]

The predominant route of entry for pharmaceuticals into the environment are waste water treatment facilities (WWTFs). By definition these sites collect the waste generated from population centers into one location to be treated and subsequently released into the environment. Common wastewater treatment methods include activated sludge, fixed film, and lagoon-based methods.[11] These methods are typically designed to decompose organic matter via aerobic processes and due to the wide range of physical and chemical properties demonstrated by pharmaceuticals, these methods are often unable to fully remove such compounds.[11] Coupled with geographically variable

pharmaceutical prescription patterns, WWTF influent composition, and therefore overall removal efficiencies and effluent composition, are highly variable.[12] For example, compounds such as ranitidine, naproxen, estrone, and triclosan often have removal efficiencies ranging from a 25-75% probability of 75% removal, while compounds such as gemfibrozil, carbamazepine, and erythromycin have a less than 25% probability of 75% removal when passing through a WWTF.[11] Considering these implications, the release of potentially damaging organic pollutants into the environment via WWTF is of significant concern. Coupled to the fact that more than 1400 WWTFs release an estimated 18 billion liters of treated wastewater into the Great Lakes basin every day, the potential for extensive environmental contamination is significant.[11]

Methodologies for improving WWTFs include membrane bio-reactors, which have shown great promise, which accelerate the degradation or retention of pharmaceuticals as they pass through WWTFs, due in part to the potential for increased bio-mass and not requiring a settlement step.[13] Further examples of methodologies currently being researched for their ability to remove pharmaceuticals from water include ultra violet/ peroxide (UV/H_2O_2) advanced oxidation process. Rosario-Ortiz *et. al.* demonstrated that for target pharmaceuticals, the percent removal of contaminants for this method ranged between 0 and >99%. However, for the vast majority of tests performed, irrespective of the UV/H_2O_2 conditions used, removal percentages of less than 50% were observed.[14] Similarly, Kimura *et. al.* reported that ionizing radiation in the form of Cobalt-60 (^{60}Co) gamma-rays was effective for the removal of select pharmaceuticals from spiked water samples.[15] However, this study was conducted at concentrations well above those seen in environmental samples, and a decrease of efficiency for the removal of pharmaceuticals was observed as concentrations were reduced. In other work, Xiao *et. al.* demonstrated that sonication of wastewater effluent was able to degrade select pharmaceuticals,[16] although hydrophobic and

hydrophilic pharmaceuticals were reported to undergo differing rates of decomposition. Also, when real wastewater was considered, due to the presence of organic matter, a significant reduction in process efficiency was observed. As a result, it is imperative that advancements in methodologies for the analysis of trace organic pollutants, such as pharmaceuticals, be made in order to facilitate comprehensive water quality monitoring and screening programs. When on-site sampling and sample analysis applications are considered, the requirement of conventional analysis methods to employ extensive sample preparation followed by chromatography limits throughput.

As with other anthropogenic compounds in the environment such as pesticides, bioaccumulation of pharmaceuticals is a critical parameter when assessing the overall impact of environmental pollution due to potentially toxic effects.[17–21] As a result, fish living in contaminated environments may play a critical role in the identification of the most adverse effects associated with exposure to pharmaceuticals as environmental pollutants. In a study conducted by *Howard et. al.*, 275 pharmaceuticals which were detected in the environment were considered for their potential persistence and chance of bioaccumulation. Of this set, 92 were considered to be potentially bioaccumulative, while 121 were considered to be persistent.[22] For this reason, targeting fish tissue may prove advantageous while performing environmental impact studies aimed at detecting pharmaceuticals in environmental surface waters, as they may be in effect pre-concentrating the pollutants of interest. The issue being, as with conventional water analysis procedures, tissue analysis is a very time consuming and tedious procedure requiring extensive sample preparation.

Solid phase microextraction (SPME), since its invention in the early 90's, has long been applied to the analysis of both simple and complex sample matrices as it combines the sampling

and sample preparative steps.[23] Certainly when compared to conventional analysis procedures, such as those applied for water analysis like liquid-liquid extraction (LLE) and solid phase extraction (SPE), SPME offers a more rapid alternative. Furthermore, SPME limits the amount of organic solvents used thereby decreasing the environmental impact of the methodologies which employ it. In addition to the obvious benefits in terms of sample throughput, SPME has additional advantages while performing tissue analysis. Compared to conventional methodologies where large portions of tissue must be removed by biopsy needle, or the sample specimen sacrificed, SPME offers a non-lethal and minimally invasive alternative.[24]

Solid phase microextraction operates based on a diffusion boundary layer through which small molecules migrate forming a concentration gradient as they sorb onto the extraction phase. This process is depicted in Figure 1.1, and describes the underlying principle of most SPME extraction techniques.[23]

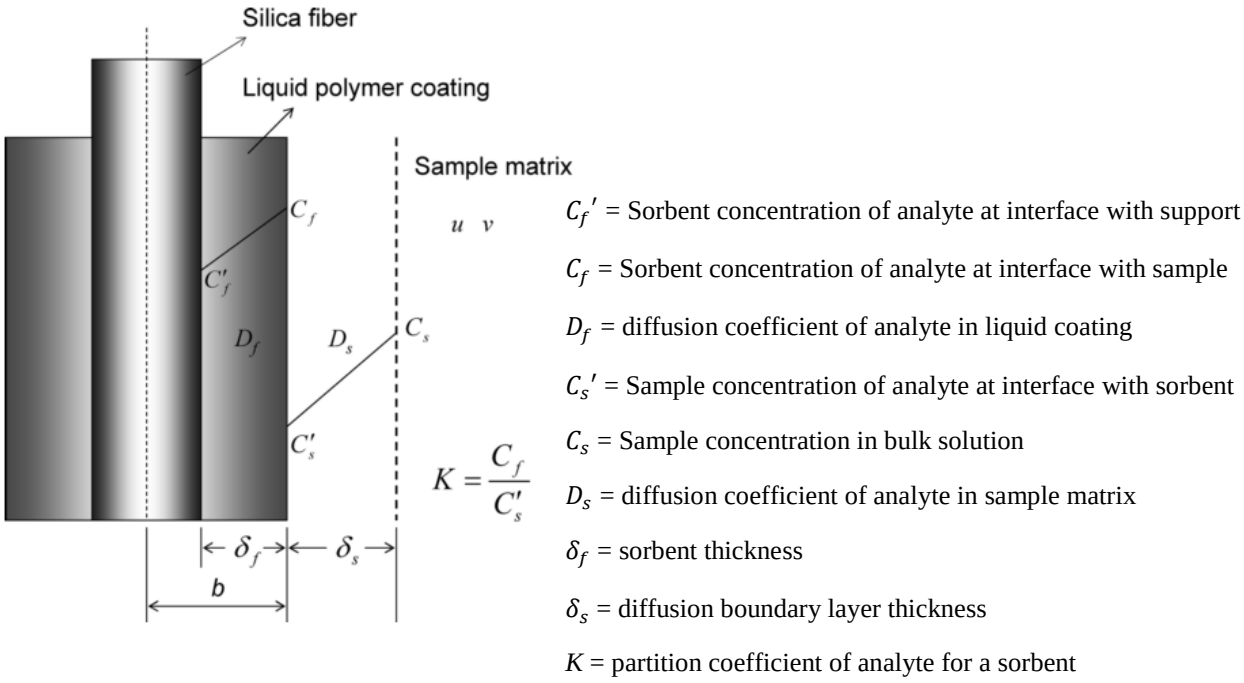


Figure 1.1: Process of SPME extraction for a liquid sorbent coating by a diffusion boundary layer which is controlled by the agitative conditions of the sample[25]

Of course, as the sample matrix changes so too does the diffusion and migratory characteristics of an analyte, though with the proper calibration techniques these can be accounted for. In addition, one of the major strengths of SPME as an extractive technology is that due to it being an affinity based extraction procedure, it demonstrates balanced coverage of analytes present in a sample matrix. What this means is that, for instance, a compound which demonstrates a high affinity for the sample matrix, say water, will tend to be at elevated concentrations over compounds which do not demonstrate this degree of affinity or solubility. As a result, if an exhaustive extraction procedure is employed, the resultant chromatograms will be dominated by these high affinity compounds. With SPME however, these high affinity compounds will demonstrate a lessened affinity for the sorbent coating while the low affinity compounds will demonstrate a larger affinity for the extraction phase. In this way, the amount of analyte extracted is balanced by its physio-chemical properties and not by the absolute amount present in a sample.[23,26] This characteristic has obvious advantages for the analysis of complex sample matrices such as environmental waters and fish tissue. As a result, the SPME technology was chosen as the extraction and sample preparation methodology to be employed in this work.

1.2 Rapid analysis: Direct to mass spectrometry

While the application of SPME to the analysis of pharmaceuticals and other anthropogenic compounds in environmental waters is well documented in the literature,[6,26–31] method throughput has to date been limited by the use of chromatography, thus causing the separation portion of the analytical method (*i.e.* GC or LC) to be a time-consuming endeavor. One potential avenue of improvement is that of the merger of SPME and direct to mass spectrometry (MS) technologies.

If successful, the analysis of trace environmental contaminants will provide a cheaper, faster, and more environmentally friendly alternative to conventional analytical techniques.

Although the direct-to-MS analysis of organic compounds is still in its infancy,[32] a wide range of methods are currently being researched due to the potential of the field.[33] Though not a comprehensive list, some examples are direct analysis in real-time (DART),[34] paper spray (PS),[35] laser ablation electrospray ionization (LAESI),[36] and desorption electrospray ionization (DESI).[37] All of these methods (and the many not mentioned here) share the same underlying purpose: to generate ionic species under ambient conditions to be introduced into a mass spectrometer for detection/identification.[38]

Paper spray was first developed and introduced by Wang *et. al*.[39] In the presented application, a piece of paper is placed in a specially designed mount, which allows for the application of a voltage onto the paper. Once in place, an aqueous sample is applied to the paper and left to dry. A desorption solution is then introduced to the paper, dissolving the analyte, and a potential difference is applied between the paper and MS inlet, initiating electrospray ionization, resulting in the formation of charged micro droplets of solvent that contain analyte. As the droplets evaporate, the charge is ultimately transferred to the analyte.[39] Notably, the use of a piece of paper with a pointed tip was found to enable the formation of a Taylor Cone, which facilitates ionization and allows for the aiming of the charged species toward the inlet of the MS.[35,39] Paper spray has been applied in a variety of applications; some examples include the analysis of blood for pharmaceuticals, and the identification of dyes in foodstuffs.[40–43]

Due to the nature of PS, pre-concentration of analyte onto the paper substrate is often difficult limiting the application of this method for trace organic analyses, like that required in environmental applications. Similarly, if direct sample analysis is considered, the presence of

matrix effects associated with un-processed samples often results in ion suppression and decreased method response. In order to eliminate both of these shortcomings, the coupling of pre-concentration techniques to direct to MS analysis has gained increasing attention. One such example is the work performed by Zhang *et. al.* and Zheng *et. al.*, where micro-SPE was successfully coupled to PS for the analysis of pharmaceuticals in blood, as well as dye components in chili products.[44,45] Other applications involving pre-concentration of analyte prior to direct to MS analysis include the work performed by Rosting *et. al.* and Zhang *et. al.* in the application of thin-film membranes; while Rosting *et. al.* demonstrated that a liquid membrane could be used as an extraction phase to pre-concentrate analyte prior to DESI, Zhang, *et. al.* showed that the application of a complex solution to the surface of a permeable membrane would result in the isolation of analytes unable to permeate the membrane on the surface [46,47]

Among these advancements, there have been a range of applications which aim to directly couple the SPME technology directly to MS. Some of these applications include in-line direct infusion of SPME fiber desorption solutions,[48] conventional SPME blades used for DESI,[49] coated mesh direct analysis in real time (DART),[50] bio-SPME fiber nano-ESI,[51] coated blade spray (CBS),[52] and more recently applications with the open port probe (OPP),[53] and dielectric barrier discharge ionization SPME.[54] Of these applications CBS was chosen as the methodology to be implemented in this work for the targeted analysis of pharmaceuticals in environmental water, as it provides the most rapid analysis procedure. In addition and the method of ionization, ESI, is well suited to the class of target compounds and the functionalities they typically possess. Images of the CBS ionization process are provided in Figure 1.2. The main difference between PS and CBS is that the blade used to produce the charged species is utilized as the sampling/sample preparation device (*i.e.* matrix clean-up and analyte enrichment can be achieved simultaneously).

CBS is performed by exposing a metal blade whose tip has been coated with a sorbent to a sample matrix. The analyte will then partition onto the sorbent, extracting it and concentrating the analyte onto the coated blade. The blade is then removed from the sample matrix, followed by application of an appropriate desorption solution to the blade to desorb the analytes from the sorbent on the blade. Once a sufficient period of time has elapsed to desorb the analyte, while limiting the loss of solvent to evaporation, the blade is placed in front of the MS inlet and a voltage is applied, resulting in analyte ionization and analysis.

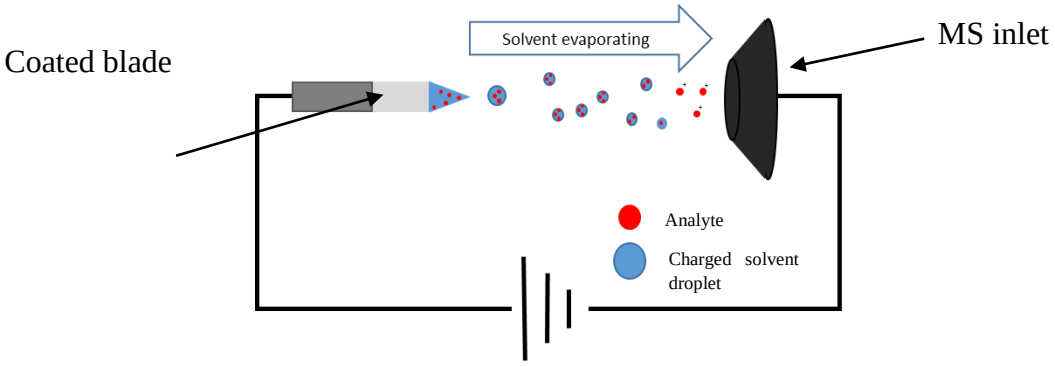


Figure 1.2: Schematic of the CBS ESI process

CBS has one main underlying benefit, in that the same piece of equipment used in ionization is also used as the sampler, greatly simplifying the methodology. In addition, as it operates using SPME principles it is able to extract analytes from the sample matrix and enrich samples prior to analysis. This extractive and concentrative property of SPME is ideally suited to direct to MS analysis techniques, as it provides a clean, concentrated extract from the sample, thereby limiting matrix effects which could decrease response.

The direct-to-MS analysis of environmental samples for pharmaceuticals is an emerging field of research. Boisvert *et. al.* have been able to demonstrate its feasibility, where the analysis of pharmaceuticals in wastewater was directly coupled to MS with the use of laser diode thermal

desorption.[55] Here, a sample is placed and dried onto a patented LazWell™ plate, where it is thermally desorbed via laser interrogation and carried through a coronal discharge by a constant flow of gas, resulting in analyte ionization followed by introduction into a MS. The devised method requires extensive sample preparation by filtration, solid phase extraction, elution, and subsequent pre-concentration of sample matrix prior to analyte ionization and analysis. However, the researchers were able to achieve limits of detection for a variety of pesticides and pharmaceuticals in the 28-162 ng/L range. Similarly, DESI has been used for the screening of cosmetic products from environmental samples.[56] In this application, tap water was analyzed through the direct use of thermally assisted ionization, where the sample target was heated during desorption electrospray ionization. Limits of detection in the ng/L range were achieved, though the range in which the analytes detected could be quantified was in the µg/L range.

1.3 Fish tissue analysis: SPME

The implementation of SPME for the analysis of fish tissue, as with any biological sample matrix, is certainly advantageous due to the minimally invasive and non-lethal ability to perform these samplings. For this reason SPME has been documented and used to great effect for the analysis of fish tissue for target compounds of interest.[57–60] However, broadly speaking traditional SPME fibers are quite fragile due to the fused silica core used as a support to bind the extractive coating.[61] Alternatives which are often employed for in-vivo applications include titanium, or nickel-titanium alloy (nitinol) supported SPME coatings. In addition, the chemical immobilization of a coating onto such a support has also been shown to be fairly robust.[62–64] However, such devices are quite flexible due to the small diameter of the supporting material; an intrinsic property of many SPME devices which may not be desirable for applications requiring the puncture of a robust

sample matrix. These limitations, namely fragility and flexibility, result in SPME fibers being augmented with sheathing needles in order to support and/or puncture sample matrices such as fish scales for in-vivo applications (Figure 1.3).[57,59,60]

Figure 1.3: SPME fiber introduction device used to puncture fish scales[65]

As such, these characteristics could be considered a detriment to the streamlined nature of SPME as a rapid and easily executed sampling and sample preparative technique as the implementation of the SPME technology becomes cumbersome and slow. For this reason it is imperative that robust SPME devices which require no additional supporting hardware or sheathing needle are developed. A device of this nature would not only facilitate efficient sampling, it would highlight the advantageous nature of SPME as a means by which to perform

chemical biopsies of tissue, thereby avoiding the invasiveness and complexity of traditional tissue sampling procedures, such as physical tissue biopsy.[24]

1.4 Summary

To conclude, the technology of SPME has been shown to be highly applicable to the analysis of environmental matrices for anthropogenic compounds, whether they be surface waters or the tissue of species which are susceptible to bioaccumulation. These traditional SPME methodologies have multiple advantages over conventional analysis methodologies due in part to the rapid sample preparative nature of SPME and its ability to provide a clean extract and balanced coverage. However, limitations to the SPME technology do exist. As a result, the thesis herein aims to address two of these limitation: 1) by applying SPME CBS for the rapid screening of target pharmaceuticals at their source, WWTF, and 2) by developing a more robust SPME device capable of performing in-vivo sampling with no protective housing or pre-puncture needle.

Chapter #2: Development of CBS for targeted environmental analysis

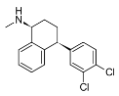
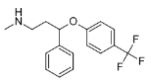
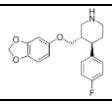
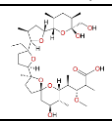
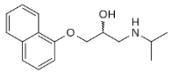
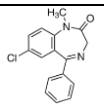
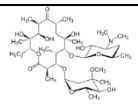
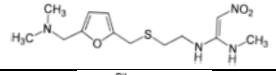
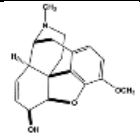
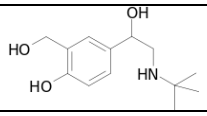
2.1 Introduction

Direct to MS techniques afford analysts the ability to perform rapid screening and quantitative procedures. Of course, as these procedures do not possess a chromatographic separation, isobars or matrix constituents which demonstrate similar select reaction monitoring (SRM) transitions cannot be resolved, imposing a limitation on the technique. With this is mind it is important to investigate these direct to MS techniques in order to identify such limitations and identify compounds which may not be suitable for this method of analysis. Technologies such as ion-mobility, or additional ion fragmentation procedures (MS^n) could be implemented in order to further resolve compounds which may not be suitable for direct to MS/MS analysis. Of course, these limitations must be identified and understood in order to take full advantage of additional mass spectrometric resolution procedures. The work to be discussed here encompasses some method development procedures for the application of the direct to MS analysis technique of CBS, for the targeted analysis of pharmaceuticals in environmental waters. Conducted experiments include an investigation of the desorption/ionization solution composition used in CBS applications, as well as the relation of sample volume and sample matrix pH towards method response.

When assembling a group of pharmaceuticals to be targeted, care was taken to select analytes which were of varied classes in terms of what they were prescribed to treat, so as to obtain a broad range of both structural and logP variations. The importance of a wide range of logP values for the target compounds cannot be understated, as the devised method may be selective for certain

compounds, and consequently, extra optimization steps may be required for highly polar analytes due to their high affinity for aqueous media relative to the sorption phase used in SPME. The target compounds to be used for method development are listed in Table 2.1.

Table 2.1: Target pharmaceuticals and their physical chemical properties

Name	Structure	Use	MW g/mol	pKa	log P	Ionization mode (adduct)
Sertraline		Selective serotonin reuptake inhibitor class	305.074	9.9	5.1	(H) +
Methadone		Synthetic opioid	309.45	8.9	4.2	(H) +
Fluoxetine		Selective serotonin reuptake inhibitor class	309.326	9.8	4.09	(H) +
Paroxetine		Selective serotonin reuptake inhibitor class	329.365	9.8	3.89	(H) +
Monensin		Ionophore, Na+/H+ antiporter	670.871	4.3	3.72	(Na) +
Propranolol		β-blocker	259.34	9.4	3.1	(H) +
Diazepam		Benzodiazepine	284.7	3.4	2.91	(H) +
Erythromycin		Macrolide	733.9	8.9	2.83	(H) +
Carbamazepine		Antiepileptics	236.27	15.9	2.67	(H) +
Ranitidine		Antihistamine, treats ulcers	314.403	8.1	1.23	(H) +
Codeine		Opiate used to treat pain	299.364	8.2	1.2	(H) +
Salbutamol		Inhaler for asthma	239.311	10.3	0.01	(H) +

2.2 Experimental

2.2.1 Materials and reagents

The pharmaceuticals propranolol, prorpanolol-d7, sertraline, sertraline-d3, fluoxetine, fluoxetine-d7, paroxetine, paroxetine-d6, methadone, methadone-d3, codeine, codeine-d3, diazepam, and diazepam-d5 were purchased from Cerilliant of Sigma-Aldrich (Roung Rock, TX, USA) while salbutamol, monensin, erythromycin, ranitidine and carbamazepine were purchased from Sigma-Aldrich (Oakville, ON, Canada) and erythromycin-d3, ranitidine-d6 were purchased from TRC (Toronto Research Chemical, Toronto, ON). For chromatographic separations and standard preparation, the MS grade methanol, acetonitrile and water were obtained from Fisher Scientific while the salts formic acid (FA) and ammonium acetate (AA) were purchased from Sigma Aldrich (Oakville, ON, Canada). Plastic 300 µL vials and amber 2mL and 10mL glass vials along with pre-pierced PTFE/Silicon septa used were purchased from Canadian Life Sciences (Peterborough, ON, Canada). Hydrophilic lipophilic balanced (HLB) particles used were obtained from Waters (Wilmslow, UK) while the C18 particles used were obtained from Supelco (Bellefonte, USA).

2.2.2 Manufacture of CBS devices

Coated blade spray devices were made of stainless steel sheets which were 5 cm in length and 0.5 cm in width, which were brought to a tip. These blades were then placed in 37 wt% hydrochloric acid and sonicated at room temperature in order to roughen the metal surface facilitating adhesion of the sorbent coating. Etching was performed for 20 minutes, during this time the blades were shook every 5 minutes so as to ensure any overlapping regions of the blades were uncovered and etching was homogeneous. Etched blades were then removed from the acid,

rinsed in water using sonication 3 times for 5 minutes, followed by a quick rinse in methanol and dried at 80 °C for 5 minutes. HLB-PAN or C18-PAN extractive coatings of thickness 10 µm and length 1.5 cm were then applied using a proprietary coating procedure.

2.2.3 Instrumental analysis

For CBS experimentation a modified TSQ Quantiva (Thermo Scientific, San Jose, USA) was used. In place of the traditional ESI source used for LC-MS/MS, a custom ionization source constructed by the University of Waterloo Science Shop was employed, and is shown in Figure 2.1. This source allows for the controlled placement of CBS devices in front of the MS inlet so as to ensure the distance between the CBS device tip and the transfer tube is reproducible from one device to the next. In order to initiate the ESI process from the tip of the CBS devices a potential difference of +4 kV was used.

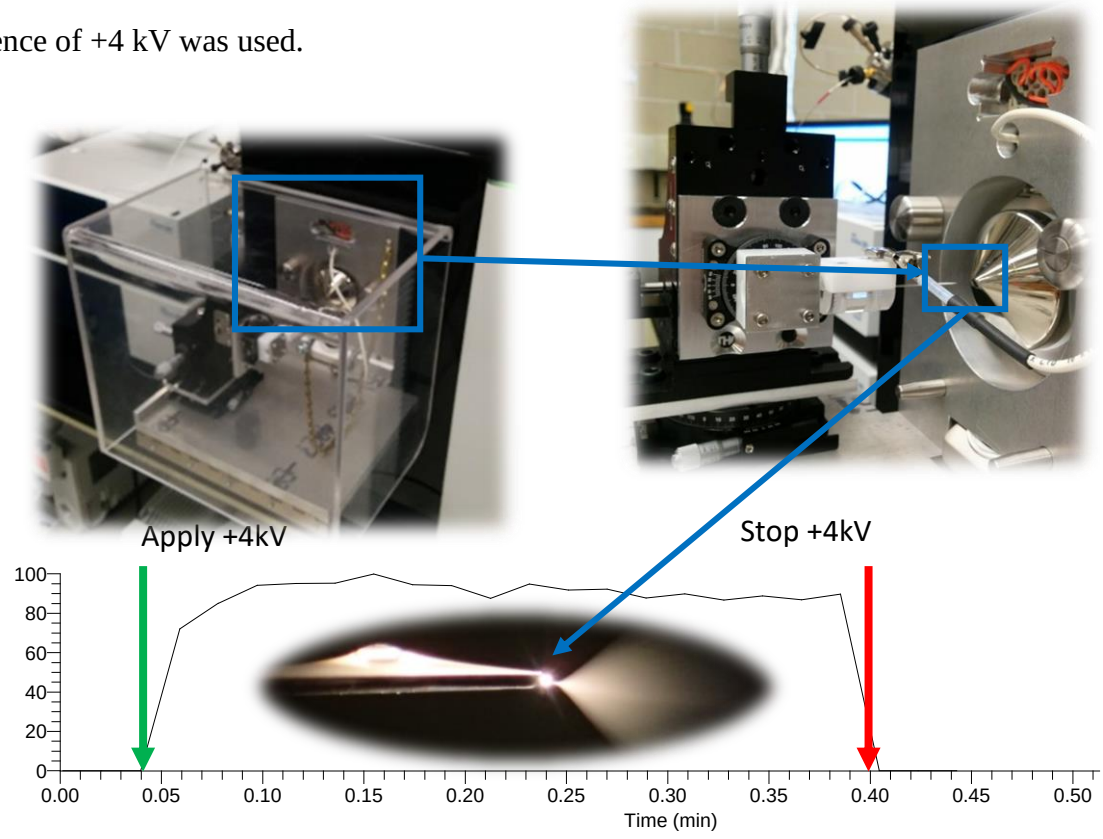


Figure 2.1: Custom CBS ionization source manufactured for the TSQ-Quantiva

As there are 22 target species to be monitored in this particular study, a 30 second spray event with a 50 ms dwell time per SRM transition was used so as to provide an adequate number of scans per transition. With this in mind, if a smaller number of targets were of interest much shorter ionization events could be applied allowing even faster analysis times.[66] Specifics relating to the MS/MS parameters as well as the SRM transitions monitored are available in Appendix #1. In all CBS experiments, unless otherwise specified, experiments were conducted in triplicate and the desorption/ionization solution was applied to the surface of the coated blades for a duration of 20 seconds in order to desorb extracted analyte prior to the initiation of ESI.

2.3 Results, discussion, and experimental specifics

2.3.1 Coating comparison, C18 v.s. HLB for the target compounds

In the discussed work HLB extractive coatings were utilized as the sorbent phase for the conducted experiments. HLB particles were observed to provide a much larger response for the target analytes, in particular carbamazepine, codeine, salbutamol and ranitidine (Figure 2.2) following a 2 minute extraction form a 1 µg/L solution in water. Though monensin did in fact demonstrate greater method response when a C18 extraction phase was implemented, the coverage of analytes extracted by HLB provided the best overall response. Of course, if monensin was the sole target of any particular application a C18 coating would be advantageous.

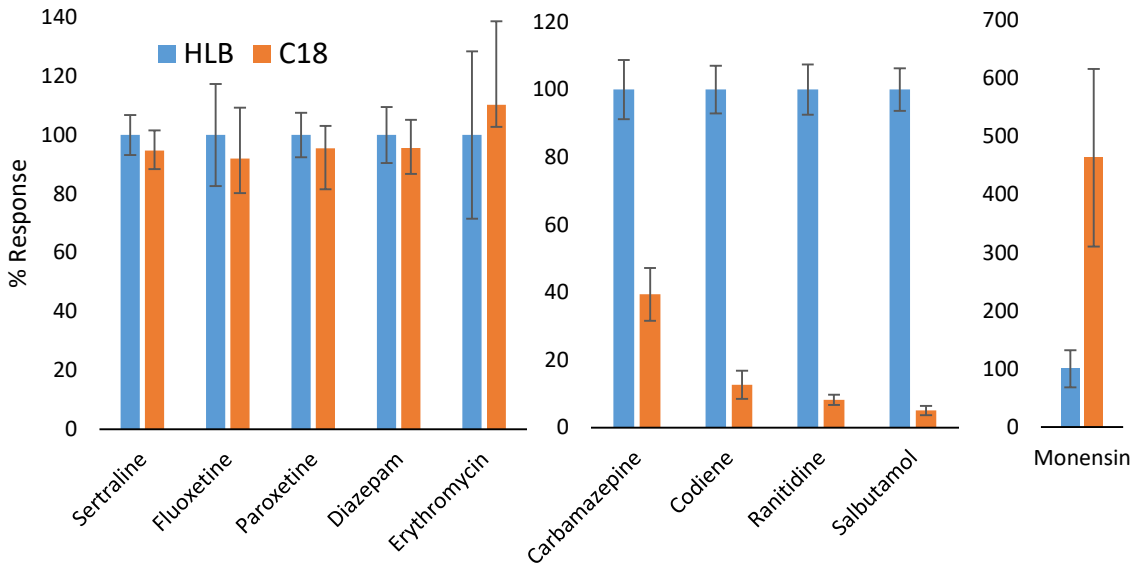


Figure 2.2: Coating comparison, HLB v.s. C18, of % response obtained for target pharaceuticals in water

2.3.2 Effect of desorption and ionization solution composition

As with any electrospray ionization process, the stability of the spray itself from the tip of the coated blade is a critical parameter when performing quantitative analysis. A stable electrospray will ensure a reproducible introduction of analyte into the mass spectrometer and hence inter blade reproducibility. As CBS uses a non-continuous flow of organic solvent to perform ESI there is depletion of the organic desorption/ionization solvent due to the electrospray process itself, but also loss due to evaporation. In order to assess the spray stability produced by CBS devices, varied desorption solvents were applied and the response of extracted analytes were monitored for 30 seconds. The base of all desorption/ ionization solutions tested was that of methanol, due to its extensive use as the desorption solution for HLB sorbents in the literature. Additives included acetonitrile and water, while the formic acid concentration was held constant at 0.1 volume percent (v%).

What was immediately apparent was that when excessively large volumes of desorption solution were used (>20 µL) the solution would quickly migrate to the tip of the device upon the application of the spray voltage, and would actually splash the MS inlet, providing irreproducible spray events. As a result, 15 µL desorption volumes were used in all further studies. Instrumental response relating to four tested compositions are provided in Figure 2.3.

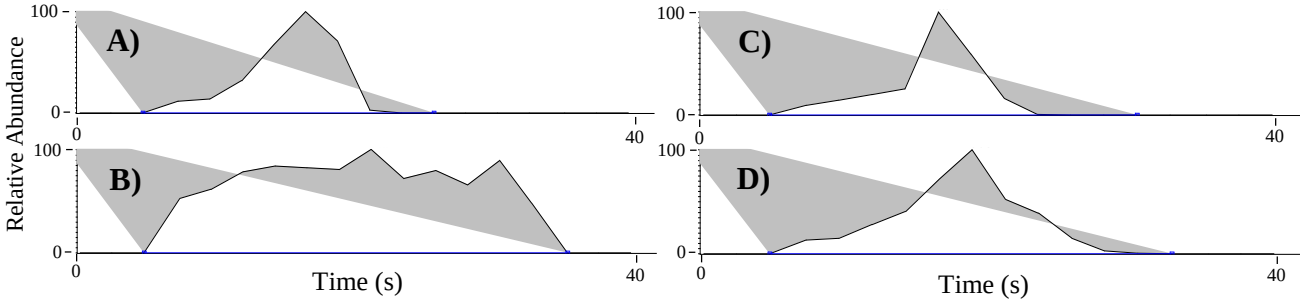


Figure 2.3: Resultant ESI spray stability for varied CBS desorption/ionization spray solutions; a) methanol, b) 5% water in methanol, c) 5% acetonitrile in methanol, d) 5% of water and 5% acetonitrile in methanol

One important observation made was that when the solution was composed entirely of organic solvent, whether it be 100% methanol, or 95% methanol and 5% acetonitrile, the solution would be exhausted before a full 30 seconds of ESI. What appeared to alleviate this was the presence of water in solution, as both compositions which incorporated water demonstrated extended ESI times. Though only one trace of each solvent composition is shown, the methanolic solution with 5% water was identified as the most reproducible, and was therefore used in all future applications.

Once a methanolic/water solution was identified as providing sufficient spray stability, methods of further improving response were investigated. The primarily focus being on the effect of additives to the desorption/electrospray ionization solution. It has been documented that the presence of ammonium in methanolic mobile phases used for LC-ESI/MS analysis often improved

the response of proton adducts in positive ionization mode. The hypothesis behind this observation being that, though in the aqueous phase there may be a larger degree of ammonium adducts, proton transfer in the gas phase from ammonium to the analyte would improve the magnitude of proton adducts observed.[67] The ammonium salt used for this purpose in the literature has typically been ammonium formate or ammonium acetate. As ammonium acetate was available at the time of experimentation, it was used in this study. Investigations employed 4 ammonium acetate concentrations, 0 mM, 6 mM, 12 mM and 24 mM in the presence of a 95:5 methanol water solution with 0.1v% formic acid. Extractions were performed for 10 minutes at 1200 rpm from a 1 µg/L aqueous solution of volume 1.5 mL.

From this study it was evident that the presence of ammonium acetate did in fact improve the magnitude of observed proton adducts for all compounds which demonstrated one, with there being no significant difference between the response of 12 mM and 24 mM solutions. For this reason, further experimentation utilized a 12 mM concentration of ammonium acetate. Furthermore, the concentration of formic acid was investigated at this ammonium acetate concentration by repeating the experiment as outlined above, however in this instance the ammonium acetate concentration was held constant at 12 mM, while the formic acid concentration was tested at 0 v%, 0.1 v% and 0.5 v%. The results of this study are presented in Figure 2.4 where it is clear that the optimum value of formic acid is that of 0.1 v% while employing a 12mM ammonium acetate concentration.

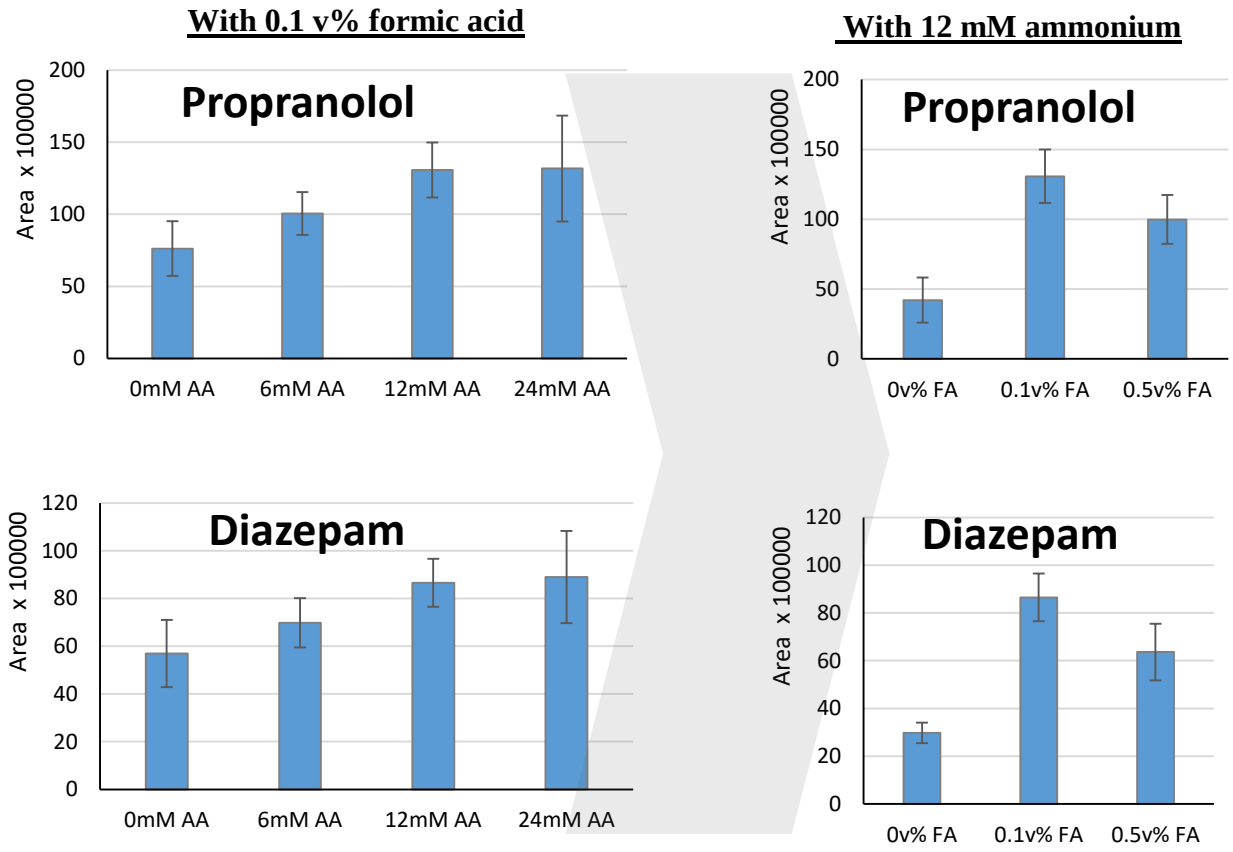


Figure 2.4: Optimization of ammonium acetate and formic acid concentratins in the CBS desorption/ ionization solution

Of course with the presence of ammonium now in the solution there would be ammonium adducts of the target compounds entering the mass spectrometer. To determine whether or not these adducts were of a significant and relevant magnitude compared to the proton adducts, the analytes were formulated at 1 ppm in a 95:5 methanol/water, 12mM AA, 0.1 v% FA. This solution was then analyzed by direct infusion using the commercial TSQ-Quantiva ESI source operated at room temperature. Of all the compounds, the only one to demonstrate an appreciable magnitude of ammonium adduct was that of propranolol. Once identified, the ammonium adduct of propranolol was tuned on the instrument and targeted while performing CBS ESI and compared

to the magnitude of the protonated adduct obtained in the same experiment. It was concluded that the proton adduct was still the most significant (2 times greater) of the two adducts observed, and as such was used as the target parent ion in subsequent analysis.

2.3.3 Sensitivity and sample volume

When performing SPME based extractions one is typically restricted in the volume of sample matrix which can be analyzed, as is the case in bio-fluid analysis. However, as environmental sample matrices are of near endless volume this is not a limitation which is imposed on environmental applications of SPME technologies. With this is mind, it is still important to provide a methodology which limits the volume of sample required so as to prevent extensive or difficult sample handling or transportation. Small volume sample analysis is, of course, advantageous when one considers the high-throughput capability of SPME as a sampling and sample preparative technique. As a result SPME can provide a unique opportunity to its users, allowing the entire analytical workflow to be automated, from sampling to instrumental analysis. For this reason sample volumes used for SPME extractions of environmental matrices should still be kept to a minimum while maintaining an appropriate method sensitivity.

Upon the initiation of the project the experimental sample volume used was that of 1.5 mL as this could allow the easy coupling of the methodology to an already existing 96-well plate system enabling high-throughput analysis. To assess the preliminary limit of detection (LOD) and limit of quantitation (LOQ) of a method employing this sample volume, real river water was analyzed. This sample was verified to be blank by external calibration and was spiked at 5, 10, 20, 50 and 100 ng/L and 0.5, 1, 5 and 20 µg/L, with available internal standards spiked at 3 µg/L. HLB coated blades were placed in the sample solution and agitated using vortex for 2 minutes. Blades

were then subsequently washed in 1.5mL of MS grade water prior to being left to dry for 5 minutes. For this application a standard CBS desorption solution of methanol with 0.1v% of formic acid was used is this study was conducted prior to the desorption solution optimization. Each calibration point was tested using two blades, using each side of the blade to perform instrumental analysis giving n=4 replicates at each calibration point. In order to correct for variation in the electrospray process and so as to calibrate total analyte concentration, response was calculated using response of analyte divided by internal standard response of that same analyte. For the analytes salbutamol and monensin, internal standards were not available so response was simply used on the y-axis as correction with the internal standard of other analytes did not improve relative standard deviation (RSDs).

In general, method performance was good, correlation coefficients (r^2) were observed to be >0.9920 for all compounds over the calibration range of 100 ng/L to 20 µg/L. However, correlation coefficients in the range 5 to 100 ng/L were much worse, with compounds having an average r^2 of 0.8100 with only six compounds (carbamazepine, propranolol, ranitidine, codeine, fluoxetine and erythromycin) demonstrating an r^2>0.9800. Of these compounds, only four were able to achieve the desired LOD (3x blank value, calibrated)[68] of less than 50 ng/L. These compounds were, propranolol (28 ng/L), ranitidine (23 ng/L), fluoxetine (22 ng/L) and erythromycin (5 ng/L). With this it was identifiable that the CBS technology could certainly meet the project goals with further development. In addition, it was clear that at elevated concentrations (>1 µg/L) analyte response was linear and did not pose any real significant challenge in terms of quantitative analysis so the focus was solely on improving linearity in the sub µg/L range, namely 1-1000 ng/L, where the target compounds would be expected in real world samples.

When dealing with SPME extractive procedures, the most accessible parameters by which to improve sensitivity are that of sampling time and sample volume. As mentioned, environmental sample matrices are of near endless sample volume and as a result its effect is worth investigating. In addition, extraction time was increased from 2 to 10 minutes so as to improve method response. Extraction time profiles were able to identify that at a 1.5 mL sample volume at 100 ng/L using a 1.5 cm HLB coated blade, the extractions reached an apparent equilibrium at ~4 minutes for all compounds. However, due to the strong sorbent nature of HLB for the target compounds this may actually be the observation of an exhaustive extraction. This hypothesis was indeed confirmed when extraction time profiles were conducted for the target analytes in 9 mL of sample at 100 ng/L using 1200 rpm, where linear uptake of analyte was observed well past 4 minutes of extraction. As a rapid screening technique was the goal of this project, extraction times of greater than 10 minutes were not investigated. The magnitude of response increase corresponding to an increase in sample volume size at 100 ng/L using a 10 minute extraction at 1200 rpm is apparent in Figure 2.5 where codeine, erythromycin and paroxetine are displayed.

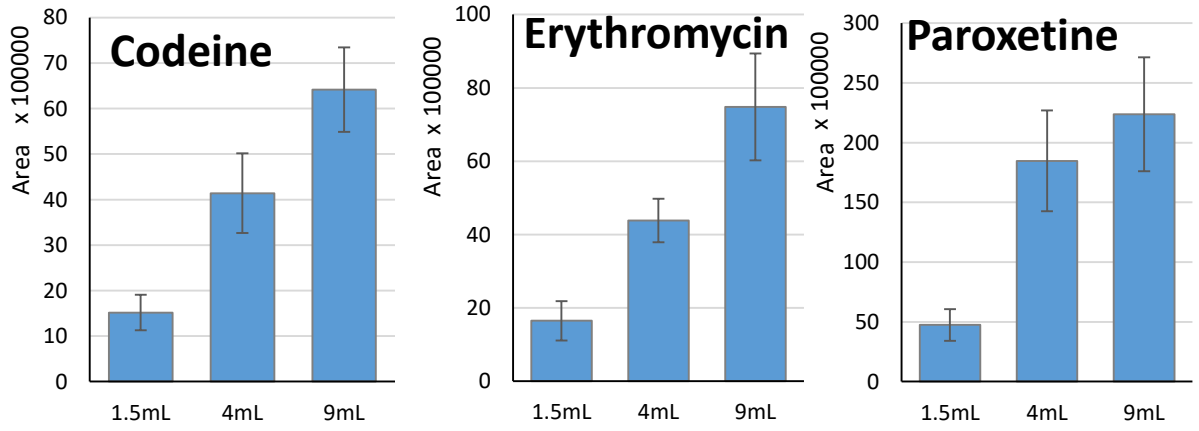


Figure 2.5: Effect of sample volume on overall method sensitivity for the target compounds codeine, erythromycin and paroxetine

As is evident, method response was the greatest when a sample volume of 9 mL was employed, though for some compounds (salbutamol, diazepam, sertraline, methadone, paroxetine and monensin) there was not a significant difference between the response generated between the 4 mL and 9 mL sample sizes. Due to this, the 9 mL sample size was employed in subsequent experiments so as to maximize response for all compounds, though a lower sample size could have been used if only the compounds listed above were of interest.

2.3.4 Effect of sample matrix pH on method sensitivity

When dealing with sample matrices which are as variable as environmental ones, it is important to understand how these variations may affect the sensitivity of a method employed. One such variable is that of sample pH. When considering conventional sample extraction procedures, or more broadly speaking, exhaustive extraction procedures, pH can be a critical parameter so as to limit the occurrence of breakthrough of a sorbent bed. By ensuring that the analyte is predominantly in its non-ionic state, the species will bind more readily to the sorbent. Of course this becomes a bit more complicated when dealing with non-exhaustive sampling procedures as the factors at play encompass not only the neutral fraction of analyte, but the affinity of the compound for the extraction phase and the effect of extraction on the equilibrium between protonated and neutral analyte species in solution. For these reasons the modification of pH and its effect on method response was an important area of investigation so as to identify if a significant boost in sensitivity was achievable by the implementation of a pH standardization procedure.

In order to assess this parameter, calibration plots were formulated in blank river water at pH's of 6, 7, 8, 9 and 10 over a concentration range of 100 ng/L to 2 µg/L. This particular pH range was selected as slightly basic conditions have been identified to provide the best results when extracting pharmaceuticals and related compounds using HLB extraction phases by SPE.[5]

In order to give a graphical representation of the data so as to visualize any trends of response *vs.* pH, the slope of the resultant calibration curves were plotted *vs.* pH so as to effectively give a response per unit concentration value on the y-axis. It is important to note that for this particular experiment, the internal standard used to correct for variation in ESI was added after the extraction had occurred. Of course, if the internal standard was present in the sample matrix during the time of extraction, calibration of the ratio of analyte to internal standard would have given the same slope for all pH's investigated. As the presence of internal standard during extraction would have corrected for the variation in amount extracted at each pH. This was accomplished by performing typical extraction procedure with one modification. After rinsing, and once the blades were dry, 10 µL of a 50 µg/L methanol solution containing the analyte internal standards was spotted onto the surface of the blade. This droplet was then allowed to dry and instrumental analysis was then performed as usual.

Interestingly, and unsurprisingly, the resultant trends were more complicated than simply being related to pKa of the target analytes. This is evident in Figure 2.6 where the trends of response per unit concentration v.s. pH are displayed for fluoxetine (pKa=9.8) and sertraline (pKa=9.9).

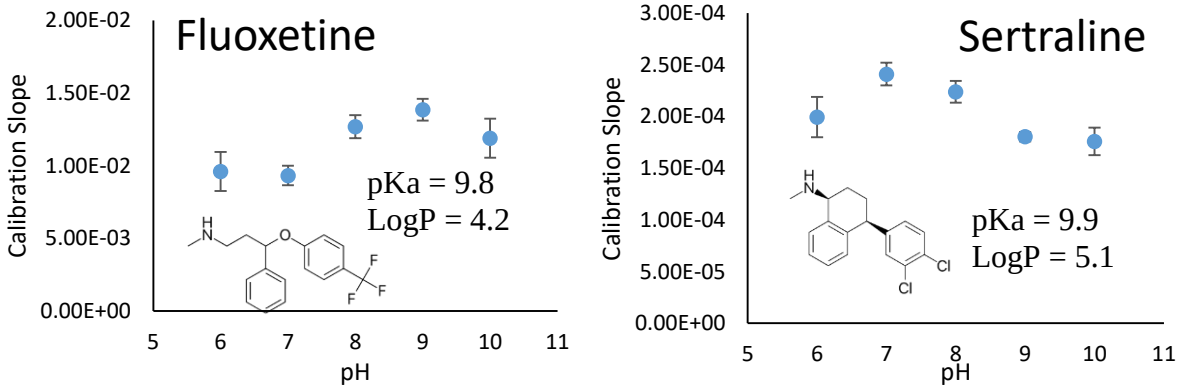


Figure 2.6: Sensitivity as a function of sample matrix pH for the target compounds fluoxetine and sertraline

Even though these two compounds demonstrate almost identical pKa's, they have different maxima of sensitivity at varied pH, with fluoxetine demonstrating its greatest response at a pH of 9, while sertraline demonstrates its greatest sensitivity at a pH of 7. As this analysis was performed in real river water, the cause of these trends could be attributed to a combination of factors. For instance, the affinity of the target analytes may vary with pH, as would the affinity of the analytes for any matrix constituents such as suspended solids or dissolved organic matter. This varied interaction with the sample matrix itself would affect the free concentration of analyte and thereby alter the extraction. With this in mind, however, the main observation of this study was that pH modification did not produce a significant increase in method response for any one analyte. Though the majority of compounds did in fact demonstrate a maximum response at a particular pH (see Appendix #2), the increase was not significant enough so as to justify an additional sample preparation step. As such, an addition to the work flow would reduce the rapid nature of the methodology, and limit its throughput capacity as a screening procedure in the real world. Additionally, as the target compounds are so varied so too are the pH's at which each of them demonstrate their greatest sensitivity. As a result, for the purposes of this study the modification of the sample pH would not provide a global improvement and was therefore not implemented in further work. It should, however, be noted that at low pH (i.e., pH < ~6.5) there is a decrease of sensitivity in almost all cases. As a result the pH of samples should be measured prior to analysis for comprehensiveness, as correction of the pH to a more neutral or slightly basic condition would in fact prove advantageous, though only for the cases in which a pH < 6.5 was observed.

2.3.5 The relationship between extraction phase thickness and method response

When dealing with SPME technologies the extraction phase thickness is of critical importance as this characteristic of the device will be a determining factor in the overall capacity of the sorbent. As an extraction phase is thickened, so too is the capacity of the coating. What is important to recognize, however, is that as thickness increases the time required to desorb the analyte is also increased as the organic solvent used for this purpose must penetrate the coating. For these reasons high affinity coatings of minimal thickness are advantageous for the majority of SPME applications, with these characteristics being of critical importance when seeking a rapid sampling and rapid analysis methodology. Of course, if traditional desorption of a SPME device is employed, where the organic solvent used is then analyzed by liquid injection into either GC or LC instrumentation, the desorption step can be modified. This then ensures complete analyte desorption and hence better sensitivity when thicker coatings are used.

In order to investigate the effect of coating thickness on method response when applying a direct to mass spectrometry approach, CBS devices were tested which had 1 layer, 3 layers and 5 layers of an HLB-PAN coating applied; corresponding to coating thicknesses of 10, 30 and 50 µm respectively. Each of the coating thicknesses was analyzed in triplicate, having each of the 9 blades used loaded with analyte by performing extractions from a 1 µg/L, 9 mL solution at 1200 rpm for 10 minutes. Analysis was then performed using the 95:5, MeOH/H_2O, 12 mM AA, 0.1 v% FA desorption solution (15 µL, 20 s desorption).

What was immediately apparent was the reduction in instrumental response as thicker coatings were used. This observation held true for all target compounds with the exception of carbamazepine and diazepam which maintained a constant response, while the response of monensin increased with coating thickness. This observation speaks to the relative complexity of

SPME extractions when a wide range of compounds are of interest. The hypothesis for the cause of this observation, of some compounds demonstrating equal or greater response as coating thickness is increased, is that of coating capacity. Simply, as more analyte was extracted, more analyte was available to be analyzed. The difficulty comes in explaining why the response dropped for the majority of the compounds tested, and the main hypothesis for this observation in the drop of instrumental response associated with coating thickness can be visualized in Figure 2.7.

Thin coating Thick coating

Figure 2.7: Visual depiction of potential phenomenon leading to reduced response with thicker extraction phases

Essentially, as a constant volume of desorption/ ionization solution was applied, the volume of solvent available to undergo ESI was less when a thick coating was used. The resultant phenomenon would be much like that observed in chromatographic peak broadening resulting from slow mass transfer in thick stationary phases. This was caused by a more significant proportion of the solvent residing within the coating and therefore it could not easily carry analyte to the tip of the CBS device in order to participate in the ESI process. This hypothesis was further corroborated by the observation that when a thick coating was used the ESI would stop prematurely or be more unstable than the ESI generated from a CBS device utilizing a thinner coating. In addition, for the 5 layer CBS devices, a 15 µL desorption volume was not sufficient and a 20 µL desorption volume was required in order to have a fully wetted surface of the device after the 20 s

desorption time had elapsed. The results of this particular study for the target analytes propranolol, codeine, and monensin are given in Figure 2.8.

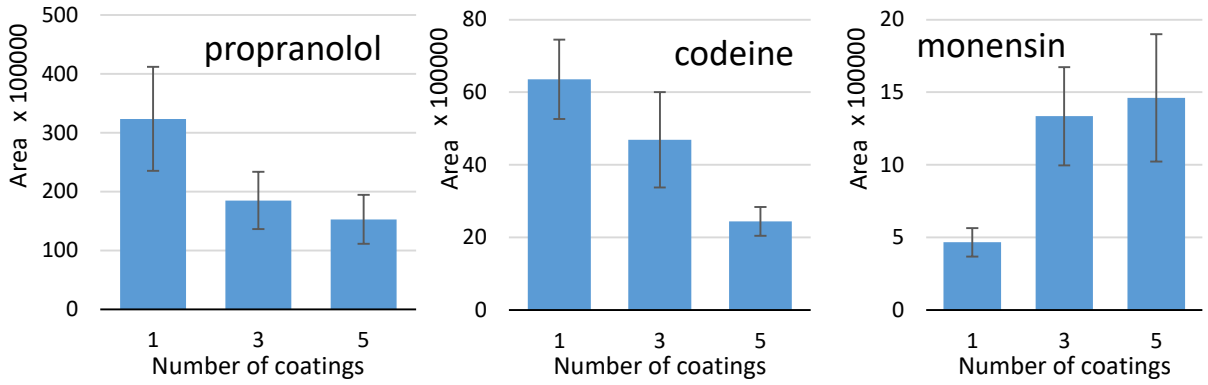


Figure 2.8: Response of the target compoounds prrapnolol,codeine and monensin as a function of extraction phase thickness

This realization, that a thinner coating in fact provides greater sensitivity than a thicker coating for CBS is of great significance. This conclusion essentially identifies the most easily produced device, as well as the device which requires the least amount of polymer coating, to be the optimal device under the conditions tested. This increases the throughput of device manufacture, and reduces consumption of the most costly portion of a CBS device, the extractive coating. Single layer HLB-PAN coatings were employed for all further experimentation.

2.4 General observations and summary of findings

Over the course of the research outlined in this chapter, some general observations were made pertaining to the CBS technology. The most significant of which being that the geometry at the tip of the device is a critical parameter in ensuring that a stable and unidirectional ESI is obtained. In order to accomplish this, after the extractive coating has been applied, a sanding step

is required. This sanding step removes any polymer coating from the tip of the device which would otherwise interfere with the formation of the Taylor cone during ESI. Additionally, this step provides a smooth surface which enables the user to better place the device in front of the MS inlet, and acts as a standardization step conducted for all CBS devices in order to ensure each sprays equivalently, or at least similarly. This tip geometry will also dictate whether a CBS device will spray equivalently from both sides.

Upon the completion of the method investigation steps, a procedure which is well suited for the rapid screening of environmental water or WWTF effluent streams for target pharmaceuticals was identified. It consisted of performing an extraction from 9 mL of sample using a 10 minute extraction time at 1200 rpm. After extraction the CBS device is rinsed with MS grade water in order to remove any attached macromolecules or matrix constituents and allowed to dry prior to instrumental analysis. Once positioned 1cm from the MS inlet, a 15 µL droplet of the desorption/ ionization solution (95:5, methanol/water, 12mM AA, 0.1 v% FA) was placed on the coating and left to rest for 20 seconds. After this desorption time had elapsed, a +4 kV potential difference was applied for 30 seconds initiating ESI and MS/MS separation and detection. Of the parameters investigated and not taken into consideration in this final method is that of the sample matrix pH. As the modification of sample pH was identified to not significantly affect the overall method sensitivity this time consuming sample preparation step was not performed so as to facilitate the rapid screening of real world samples. Additionally, as the calibration is performed by ratio of analyte signal to spiked internal standard signal, the pH will not effect the calibrated value. In order to demonstrate the ability of the method to perform as required, a real river water sample was obtained using a 1 L amber glass bottle and stabilized with 0.2 g/L of sodium azide and 0.05 g/L of ascorbic acid. The sample had a pH of 7.7 and which was verified to be blank by

the use of external calibration, characterization such as total organic carbon determination and sample conductivity were not performed as this was a proof of concept. This sample was then spiked with the target analytes over the concentration range of 1 to 1000 ng/L and LOD and LOQ values were calculated. Resultant LOD and LOQ values for the spiked river water, as well as LOQ values for a calibration curve generated in MS grade water are provided in Table 2.2.

Table 2.2: Resultant LOD and LOQ values for the CBS methodology in real river water to be applied in the WWTF effluent screening study

Compound	MS Water	River water	
	LOQ (ng/L)	LOQ (ng/L)	LOD (ng/L)
Ranitidine	1	32	7
Codeine	7	116	24
Salbutamol	4	36	8
Carbamazepine	4	35	9
Diazepam	13	128	32
Propranolol	6	16	4
Erythromycin	1	4	1
Paroxetine	3	64	16
Methadone	2	8	2
Fluoxetine	19	163	39
Sertraline	79	188	44
Monensin	35	98	22

As shown, in a real river water sample the LOD for all target compounds was below a value of 50 ng/L, having only 4 compounds, codeine, diazepam, fluoxetine and sertraline demonstrate a LOQ larger than 100 ng/L. With this, it can be concluded that the method used is capable of performing the rapid screening of real world samples for target pharmaceuticals in the low ng/L range.

Chapter #3: Targeted analysis of pharmaceuticals in WWTF final effluent

3.1 Introduction

Waste water treatment facilities offer environmental agencies an interesting opportunity when gauging the magnitude of environmental pollution and its sources. Unlike other anthropogenic compounds such as pesticides, which enter the aquatic environment by means of leaching from contaminated soils during rain events or irrigation, the sources of pharmaceuticals are much more concentrated. By definition, WWTF concentrate the waste water generated over a relatively large area and release the treated effluent at a single location, effectively forming a point source of environmental pollution.

Currently, all accredited methods employed for the analysis of pharmaceuticals are very similar in methodology and highly dependent on the sample matrix. These methods typically rely on filtration and subsequent SPE, or LLE to concentrate the target analytes. These concentrated samples then undergo chromatographic separations, either GC or LC, in order to isolate each target analyte from other sample matrix constituents.[69,70] In essence, the accredited methods are time-consuming and environmentally taxing due to the extensive sample preparation often required, long chromatographic analyses, and the use of organic solvent. As a result, these methods cannot be used for rapid screening and quantitation of pharmaceuticals in environmental samples due to the slow turn-around time between sampling and resultant analytical data.

In order to demonstrate the effectiveness of the direct to MS technology CBS towards real world applications, it was applied as a screening procedure to the final effluent of 6 WWTF. In addition to the CBS determination of the target pharmaceuticals in the final effluent samples, a

traditional SPE methodology which has been used in the literature for the analysis of pharmaceuticals in similar sample matrices was used as a comparison. In order to test the agreement of the CBS technology with the conventional SPE technique, CBS was applied to the WWTF samples using two calibration schemes; 1) external calibration and 2) standard addition calibration. The SPE extracts were calibrated using external calibration. In all cases the method response was calibrated as a ratio of analyte response to spiked internal standard so as to correct for matrix effects and provide a total concentration determination, allowing the direct comparison of SPE to SPME technologies.

3.2 Experimental

3.2.1 Sampling

The final effluent of 6 WWTFs were sampled for the CBS/SPE comparison study. Sites #1, #2 and #3 were WWTFs located in urban environments employing a conventional activated sludge treatment process followed by clarification, nitrification and treatment with ultra-violet (UV) light prior to discharge. Site #3 operated using the same processes at sites #1 and #2, however it had an additional sand filtration step prior to UV treatment. Sites #4, #5 and #6 were in rural environments and employed varied treatment processes. Site #4 operated using an activated sludge process followed by chlorination and subsequent de-chlorination, while site #5 employed a sequence batch reactor followed by sand filtration and UV treatment. Finally site #6 is an extended aeration facility with sand filtration followed by ozone disinfection prior to discharge. At each of the WWTFs final effluent was sampled using a 1 L amber glass bottle and stabilized with 0.2 g/L of sodium azide and 0.05 g/L of ascorbic acid. Sample pH's were 7.0, 6.6, 7.2, 6.9, 7.4, and 6.9

respectively. Characterization such as total organic carbon determination and sample conductivity were not performed as this was a proof of concept study designed to compare CBS to SPE. Samples were then stored in a cooler on ice with a tightly sealing lid so as to ensure no light exposure. After sampling, the final effluent grab samples were taken back to the lab where the pH was measured followed by spiking of the standards and internal standards at the concentrations used for the calibration method to be employed. Of the 6 WWTF final effluents sampled, the pH was measured to be greater than 6.5 or less than 7.5 and was not adjusted. Samples were then stored at 5°C overnight followed by extraction and analysis the next day.

3.2.2 WWTF effluent sample analysis method: SPME/ CBS

The CBS devices used for this analysis are those described in section 2.2.2. Sample volumes of 9 mL were used in this study. Extractions were performed for 10 minutes using an agitation rate of 1200 rpm by the use of a bench top mixer. After extraction, blades were rinsed in MS grade water for 5 seconds using vortex agitation and allowed to dry prior to instrumental analysis. A desorption solution of composition 95:5, methanol/water with 12 mM ammonium acetate and 0.1 v% formic acid was used. Once CBS devices were oriented in front of the mass spectrometer, 15 µL of the desorption/ ionization solution was applied to each blade and left to rest for 20 seconds prior to the initiation of a 30 s ESI event by application of a +4 kV potential difference. The MS parameters and target compounds are the same as those mentioned previously and information such as SRM transitions monitored are available in Appendix #1.

3.2.2.1 Standard addition calibration

In order to provide a matrix match calibration for comparison to external calibration methodologies, standard addition calibration (SA) was employed. Calibration curves were formulated in the sample matrices immediately after sampling by spiking the target analytes outlined in the previous chapter at 0, 10, 50, 100, 250, 500, 1000, and 2000 ng/L with internal standard at 100 ng/L in all samples. For the target compounds carbamazepine, ranitidine, and codeine calibration was performed using all standard addition calibration points. For all other target compounds, the range of 0-250 ng/L was employed as two apparent regions of linearity were observed. Salbutamol was calibrated over the range of 0-100 ng/L as this phenomenon was more pronounced as no internal standard correction was performed as this internal standard was not available at the time of experimentation. At each point of the calibration curve analysis was performed in triplicate with the exception of the zero point spiked with internal standard, where 5 replicate extractions were performed. In this case both sides of the CBS devices were analyzed giving n=10 replicates. When performing calibration, the concentration was determined by extrapolation to a y-value associated with the response of a blank rather than a y value of 0 corresponding to the x-intercept of the line of best fit as many compounds demonstrate a relevant non-zero blank response.

3.2.2.2 External calibration

External calibration (EXT) is often favorable as one calibration curve can be used to calibrate multiple samples. For this reason it was employed in this study so as to identify whether it is a viable alternate to matrix match calibration, which is more time consuming to perform and requires a larger total sample volume. Of course, with a sample matrix such as WWTF effluent,

analyte sorption to suspended particulate or interaction with other dissolved substances will affect the free concentration of analyte sampled by SPME devices. To account for this, while performing external calibration it was imperative to have internal standard spiked so as to correct for these sample characteristics and matrix effects and facilitate the determination of total concentration allowing for a comparison to the other methodologies used (matrix match SPME, SPE).

External calibration curves were formulated in MS grade water which used the same extraction and analysis procedure as outlined in the standard addition calibration method. Calibration points were spiked at 1, 5, 10, 50, 100, 500, 1000 and 2200 ng/L with internal standard spiked at 100 ng/L. Calibration curves were formulated by taking the ratio of analyte response to internal standard response and weighed by least squares as the error was observed to be heteroscedastic with concentration. The ratio of signal to internal standard of the zero spike point used in the standard addition curves was then calibrated using this external calibration line of best fit.

3.2.3 WWTF effluent sample analysis method: SPE

The SPE methodology used was applied in triplicate and is that of a common SPE procedure which is documented to have been used for this purpose in the literature.[5] The sample volume utilized in this particular application is 100 mL spiked at 400 ng/L with internal standard. Waters Oasis-HLB, 60 mg, SPE cartridges were first preconditioned using 5 mL of methanol followed by 5 mL of water using vacuum assistance. The 100 mL sample was then passed through the SPE cartridge using vacuum assistance at a controlled draw rate. SPE cartridges were then dried by vacuum and eluted using 5 mL of methanol. This methanol concentrate was then evaporated to dryness under nitrogen flow, followed by reconstitution in 1 mL of a 50:50

methanol/water solution. This reconstituted sample was then analyzed using LC-MS/MS. Response was calibrated by ratio of analyte to internal standard and was compared to a calibration curve generated in spiked organic solvent (50:50 methanol/water) over the range of 10 ng/L to 2.2 µg/L. Specifics such as LC method and MS parameters are available in Appendix #1. Samples were verified to have not degraded due to microbial or other processes by comparison of internal standard response obtained from the WWTF samples spiked after sampling to that of MS grade water which was spiked the day all sample SPE extractions were performed.

3.3 Results and discussion, SPME v.s. SPE

In general, good agreement between the analysis methodologies was observed. Of the target compounds, all were detected by the SPE methodology with the exception of monensin. Though monensin was detected by the use of the CBS methodology, it is not reported here as this was a comparative test and no confirmation could be made that monensin was in fact in the sample. Of the compounds targeted, carbamazepine, codeine and ranitidine were observed to have the highest concentrations of all target compounds at each of the test sites with the exception of site #6. The analysis of site #6 only yielded a positive result for carbamazepine, which was detected at a concentration of 19 ng/L by SPE, and had a value around the LOD of 9 ng/L for the CBS methodology. This result could in part be explained by the fact that Site #6 was the only WWTF sampled which employed ozone disinfection, which may have played a role in pharmaceutical degradation. Of sites #1-5, the next most prevalent and quantifiable of the target pharmaceuticals were propranolol, methadone, salbutamol and erythromycin. While paroxetine, fluoxetine, and sertraline were below the LOQ of the method but detectable by CBS and verified to be present by SPE. A summary of the results can be seen in Table 3.1. For comparative sake, as the error associated with the quantification by a standard addition curve is extrapolated and given as a

confidence interval, the error associated with the CBS external calibration was also converted to a 95% confidence interval by multiplication of the standard deviation by the appropriate two tail, α=0.05, t-value.[71] The error associated with the SPE determination was left as a standard deviation as the value determined by this method was assumed to be correct. Diazepam was not included in the results table as only a very small peak associated with it was found in the SPE extracts, making quantification difficult and unreliable. This low response was corroborated by the observation of above blank response when performing CBS analysis of the WWTF effluent samples, though none were above the method LOD of 33 ng/L with the exception of the standard addition calibration of Site #4, which yielded 42 ng/L, with a +/- 2 ng/L 95% confidence interval (CI). Interestingly, the region of the chromatogram in which diazepam would be detected by LC-MS/MS analysis of the SPE extracts is in a region of high noise, as evident in Figure 3.1.

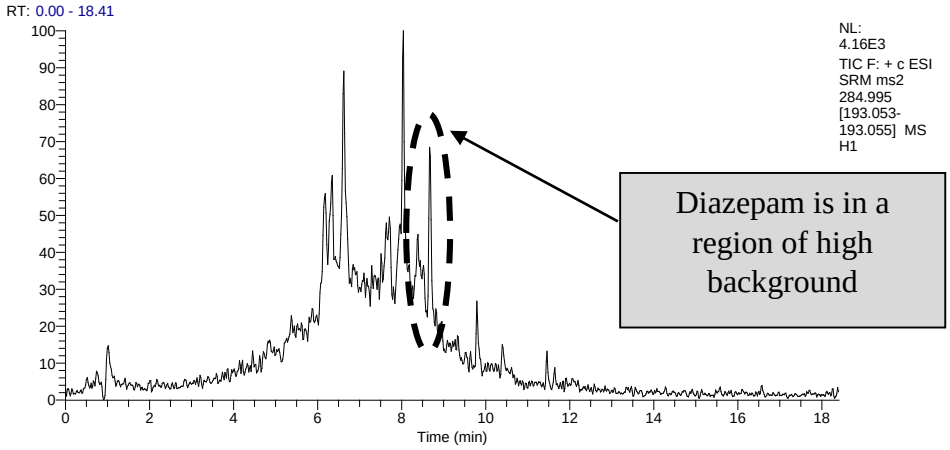


Figure 3.1: Chromatographic background for the SRM transition of m/z 285^+ to 193^+, relating to the MS/MS fragmentation of diazepam

This of course will effect direct to MS determinations of diazepam as well, and is the likely reason why an LOQ of less than 100 ng/L was not attainable for the CBS methodology under these conditions.

One interesting observation was that upon LC separation of the SPE extracts it was noted that there were multiple compounds that achieved chromatographic separation which demonstrated the same SRM transitions used to target propranolol and paroxetine. As CBS is a direct to MS approach, it is unable to differentiate these compounds from one another. As a result, if truly quantitative determination of these compounds is to be conducted using direct to MS approaches additional separation procedures are required such as ion mobility spectrometry (IMS), or additional ion fragmentation (MS^n). For example, Figure 3.2 demonstrates a side by side comparison of a paroxetine standard to the SPE extract of Site #1, while Figure 3.3 demonstrates a side by side comparison of a propranolol standard to the SPE extract of Site #2.

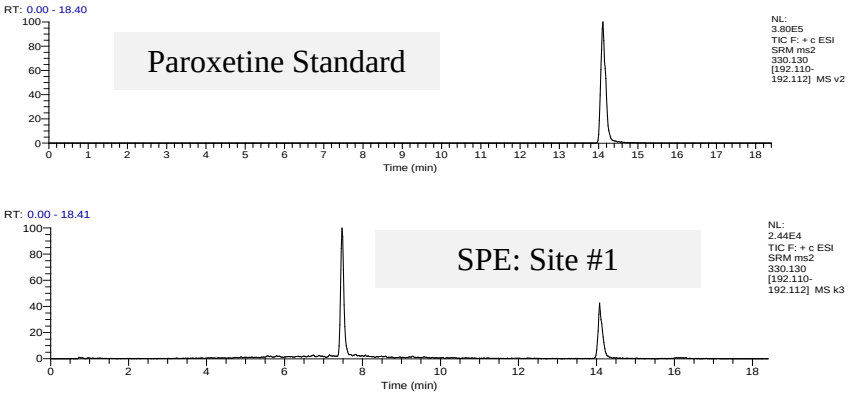


Figure 3.2: Chromatographic background for the SRM transition of m/z 330^+ to 192^+, relating to the MS/MS fragmentation of paroxetine

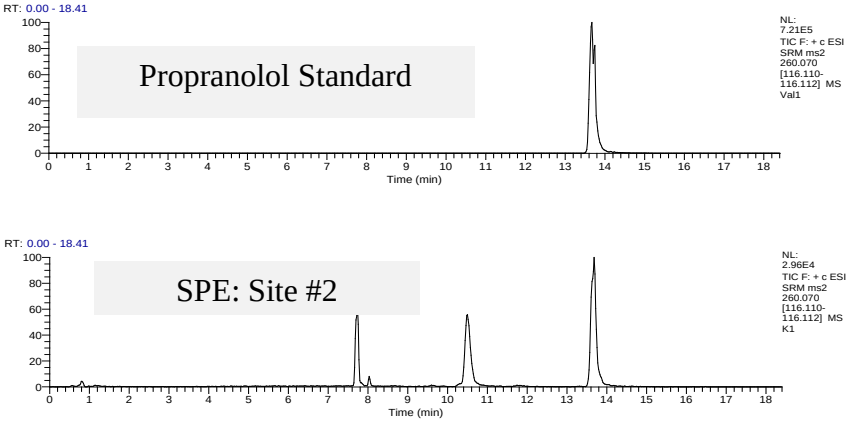


Figure 3.3: Chromatographic background for the SRM transition of m/z 260^+ to 116^+, relating to the MS/MS fragmentation of propranolol

		Ranitidine	Codeine	Carbamazepine	Propranolol	Paroxetine	Methadone	Fluoxetine	Sertraline	Salbutamol	Erythromycin
Site #1	SPE	1063(1)	1769(30)	287(7)	13(1)	15(3)	61(1)	D	57(2)	83(5)	35(5)
	SA	826(156)	1628(223)	370(30)	42(8)	D	27(12)	D	D	43(19)	83(14)
	EXT	939(60)	1318(252)	362(20)	12(5)	D	75(3)	D	D	N/A	20(9)
Site #2	SPE	808(12)	875(136)	235(62)	19(7)	24(3)	14(1)	D	62(1)	57(3)	44(2)
	SA	769(110)	857(184)	276(56)	45(11)	D	17(14)	D	D	55(15)	60(22)
	EXT	757(61)	643(136)	354(13)	21(2)	71(26)	29(3)	D	D	N/A	16(9)
Site #3	SPE	1096(9)	997(2)	509(5)	24(1)	D	109(3)	D	59(3)	103(6)	38(4)
	SA	1159(75)	1062(134)	720(84)	48(8)	D	141(14)	D	D	54(17)	33(17)
	EXT	1028(164)	744(204)	593(92)	23(21)	D	141(37)	D	D	N/A	19(8)
Site #4	SPE	101(1)	1569(30)	573(15)	58(2)	12(2)	45(1)	D	87(2)	103(1)	64(14)
	SA	113(41)	1427(228)	649(161)	141(24)	D	104(31)	D	D	50(16)	100(43)
	EXT	90(19)	993(361)	651(28)	32(18)	D	57(5)	D	D	N/A	19(14)
Site #5	SPE	593(12)	504(2)	350(4)	30(1)	D	21(2)	D	45(1)	61(4)	85(3)
	SA	547(81)	564(92)	416(29)	70(17)	D	62(34)	D	D	35(12)	93(23)
	EXT	678(75)	523(63)	438(30)	23(6)	D	33(3)	ND	D	N/A	52(14)

Table 3.1: SPE vs SPME comparison for the determination of target pharmaceutical at 5 WWTF sites

- +/- error in brackets represents 95% C.I. for CBS standard addition(SA) and CBS external calibration(EXT), for SPE it is standard deviation
- D = >LOD, SPE extract listed as D has peak in sample, but quantified at a value less than the lowest calibration point
- ND = not detected
- As no internal standard was available for salbutamol SPME determinations of this compound, which are not a matrix match calibration, would determine free concentration and would therefore not be comparable.

Upon literature review, a potential candidate for the compound which is demonstrating the same SRM transition as paroxetine is that of reticuline. Both compounds demonstrate the 330 to 192 transition, with literature references even using this transition for the monitoring of reticuline in real samples.[72,73] More strikingly is that reticuline is known to occur in the urine of opium users and those who have used codeine or morphine.[74] As codeine was detected in the WWTF effluent, and even though we did not target reticuline in this study, one would expect to find this compound in such a sample matrix.

In the case of propranolol, no specific compounds have been identified which may account for these particular peaks associated with the SRM transition of m/z 260^+ to 116^+. However, propranolol is a member of a group of β–blocker pharmaceuticals including atenolol, metoprolol, oxpremolol and pindolol, all of which having the ability to produce a m/z = 116^+ daughter ion, as demonstrated in Figure 3.4 due to their shared functionality.

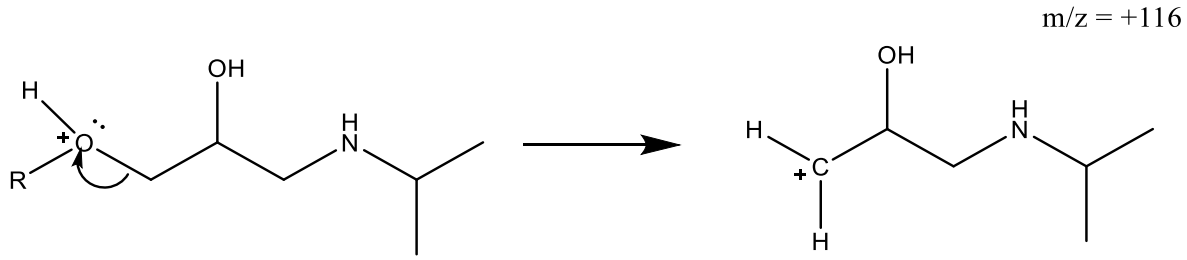


Figure 3.4: Formation of the m/z = 116^+ daughter ion in propranolol like molecules

What could be occurring here is that metabolites or environmental reaction products associated with any of these compounds are able to form a 260^+ ion, enabling it to pass the first quadrupole and form the 116^+ daughter ion. Of course, alternate SRM transitions could be selected which are specific to the differentiating region of these molecules but as this occurrence has not been reported, the m/z = 116^+ was selected as it was the most prevalent daughter ion. With this in

mind, and what is perhaps more likely to be occurring is that the metabolites of propranolol themselves are present in the sample and are undergoing in source fragmentation, generating propranolol and hence the m/z = 260^+ ion. This hypothesis is supported by the knowledge that propranolol is known to have a large number of metabolites, many of which possess attached functionalities such as glucuronide.[75] These metabolites are more polar than propranolol and hence are less retained by the column and elute earlier than propranolol, as observed in the SPE extract separations.[75] This explanation may also hold true for the similar observation made of paroxetine chromatograms if the compound is in fact not reticuline.

3.4 Summary

Coated blade spray can certainly offer a rapid and quantitative methodology for the screening of target pharmaceuticals in surface waters and the final effluent streams of WWTFs. In the application discussed above, the merits of CBS as a technique were underlined by its truly rapid nature and requirement for limited sample preparation compared to the conventional method, SPE, which it was compared to. When residual pharmaceuticals were in the quantifiable range, agreement between the CBS methodologies and the SPE determination was observed. Additionally, where SPE could validate that a target compound be present, CBS was able to detect it given the achievable LOD's for all targets being below the 50 ng/L level. Of course, some limitations of CBS were identified. Perhaps the most significant one being the observation of compounds in the samples which demonstrated the same SRM transitions as those monitored for the compounds targeted. Of course, with the implementation of a more comprehensive screening of SRM transitions this issue may be addressed. Alternatively, with the implementation of ion mobility or MS^n these compounds could be differentiated from one another while still providing a direct to MS procedure for the rapid quantitation of target analytes.[53,76]

Chapter #4: Development of a recessed extraction phase device for the sampling of tissue

4.1 Introduction

When investigating complex sample matrices, direct immersion (DI)-SPME is typically employed as a broad range of analytes are often of interest for both targeted and untargeted studies. Of course, there are a range of difficulties associated with DI-SPME, including the co-extraction of undesirable compounds resulting in matrix effects and/or fouling of the extraction phase causing poor extraction efficiency and reproducibility.[31] Methodologies by which to protect SPME fibers from the chemical complexities of DI samplings include membrane protected SPME where, for example, a hollow cellulose or polypropylene membrane is placed over an SPME fiber in order to limit the extraction of high molecular weight compounds which could interfere with analysis.[77,78] In addition, the application of protective over-coatings such as polydimethylsiloxane (PDMS) on top of polydivinylbenzene (DVB)/PDMS SPME fibers have demonstrated the ability to provide a smooth fiber surface limiting coating fouling and adhesion of matrix constituents onto the fiber resulting in increased fiber lifetime and reproducibility.[31,79] These developments, though significant, do little in the way of preventing mechanical damage to an SPME fiber resulting from the puncture of, or passage through, tough protective tissue. Methods by which to physically protect sorbent coatings from mechanical damage include "in-groove" SPME, where an extraction phase is glued into a depression on a wire as to protect it from smearing onto the inner wall of a protective housing used to puncture vial septa.[80] Alternatively, the SPME-Arrow incorporates an extraction phase which has been enlarged in order to increase method sensitivity and incorporated into a telescoping region which is shielded inside a protective tube during septa puncture.[81,82]

Though both devices may be well suited for high-throughput applications, their size and continued incorporation of a sheathing needle do not prove advantageous for in-vivo samplings where the sampler should be simple to operate and as small as possible in order to limit damage to the target tissue. For these reasons no available device was suitable for the desired application, that of the rapid administration of an SPME device through fish tissue, for the targeted analysis of anthropogenic compounds in fish tissue.

Presented herein is a device which is miniature in nature and able to directly puncture robust sample matrices with no additional supporting hardware. Due to this, the device in question is able to directly puncture fish scales, enabling the sampling of wild fish for a range of purposes including environmental exposure studies. This was accomplished by applying a sorbent into a recession around the circumference of a stainless steel support, as shown in Figure 4.1. The device was designed to have the surface of the coated region possess a diameter less than or equal to that of the solid support in order to protect the leading edges of the coating during puncture into, and withdrawal out of, tissue.

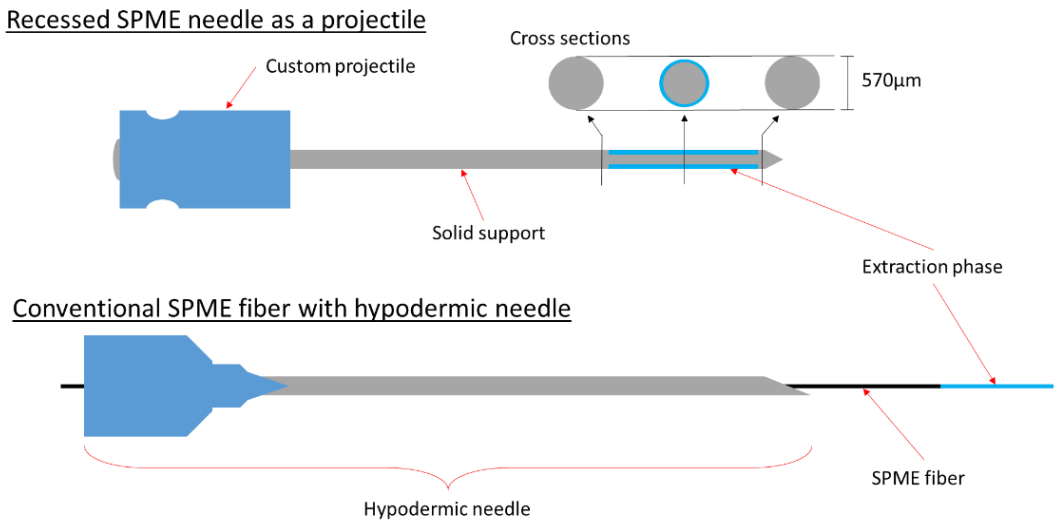


Figure 4.1: Side by side comparison of the recessed SPME needle as a custom projectile to that of the traditional method of insertion of an SPME fiber through protective tissue

This development enables a maximum surface area of sorbent per unit of puncture hole diameter to enter the sample matrix as a larger sheathing needle support is no longer required. This characteristic maximizes extraction phase surface area, and therefore sensitivity. Which is essential for shorter, more timely, extractions thus minimizing the invasiveness of the sampling. For example, even though a traditional nitanol based bio-SPME fiber possesses a relatively small outer diameter of 200 µm, a much larger 720 µm sheathing needle is required to guide said fiber through protective biological tissue such as fish scales.[57,59,60] Hence, without the need of an external protective housing, the SPME needle was enlarged to the size of a traditional 24 gauge (570 µm) hypodermic needle providing more surface area which could translate into much shorter sampling times than the traditional bio-SPME fiber while still reducing the size of the resulting puncture hole.[83]

The device was further applied to the on-site sampling of wild muskellunge in order to demonstrate the rapid sampling ability of the developed device. This particular species of fish was selected for this demonstration due in part to their relatively long life expectancy, identifying this particular species as a prime target given that fish in general have already been identified as susceptible to bioconcentration of select pharmaceuticals.[20,84]

4.2 Experimental

4.2.1 Materials and reagents

The pharmaceuticals paroxetine, diazepam, and codeine were purchased from Cerilliant of Sigma-Aldrich (Roung Rock, TX, USA) while salbutamol, monensin, erythromycin, ranitidine and carbamazepine were purchased from Sigma-Aldrich (Oakville, ON, Canada). The polyunsaturated fatty acids (PUFAs) used, arachidonic acid (ARA), docosahexaenoic acid (DHA)

and eicosapentaenoic acid (EPA) were purchased from Cayman Chemical Company (Ann Arbour, MI, USA). Stainless steel needles to be converted into SPME needles were obtained from a local craft shop, while the 1.5 cm C18-mixed mode SPME fibers used in the comparative studies were kindly provided by Supelco (Bellefonte, PA, USA) and modified to possess a coated region of 1 cm. SPME needles and the mixed mode SPME fibers both had a coated region length of 1 cm. For chromatographic separations and standard preparation the MS grade methanol, acetonitrile, and water were obtained from Fisher Scientific while the salts formic acid and ammonium acetate were purchased from Sigma Aldrich (Oakville, ON, Canada). The dimethylformamide (DMF) used in the over coating procedure was purchased from Sigma Aldrich. Plastic 300 µL vials and amber 2mL glass vials along with pre-pierced PTFE/Silicon septa used in puncture tests were purchased from Canadian Life Sciences (Peterborough, ON, Canada). For sample matrices, whole fish and salmon steaks were purchased from a local grocery store. HLB particles used were obtained from Waters (Wilmslow, UK) while the C18 particles used were obtained from Supelco.

4.2.2 Manufacture of recessed SPME devices

Recessed SPME devices were manufactured so as to possess a 1 cm coated region by protecting all regions of the stainless steel pins used with silicon, leaving exposed only the region which is to be recessed. The protected pins were then placed in 37wt% hydrochloric acid and sonicated at room temperature. The duration of sonication was used to determine the depth of recession formed into the steel pins, and would depend on the thickness of the coating to be applied within this recession. As the coating thickness used for the devices investigated here was that of 10 µm, recessions of 10-15µm were formed using a 10 minute sonication time. Etched pins were then removed from the acid, rinsed in water using sonication 3 times for 5 minutes, followed by a

quick rinse in methanol and dried at 80°C for 5 minutes. HLB-PAN or C18-PAN extractive coatings were then applied using a proprietary coating procedure. After coating, the extraction phase on the tip of the device, which was not recessed, was removed with a razor blade.

4.2.3 Analysis of Pharmaceuticals, device validation

Prior to use, all recessed SPME needles and mixed mode fibers were pre-conditioned in 1.5 mL of 50:50 v/v water-methanol for 30 minutes at 1500 rpm. Extractions for the comparison of SPME needles which were and were not punctured through septa (5 of each) in order to access the sample solution were performed from 1.6 mL of MS grade water spiked with sertraline, fluoxetine, paroxetine, diazepam, salbutamol, ranitidine and codeine at a concentration of 15 µg/L. Extractions were performed for 2 minutes using vortex agitation. Once completed, the SPME needles were removed from the sample solution, washed for 5 seconds in MS grade water under vortex agitation and desorbed into 300 µL of a methanolic solution at 1500 rpm for 10 minutes. No carry-over of analyte on the SPME devices was verified by performing a second desorption. For the intra needle reproducibility study, all recessed SPME needles and mixed mode fibers were pre-conditioned in a 50:50 water-methanol solution for 30 minutes at 1500 rpm. Extractions were subsequently performed by pushing the needle through the septa in order to access the sample solution which was 1.6 mL of pH = 7.4, 1 M phosphate buffer solution made with MS grade water spiked with sertraline, fluoxetine, paroxetine, diazepam, salbutamol, ranitidine and codeine at a concentration of 30 ng/mL. Extractions were performed for 2 minutes using vortex agitation. Once completed, the SPME needles were removed from the sample solution, washed for 5 seconds in MS grade water under vortex agitation and desorbed into 1.5 mL of MS grade methanol (Fisher)

at 1500 rpm for 10 minutes. No carry-over of analyte on the SPME devices was verified by performing a second desorption.

4.2.4 Analysis of fatty acids in salmon, an in lab proof of concept

Fresh, refrigerated salmon steaks were purchased from a local grocery store and left to reach room temperature for one hour prior to extraction. HLB-PAN, C18-PAN SPME needles, and mixed mode SPME (C18 with strong cation exchanger) fibers were conditioned as described above prior to being inserted into the salmon steak and left for one hour, having three of each directly inserted into the muscle tissue. In order to take full advantage of the robust SPME needle, an additional three HLB-PAN SPME needles were pushed through the protective flesh into the underlying tissue for one hour. After extraction, the devices were washed 2 times for 5 seconds in MS grade water using vortex agitation and desorbed in 100% ACN for one hour at 1500 rpm.[85] No carry-over of analyte on the SPME devices and mixed mode SPME fibers was verified by performing a second desorption. Newly fabricated and never used SPME needles were used for each extraction unless otherwise specified. Quality control injections were used to correct experimental data to the day of instrument calibration.

4.2.5 On-site sampling of angled muskellunge

Wild muskellunge were sampled from two locations in southern Ontario in an effort to obtain data which would be able to differentiate the two populations from one another based on the presence or absence of contaminants in the sampled specimens. Sampling was performed in accordance with the proper animal care protocols (AUPP# 14-06). The SPME needles used in this application possessed a HLB-PAN coating, and were stored in 50:50 methanol/water prior to use. Sampling at the Georgian Bay location was performed by pushing the devices into each of the fish

(n=2, 4 needles per fish), while at the Ottawa River site SPME needles were administered as a projectile (n=2, 5 needles per fish) so as to expedite the sampling process. The extractions were performed mid-way along the back of each fish, and extractions were performed for 10 minutes. Once complete, the devices were removed and rinsed in 1.5 mL of MS grade water and subsequently stored in 300µL glass vials on ice. Upon returning to the lab, samples were stored at -80°C until the time of desorption. SPME needles were desorbed in 250 µL 80:20 methanol/water for 3 hours at 1200 rpm.

4.2.6 Instrumental analysis

4.2.6.1 Instrumental method #1: Pharmaceuticals

Samples were run on a TSQ-Vantage coupled to an Accela Auto sampler and LC pump (Thermo Scientific, San Jose, USA). Separations were performed on an Ascentis Express F5 PFP HPLC column (2.1mm x 100 mm, 2.7µm). A ternary mobile phase system was employed. Mobile phase A B and C were 100% water, acetonitrile and methanol respectively. All mobile phases contained 0.1 v% formic acid. Specifics such as solvent gradient, ionization conditions and SRM transitions can be seen in Table 4.1.

Table 4.1: Chromatographic solvent gradient along with positive H-ESI ionization conditions with SRM transitions for the analysis of select pharmaceuticals[86]

Mobile phase gradient				
Time (min)	Percent composition			Flow (µL/min)
	A	B	C	
0	90	5	5	300
0.5	90	5	5	300
7	0	50	50	300
12	0	25	75	300
15.3	0	25	75	300
15.5	90	5	5	300
17.5	90	5	5	300

Mass spectrometer conditions
Spray voltage: 1300V Vaporizer temperature: 275°C Sheath gas: 30 Auxiliary gas: 30 Sweep gas: 2 Capillary temperature: 275°C

SRM parameters by compound				
Compound	**Parent (m/z)**	**Daughter (m/z)**	**Collision energy (eV)**	**S-Lens**
Diazepam	285.056	193.079	30	115
Ranitidine	315.128	176.024	16	92
Codeine	300.134	152.059	60	120
Paroxetine	330.129	192.097	19	133
Fluoxetine	310.121	44.157	13	76
Sertraline	306.057	158.955	29	75
Salbutamol	240.147	148.071	16	79

4.2.6.2 Instrumental method #2:PUFAs in Salmon

Samples were run on a TSQ-Vantage coupled to an Accela Auto sampler and LC pump (Thermo Scientific, San Jose, USA). Separations were performed on an X-Bridge C18 HPLC column (2.1mm x 150 mm, 2.5µm). A binary mobile phase system was employed. Mobile phase A consisted of 90% water and 10% methanol while mobile phase B was composed of 80% methanol and 20% acetonitrile. Both Mobile phase A and B were modified to contain 5 mM ammonium acetate. Specifics such as solvent gradient, ionization conditions and SRM transitions can be seen in Table 4.2.

Table 4.2: Chromatographic solvent gradient along with negative H-ESI ionization conditions with SRM transitions for the targeted analysis of select PUFAs[85]

Mobile phase gradient			
Time (min)	Percent composition		Flow (µL/min)
	A	B	
0	40	60	300
1	40	60	300
2	5	95	300
5	0	100	300
6	0	100	300
6.5	40	60	300
8	40	60	300

Mass spectrometer conditions
Spray voltage: -2600V Vaporizer temperature: 275°C Sheath gas: 30 Auxiliary gas: 30 Sweep gas: 2 Capillary temperature: 275°C

SRM parameters by compound				
Compound	**Parent (m/z)**	**Daughter (m/z)**	**Collision energy (eV)**	**S-Lens**
Arachidonic acid	303.431	260.610	17	132
Docosahexaenoic acid	327.098	284.672	16	134
Eicosapentaenoic acid	301.103	263.631	16	133

4.2.6.3 Instrumental method #2: HRMS analysis of muskellunge extracts

Samples were run on an Exactive coupled to an Accela Auto sampler and LC pump (Thermo Scientific, San Jose, USA). Separations were performed on a Discovery HS F5-3 column (2.1mm x 100 mm, 0µm). A binary mobile phase system was employed. Mobile phase A consisted of water while mobile phase B was composed of acetonitrile. Both Mobile phase A and B were modified to contain 0.1 v% formic acid. Specifics such as solvent gradient, ionization conditions and MS conditions can be seen in Table 4.3. Instrumental response was verified to be stable by the repetitive injection of a Pooled QC sample over the course of the experimental run.

Table 4.3: Chromatographic solvent gradient along with positive H-ESI ionization and HRMS conditions for the untargeted analysis of muskellunge extracts

Mobile phase gradient			
Time (min)	Percent composition		Flow (µL/min)
	A	B	
0	100	0	300
3	100	0	300
25	10	90	300
34	10	90	300
35	100	0	300
40	100	0	300

Mass spectrometer conditions
Spray voltage: +3900V Vaporizer temperature: 275°C Sheath gas: 35 Auxiliary gas: 5 Sweep gas: 0 Capillary temperature: 275°C

HRMS parameters				
Scan range (m/z)	**Lock mass (m/z)**	**Inj. Time (ms)**	**Resolution**	
100-1000	391.2849	100	50 000	

4.2.6.4 SEM and EDX characterization

Scanning electron microscope (SEM) images and energy-dispersive x-ray (EDX) spectra were obtained using a Merlin SEM and an Apollo XL-SDD detector respectively operated by WATLab at the University of Waterloo, samples were deposited onto adhesive carbon tape and coated in gold prior to analysis.

4.3 Results and Discussion

4.3.1 Demonstration of rigidity of the recessed device

Preliminary experimentation with the recessed SPME needles employed HLB particles suspended in a PAN glue as extractive coatings due to the wide variety of compounds which demonstrate affinity for HLB.[86] In these experiments SPME needles were used to puncture fish scales, cardboard, and plastic. For these tests, in order to fully test the robustness of the SPME needle as well as to facilitate rapid, one-handed sampler introduction, SPME needles were

incorporated into custom projectiles which were fired from an unmodified airsoft gun. The firing speed was approximately 90 m/s to simulate aggressive handling and demonstrate the ability of the technology to be used as a rapid sampling tool for in-vivo applications. It was quite evident that needles which did not incorporate a recession along the coated region were more susceptible to damage upon impact and puncture, as demonstrated in Figure 4.2.

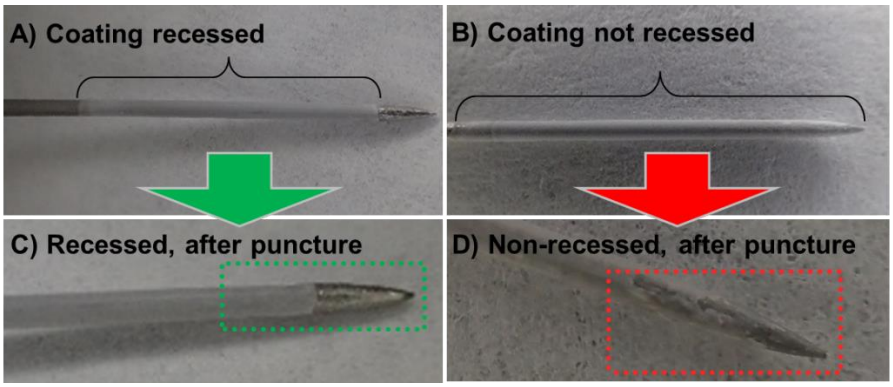


Figure 4.2: Comparison of recessed and non-recessed coating damage resulting from the rapid, gun-assisted, puncture of fish scales. A) recessed coating before puncture, B) non-recessed coating before puncture, C) recessed coating after puncture, D) non-recessed coating after puncture

Upon the determination that the SPME needle did in-fact provide a mechanically robust sampling device, the process of puncturing was further validated to not affect extraction of target analytes. To assess this parameter, two sets of five SPME needles were used to extract seven pharmaceuticals (sertraline, fluoxetine, paroxetine, diazepam, salbutamol, ranitidine, and codeine) at 15 ng/mL in water with one set first being forced through septa in order to access the sample. As shown in Figure 4.3 method response for the targeted analytes was generally comparable based on visual magnitude for both sets of SPME needles studied. Interestingly statistical analysis by

ANOVA ($\alpha = 0.95$) was inconclusive, with only paroxetine ($p = 0.45$), diazepam ($p = 0.10$), and salbutamol ($p = 0.38$) demonstrating p values greater than 0.05, identifying method response of both treatments to be statistically similar. This observation hints towards some disagreement, which is addressed and explained by Figure 4.6 and the subsequent discussion.

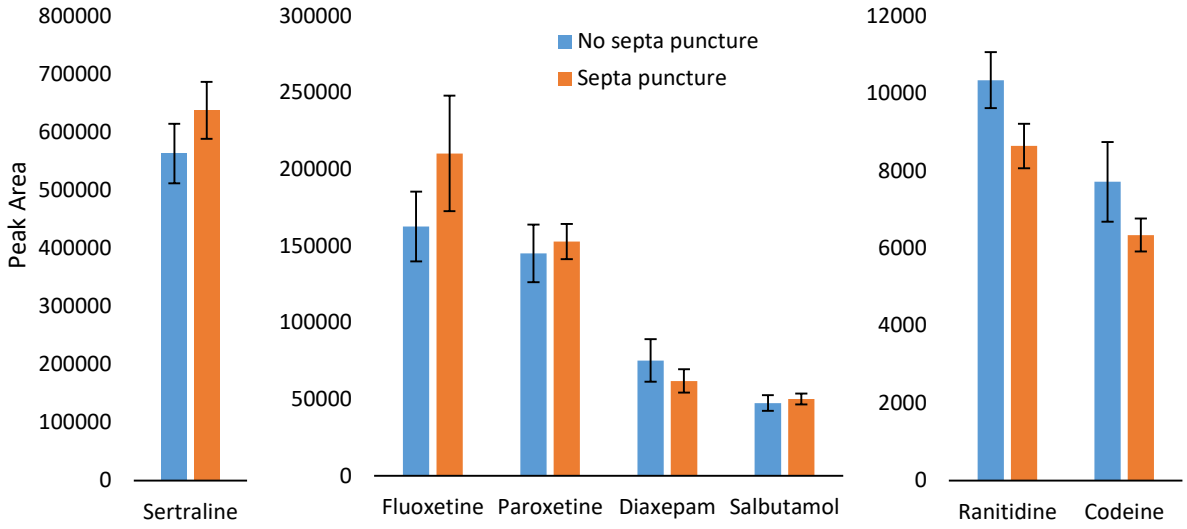


Figure 4.3: Comparison of HLB-PAN - SPME needles with and without septa puncture in order to access aqueous solution of pharmaceuticals with no internal standard correction

To further determine the effect of septa puncture of a pre-slit septa on the extraction of analyte an inter-SPME needle reproducibility study was conducted. This experiment employed 5 SPME needles which were pushed through the protective septa of the sample vials in order to access the sample solution. With no internal standard correction, no observed RSD was greater than 14%. An example of the resultant data for the target compounds ranitidine and codeine are given in Figure 4.4.

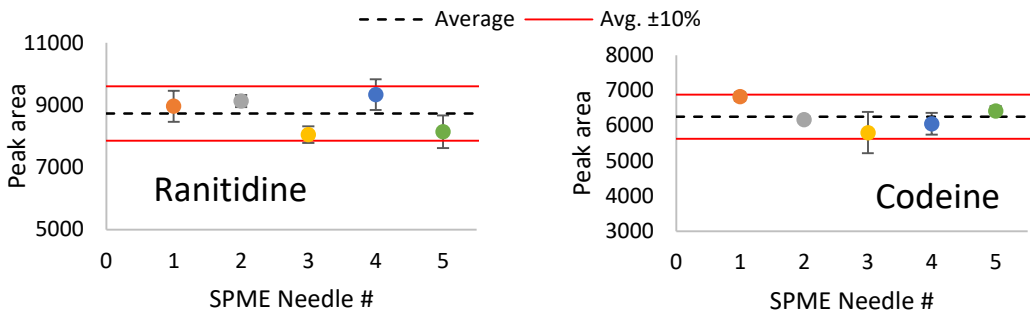


Figure 4.4: Inter device reproducibility of HLB-PAN recessed SPME needles for the targeted analytes ranitidine and codeine

In addition, intra-SPME needle reproducibility was observed over 15 pre-slit septa punctures giving a % RSD <13% for all compounds tested using no internal standard correction. The exception to this observation was that of salbutamol, which demonstrated an intra-needle RSD of 21%, though this observation was in part explained by the results of the following experiment. An additional observation of this particular intra needle study being that over the course of 15 septa puncture events, no physical damage was observed to occur to the extraction phase.

After the initial 15 septa punctures, the SPME needles were then pushed through non-pre-pierced septa in order to determine whether this, more aggressive treatment, would affect the extraction of the target analytes or induce mechanical damage to the coating. To accomplish this, the same experimental procedure was followed as with the initial 15 puncture events for a total of an additional 35 septa punctures. After the conclusion of these 50 total septa punctures, an additional 3 extraction, wash, and desorption steps were performed using pre-slit septa in order to see if the SPME devices could be regenerated in the event a decrease in response was observed.

After the conclusion of this study, only slight mechanical damage was noted to have occurred to the extractive coatings, as shown in Appendix #3. It should be noted however that this damage occurred after 25 puncture events, well past the number of puncture events expected in

any future application. Interestingly, though, was the observation that there was a drop in method response over the course of the study. This reduction was compound dependent and appeared to be related to the polarity, or logP, of the target compounds. Of the target compounds salbutamol (logP=1.4), codeine (logP=1.2), and ranitidine (logP=0.8) were observed to have a reduced method response after puncture through the non-pre-pierced vial septa. While, the target compounds diazepam (logP=2.8), sertraline (logP=5.2), fluoxetine (logP=4.2), and paroxetine (logP=3.2) were observed to maintain a constant response over all 50 puncture events. This phenomenon is presented in Figure 4.5, where the traces for diazepam and carbamazepine are provided. Also of note, the 51st, 52nd, and 53rd samplings through pre-pierced septa were not able to recover the coating, leading to the conclusion that alteration to the coating was irreversible under the experimental conditions used.

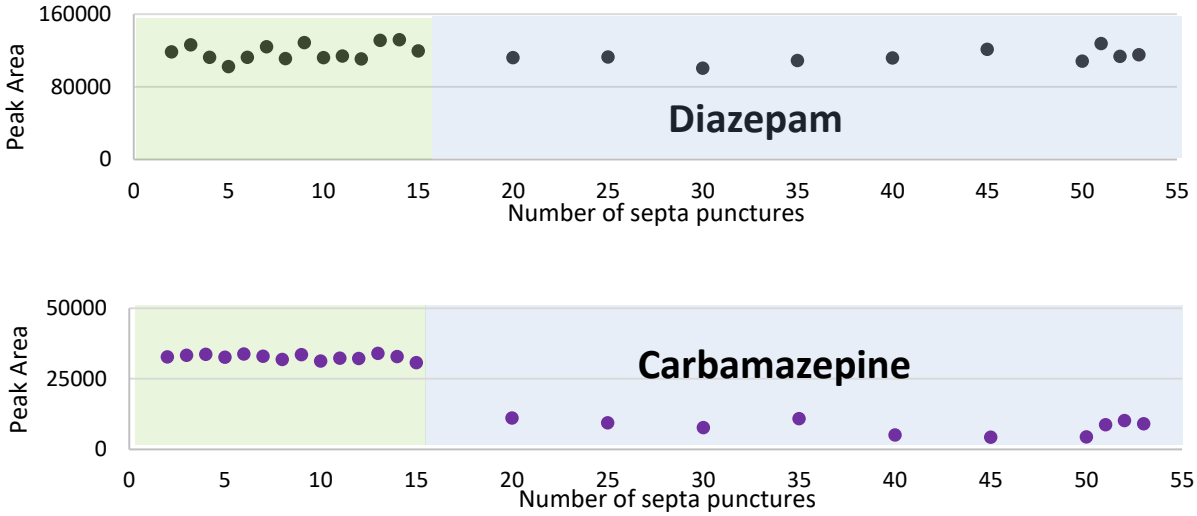


Figure 4.5: Inter needle reproducibility for the target analytes diazepam and carbamazepine over 15 punctured through pre-pierced septa followed by 35 puncturess througha non-pre-pierced septa in order to access an aqueous sample solution

Mechanical damage was quickly ruled out as the cause for this observation, as if this were the case there would have been a reduction in response for all of the target compounds, rather than

a select few with similar characteristics (logP). As a result, the cause of this reduction was likely the result of the chemical modification of the coating itself, as this would allow for the observation that compounds with different chemical characteristics would behave differently. In order to gain a better understanding of this, SEM images were taken of the surface of the extraction phase for a new SPME needle, and one which had been through the 53 septa puncture study. What was immediately apparent was the modification of the surface of the extraction phase by the deposition of some substance. It was clear that the coating itself was not being removed, as the HLB particles were still observable, but rather covered. In order to identify the chemical composition of the substance EDX was performed of the coating surface of each device. Unsurprisingly, the EDX revealed the substance which had been deposited was composed primarily of silicon and oxygen. This of course is the composition of the siloxane septa used, and as such, the conclusion was drawn that the septa itself was smearing onto the surface of the extraction phase as it passed through the vial septa. Figure 4.6 provides SEM images and EDX spectra of the devices tested.

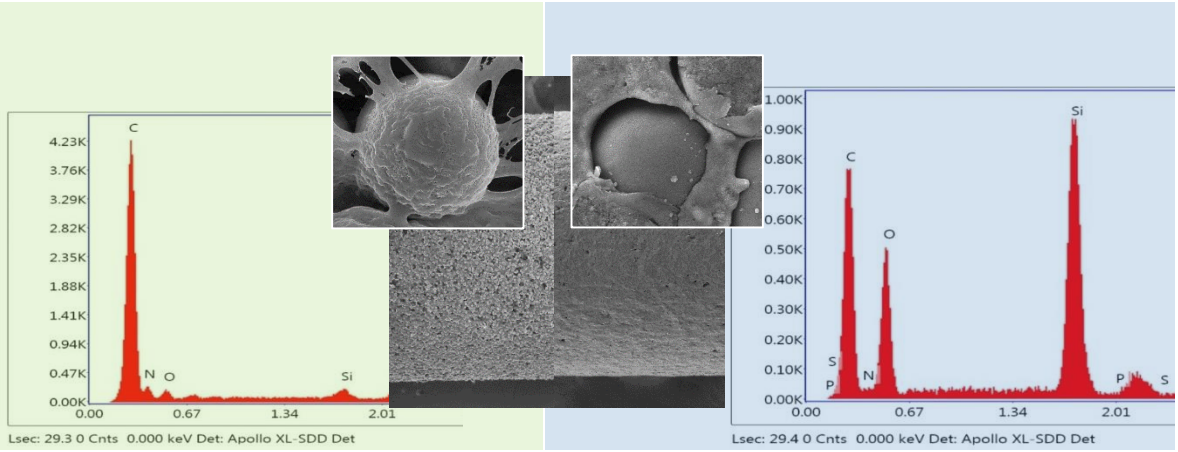


Figure 4.6: SEM images and EDX spectra of SPME needles used in the septa puncture study

Interestingly, the presence of silicon is noted in the EDX of the needle which was new and pushed through a pre-pierced septa for storage. This observation leads to the conclusion that there

is deposition of silicon onto the coating surface even when pre-pierced specta are used. However, this deposition occurs to a much lesser extent than non pre-pierced septa, and does not appear to greatly affect analyte extration. What this determination does not clearly explain is why, when non pre-pierced septa were used, the extraction of some of the target compounds was not affected. This is an interesting observation, as one can clearly see the alteration to the extraction phase is quite significant. The initial conclusion to be drawn is that perhaps the extraction of compounds by the sorbent is occurring by two phenomenon. In the case of highly polar compounds, such as those where a drop in response was observed, the sorptive process is dominated by the interaction of the polar functionalities with the HLB extraction phase. In this case, as these polar functionalities of the HLB are now covered with a chemically different siloxane, reducing extraction. However with less polar compounds the extraction of analyte is more related to non-polar interaction, and as a result, the siloxane deposited onto the coating by passage through the septa is participating in the extraction of analyte.

As high-throughput applications requiring septa puncture events were not the targeted application of the recessed SPME device, but rather a demonstration that the device was mechanically robust and able to protect the extractive coating from chipping, peeling or scratching, no steps were taken to prevent the attachment of the silicon onto the coating surface. If this type of application does become an area of interest for such a device, the application of a smooth over-coating may alleviate this attachment. However, for high-throughput applications requiring the puncture of vial septa, technologies such as the SPME-Arrow would likely be advantageous. In this case the sorbent would never come in contact with the vial septa. As a result, the smearing of siloxane, or other media, onto the extraction phase would not need to be considered as the extraction phase is protected by a sheathing needle.

4.3.2 Proof of concept, targeted analysis of PUFAs in salmon tissue

Upon the conclusion of the above proof of concept experiments, the recessed SPME needle was identified as suitable for the intended purpose of in-vivo sampling. Due in part to its miniature size, robust nature, and requirement of no protective housing or sheathing needle. To further demonstrate this, the device was applied to the targeted analysis of the poly unsaturated fatty acids (PUFAs) docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (ARA) in salmon. In order to provide a comparison of the SPME needle to a conventional SPME device, HLB-PAN and C18-PAN coated SPME needles were compared to mixed mode (MM) SPME fibers. Initial studies were able to demonstrate that the HLB sorbent coated needles extracted 3 to 4 times more PUFAs than C18 coated SPME needles and MM SPME fibers (n=3, Figure 4.7).

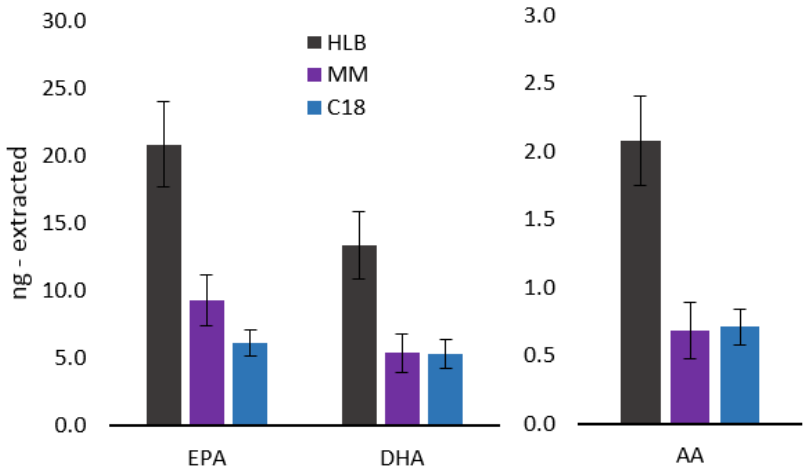


Figure 4.7: Comparison of HLB, C18 SPME needles and mixed mode SPME fibers for the direct analysis of salmon by the direct puncture of fish muscle tissue for the targeted analysis of poly-unsaturated fatty acids.

In addition, the process of forcing the HLB coated needles through the protective outer skin was validated to not affect PUFA extraction (Figure 4.8) by comparing SPME needles (n = 3)

which had been forced through the protective tissue to those which had been pushed into muscle tissue directly (ANOVA, $\alpha = 0.95$, EPA $p = 0.68$, DHA $p = 0.56$). Interestingly, the SPME needles which punctured the skin appear to extract a somewhat greater amount of arachidonic acid than those which were pushed into the muscle tissue directly (n = 3). This difference was identified to be significant (ANOVA, $\alpha = 0.95$, $p = 0.0015$), and one potential hypothesis for this observation is that the levels of arachidonic acid may be greater closer to the surface of the tissue, or rather, closer to the scales. In any case, it is quite clear that the SPME needle can provide an easy to use and comparably reproducible method to traditional SPME fibers for the analysis of whole, unmodified tissues and hence in-vivo applications.

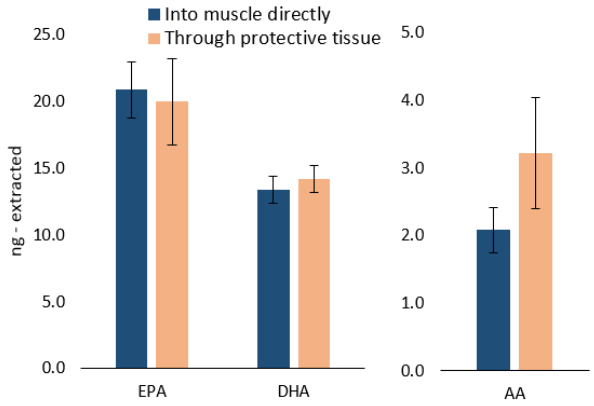


Figure 4.8: Comparison of HLB SPME needles which had and had not been pushed through the protective flesh of salmon in order to sample the underlying tissue for the PUFAs eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (AA).

So as to identify the true robustness of the device, new SPME needles were used to perform repetitive extractions from salmon by directly puncturing through the protective tissue. The experiment utilized five separate SPME needles which were each used for nine successive samplings through the protective skin of a freshly purchased section of salmon. Over the course of this study no mechanical damage was observed to occur and this was verified by microscope

images taken after each extraction. What was observed however was the general darkening of the extraction phase over the course of the experiment. In addition, blemished or severely darkened regions were observed to occur on the surface of the extraction phase, as evident in Figure 4.9.

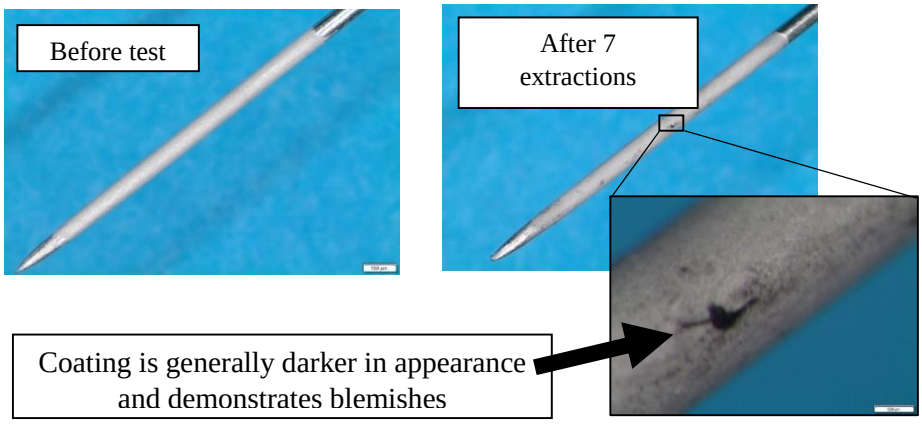


Figure 4.9: Visual comparison of newly made SPME needle compared to the same needle after 7 extractions from salmon tissue were performed.

Upon analysis, this progressive darkening was noted to occur in conjunction with a gradual decrease in the amount of the target PUFAs extracted (Figure 4.10). As no mechanical damage to the coating was noted, as with the septa puncture event, the initial hypothesis for the cause of this observation was that of coating surface alteration. More specifically, the attachment of matrix constituent biomolecules causing bio-fouling. In order to determine if bio-fouling of the extraction phase had occurred, SEM images and EDX spectra of the devices used in this study were obtained.

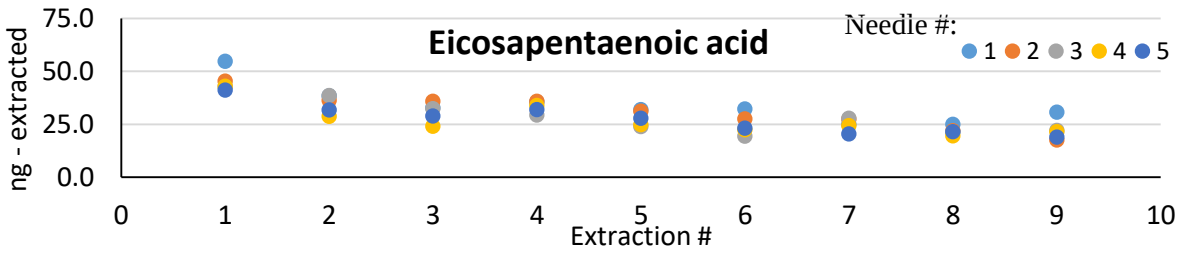


Figure 4.10: Intra SPME needle reproducibility and robustness test for the targeted and repetitive analysis of poly unsaturated fatty acids in salmon tissue.

Immediately apparent when viewing the coatings under SEM was the gradual deposition of a substance onto the surface of the extraction phase, as evident in Figure 4.11. Upon analysis of the EDX spectra of the coating surface, it was clear that the abundance of oxygen was increasing over successive exposures to the biological sample. This agrees with literature reference where the amount of oxygen is observed to increase on the surface of SPME coatings as a result of a bio-fouling process, where bio-molecules possessing oxygen contained in the sample are deposited onto the surface of the extraction phase.[87]

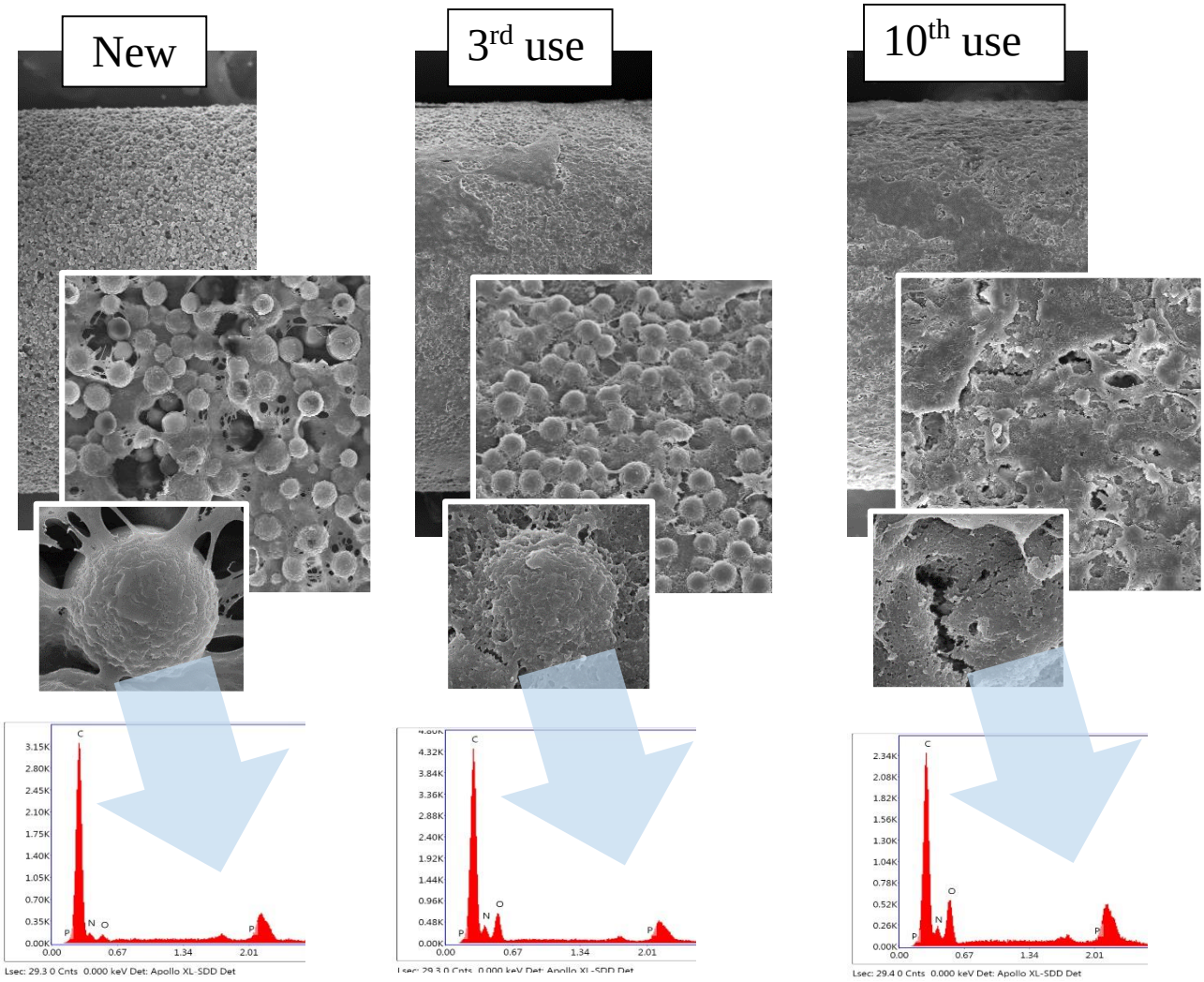


Figure 4.11: SEM images and EDX spectra of a newly prepared HLB-PAN coating compared to devices which were used for the repetative extraction from salmon tissue 3 and 10 times.

In order to verify that this bio-fouling was associated with the surface of the coating and was not altering the bulk chemistry of the extraction phase further testing was required. To accomplish this, the extraction phase of an SPME needle which had been used for 10 extractions in salmon tissue was stripped off of the solid support using a razor blade. EDX spectra were then obtained for the surface of this coating and for the region of the coating which would have been in contact with the solid support, and would not have had direct exposure to the biological sample matrix. The results of this are presented in Figure 4.12, and clearly show that the underside of the coating does not demonstrate elevated oxygen levels, and is rather similar to a never used coating. With this, the conclusion could be drawn that yes, bio-fouling was occurring as a result of the deposition of matrix constituents containing oxygen onto the surface of the extraction phase.

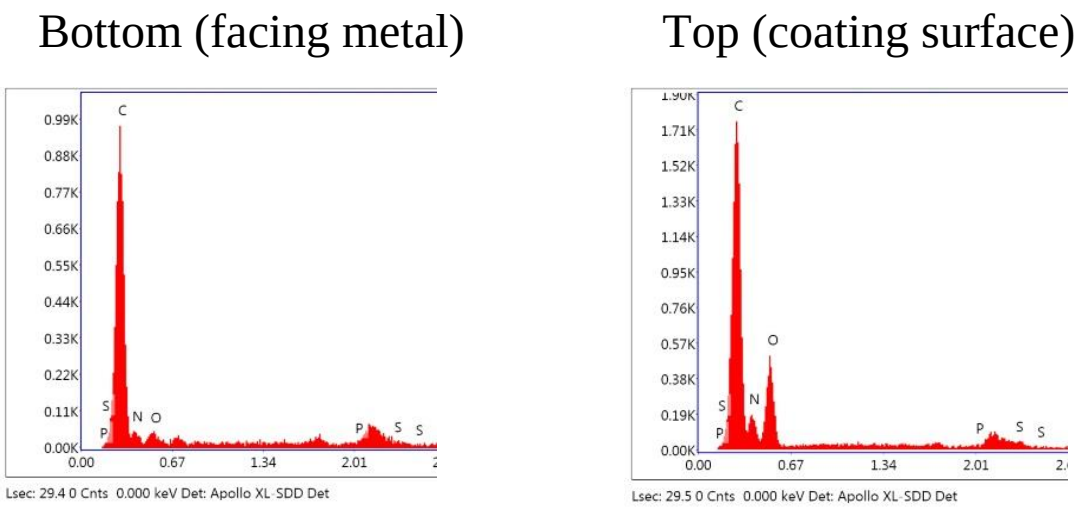


Figure 4.12: EDX spectra of top and bottom of bio-fouled SPME coating demonstrating the attachment of bio-molecules to the coating surface

4.3.3 Over-coating procedure development

In an effort to limit the bio-fouling of the presented device, it was important to investigate a methodology which would alleviate such a process from occurring. One such application which

has been used to great success in food analysis, as well as bio-analysis, is that of the application of a smooth over-coating.[31,88] For the purposes of this particular application, poly-acrylonitrile was chosen as the desired over-coating chemistry to be employed as it has already incorporated into the extractive coating as the glue which binds the HLB extraction phase into place, and is known to be a biocompatible polymer.[87] The general process used to apply the HLB-PAN over-coating was as follows: this device would have the extractive coating applied as usual, and then have an appropriate over-coating procedure applied to the device.

The solution used to perform the over-coating procedure was a 10 wt% solution of PAN in the solvent DMF. This solution was brought to 90 °C for 60 minutes, providing a homogeneous solution of PAN/DMF. A dip coating procedure was employed to apply the PAN over-coating, the thickness of which was controlled by variation of the withdrawal speed from the over-coating solution mixture (due to viscosity, fast withdrawal results in more deposited material). Upon withdrawal from the solution the device was immediately cured at 120 °C for 1 minute so as to evaporate the DMF and polymerize the PAN. The application of multiple layers of the PAN over-coating was also investigated to determine whether this could perhaps provide a more uniform surface. Table 4.4 outlines a selection of the procedures tested as a part of this study, along with corresponding total coating thickness while Figure 4.13 shows SEM images of the over-coated surfaces.

Table 4.4: Over-coating application parameters and resultant overall coating thicknesses

Over coating Process	Metal (µm)	Metal + Coating (µm)	Metal + Coating in recess region (µm)	Total coating thickness (µm)
No OC	594	605	570	6
1x 0.5mm/s	592	603	564	6
3x 0.5mm/s	593	604	576	6
5x 0.5mm/s	593	606	580	7
1x 1mm/s	597	606	579	6
3x 0.1mm/s	586	598	574	6

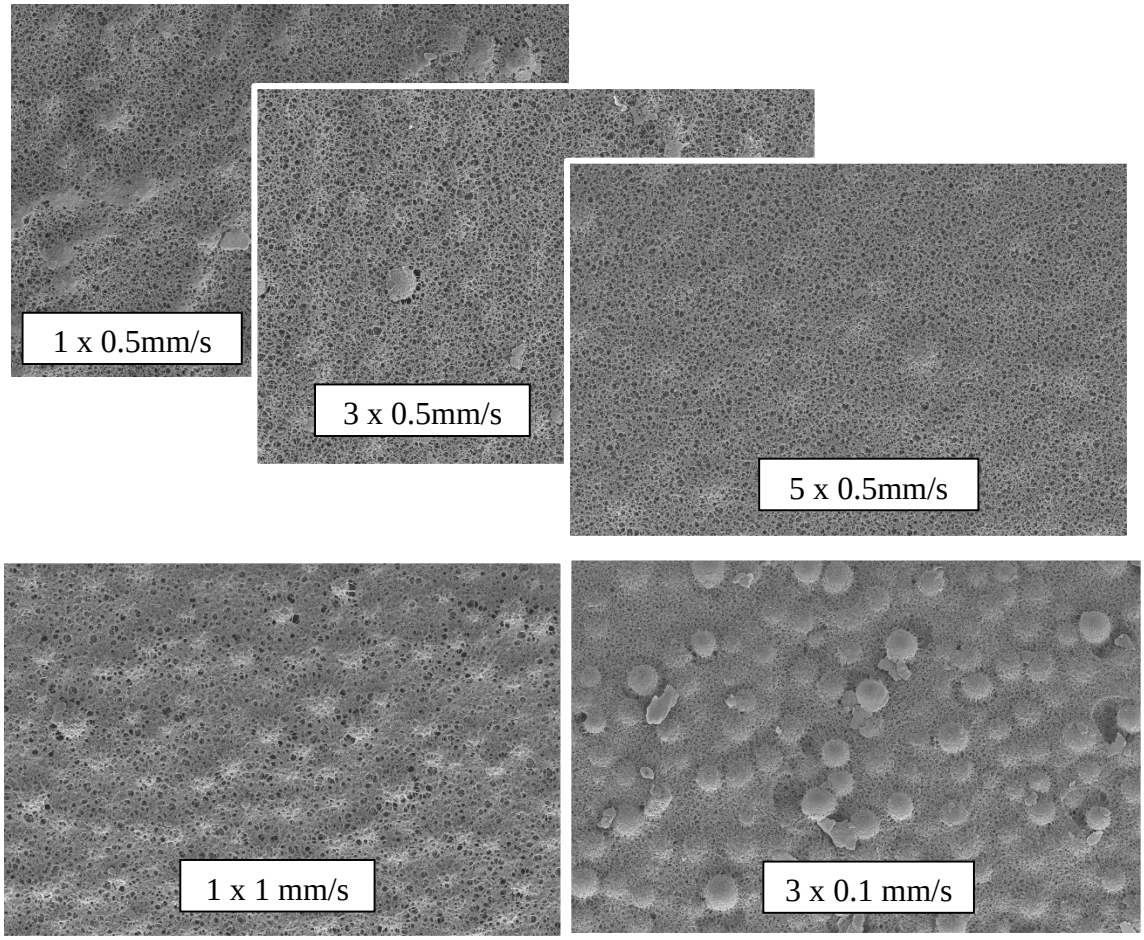


Figure 4.13: SEM images of select PAN overcoatings prepared using different methodologies

Interestingly, when considering the 0.5 mm/s withdrawal over-coatings procedure, the application of multiple layers of PAN did not significantly increase the thickness of the resultant coating. For instance, even with a total of 6 layers of PAN or PAN-HLB, the coating thickness was only observed to be 7 µm. As is evident by the SEM images in Figure 4.13, the 5 µm HLB particles themselves become less visible as successive over-coating layers are applied demonstrating that the application of multiple layers was beneficial. Most importantly, as more layers were applied the smoother the coating became. The hypothesis for these observations is that of, when the already coated device is placed into the PAN/DMF mixture for over-coating some or perhaps a significant amount of the PAN glue already deposited is partially dissolved. However, due to the viscosity of the solution, it will not migrate away from the surface of the coating, but rather be redistributed around the particles when the next layer is applied. Alternatively, the next layer of PAN over-coating is preferentially being deposited into the valleys of the existing coating giving the same result of a smoother coating surface. When coating withdrawal speeds are compared, it is evident that the 0.1 mm/s tests were not of any significant benefit, while the 0.5 mm/s procedure was able to provide a more homogeneous surface topography. Most importantly, however, was that of the 1 mm/s withdrawal procedure, where a more thick and continuous PAN layer was deposited, compared to the somewhat porous nature of the 0.5 mm/s procedure. As a result, the over-coating procedure applied in the preliminary over-coating evaluation studies was as follows: 5 layers of 0.5 mm/s was applied so as to provide a smooth surface, followed by a 1 mm/s over-coat in order to achieve a non-porous surface. Figure 4.14 provides a visual representation of what this resultant over-coating procedure provided.

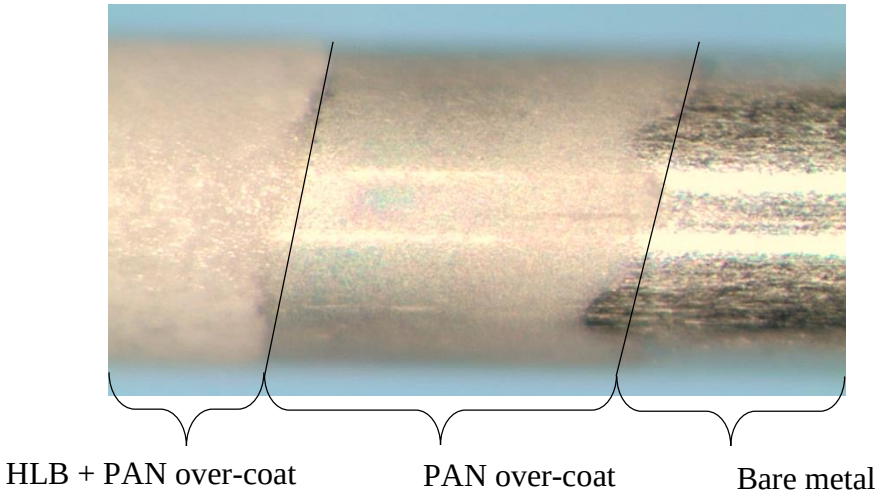


Figure 4.14: Visual representation of the final PAN overcoating employed for the recessed SPME devices

In order to assess the ability of the developed over-coating procedure to protect the recessed SPME coating from bio-fouling, the same experimental conditions which resulted in bio-fouling were replicated with over-coated devices. Teflon over-coated devices were also incorporated into this study, the procedure for application being developed by Dr. Emanuela Gionfriddo and Dr. Ezel Boyaci. In addition, a variation of the post extraction washing step was implemented. Rather than 100% water, 10% acetone in water was used as this had been identified to prove beneficial in some, unpublished, cases when performing extractions from complex sample matrices using SPME. For this study, 3 devices were used for each permutation of experimental conditions totaling 36 devices. For example, 3 device with PAN OC had the water wash procedure, and 3 devices with PAN OC had the acetone wash procedure. The results of this study for the target compound EPA are presented in Figure 4.15.

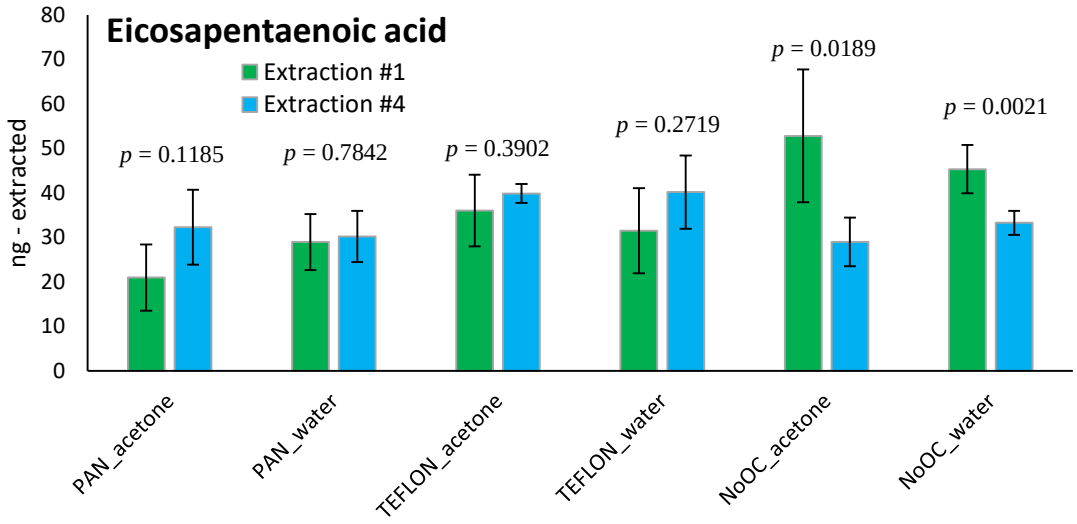


Figure 4.15: Comparison of non over-coated, PAN, and Teflon over-coated recessed SPME devices for the targeted analysis of PUFAs in salmon with varied wash procedures.

What was immediately apparent was that there was no significant difference between devices which possessed an over coated region (ANOVA, $\alpha = 0.95$). This conclusion was valid for all over coating procedures, p values of greater than 0.05 were observed in all cases, as shown in Figure 4.15, when the replicate first and fourth extractions were compared. In addition, the drop in amount of PUFAs extracted resulting from the bio-fouling of the surface of non-over coated devices was identified to be significant ($p < 0.05$), irrespective of the washing conditions used.

4.3.4 Preliminary Results: application of recessed SPME to angled muskellunge

In conjunction with the aforementioned device development and validation was the implementation of the SPME needle for the targeted analysis of anthropogenic compounds, specifically pharmaceuticals, in wild muskellunge tissue. Upon inspection of the recessed devices used in this application, some bending and device deformation was noted to occur during the Georgian Bay samplings while this deformation did not occur during the Ottawa River samplings. The hypothesis for this being that, for the Ottawa River samples, the devices were administered

using the projectile form and were therefore applied rapidly and hence more forcefully. This limited the potential for the force of insertion to be applied at a strange angle causing bending. For this reason, all future samplings of fish should be conducted using the projectile form of the SPME needle, as the rapid (more forceful, see equation 1) puncture of fish scales appears to provide a more uniform and repetitively successful sampler administration.

Equation #1: $F = ma, where, m = {}^{p}/_{v}$ F = force, m = mass, a = acceleration; p = momentum, v = velocity

Of significance however, is the demonstration that the SPME needle can be applied as a projectile through fish scales and into the underlying muscle tissue in on-site applications. Total time of sampler administration was measured on the order of single seconds using one hand, rather than the minute or so typically taken to introduce bio-SPME fibers using the syringe device, which requires two individuals for safe operation.[65]

Somewhat unfortunately, though good for the fish sampled, no target anthropogenic compounds were identified to be present in the samples from either site. This may be a result of the limited sampling time of 10 minutes and as a result longer sampling times should be applied in future applications. A 10 minute sampling time was employed rather than the typical 30 to 60 minutes used in other SPME samplings of live fish, as the fish in this study were not anesthetized. In addition, once captured the fish were placed in a net which was hung from the side of the boat used, meaning that prolonged retainment of the captured fish could have been detrimental to the fish and logistically difficult given the circumstances.

Screening of the resultant LC-HRMS data was conducted using XCMS online software, and more specifically its cloud-plot function. The cloud plots generated are based on box-plots

formulated during the analysis process which compare average response and variation for each significant feature at each site. The m/z ratios identified as significant features were then input into online databases such as t3db.com, and metlin.scripps.edu.[89,90] Though no pharmaceuticals or pesticides have yet to be tentatively identified to be present in the samples, data processing pertaining to significant features is still ongoing.

4.4 Summary

Developed and presented in this chapter is an SPME device which is capable of directly puncturing fish scales or other protective tissue with no additional support or protection. Such a device facilitates the rapid administration of the SPME technology and will certainly see further application in a range of studies. The device was validated to be reproducible by the targeted analysis of pharmaceuticals spiked in water. It was also observed that the direct puncture of vial septa which are not pre-pierced resulted in the irreversible deposition of siloxane onto the coating surface under the experimental conditions used. The device was further applied to the targeted analysis of PUFAs in salmon tissue, and was compared to commercially available bio-SPME fibers where HLB-PAN SPME needles were found to provide the greatest sensitivity. Additionally, the process of puncturing protective fish tissue was validated to not affect the extraction of PUFAs from salmon. Finally, the device was incorporated into a projectile and applied to the analysis of wild muskellunge in an effort to identify anthropogenic compounds resulting from bioaccumulation. This study has yet to yield any positive result for these compounds, however further data analysis of this sample set is on-going.

Chapter 5: Summary

Two forms of the SPME technology have been applied to the targeted analysis of pharmaceuticals in environmental water. In the first case, a method was developed by which to apply the CBS technology to the rapid screening of WWTF effluent streams for target pharmaceuticals. Method LOD's for all target compounds were confirmed to be less than 50 ng/L in river water, with LOQ's for 75% of compounds being less than 100 ng/L. This methodology was then compared to a conventional SPE technique in a study of the final effluent streams of 6 waste water treatment facilities where good agreement was observed. As a part of this study some limitations of the direct to MS technique of CBS were identified. It was observed that for the compounds propranolol and paroxetine there were multiple species in the sample solution which demonstrated the SRM transition monitored during the MS/MS detection and quantification of these species. This observation was validated by LC separation of the SPE extracts. Though these compounds are likely the metabolites of these target compounds, further work is certainly needed to resolve this issue for future applications of the technology for this purpose. For instance, ion mobility spectroscopy could be employed prior to MS/MS separation, or MS^n could be employed in order to provide better compound resolution.

The second technology developed was that of the recessed SPME needle. With the realization of this technology, SPME sorbent phases can now be protected from the physical damage induced by puncturing protective tissue without the need of an additional protective housing or sheathing needle. The device was successfully applied to proof of concept studies extracting the PUFAs EPA, DHA, and ARA from salmon. In addition, the SPME needle was applied to the proof of concept on-site sampling of wild muskellunge where no mechanical damage

to the coating was observed and device deformation did not occur when administered as a projectile.

To conclude, the SPME technology has been advanced in two main ways. Firstly, with the comparison of CBS to SPE for the analysis of WWTF effluent, a rapid SPME method has now been confirmed as a viable method by which to perform the rapid screening of WWTF effluents for pharmaceuticals. In addition, with the introduction of recessed SPME coatings, rapid sampler introduction is now possible as protective housings and sheathing needles are not required, simplifying the device administration process.

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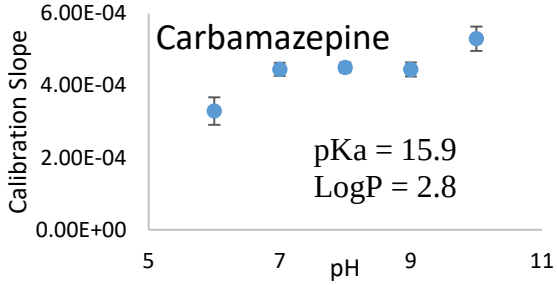
Appendix #1: Table of LC conditions and MS/MS parameters for analysis of pharmaceuticals

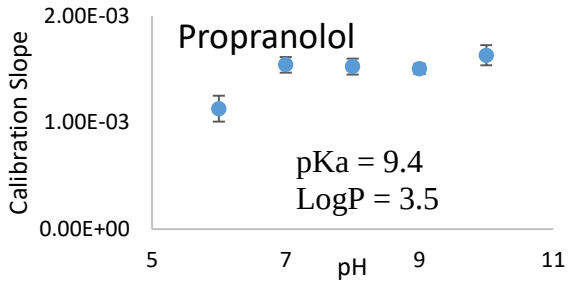
LC separation conditions			
Time (min)	Percent composition		Flow (µL/min)
	A	B	
0	10	90	300
0.5	10	90	300
10	100	0	300
17.5	100	0	300
17.9	10	90	300
18.9	10	90	300

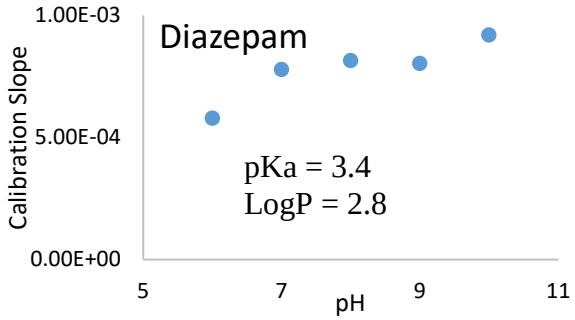
LC-MS conditions	CBS-MS conditions
Spray voltage: 1300V	Spray voltage: 4000V
Vaporizer temperature: 275°C	Vaporizer temperature:
Sheath gas: 30	Sheath gas: 0
Auxiliary gas: 30	Auxiliary gas: 0
Sweep gas: 2	Sweep gas: 0
Capillary temperature: 275°C	Capillary temperature: 350°C
Q1 Resolution: 0.7	Q1 Resolution: 0.7
Q2 Resolution: 0.7	Q2 Resolution: 0.7
CID Gas (mTorr): 2	CID Gas (mTorr): 2
Dwell Time (ms): 50	Dwell Time (ms): 50

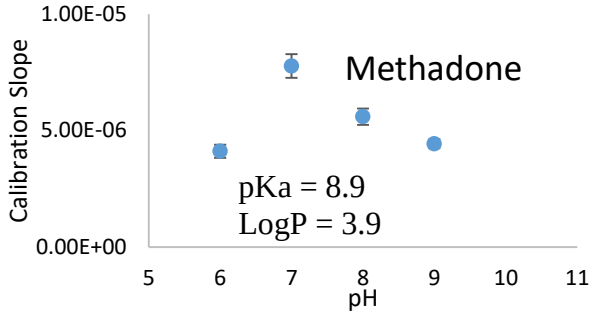
SRM parameters by compound				
Compound	**Parent (m/z)**	**Daughter (m/z)**	**Collision energy (eV)**	**RF-Lens**
Diazepam	285.056	193.079	30	115
Diazepam_d5	290.090	198.111	32	86
Ranitidine	315.128	176.024	16	92
Ranitidine_d6	321.183	130.040	25	49
Codeine	300.134	152.059	60	120
Codeine_d3	303.122	165.071	41	82
Paroxetine	330.129	192.097	19	133
Paroxetine_d6	336.157	198.111	21	71
Fluoxetine	310.121	44.157	13	76
Fluoxetine_d6	316.059	154.087	10	50
Sertraline	306.057	158.955	29	75
Sertraline_d3	309.03	158.929	31	106
Salbutamol	240.147	148.071	16	79
Propranolol	260.070	116.111	18	62
Propranolol_d7	267.137	116.111	19	61
Diazepam	284.995	193.054	32	82
Diazepam_d5	290.090	198.111	32	86
Methadone	310.048	265.007	15	57
Methadone_d3	313.272	258.166	15	57
Erythromycin	734.483	158.111	28	84
Erythromycin_d6	740.483	164.111	28	82
Monensin	694.391	676.419	35	187

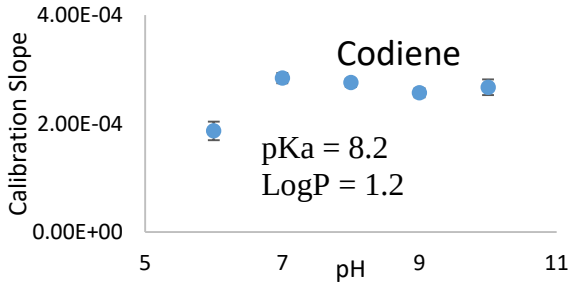
Appendix #2: Target compound sensitivity dependence on sample pH

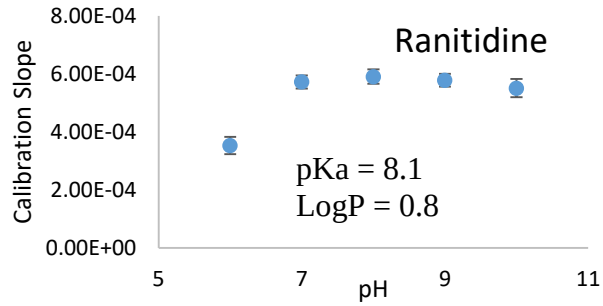


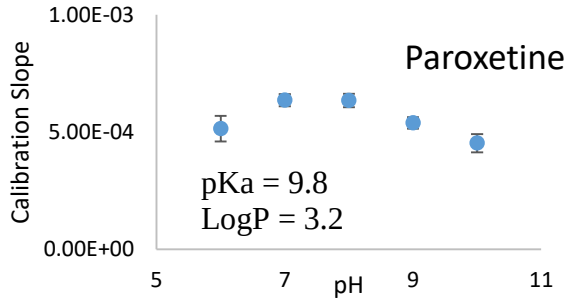


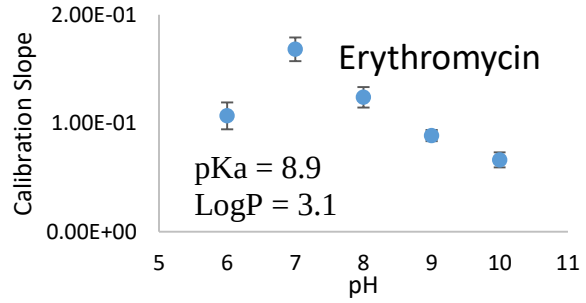


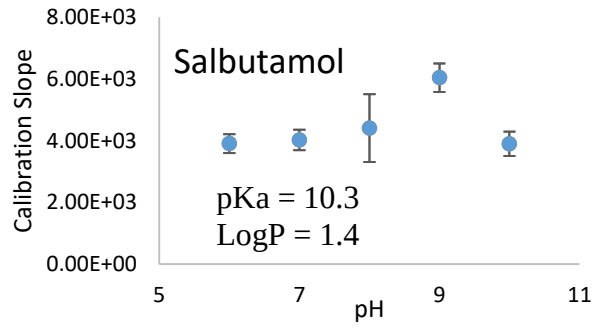


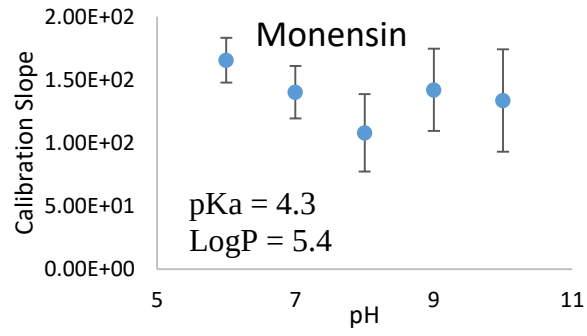












Appendix #3: Septa puncture damage induced to SPME needle coatings

First instance of damage for each needle: