Optimization and evaluation of the performance of thin-film molecularly imprinted polymers for the analysis of cotinine in fluids from human subjects

By
©Adam G. Beaton

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Abstract

Molecularly imprinted polymers (MIPs) are cross-linked synthetic polymers that can selectively take up target analytes from a solution. They are often used in bulk format for solid phase extraction and HPLC. In this work, the main goal was to develop MIPs in a thin-film format for direct analysis of analyte species by desorption electrospray ionization-mass spectrometry (DESI-MS). A cotinine template was used with methacrylic acid (MAA) monomer and ethyleneglycol dimethacrylate (EDGMA) cross-linker to synthesize these MIPs. Cotinine is the primary metabolite of nicotine and was chosen as the template due to its high concentration in biological fluids from smokers and non-smokers. Optimization of the ratios of polymer components (template:monomer:cross-linker) and porogen was completed using a modified Box-Behnken experimental design. Each composition tested was assessed for polymer robustness, imprinting factor and sorption capacity. The optimal molar ratio was 1:2:22.5 (template:monomer:cross-linker), with 239 µL porogen added for each 0.02 mol of template. Template removal from the MIP was studied and reduced from 2 h to 100 min. For optimal analyte uptake, MIPs were placed for 90 min in samples buffered at pH 7.0. Cotinine was quantified in extracts using GC-MS. Myosmine, B-nicotyrine, 1-phenyl-3-pyrazolidinone and nicotine-N-oxide were used as pseudo-templates to overcome template bleed were studied but with little success due to the lack of uptake by these pseudo-templates. Scanning electron microscopy showed that the MIPs were porous and

up to approximately 0.05 μm in diameter. Cotinine calibration curves for human urine spiked with cotinine gave results of R^2=0.6 with n=2. Testing with saliva samples did not produce any promising results. Proof of principle was demonstrated for detection of cotinine using MIPs with DESI-MS.

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List of Abbreviations and Symbols

AcOH	Acetic acid
APCI	Atmospheric pressure chemical ionization
ATR	Attenuated total reflectance
DAD	Diode array detector
DCM	Dichloromethane
DESI-MS	Desorption electrospray ionization-mass spectrometry
DMPA	2,2-dimethoxy-2-phenylacetophenone
EGDMA	Ethyleneglycol dimethacrylate
ELISA	Enzyme-linked immunosorbant assay
ESI	Electrospray ionization
FID	Flame ionization detector
GC-MS	Gas chromatography-mass spectrometry
GC-NPD	Gas chromatography-nitrogen phosphorous detection

HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HPLC-APCI	High performance liquid chromatography-atmospheric pressure chemical ionization
HPLC-DAD	High performance liquid chromatography-diode array detector
HPLC-ESI	High performance liquid chromatography-electrospray ionization
HPTLC	High performance thin layered chromatography
LC	Liquid chromatography
LC-ESI-MS/MS	Liquid chromatography-electrospray ionization-tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
MAA	Methacrylic acid
MeOH	Methanol
MIP	Molecularly imprinted polymer
MISPE	Molecularly imprinted solid phase extraction

MS	Mass spectrometry
NIP	Non-imprinted polymer
RIA	Radioimmunoassay
RIFS	Reflectometric interference spectroscopy
SALDI	Surface-assisted laser desorption/ionization
SHS	Second hand smoke
UV	Ultraviolet

Chapter 1. Molecularly imprinted polymers – An introduction

1.1. Introduction

Molecularly imprinted polymers (MIPs) are becoming more widely used in sensors for a variety of applications, such as water analysis and medical diagnostics. Theoretically MIPs can be used in the detection any compound in complex matrices due to their high selectivity. The most commonly used form of MIPs has been bulk polymers which can be used in SPE.[1] Lately, attention has been given to thin-film MIPs due to their ease of preparation, potential for use in sensors, and simplicity. This thesis will focus on the optimization and evaluation of cotinine MIPs in thin-films for the analysis in human fluids.

1.2. Molecularly imprinted polymers

MIPs are highly cross-linked synthetic polymers that are nominally synthesized from a functional monomer and a cross-linker in the presence of an analyte molecule, also known as the template.[1] Once the MIP has been synthesized, the template is removed from the imprinted polymer by various methods, leaving an empty cavity with binding sites in the polymer that are highly selective for the template and molecules of similar shape and functionality. Biological receptors are superior to MIPs in terms of selectivity,

but are less chemically and physically stable. This allows MIPs to be used in more extreme environments and conditions that would inactivate many bioreceptors, e.g., higher temperatures and broader pH range.[2] The biggest analytical advantage of MIPs is that theoretically any molecule can be used as the template if the appropriate monomer and cross-linker are found. Thus, MIPs can be used in a variety of applications such as: selective materials for pre-concentration using solid-phase extraction,[3] uses in drug delivery,[4] in analytical separations for environmental analysis,[5] and in thin-films for sensors.[6]

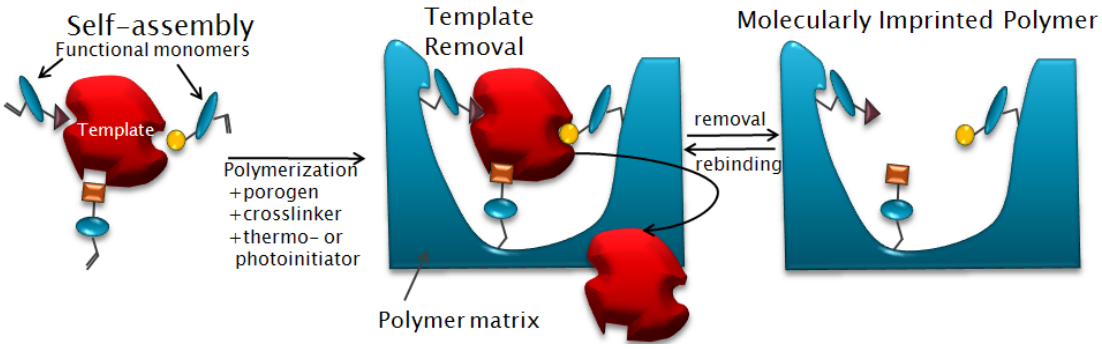

Figure 1.1. Conceptual interpretation of the synthesis of molecularly imprinted polymers. Property of Dr. Christina Bottaro

A general scheme for the synthesis of MIPs is presented in Figure 1.1. The main components of the MIP are: a template molecule, a functional monomer(s), a cross-linker, and an initiator. All four of these components must be soluble in the same solvent, which

is referred to as the porogen due to its role in the resulting porosity of the polymer. The synthesis of MIPs requires several steps. Initially, complexation of the template occurs with functional monomers via self-assembly in a pre-polymerization solution containing all compounds. In most MIPs the self-assembly occurs through non-covalent interactions, such as hydrophobic interactions, hydrogen bonding, and ionic interactions. Next, the polymerization is induced between the monomers surrounding the template and a cross-linker with the assistance of a thermal or photochemical initiator. Lastly, the template is removed from the MIP, which opens an empty binding site with complementary geometry and functionality to the template molecule. This newly vacant binding site can then be used to uptake the analyte of interest which can be either the template molecule used initially, or a molecule with analogous geometry and chemistry.[7]

MIPs are generally very robust and stable, therefore the transition from bulk polymerization to thin-films is conceptually simple. When made in the thin-film format, MIPs must be very robust and mechanically stable after polymerization or else their performance will be affected and reproducibility will be difficult to achieve. Most molecularly imprinted polymers rely on hydrogen bonding between the monomer and the template, for example, cotinine can undergo hydrogen bonding with the hydroxyl group of the methacrylic acid monomer. The next most widely exploited type of non-covalent interaction is π-π interactions. An example of this would be the π-stacking and electrostatic interactions between 4-vinylpyridine and 2,4-dichlorophenoxyacetic acid.[6]

1.3. Imprinting in MIPs

Though the most commonly used method of imprinting in MIPs is non-covalent imprinting due to its ease of template removal for analysis. Covalent and semi-covalent imprinting have also been implemented. Non-covalent imprinting has advantages over other imprinting methods with its relatively simplistic synthesis process and broad range of chemical functionalities that can be used. Problems can arise in non-covalent imprinting with the heterogeneity of binding site distribution but in general this problem can be minimized with careful optimization.[8,9]

Unlike non-covalent imprinting, covalent imprinting relies on very selective reactions between the template and monomer, which significantly lowers the non-specific interactions and thus potentially provides more accurate and better results. Unfortunately, selection of the template removal and rebinding processes for covalent interactions are difficult and make covalent interactions non-practical in most MIP applications. Research presented by Hashim *et al.* has shown that MIPs for stigmasterol provide much better retention with the covalent imprinting fabrication method compared to the non-covalent method with minor drawbacks in this particular case.[9] The biggest drawback of covalent imprinting is the difficulty in finding an acceptable monomer and optimizing the whole process can be extremely time consuming and not necessarily guaranteed to succeed.

A third option called semi-covalent imprinting has also been employed and is a meeting point between the use of covalent and non-covalent interactions. The advantage semi-covalent binding offers over other methods is that the rebinding process is non-covalent in nature, thus providing benefits of both covalent and non-covalent binding. The imprinting step usually involves a covalently bound complex whereas the rebinding step is non-covalent allowing for easier re-uptake of the template. In a study by Peipei Qi *et al.*, MIPs were synthesized for 4-chlorophenyl (4-vinyl)phenyl carbonate using both semi-covalent and non-covalent imprinting.[8] The results showed that semi-covalent imprinting gave superior selectivity for phenols. One issue that arose was that residual template was covalently bound to the polymer, thus limiting the number of binding sites, but it did not show template bleeding as many non-covalent MIPS do.

The imprinting factor is one of the most commonly used methods to determine selectivity of MIPs; it is a comparison of uptake by the MIPs to that of the NIPs. The imprinting factor may be calculated from the following equation

$$Q_{MIP}/Q_{NIP} = \text{Imprinting factor} \qquad (1.1)$$

Where $Q_{MIP/NIP}$ = Mass of analyte taken up by MIP/NIP

This ratio tells us how well the MIP performs relative to the NIP and is a meaningful way of determining if an imprinted polymer is selective or not. The larger the value, the more selective the binding is relative to non-selective interactions.

1.4. Polymerization formats and applications

Traditionally, MIPs were prepared in bulk as a monolith polymer using a large volume of pre-polymer solution polymerized in a glass tube. The polymer was then crushed and particles of specific sizes usually ranging from 25 to 50 µm were collected via sieving.[7] This is the most widely used method for the preparation of MIPs, primarily due to its simplicity. As with all methods, bulk polymerization has its drawbacks. For example, a lot of material is wasted during this procedure, sometimes with as little as 20% of the material will be recovered and usable.[10] MIPs in the bulk format are also not suitable for use in sensors or directly with ambient desorption ionization mass spectrometry methods, such as desorption electrospray ionization mass spectrometry (DESI-MS), whereas MIPs in the thin-film format are ideal for those types of applications. Thermal initiation using azobisdimethylvaleronitrile in bulk monolith MIP formation on a ZnSe attenuated total reflectance (ATR) crystal has also been used but suffers from problems corresponding to overwhelming non-specific interactions.[11] Due to this issue the MIP application showed limitations such as non-linear results at concentrations above 0.200 mg/L and mainly non-specific adsorption at even higher concentrations of 0.550 mg/L.[12]

MIPs in the thin-film format is employed in the research reported in this thesis and has been widely used in the Bottaro research group for environmental applications with polyaromatic hydrocarbons,[13,14] caffeine,[15] and 2,4-dichlorophenoxyacetic acid.[6] Solvent

cast thin-film MIPs are synthesized on a derivatized glass slide and have advantages over bulk monolith format, for example, the final extraction of the analyte loaded from the sample, as is needed before analysis with gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS), can be avoided and the film can be directly interrogated by DESI-MS for analyte detection.[6] Thin-film MIPs can be readily incorporated into sensor applications and used in on-site analysis where use of bulk format polymers would be more laborious. Analytes in thin-film MIPs have also been analyzed directly using a variety of techniques such as infrared evanescent wave spectroscopy and Raman spectroscopy.[11,16] Some problems that can arise with thin-film MIPs include the requirement of having a very robust polymer that will not separate from the support, including derivatized glass slides under harsh experimental conditions and reproducibility problems. Reproducibility is usually related to inconsistencies arising during fabrication since determining the exact amount of polymer produced and proportion of binding sites present can vary greatly with each thin-film MIP.

Boysen *et al.* looked at thin-film molecularly imprinted polymers as patterned polymers with proton NMR as the validation method.[17] Patterned polymers described by Boysen *et al.*, are thin-film MIPs spin coated on a silicon wafer and polymerized separately in two layers; one later is based on a 4-vinylpyridine monomer and the other is based on a methacrylic acid monomer. The result is a MIP sandwich with nm to μm thicknesses. The comparison of the non-imprinted polymer to the MIP double-layered

polymer showed a good imprinting factor of 6.3 (SD = 0.4). A selectivity of the analytical method was based on fluorescence spectroscopy. Fluorescence spectroscopy is an alternative method if techniques such as mass spectrometry are unusable to determine the amount of template.[17]

MIPs in the thin-film type format coated onto a quartz crystal microbalance sensor have also been used. One group developed MIPs for the analysis of digestive proteins in saliva.[18] MIPs used for this application are more cost effective and robust compared to using natural antibodies. MIPs were more selective than non-imprinted polymers and limits of detection (LOD) of 0.1 mg/mL were found for the three main lysosome targets of this analysis of saliva. An imprinting factor of 2 was also obtained for this work, which is moderately low.

The morphology of spin-coated thin-film MIPs was studied by Belbruno *et al.*[19] In their work, non-imprinted control polymers gave a very complex "honeycomb" structure compared to the imprinted polymers. This occurred in fructose imprinted polymers made with polyvinylphenol. It was also found that morphology is very dependent on the template molecule as using 4-methyl nitrobenzoate creates elongated pores compared to the honeycomb pores from fructose template imprinting. These MIPs were applied to nylon films as a substrate but were unsuccessful in yielding good selectivity.[19]

Belmont and co-authors used spin-coating to fabricate thin-film MIPs for the herbicide atrazine and reported that polyvinyl acetate was necessary to obtain films with

good uptake for their MIPs when analyzed by reflectometric interference spectroscopy (RIfS) measurements using toluene as a solvent. Using PVAc seems to cause what the authors call “false pores” in the polymer complex, which may lead to higher uptake of analytes when they become confined in these pores, but may not necessarily give better selectivity of binding sites.[20]

Recently thin-film MIPs were used for the binding of phenol and alkylphenols from water. These MIPs showed only a small degree of selectivity over their non-imprinted counter parts due to the suppression of hydrogen bonding in the aqueous environment. The highly hydrophobic solvent used in the pre-polymerization solution and the very polar and protic water binding system also negatively impacted selectivity. MIPs generally perform better when the solvent used during formation of the polymer is similar to the solution used during re-binding of the analyte. Considering all the factors going against these phenol MIPs, they performed well.[14]

1.5. Cotinine

Cotinine is the most common biological marker for nicotine exposure in both smoking and non-smoking individuals. It is the primary metabolite of nicotine. The half-life after a single transdermal application of nicotine and cotinine are approximately 4 hrs and 20 hrs, respectively.[21] As the half-life of cotinine is longer than that of nicotine, it is

given priority when used as a marker of tobacco smoke exposure. Cotinine is excreted from the body directly in urine; this allows urine to be used to reliably assess nicotine exposure in smokers and non-smokers.[22]

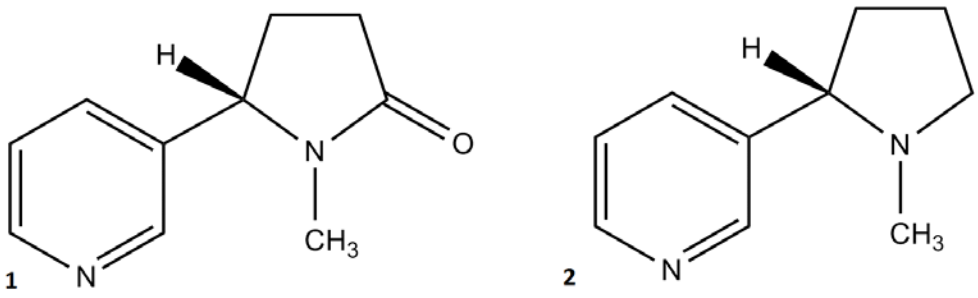

Figure 1.2. Structures of 1:cotinine and 2:nicotine.

Nicotine and cotinine are oxidized primarily by CYP 2A6 in human liver microsomes; the exposure and excretion of these compounds are highly correlated.[22] The primary pathway of clearance for cotinine is to trans-3′-hydroxycotinine but it can also undergo glucuronidation and N-oxidation.[22] Saliva and plasma concentrations of cotinine have also been found to be correlated. Urine and saliva samples are preferred over serum since obtaining samples is a less invasive method and desirable results can still be obtained.

Testing of nicotine as a biomarker is problematic because it is toxic to everyone, even in relatively low doses. Sampling over 8 hr periods and intravenous administration

due to the extensive first-pass metabolism can cause medical problems. Cotinine can be administered orally, for research, to non-smokers with a bioavailability close to 100%.[23] It should be noted that blood, urine and saliva are the most commonly used bodily fluids for the analysis of cotinine, but also breast milk and cervical mucus can be used in situations where the more common methods cannot be used, or when other exposure routes are being studied.[24] In an older study from Sepkovic and Haley, the decline in cotinine concentration in serum and urine decreased to non-smoking levels in only 3 days, whereas salivary cotinine levels drop from 600 ng/mL to 300 ng/mL over the same interval and then showed no change for up to a week. It can be stated that saliva might be better for determining nicotine exposure due to the long retention time for cotinine and the collection of saliva is the least evasive method of sample extraction.[25] More recent studies show that salivary cotinine half-life to be no different than serum or urine samples, which still supports using the least evasive and more convenient sample for cotinine analysis.[24] Due to the above reasons, alternative methods are extensively studied in order to avoid toxicity and invasive problems.

As a biomarker for environmental tobacco smoke, cotinine is widely used to determine the concentrations in non-smokers. However, for purposes such as this, very selective and sensitive methods must be used to accurately obtain results at the desired concentrations in these complex matrices.

1.6. Methods of analysis for cotinine

As with all compounds being analyzed in a complex matrix, the primary issue is due to signal suppression and more specifically, ion suppression for mass spectrometry.[26] This can be corrected for by separating the analyte of interest from the complex matrix and then doing the analysis of the compound alone, or with significantly less interference. Many analytical methods have been employed for the analysis of cotinine in human urine of non-smokers such as high performance thin-layer chromatography (HPTLC)[27], gas chromatography nitrogen phosphorous detection (NPD)[8], HPLC with UV detection,[28,29,30,31] radioimmunoassay (RIA),[32] GC,[33,34] and enzyme-linked immunosorbant assay (ELISA)[30]. The significant advantages of ELISA and RIA are the small sample volume (1 mL) and limited manipulation required to do the analysis. Unfortunately, cross-reactivity toward anti-cotinine (an antibody commonly used with ELISA) and trans-3′-hydroxycotinine is a major concern as they interfere drastically with the analysis. Furthermore, the issue of high amounts of radioactive hydrogen (during RIA) and very poor sensitivity decreases the desirability of these methods. In a study by Kuo *et al.*, urinary and salivary cotinine was analyzed using GC-NPD, HPLC, and ELISA.[28] Pre-treatment for these methods used liquid-liquid extractions to separate the cotinine from the urine or saliva. Calibration curve reproducibility was very good but the real sample analysis had very poor reproducibility without excessive time and effort

expelled to optimize the technique (due to matrix effects), therefore new methods need to be developed such as using MIPs for a quicker analysis.[35]

A clinical study was done to determine the interpersonal and temporal variability of urinary cotinine in elderly subjects using a Hitachi 7600 clinical analyzer, a biochemical analyzer capable of running 800 samples per hr with 80 analytes by way of spectrophotometry.[36] Results showed that single measurements were very inaccurate and that multiple measurements are necessary to obtain acceptable results. Analysis concentrations were found as cotinine-creatinine ratios with an LOD of 1.0 ng/dL. Urinary cotinine levels were measured using a microplate enzyme immunoassay, which is determined by competitive reactions with enzyme-bound and non-enzyme-bound antigens.

Preference is given to chromatographic methods such as LC and GC since they can be more selective, sensitive and accurate, on the provision that the extraction and clean up procedures (solvents, SPE sorbents, etc.) are effective, especially if coupled with mass spectrometers. A commonly used pre-treatment/clean up methods are solid phase extraction (SPE) and solid phase micro-extraction (SPME). Often it is coupled with LC-MS as was done in a study by Kataoka *et al.*[37] In this study, SPME was used in human urine and saliva samples for the analysis of nicotine, cotinine, nornicotine, anabasine, and anatabine. Separation and detection was achieved with LC-MS in 7 mins and the method showed at least 83% recovery. Unfortunately, most clean up methods use a lot of

consumables and each sample must be treated individually leading to massive waste. Achieving the necessary low LOD with multiple steps of sample preparation is uncommon, especially to measure concentrations as low as 1 ng/mL, which are typical of second hand smoke (SHS) exposure. Internal standards can assist with accuracy and reproducibility problems as in the study by Tsiniszeli *et al.,* in which deuterated versions of cotinine and nicotine standards were used but, this has the drawback of being expensive.[38] HPTLC does not have the sufficient sensitivity for accurately obtaining low concentration SHS exposure.[27] Common chromatography systems such as gas chromatography-flame induced detection (GC-FID) and HPLC with ultraviolet (UV) use less sensitive detection systems that require high pre-concentration factors, which typically means a large sample volume is needed to obtain the desirable sensitivity. This can be problematic since there are usually restrictions when it comes to obtaining large volumes of fluid from human subjects.[39,40]

The most effective method for high sensitivity detection is mass spectrometry, since it provides excellent selectivity and sensitivity, particularly when coupled with GC or LC for separation of analytes. High performance liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (HPLC-APCI-MS) has been used to analyze cotinine in human plasma in non-smokers and also for urinary cotinine in both smoking and non-smoking volunteers with moderate success.[39] HPLC-ESI-MS is also an effective method for the analysis of cotinine, more specifically for rat plasma,[41] human

hair,[42] and human urine.[43] LC-ESI-MS/MS has been used to analyze cotinine and nicotine in non-smokers for SHS exposure with good success.[35] GC-MS has been used with non-smoking subjects with success and is highly viable for evaluation of smoking status and SHS exposure as well. Cotinine is volatile by nature and is a good compound to be analyzed by GC-MS with good sensitivity and short retention times. In 2009, a study was completed which used surface assisted laser desorption/ionization-time of flight-mass spectrometry (SALDI-TOF-MS) in positive reflectron mode. This study looked at latent fingerprints on glass and metal surfaces. A simple method was developed for detecting nicotine and cotinine using sub-micron sized hydrophobic silica particles doped with carbon black for the pre-treatment. This method worked well for determining nicotine but only worked a handful of times for cotinine as confirmed using SALDI-TOF-MS.[44]

1.7. Limitations of current common methods for cotinine analysis

There are various limitations of current methods for cotinine analysis. SPE is one of the most commonly used methods to separate compounds from complex matrices. One limitation of SPE is that it takes a long time to load large volumes of samples and low flow rates required for quantitative extraction. Adding to the time and sample handling is the necessity of pre-filtering to remove solid matter from urine, saliva, and wastewater, which can also lead to loss of analyte. Complex matrices can take a long time to load due

to the low flow rate required for efficient extraction. Long runs and multiple steps can create large errors in the analysis.[46]

Another common separation method used for cotinine is liquid-liquid extraction. This method can be inaccurate due to incomplete separation i.e. non-quantitative. Often some of the desired analyte still resides in the original sample and it can take many extraction steps to achieve complete separation. Complex matrices also cause issues with non-target compounds also transferring with the desired compound, which may require further liquid-liquid extractions with a new solvent system or other steps to remove interfering compounds.

MIPs are used to selectively remove the compound of interest from the matrix, binding it within the polymer. The analyte is then removed into fresh solvent producing a solution containing only the desired compound, at least under ideal conditions. Studies by Liang *et al.*[48], and Man *et al.*[47] used LC-MS and GC-MS, respectively, without any treatment to the urine or plasma samples. Using instrumentation to directly analyze the samples is a quick method of obtaining data but suffers from poor reproducibility and limited sensitivity issues. Separating the targeted compound from the matrix is ideal but not always a possibility; MIPs can improve efficiency of extraction from complex matricies.[47,48] GC-MS has poor sensitivity towards some aromatic amines, such as cotinine, causing errors in the analysis.

DESI-MS has been chosen as the desired method of analysis over LC-MS and GC-MS. This is due to the ability to reduce sample handling and eliminate the final step in the procedure in which the analyte is removed, allowed to evaporate, and redissolve in a solvent for injection into an LC or GC. Eliminating these steps allows for better accuracy since many errors can be introduced during the evaporation and dissolution steps with loss of analyte. As with most techniques, there are always some disadvantages as well. DESI-MS is an ambient technique and requires a very robust polymer for the analysis or detection will be problematic because of polymer flaking. MIPs suitable for GC-MS analysis can be less robust polymers. DESI-MS also suffers from background noise problems, especially when analyzing targets in very complex matrices and even when MIPs are used with only one analyte, but this can be corrected using MS/MS techniques.[6]

1.8. MIPs for the analysis of cotinine

Previous work on MIPs for the analysis of cotinine has been successfully shown by R.V. Vitor *et al.*[49], and J. Yang *et al.*[50] in which MIPs in the bulk format made traditionally covalent interaction based monomers such as methacrylic acid and 4-vinylpyridine with non-covalent interactions. Publications on cotinine MIPs are few and far between, allowing for a meaningful opportunity for research in this field. Many

studies have been done using nicotine as the template and using cotinine as a comparison template. Most applications of these MIPs have been in the SPE format; i.e., molecularly imprinted solid-phase extraction (MISPE) and usually combined with high-performance liquid chromatography (HPLC).[49] Vitor *et al.* used MISPE to analyze cotinine in saliva samples of smokers. The study used cotinine as the template, methacrylic acid (MAA) as the functional monomer, ethylene glycol dimethacrylate (EGDMA) as the cross-linking agent and 2,2′-azobisisobutyronitrile (AIBN) as the thermal radical initiator, and dichloromethane (DCM) as the porogenic solvent. The polymer was prepared in the traditional bulk format using a thick-walled glass polymerization tube and sufficient heating for complete polymerization. Template removal was achieved by 9:1 (v/v) methanol/acetic acid washings in an ultrasonic bath for 1.5 hrs. No set amount of time was used for template removal but instead each wash was analyzed by the same method for the presence of cotinine until it was no longer detected. Sample preparation was done by mixing 1.5 mL aliquots of saliva (or spiked saliva) with 1.5 mL of 0.1 mol L^{-1} sodium acetate buffer at pH 5.5. Prepared samples were loaded on the MISPE cartridges after conditioning at a flow rate of 1 mL min^{-1}. Analyte was eluted with 3 mL methanol/water (97.5:2.5, v/v) and then evaporated to dryness under a nitrogen stream. Residue was redissolved in 300 µL of mobile phase and 100 µL injected into the HPLC-DAD for analysis. The MISPE cartridge was also shown to be reproducible up to 100 extractions, which is uncommon for most MIPs. Results showed that concentrations of less than 10 ng mL^{-1} are representative of passive smokers, and 10 to 100 ng mL^{-1} reflect passive

and irregular smoking while values above 100 ng mL^{-1} indicate regular and active smokers. The LOD and limit of quantification (LOQ) were 10 and 30 ng mL^{-1} respectively. One of the major advantages of these MIPs was the low bleeding effects, from which most MIPs suffer.[49]

Studies done by Yang *et al.* using MIPs with HPLC-UV analysis have shown that loading of cotinine in both MIP and NIP polymers show retention of cotinine.[50] They attribute the retention of cotinine in NIPs to non-specific binding in the polymers. It is suggested that optimization of the washing steps is critical to disrupt the non-specific interactions between the analyte and the polymer. The MIPs showed higher selectivity towards cotinine compared to structurally similar and unrelated compounds. Some structurally related compounds such as nicotine, nornicotine show some molecular recognition and may be used as an internal standard with the MIP. The results of the selectivity tests can be used to speculate on how the molecular recognition mechanisms work. Yang *et al.* states that the lactam group in cotinine is likely to be the predominant interaction with the functional monomer, MAA. Analysis of urine spiked with cotinine gave a recovery of 90.2% with these MIPs.[50]

As mentioned previously, MIPs for nicotine are much more common than for cotinine.[49,50,51] A study by Zander *et al.* has shown promising results for the analysis of nicotine and its oxidation products, such as cotinine in nicotine chewing gum by MISPE.[51] These MIPs were synthesized in the traditional bulk polymerization fashion

using UV light to initiate polymerization. Polymers made with nicotine and cotinine together as the templates showed recognition for both templates during uptake procedures. The nicotine MIPs could pick up a range of similar compounds such as nicotine *N*-oxide, cotinine, myosmine, and beta-nicotyrine. These structurally analogous compounds may be useful as pseudo-templates or in-polymer internal standards. The biggest drawback of MIPs reported in this study was reproducibility due to template bleeding issues.

1.9. Analogues of cotinine as potential pseudo-templates in MIPs

Cotinine is part of a large class of compounds with similar properties. Nicotine is the primary compound in this class and is very commonly found and has been extensively researched.[51] Most of the analogous compounds to cotinine are nicotine oxidation products and have very similar structures and as such could be useful as pseudo-templates (Figure 1.3). However, there may be abundance issues with cross-selectivity if present in bodily fluids. The oxidation products studied in this paper were nicotine, cotinine, myosmine, β-nicotyrine, nicotine-trans-*N*-oxide, and nicotine-cis-*N*-oxide.

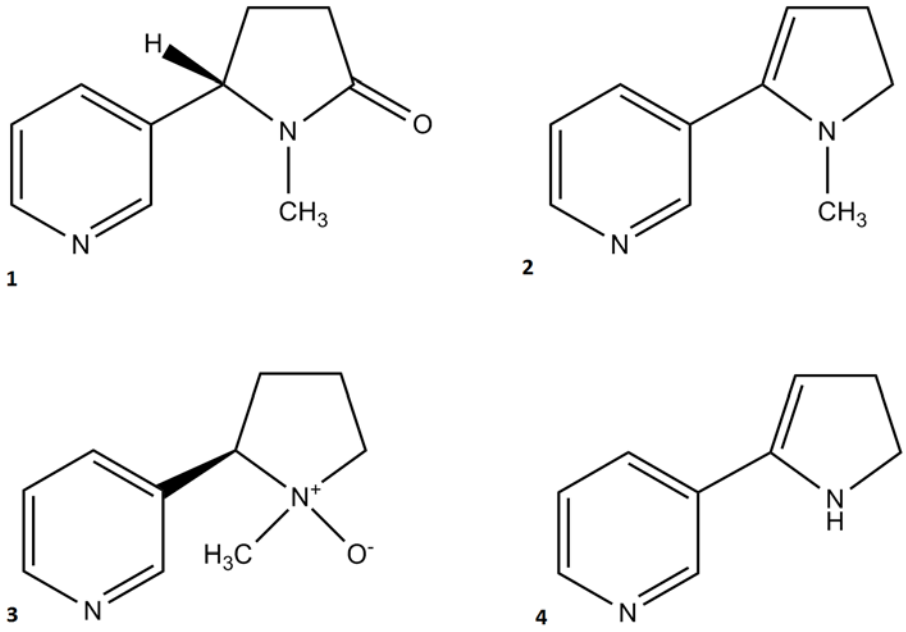

Figure 1.3. Cotinine and analogous compounds (1:cotinine, 2:nicotine, 3: nicotine-N-oxide, and 4: myosmine).

Nicotine imprinted polymers were used to analyze the oxidation products of nicotine to determine their selectivity. As would be expected, MIPs showed the highest degree of selectivity toward nicotine followed by nicotine-*N*-oxide. Myosmine and β-nicotyrine showed moderate uptake with cotinine giving the lowest uptake for nicotine MIPs.[51] Slight variations were observed based on the composition of the mobile phase and pH values. These results show that the structure and functionalities of the imprinting molecules and targets have a profound effect on MIP behavior. It is also clear that the

lactam ring is a key feature of the molecular recognition mechanism. This information can be used in choosing a pseudo-template for cotinine MIPs to prevent template bleeding issues.

1.10. Desorption electrospray ionization – mass spectrometry

DESI-MS is an ambient ionization technique which can be used to analyze chemicals without transferring them to solution or infusing them into an enclosed ionization source. DESI occurs at atmospheric pressure and can be used to ionize gases, liquids, and solids in the open atmosphere making it ideal for the analysis of analytes bound in MIPs. DESI is more common than other ambient techniques such as direct analysis in real time (DART), mainly due to its versatility and unique ionization mechanism. This technique, which is a combination of electrospray (ESI) and desorption ionization, is a relatively recent invention, developed in 2004 by Professor Graham Cooks and co-workers.[52] Ionization occurs differently in high and low molecular weight molecules. The solvent used also plays a role in how well the ionization occurs as solvents like a mixture of methanol and water can be used for positive and negative ionization without issue[56]. Acid can also be used in the spray if positive ions are desired for the analysis. For low weighted molecules ionization occurs by a charge transfer of a proton or electron from a solvent ion and the analyte or by a gas phase ion and the

analyte. The ionization process for high molecular weighted analytes via DESI has been described as follows. Initially, electrospray droplets are directed towards the sample at the incident angle, α. Once the initial droplets wet the sample, secondary droplets impact the spot, which desorb charged droplets containing analytes from the surface. The desorption of the droplets is aided by electrostatic repulsion. As the droplet travels toward the MS inlet at the collection angle, β, ions are formed through the usual ESI mechanisms (solvent evaporation, droplet fission, ion ejection and ion desolvation through collisions) and are swept into the inlet of the mass spectrometer by vacuum. This technique does not require the use of a specialized matrix, which is advantageous with porous surfaces. The following diagram shows a simplified diagram of how DESI operates.

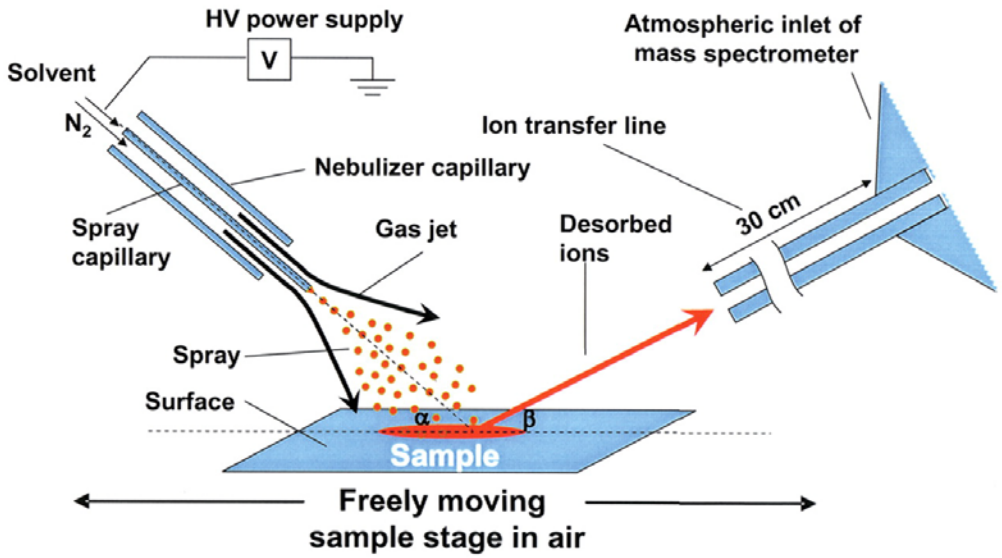

Figure 1.4. Diagram of the DESI system[52]

The use of DESI-MS for the analytes removed from MIPs is highly desirable since it can be used to provide surface analysis of these polymers with good selectivity as previously shown by Van Biesen *et al.* and Takáts *et al.*.[6,52] In this work it was found that analyte extraction from the polymer was dependent on stirring rates and diffusion into the polymer which can be highly irregular. The pore formation may be different for each polymer as well causing discrepancies in the diffusion of analyte from the pores during extraction. This is a fabrication issue that is difficult to correct for, but internal standards assist greatly. DESI-MS also has the advantage of being much faster than previous methods as the final removal step would be eliminated and the DESI can directly desorb analytes in the MIP film for MS analysis. Thin-film format MIPs are ideal and better suited for ambient techniques due to their ability to be easily transportable on a glass slide.

1.11. Project objectives

The objective of this project was to optimize the composition and evaluate the performance of thin-film MIPs for cotinine in a thin-film coating. The starting point for the composition was that of the MIP originally developed in bulk monolith format by Yang et al.[50]

Their work showed that cotinine MIPs are selective compared to their NIP counterparts. This is the single most important point for MIPs because if the polymer does not have good selectivity compared to its NIP, then the results are almost guaranteed to be poor. Selectivity in comparison with other compounds of similar structure such as nicotine, nornicotine, and caffeine, showed that recognition was much lower than that for the template molecules but some recognition was seen. This meant that finding a suitable internal standard for our study should be possible. Loading in aqueous environments showed selectivity for both MIP and NIP via non-selective binding, but ultimately, selective binding would prevail. Studies were completed on human urine samples spiked with cotinine which showed good results and therefore gave promise to future studies such as those described in this thesis.

Studies were continued using bulk cotinine MIPs by Vitor *et al.* which used similar polymerization steps but the porogen was changed from dichloromethane to acetonitrile.[49] Template bleeding was determined to not be a problem in this study after 6 washes of acid and methanol for removal of the template. Optimization was only shown to be done on template removal, leaving much work to be done before complete optimization of cotinine MIPs was completed. Since the study was completed on bulk MIPs, the optimization may be vastly different for thin-films but still shows potential for the current study on the different MIP format.

The main objective of my work was to develop a new MIP for cotinine in thin-film format that would be compatible with DESI-MS. This would allow a quicker analysis time due to the lack of final wash step that would be required before the analyte could be detected on an instrument such as GC-MS. The hypothesis for the current thesis was that "*Thin-film MIPs can be analyzed directly under ambient conditions in mere minutes with good sensitivity using DESI-MS*".

Systematically optimizing the composition and synthesis conditions to form robust thin-film polymers that can also uptake cotinine favorably is a complex task. The easiest and most important place to start is with the composition. The effects of composition on MIP efficiency and selectivity must be optimal, but this must be balanced with the need for a stable polymer that will not break down when introduced to harsh removal techniques required to disrupt hydrogen bonding. MIPs can vary greatly in their robustness based on which porogen is used in the pre-polymerization complex. Due to phase separations with thin-film MIPs, porogens that are volatile will evaporate rapidly and may cause issues with the robustness of the MIP. This ends up being a significant challenge due to the key requirement that the components be soluble in the solvent system, and therefore requires extensive trial-and-error optimization. Template bleeding is a known problem with MIPs which occurs because of incomplete template removal. The template removal is generally carried out by an acidified solvent wash, but it is typical that it is not possible to remove all of the analyte (template). Studies were done to

see how problematic template bleeding is, and if the wash/extraction time requirement could be reduced. Once the template is removed from a MIP, reuptake will occur in a solution containing the analyte, such as a spiked buffer solution. In the current research, calibrations were completed with urine and saliva samples, which were spiked with cotinine, to determine the effect of uptake from complex biological matricies. Due to the complexity of human samples, a minimal treatment will be the use of a buffer solution for optimization. GC-MS was used to optimize the steps for MIP synthesis and analysis before DESI-MS was used to analyze MIPs which had been exposed to human samples spiked with cotinine.

Chapter 2. Methods and materials

In this chapter, a detailed explanation of the synthetic procedure of MIPs will be presented. The procedure for MIP synthesis and use is very specific but simple in nature. Much of the time spent making and using MIPs involves waiting for extractions and uptake with little need for human intervention. Instrumentation parameters will also be presented in detail. Keep in mind that these parameters and the procedure are the starting point of the work, much optimization was carried out afterwards and will be described later in the thesis.

2.1. Synthesis of thin-film MIPs

The initial procedure used to synthesize thin-film MIPs was developed and published by the Bottaro group using a sandwich-type polymerization, also known as drop-casting.[6, 14] The MIP components used in this project were selected due to their previous use in bulk monolith MIPs for cotinine with success.[49, 50] Methacrylic acid (MAA) (Sigma-Aldrich Inc., St. Louis, MO, USA) was chosen as the functional monomer because of its ability to form hydrogen bonds with cotinine. EDGMA (Sigma-Aldrich Inc., St. Louis, MO, USA) was used as the cross-linker in this study.

Myosmine (Sigma-Aldrich Inc., St. Louis, MO, USA) and nicotine (Sigma-Aldrich Inc., St. Louis, MO, USA) were used as pseudo templates and internal standards due to their analogous geometry to the analyte molecule. All procedures and experiments were performed at room temperature unless otherwise indicated.

The synthesis of MIPs was carried out on derivatized glass slides. These slides were standard microscope slides (Fisherbrand Products) cut in 3 roughly equal pieces and marked by a number in the corner for tracking purposes. The slides were cleaned by immersion in a solution of HCl/MeOH (1:1) for 30 mins. After immersion, the slides were rinsed with deionized water and 95% ethanol and dried in an oven or incubator at 60 °C for 1 hr. Derivatization was done overnight, in a solution of 2% (v/v) of 3-(trimethoxysilyl) propylmethacrylate (Sigma-Aldrich Inc., St. Louis, MO, USA) in toluene (Sigma-Aldrich Inc., St. Louis, MO, USA). The reaction that corresponds to the derivatization of the glass surface is presented in Fig 2.1. The container holding the slides and derivatizing solution was covered with tin foil to prevent exposure to light which could initiate premature polymerization. The next day, derivatized glass slides were rinsed with 95% ethanol and blown dry with N_2. Slides were kept in the dark or covered in aluminum foil until use (usually within 24 hrs).

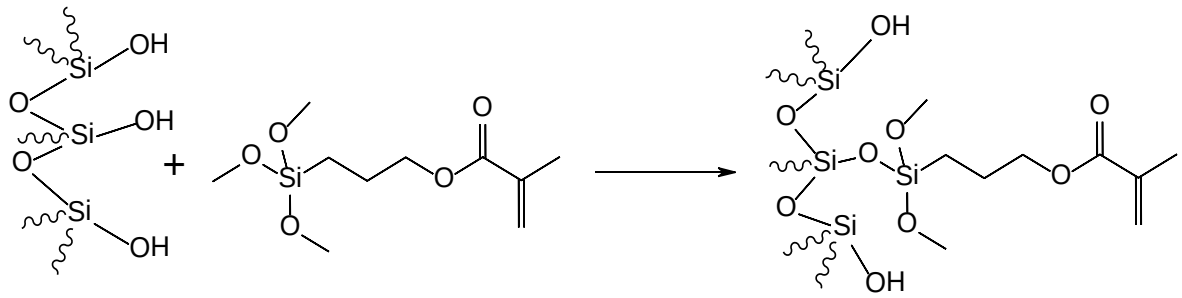

Figure 2.1. Derivatization of glass surface with 3-(trimethoxysilyl) propylmethacrylate.

The pre-polymerization solution was prepared by combining the template, functional monomer, cross-linker, photo-initiator and porogen in a 2-mL vial (see Table 2.1 for proportions). 2,2-dimethoxy-2-phenylacetophenone (DMPA) (photo-initiator) (Sigma-Aldrich Inc., St. Louis, MO, USA) and cotinine (Sigma-Aldrich Inc., St. Louis, MO, USA), both solids, were weighed into the vial, then the solvent/porogen ($MeOH/H_2O$, 4:1) was added using a micropipette. Lastly, appropriate volumes of the monomer and the cross-linker, both liquids, were added to the mixture of cotinine and DMPA using a micropipette. After all components were added to the vial, the mixture was vortex mixed to obtain a homogenous solution and sonicated to remove dissolved air (N_2, O_2, etc) as the presence of oxygen can interfere with polymerization. Comparison materials called NIPs were prepared in an identical manner, except no template was added to the pre-polymer solution. NIPs were used to study the imprinting efficiency.

Table 2.1 Composition of stock pre-polymerization solution before optimization

Compound	Function	Amount (mmols)	Amount (mass/volume)
Cotinine	Template	0.04	7.05 mg
Methacrylic Acid	Monomer	0.16	13.6 μL
Ethyleneglycol dimethacrylate (EGDMA)	Cross-linker	0.80	151 μL
2,2-Dimethoxy-2-phenylacetophenone (DMPA)	Initiator	0.010	2.6 mg
MeOH/H_2O (4:1)	Solvent/Porogen	N/A	224 μL

2.2. Fabrication of the thin-film MIP

An 8-μL aliquot of the MIP pre-polymerization solution was deposited on each derivatized glass slide using a micropipette. The deposited solution was then covered with a clean glass cover slide. The glass cover slide was previously cleaned with 95% ethanol and dried under N_2. The cover was placed quickly and strategically to ensure no air bubbles were trapped underneath. When air bubbles form, slight pressure can be applied

to remove them without causing the solution to be forced from beneath the cover slide. The mixture was then exposed to UV light (254 nm – 8 W) for 30 mins using a UV lamp (λ = 254 nm, 6 watt). The distance between the lamp and the cover slide was kept at approximately 0.5 cm. Following irradiation, the cover slide is gently lifted from the polymer using a sharp blade as to avoid damaging the the polymer on the slide. The resulting films are typically white and opaque in appearance, as shown in Figure 2.2. (transparency depends on the porogen used) and are robust, i.e., mechanically stable.

The removal of the cotinine template was achieved by immersing up to 8 slides in a large petri dish with 100 mL of MeOH/AcOH (9:1) and stirring for 1-3 hrs (initially 3 hrs was used but time was reduced during optimization studies). This procedure was repeated twice more and then a final 1 hr wash in MeOH. All extractions are done in a different MeOH solution to ensure no analyte is present.

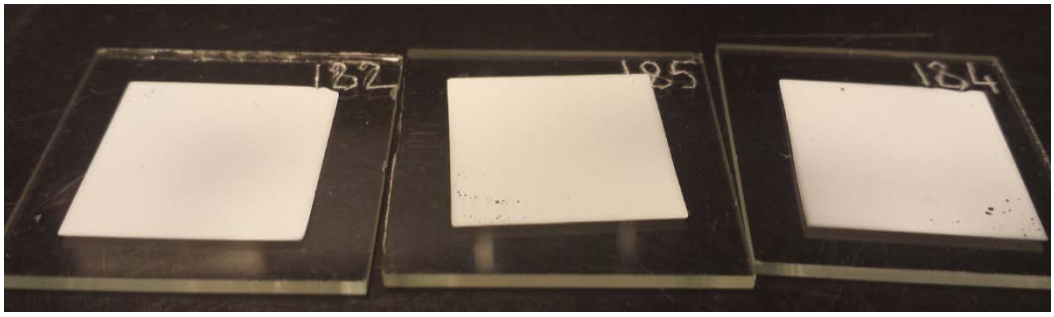

Figure 2.2. Image of MIPs in thin-film format.

2.3. Uptake and extraction of cotinine

The uptake process was studied and the following procedure is the optimized result. Two MIPs are placed in a large petri dish containing 2 mM Na_2HPO_4 buffer solution. The buffer solution was made by mixing appropriate amounts of monosodium phosphate (Sigma-Aldrich Inc., St. Louis, MO, USA) and disodium phosphate (Sigma-Aldrich Inc., St. Louis, MO, USA) with phosphoric acid (Sigma-Aldrich Inc., St. Louis, MO, USA) or sodium hydroxide (Sigma-Aldrich Inc., St. Louis, MO, USA) to adjust to a pH of 7. The uptake solution is spiked with appropriate amounts of cotinine standard to obtain the desired concentrations in 100 mL of buffer solution. For example, addition of 67 µL of a 1492 mg/L cotinine stock solution up to 100 mL of buffer yields a 1 mg/L solution. This solution was then stirred at high speed for 1.5 hrs, while ensuring no splashing occurs. After uptake, MIPs were rinsed with deionized water and allowed to air dry. The template was extracted by stirring from the MIPs using MeOH/AcOH (9:1) in a 50 mL beaker, for 1 hr. The MIPs were removed from the beakers and rinsed with MeOH, which was collected in the beakers containing the extract. The beakers were left in a fume hood overnight to allow all the solvent to evaporate. The residue was redissolved in 1.00 mL of dichloromethane and analyzed via GC-MS.

2.4. GC-MS Instrumentation

All samples from optimization and other studies were analyzed using an Agilent Technologies 6890N Network GC system equipped with an Agilent 7683 Series Injector and Agilent 5973 inert Mass Selective Detector (MSD). The GC capillary column used was an Agilent 19091S-433 HP-5MS (0.25 mm diameter x 30 m length x 0.25 µm film thickness). The carrier gas was He and the head pressure was set at 11.99 psi and splitless mode was used. The injection volume was 1.0 µL, the injection temperature was 280 °C and the transfer line temperature was 280 °C. The oven temperature program (as shown in Table 2.2.) was: solvent delay of 3 mins, initial temperature at 70 °C held for 1 min; temperature was increased at a rate of 30 °C/min until 190 °C; then 5 °C/min until 230 °C, and finally 25 °C/min until 290 °C. The post run over temperature was 315 °C held for 2 mins. The total run time was 15.40 mins. Cotinine was detected using selected ion monitoring (SIM) of signals at *m/z* value of 98, 118, and 176. Quantitative analysis is done using *m/z* 98 while 118 and 176 are for confirmation of analyte.

Table 2.2 GC-MS parameters for cotinine analysis

Carrier gas	He
Head pressure	11.99 psi
Injection volume	1.0 µL
Injection temp	280 °C

Solvent delay (mins)	3 min
SIM (m/z)	98, 118, 176
Temperature Program	
T_i	70 °C for 1 min
T_{ramp1}	30 °C/min to 190 °C
T_{ramp2}	5 °C/min to 230 °C
T_{ramp3}	25 °C/min to 290 °C
T_{post}	315 °C for 2 min

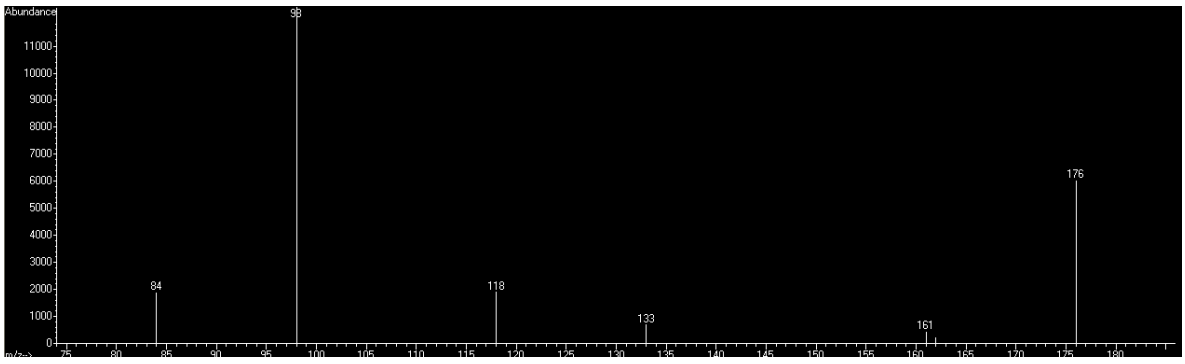

Figure 2.3. GC-MS spectrum of a Cotinine MIP extract.

2.5. Desorption electrospray ionization – Mass spectrometry instrumentation parameters

Ambient ionization studies were also completed on a Xevo TQ-S tandem quadrupole mass spectrometer (Waters, Mississauga, Ontario, Canada) with a 2D DESI source (Prosolia, Indianapolis. IN, USA). The spray solvent used was equal parts methanol and aqueous with 0.1% formic acid. Spray gas pressure was set at 100 psi N_2 with a solvent flow rate of 2 µL/min. The voltages of the capillary and the cone were 2.75 kV and 50 V, respectively, with an ion source temperature of 150 °C. The tip-to-surface distance was 4 mm and the MS inlet-to-surface distance was 2 mm. The incident angle (α) and collection angle (β) were 55° and 5° respectively. Conditions were not optimized for cotinine MIPs but rather a method for MIPs within the Bottaro group was used. The m/z desired was 177 as M+1 and M is 176 m/z with fragments at 69 and 113 m/z. A mass spectrum of a cotinine MIP can be found in Figure 2.4.

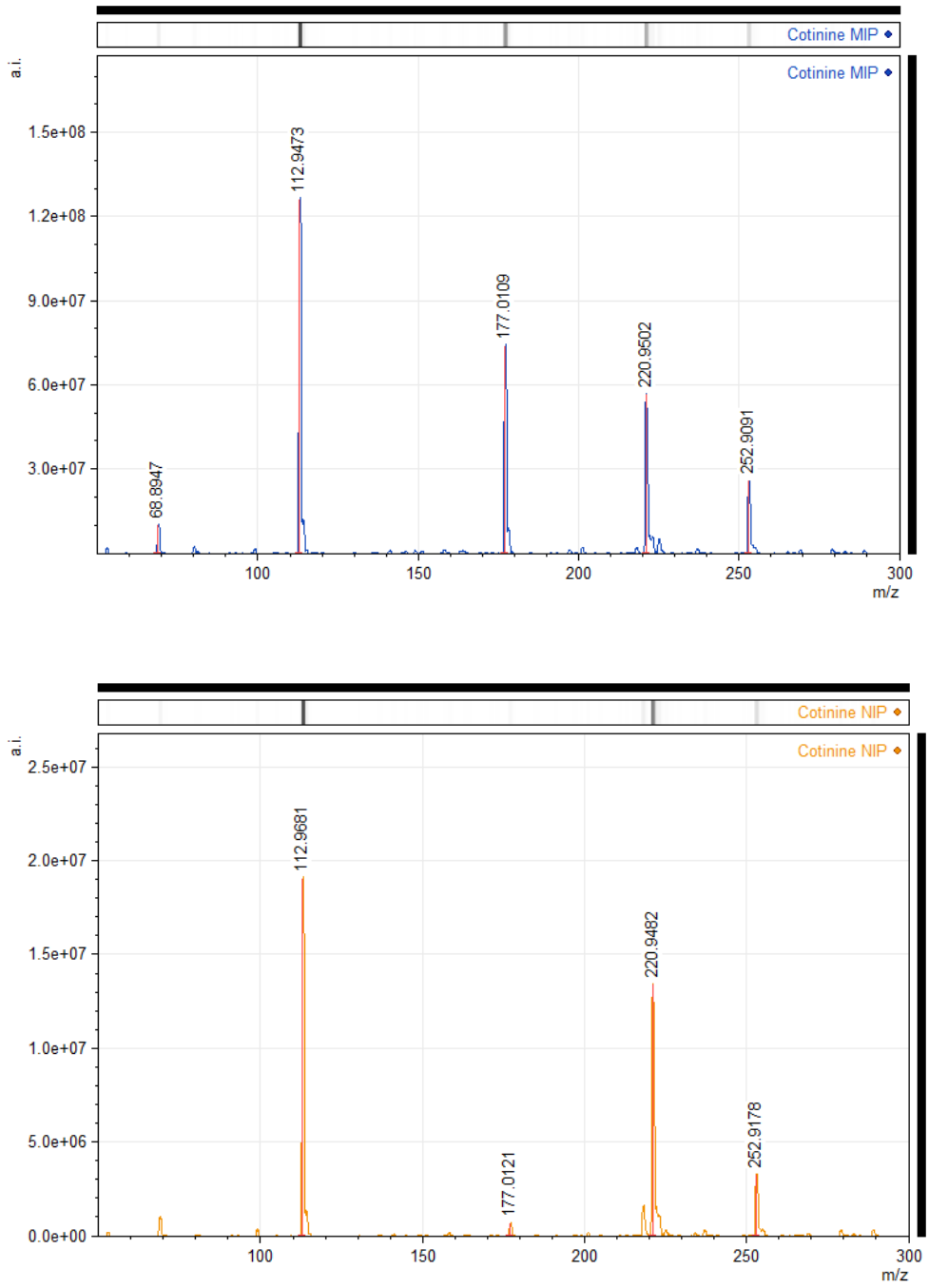

Figure 2.4. DESI mass spectrums of a Cotinine MIP and NIP.

Chapter 3. Optimization of cotinine MIPs

This section will explain the steps that were taken to optimize cotinine MIPs. Optimization of the composition is generally the first step in developing a material and needs to be done correctly in order to optimize other analysis parameters. Stoichiometric ratios between the template and the monomer are important as too little monomer will not provide enough non-covalent interactions with the template to produce a selective polymer. Using Box-Behnken experimental design (see Section 3.2 for further details), parameters can be adjusted in order to find a composition which gives a high amount of analyte uptake and a robust polymer. To finalize the optimization, various steps involving template extraction and uptake can be adjusted to allow for better template extraction and reduced time.

3.1. Selection of the porogen and proportions

Before the pre-polymerization composition can be optimized, the best porogen for thin-film fabrication of water compatible cotinine MIPs must be found. Previous studies suggested that dichloromethane would be the best solvent because it cannot hydrogen bond with itself or with cotinine, allowing cotinine and methacrylic acid to have much stronger hydrogen bonding and thus, allowing for more specific interactions.[49,50] In a

previous MIP study with 2,4-dichlorophenoxyacetic acid as the analyte, a porogen composition of MeOH:H_2O (4:1, v/v) was determined to be ideal for water samples since MIPs perform better when the composition of the uptake solution is similar to that in which the polymer was created.[6] Another study found that MIPs made using 1-octanol as a porogen were very robust and porous, which is not true when volatile solvents such as DCM are used.[13] Thus, octanol was also tested.

For each porogen, four MIPs were made and were used for uptake of cotinine at a concentration of 1.00 mg/L in a phosphate buffer solution at pH 7. As can be seen from the results in Figure 3.1, 1-octanol MIPs gave the highest uptake of cotinine compared to DCM and 4:1 MeOH:H_2O but along with the DCM polymers, suffered from poor reproducibility. The 4:1 MeOH:H_2O MIPs gave lower uptake values, but had good reproducibility in comparison. These polymers also were very robust, which is desirable for thin-film MIPs to ensure a high precision and accuracy of the analysis. Therefore, the 4:1 MeOH:H_2O solvent was chosen as the ideal porogen for this study. Figure 3.2 clearly shows the comparison of robustness between the three different porogens. One of the DCM polymers were fragile and separated easily from the glass slide and the 1-octanol polymers had a minimal amount of flaking.

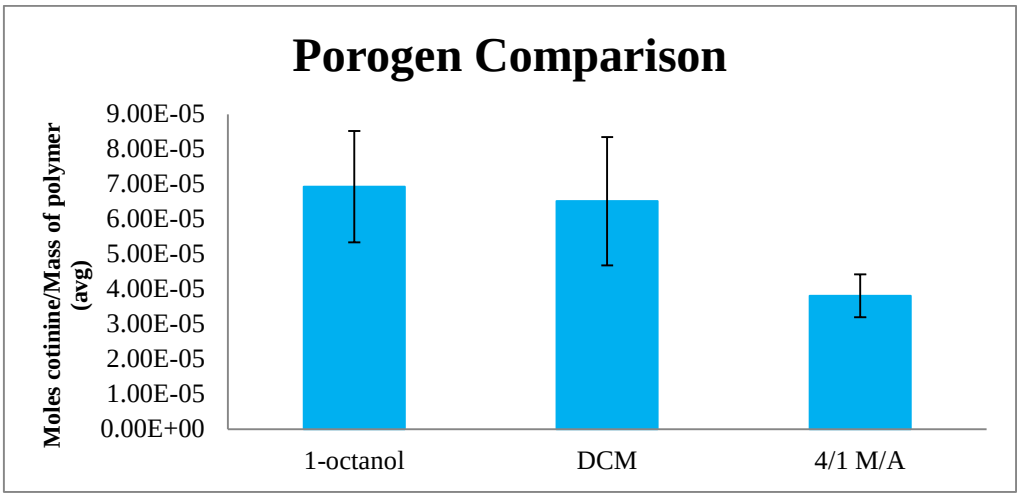

Figure 3.1. Comparison of cotinine uptake for cotinine MIPs made using various porogens (M/A: methanol/aqueous).

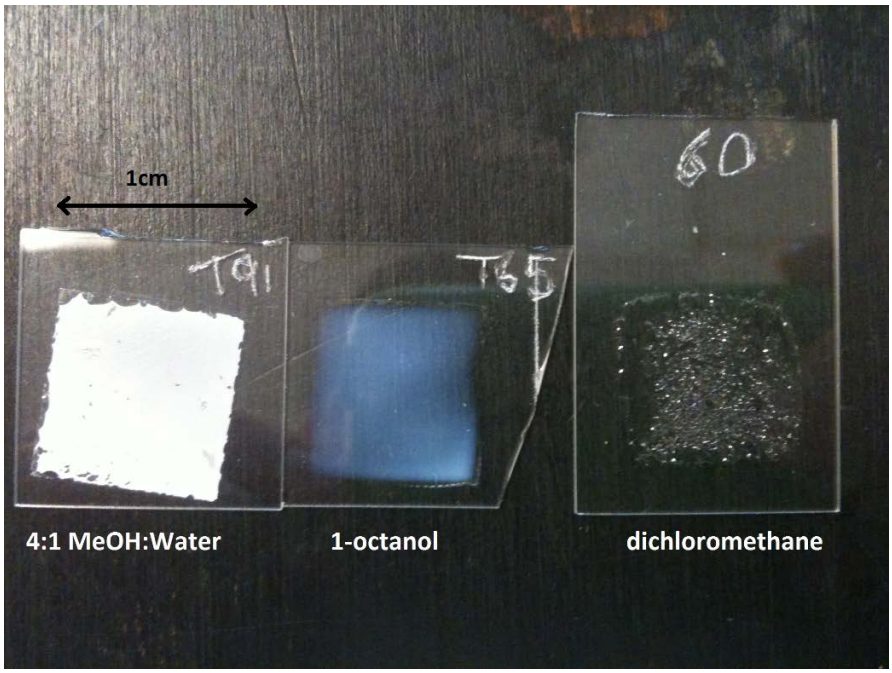

Figure 3.2. Thin-film MIP surface appearance using various solvents

3.2. Optimization of the component ratios

Previous studies done by Yang *et al*. and Vitor *et al*.,[49,50] reported a standard ratio of 1:4:20 of template:monomer:cross-linker with Yang using dichloromethane and Vitor using acetonitrile as the porogen.

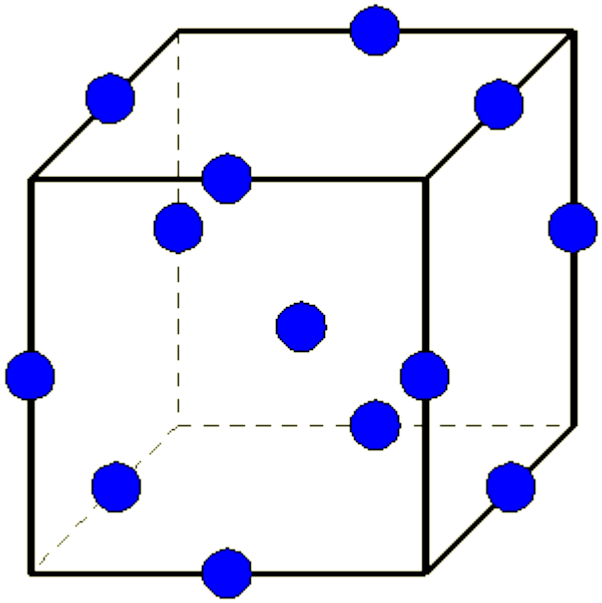

Figure 3.3. 3-D cube plot of Box-Behnken design for 3 factors (blue balls are independent variables)

Based on preliminary studies and knowledge of the roles of each polymer component the monomer, cross-linker, and porogen were the factors optimized in the pre-polymerization solution. To assist with the optimization study, Minitab™ software package (v.7, Minitab 16 Inc.) was used to develop the experimental design and evaluate

the results. Box-Behnken experimental design was used to obtain the optimal composition for maximum uptake of cotinine from a standard solution with 1 ppm cotinine (the solution of interest). Box-Behnken designs are experimental designs to achieve the goals of having independent variables (three in this case) which fit a quadratic model and can be analyzed to find axial points which can represent maximums and minimums (see Figure 3.3).

For each composition, four MIPs were made and analyzed for their uptake of cotinine. Values for the monomer and cross-linker are relative to the moles of template which is set at 1. The ratio between the template and the functional monomer is important for maximum selectivity. Depending on the analyte being used, different maximum ratios will exist due to non-covalent interactions. According to Figure 1.2 and 1.3, there are two available spots for hydrogen bonding and one location that is possible but would be very strained due to the methyl group attached to the lactam nitrogen. From this we expect a 1:2 or 1:3 ratio of template:monomer. The cross-linker is generally the most abundant component of the polymer and is essentially the supporting structure for the imprinted sites. Too little cross-linker will create a flaky polymer but too much may lead to a non-selective sorbent; therefore, we need to find the ideal ratio to obtain the most robust polymer with controlled porosity dictated by the phase separation process during polymerization. It should be noted that the slightly aqueous porogen assists heavily with phase separation for these MIPs. The porogen volume was varied as needed to

accommodate the change in amounts of components which requires a volume change to allow for dissolution. To determine the highest amount of cotinine these polymers could uptake, the data was expressed as the ratio of moles of cotinine per mass of polymer. This is a common approach for sorbants and compensates for variations in polymer mass related to the sandwich fabrication method. The following table (Table 3.1) summarizes the various compositions used in the optimization study.

Table 3.1. Composition of pre-polymer solution for optimization experiment.

Monomer to Template Ratio (molar ratio)	Cross-Linker (mol)	Porogen (μL)	Moles Cotinine/Mass Polymer (mol/g)
2	10	230	0.55
2	20	160	0.16
2	20	300	0.41
2	30	230	0.79
4	10	160	0.16
4	10	300	0.07
4	20	230	0.31
4	20	230	0.40
4	20	230	1.23
4	30	160	0.52
4	30	300	0.00
6	10	230	0.05
6	20	160	0.32
6	20	300	0.36
6	30	230	0.02

Once the study was completed, data analysis was carried out using Minitab to determine the optimal values for each factor, which should give a polymer with the highest capacity for cotinine uptake. The output from Minitab is presented in Figure 3.4. The optimal ratio was determined to be 1:2:22.5:239.19 (template:monomer:cross-linker:porogen) all in moles except the porogen, which is given in µL. Other than analyte uptake other important factors were considered, such as the imprinting factor and the robustness. Some compositions resulted in a low degree of robustness for the polymers with varying sorption capacities. The ratio of monomer did not change from the results obtained by Vitor *et al*. which confirms his findings.[49] Cotinine can only non-covalently bond to two methacrylic acid molecules therefore a molar ratio of greater than 2:1 monomer:template will give an excess of functional monomers. This did not seem to affect the uptake. It also further supports the theory that the methyl lactam functionality is too sterically hindered to provide a third hydrogen bonding site. The amounts of cross-linker and porogen that were optimal increased slightly from 20:1 cross-linker:template to 22.5:1 and 225 µL to 239.19 µL. A difference here is not unexpected since the initial ratio used were based on a different polymer composition.[6] The amount of photo-initiator, template and the concentration of cotinine available for upload were the only two factors kept constant.

Since the initiator is responsible for starting polymerization, studies were completed to determine if increasing the amount of initiator present in the pre-polymerization

solution would result in increased robustness and maintain the high degree of uptake shown by the optimized ratios. Initially the amount of initiator used was 0.012 mmol (3.1 mg). New amounts chosen were 0.016 mmol, 0.020 mmol, 0.024 mmol, and 0.050 mmol. Pre-polymerization solutions were made up with varying amounts of photo-initiator, while keeping constant optimized ratios of the other components. No change was observed in varying the amount of photo-initiator since all MIPs were very robust. All future studies were completed using this optimized pre-polymerization solution. One factor that seemed to affect synthesis of robust MIPs is the age of the reagents, more specifically the MAA. One of the biggest issues found was that MAA has a shelf-life of approximately 6 months after opening. Thus, MIPs made with older MAA were found to be very flaky and generally of poor quality.

The following figure (Fig 3.4) shows the maximum results calculated using Minitab software. The y value relates to the theoretical maximum of analyte taken up by the polymers and the d value relates to the desirability of these ratios since we chose the middle values to be more desirable than the high and low values since they already worked well for creating cotinine MIPs. The cross-linker and porogen data shows a clear peak at the maximum, whereas the monomer data was actually already at a maximum due to the lack of binding sites on cotinine.

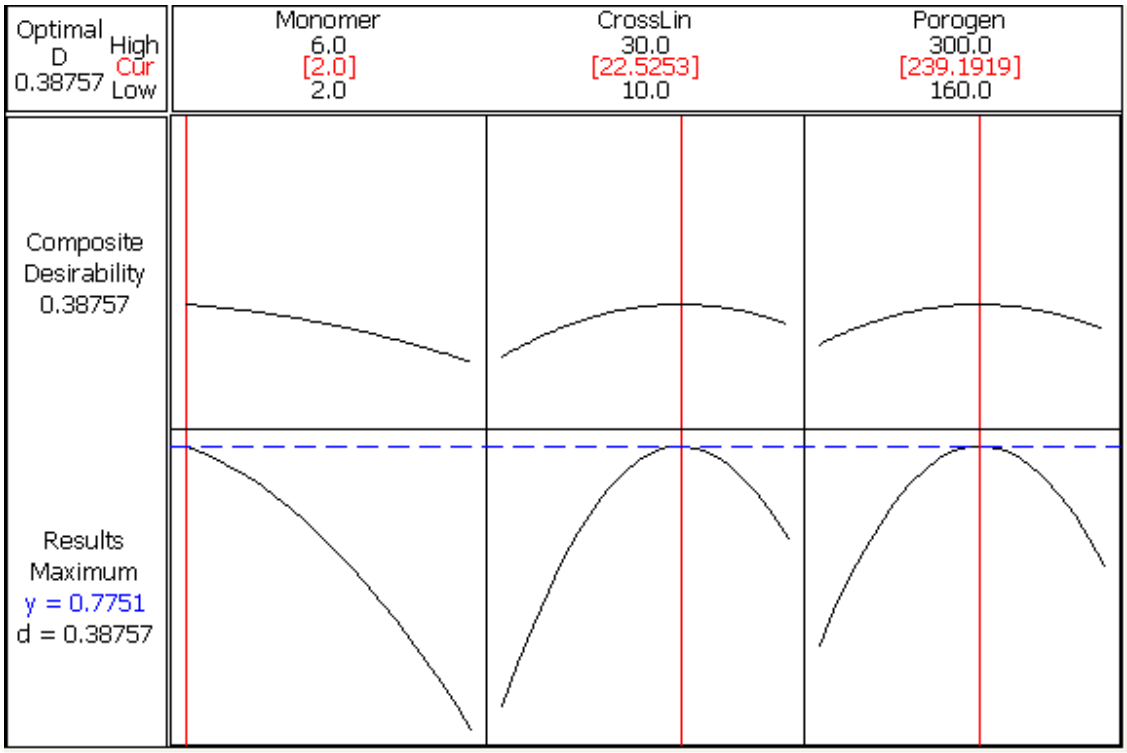

Figure 3.4. Optimization plot from Minitab to determine the optimal ratios in the pre-polymerization solution.

3.3. Optimization of the template removal procedure

3.3.1. Equilibration time

In previous studies,[6,49,50] the template removal step was the longest part of the MIP analysis, with extraction times as long as 25 hrs to ensure complete removal. An underlying problem with all MIPs is the possibility of template bleeding, which is due to incomplete template removal resulting in the potential for positive bias or diminished uptake capacity. Studies were completed to determine the actual time necessary before equilibrium of cotinine between the MIP and the template removal solution. In this study, a 50-mL portion of MeOH:HOAc (9:1, v/v) was exposed to a MIP with template intact and analyzed in duplicates. Every 10 mins, 0.1 mL of extraction solution was removed and analyzed by GC-MS, including a blank before any MIPs were added. This experiment also included analysis of the template removal solution prior to exposure to the MIP.

As can be seen from the data shown in Figure 3.4, cotinine very rapidly desorbs from the polymers into the solution, and slowly increases in concentration until leveling off around 100 mins into the template removal procedure. Therefore, we can safely state that at approximately 100 mins the cotinine in the acidic solution is in equilibrium with that in the polymer and a new solution is needed to remove further cotinine. For all future

template removal procedures, 100 min intervals were used instead of the three hrs reported previously.[6,49,50] This makes MIP preparation significantly faster.

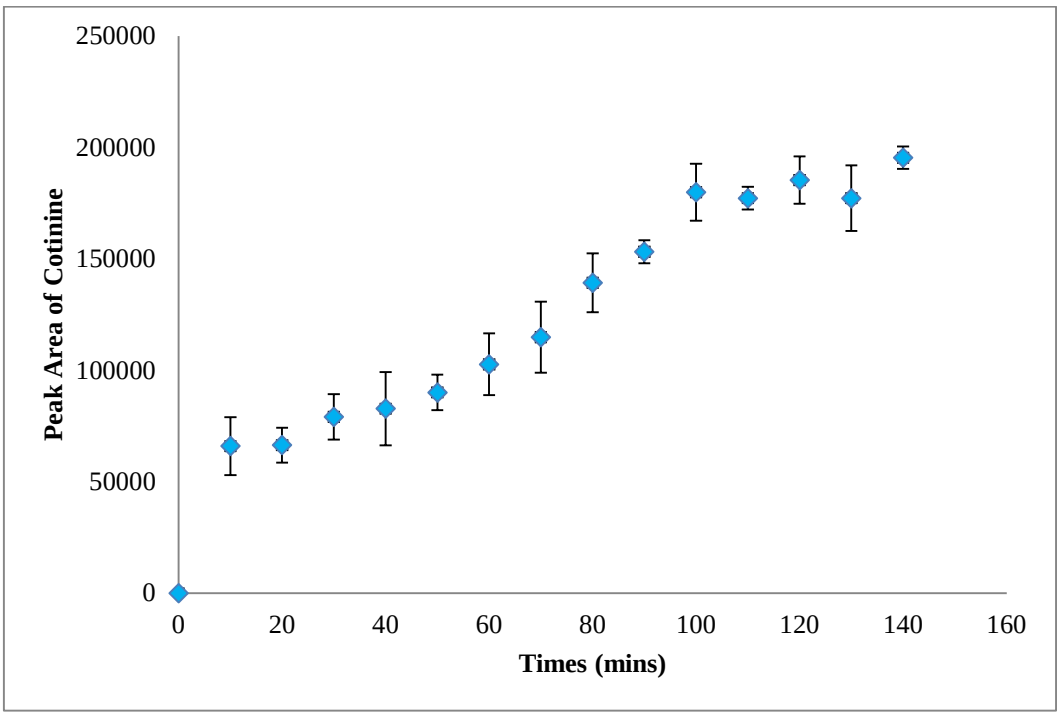

Figure 3.5. Template removal equilibrium study at 10 min intervals, analysis carried out by GC-MS in SIM mode (n=2).

3.3.2. Template removal studies for minimal wash cycles

To determine how many 100-min extractions were needed to remove a satisfactory amount of the template, a single MIP was placed in 50-mL of 9:1 MeOH:HOAc solution with stirring for 100 mins, rinsed with MeOH, and then placed in 50 mL of fresh template removal solution for each new extraction step. Before each rinsing step, a 2-mL aliquot of the solution was taken, evaporated in a scintillation vial, redissolved to 1.00 mL in a volumetric flask using DCM and analyzed by GC-MS in SIM mode. This was repeated six times to ensure sufficient data to evaluate template bleeding. The following graph, Figure 3.6, shows the amount of cotinine detected in each of the 50-mL solutions.

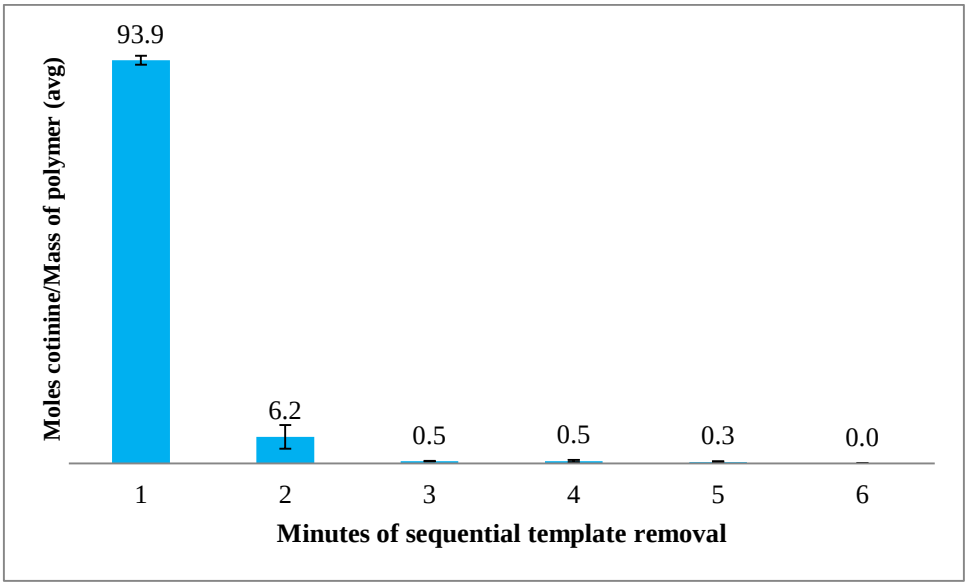

Figure 3.6. Template removal study, change in cotinine over 100 min intervals with 9:1 MeOH:AcOH as extraction solvent (n=2)

No cotinine could be detected in the sixth extraction solution. After 3 extractions the amount of cotinine in the solution was barely detectable. For further template removal procedures only three 100 min extractions were used for template removal. 100-mL extraction solutions were used since that amount covered the slides complete in the petri dishes used. A final 1-hr template removal step was also added using only methanol to ensure removal of residual acetic acid which aided in disrupting hydrogen bonds within the MIP, but may lower the amount of cotinine up taken during the following steps.

3.3.3. Sonication of MIPs for template removal.

At least one other study has used sonication as an extraction technique for bulk MIPs.[53] This method was applied to thin-film MIPs for the first time by the Bottaro group during the current research. The MIP films were placed into 50 mL beakers with 20 mL of 9:1 MeOH:HOAc for 1 hr intervals. A similar study was done parallel to this but instead using the more common stirring method for comparison (see Figure 3.7.).

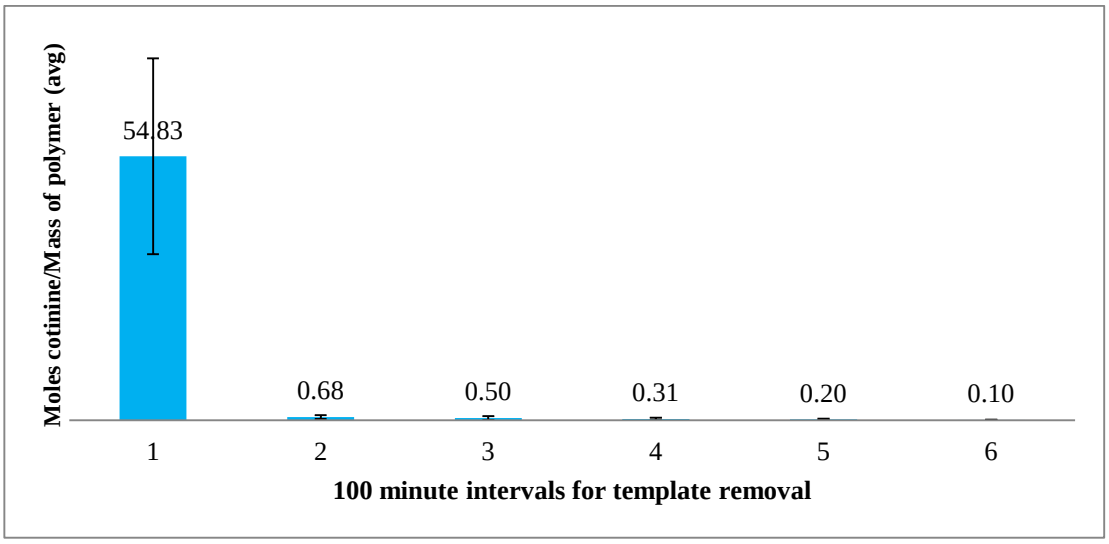

Figure 3.7. Sonication template removal at 100 min intervals

Comparing the results in Figures 3.6 and 3.7, it is clear that more template is removed using the traditional stirring method over the sonication method. Both methods initially remove the majority of cotinine in the first extraction, dropping off drastically in subsequent extractions with only minimal template bleeds occurring. Interestingly, the first extraction had a very large standard deviation which shows the uptake discrepancy between polymers. However, sonication also damaged the polymer by causing most of the polymer to flake off due to the aggressive sonic disruption. For this reason, all future template removal procedures relied on stirring to ensure efficient template removal without causing damage to the polymer structure. This also suggests that sonication may be better for bulk polymers compared to thin-films due to the lack of necessary binding of

the film to a glass substrate. Stirring based extractions are demonstrated as being superior to sonication techniques for thin-film MIPs.

3.4. Template bleeding in cotinine MIPs

Template bleeding is the term used to describe the transfer of small amounts of the template that remains in the MIP after template removal, which extracts into the final sample. This small amount of residual template is present in almost all non-covalent molecularly imprinted polymers and is one of the biggest drawbacks of MIPs. Bleeding causes error in the analysis when residual template is detected during analysis. This can give positive errors, especially when low concentrations are being measured. A simple solution for template bleeding is to use an isotopically labelled template to distinguish between template bleed and actual analyte being picked up by the polymer. Generally, isotopically labelled compounds are expensive. Isotopically labelled cotinine is priced at ~ $529 CDN for 100 mg at the time this thesis was written. Structurally similar analogues to the target analyte called pseudo-templates can be used to synthesize the MIP with the analyte being used only for uptake. This causes no doubt in the data measured for the analysis.

Template bleeding was assessed for thin-film cotinine MIPs by subjecting eight MIPs were subjected to the previously described template removal procedure. Four MIP

slides were placed in 100 mL of phosphate buffer adjusted to pH = 7.00 spiked to obtain 1.00 mg/L cotinine. The remaining four were placed in the same buffer solution with no cotinine. Following uptake these MIPs were placed in separate 50 mL beakers with 10.00 mL of 9:1 MeOH:HOAc with stirring for 60 mins to extract the cotinine. The MIPs were removed and rinsed with MeOH and the resulting solution was allowed to evaporate, the residue was redissolved in 1.00 mL of DCM and analyzed by GC-MS in SIM mode (See Section 2.4 for details). The cotinine left in the polymer that was detectable as template bleed was calculated as 0.451 mg/L from a calibration curve. This result showed that template bleeding is a significant issue for these cotinine MIPs and needed to be resolved before further studies were completed.

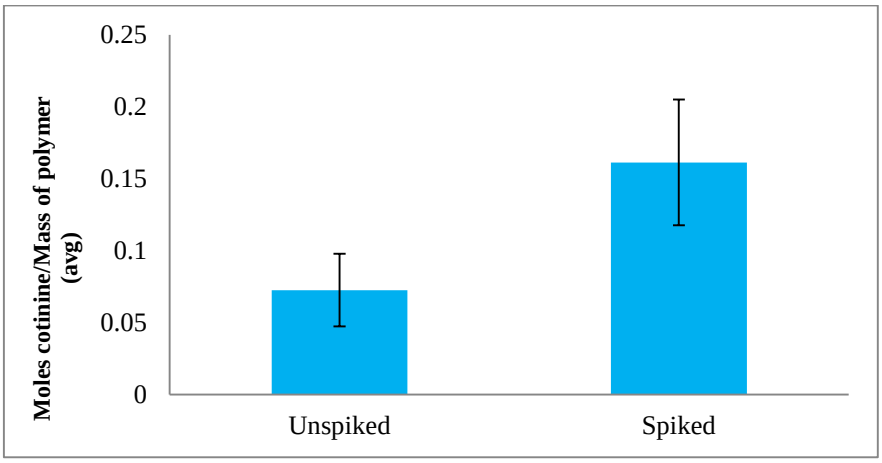

Figure 3.8. Assessment of template bleeding in cotinine MIPs by comparison of uptake from spiked and unspiked solutions (n=4).

3.4.1. Potential pseudo-templates for cotinine MIPs

A pseudo-template is required for most MIPs to correct for the problematic template bleeding issue. A pseudo-template may be any compound that has a very similar structure and functional groups to the analyte, ideally a deuterated version of the analyte or structural analogues. For cotinine, some of the possible pseudo-templates are shown in Figure 3.9: nicotine, myosmine, nicotyrine, nicotine-1′-*N*-oxide, and deuterated-cotinine. Nicotine would not be suited for this pseudo-template since real samples which should contain cotinine, will also contain nicotine since it is the precursor in metabolic systems. Measurement of nicotine is also of interest and its presence may preclude use of the MIP for detection of smoke exposure. Myosmine and nicotyrine do not have a carbonyl group, which is expected to be an important site for hydrogen bonding of cotinine by MIPs. Nicotine 1′-*N*-oxide and d-cotinine both have a carbonyl group, but are more difficult and expensive to obtain.

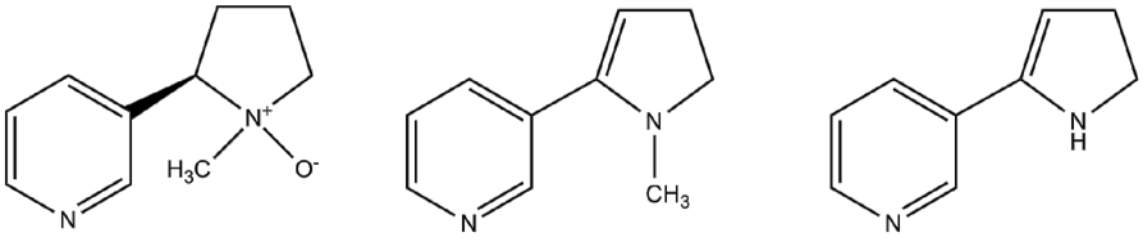

Figure 3.9. Structures of possible pseudo-templates (from left to right: nicotine-N-oxide, β-nicotyrine, myosmine)

3.4.2. Myosmine as a pseudo-template

Based on cost, availability and most importantly, the likelihood the simple structure of myosmine will produce an MIP with cross-reactivity toward cotinine; myosmine was chosen as a pseudo-template. Ideally a new full optimization protocol should be performed for myosmine, however, it was decided to proceed with optimization only if preliminary experiments were successful. Other than modifying mass ratios to be consistent with the molar mass of myosmine, no other changes were made to the procedure, described in Section 2.1. Also, to check the selectivity of these myosmine MIPs, 4 MIPs and 4 NIPs were made; uploaded in a standard solution containing 1 mg/L cotinine, analyzed by GC-MS in SIM mode and compared, as shown in Figure 3.10.

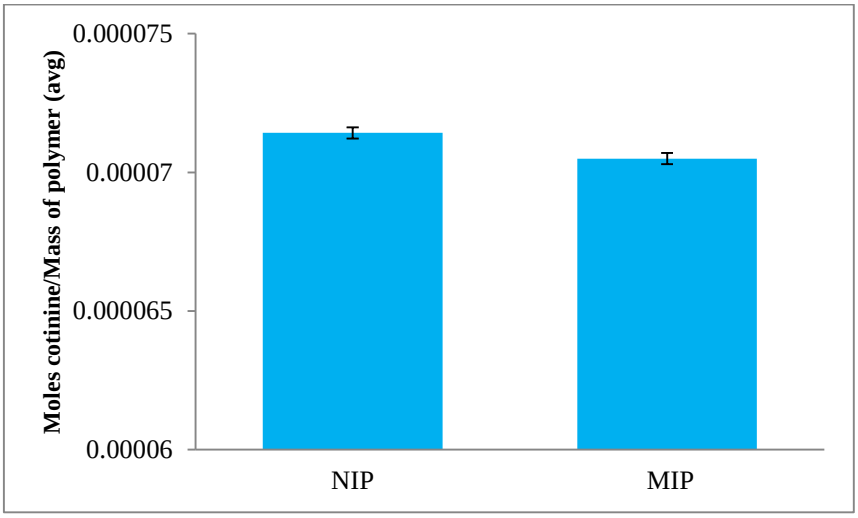

Figure 3.10. Comparison of MIP and NIP after uptake of cotinine when myosmine is used as a pseudo-template. (n=4)

The results show that the amount of cotinine taken up by both the MIP and the NIP were nearly identical. One promising result from this study compared to the cotinine MIPs is that myosmine MIPs showed much better reproducibility, which was one of the biggest limitations for cotinine MIPs. The amount of template bleeding while using myosmine MIPs is inconsequential since all the cotinine detected in the polymer was from the uptake solution. The data also suggests that the myosmine MIPs did not show selectivity towards cotinine compared to cotinine MIPs, therefore the carbonyl group is an important feature for molecular recognition by these MIPs, likely through hydrogen bonding between the monomer and cotinine at the carbonyl. Therefore, the next selection of a pseudo-template was made to ensure there was an available carbonyl group for hydrogen bonding.

3.4.3. 1-Phenyl-3-pyrazolidinone as a pseudo-template

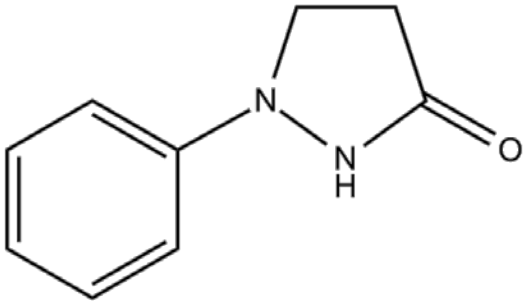

Figure 3.11. Structure of 1P3P

To meet this goal 1-phenyl-3-pyrazolidinone (1P3P) (Figure 3.11) was chosen as a pseudo-template due to the carbonyl group present which cotinine also has and it is arguably the determining factor in cotinine MIPs. Unfortunately, 1P3P was not a successful pseudo-template; no cotinine was picked up by the 1P3P MIPs. This could be because the positions of nitrogen atoms together on the five-membered ring have electro-static and steric effects.

3.5. Optimization of the uptake solution

3.5.1. Study of the length of time required to maximize cotinine uptake

Previous studies using thin-film MIPs used an uptake time of 1.5 hrs.[6] This uptake study was completed using the optimized conditions reported in this thesis, only the length of uptake was changed. Two MIPs prepared with cotinine were placed in a petri dish with 100 mL of the 7.00 pH phosphate buffer uptake solution spiked to 1.00 mg/L of cotinine. The time of uptake was varied from 15 to 120 mins. The extracts were analyzed by GC-MS in SIM mode and the results are shown in Fig 3.12. Uptake seems to reach an equilibrium within the first 30 mins of exposure but some increase was detected at 120 mins. The second study showed an increase in cotinine uptake with the length of time. Due to inconclusive results and the fact that the previously used 1.5 hrs gave good results, no further tests were completed to change the uptake time. However, it was also concluded that if it was necessary to streamline the method, 30 mins would be satisfactory based on these results.

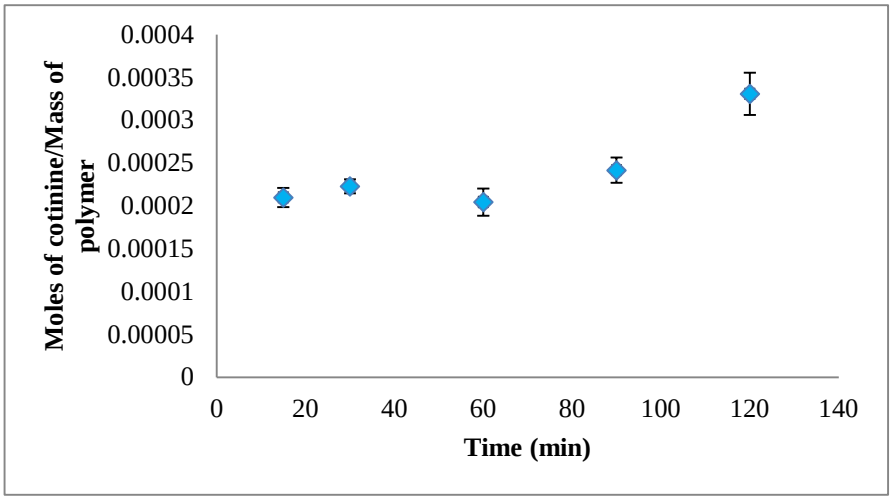

Figure 3.12. Study of the optimal length of uptake time for cotinine MIPs

3.5.2. Varying pH of buffer solution for uptake of cotinine

To study the selective non-covalent interactions between the analyte and the monomer, the pH of the uptake solution was varied to determine which pH would give a better result. At an acidic pH, it would be expected that hydrogen bonding would be minimized due to the protonation of the carbonyl in cotinine. This study can help us understand how strong the hydrogen bonding interactions are between cotinine and the MIP compared to other various competing interactions such as π-π interactions and van der Waals forces. Two buffers were chosen to represent acidic and neutral pH at 4.69 and

7.00. See Section 3.1 for details on the preparation of the buffer solutions. Sodium acetate and phosphate buffer are readily available and were therefore chosen as the buffer reagents for pH 4.69 and pH 7.00, respectively. No basic buffer was chosen for this study since we are primarily studying the effect of hydrogen bonding which should not be impacted at a basic pH. This study was completed using MIPs and NIPs to determine the degree of non-specific interactions.

In Figure 3.13, it can be seen that using the phosphate buffer at pH = 7.00 gives a much higher uptake of cotinine than from a sodium acetate buffer at pH = 4.69. As expected, the MIPs gave much higher results than the corresponding NIPs showing selective interactions and only a small amount of non-selective interactions from the NIPs. Since the phosphate buffer at a neutral pH gives a much higher uptake compared to the slightly acidic pH buffer it can be assumed that hydrogen bonding does not occur in the sodium acetate buffer or is diminished and it is the primary mode of binding in the phosphate buffer. Although selectivity is, in fact, better in acidic conditions suggesting that much of the non-selective binding is through hydrogen-bonding. These results may also suggest that the methanol only washing step in the template removal is necessary to remove the acetic acid from the MIPs before uptake, as the presence of the acid might interfere with the hydrogen bonding interactions.

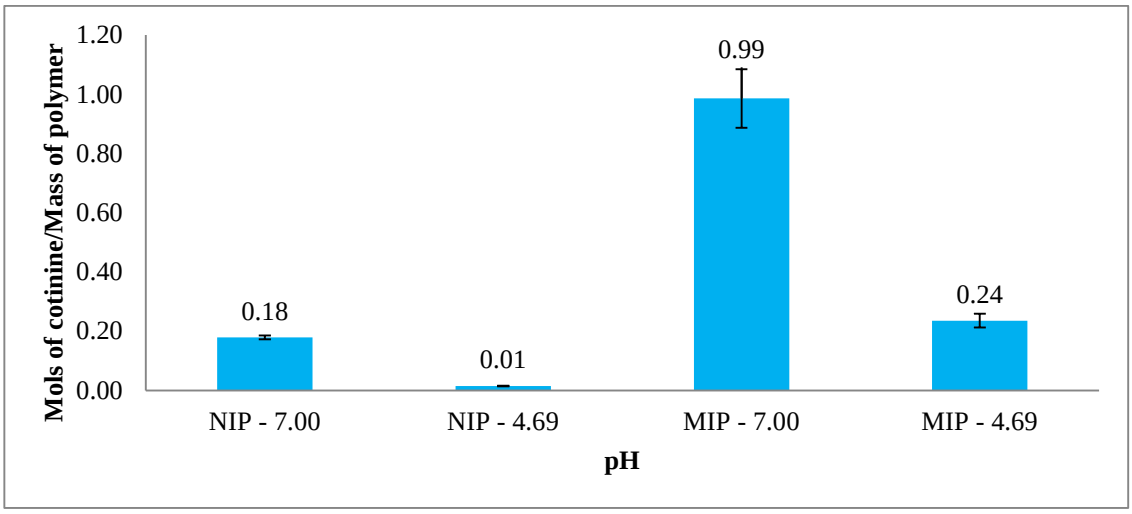

Figure 3.13. Comparison of uptake solution pH to study non-covalent interactions when analyzed by GC-MS (n=4).

Table 3.2. Optimized parameters for cotinine MIP analysis

Template (mols)	1
Monomer (mols)	2
Cross-linker (mols)	22.5
Porogen (µL)	239.2
Wash time (mins)	100
# of wash cycles	3
Uptake time (mins)	90
Uptake pH	7.00 (phosphate buffer or biological sample)
Final removal (mins)	60

Chapter 4. Analysis of cotinine in urine using fully optimized molecularly imprinted polymers

After much optimization of the MIP method, the goal of this thesis is to apply the results of using these fully optimized cotinine MIPs for the uptake of cotinine from human urine. This chapter will focus on the comparison of these fully optimized MIPs with their NIP counter parts in terms of key performance indicators, such as their imprinting factor. The imprinting factor, IF, is calculated as the ratio of the cotinine uploaded to an MIP *vs*. an NIP. Scanning electron microscopy was also completed for qualitative assessment of porosity within the polymers. Finally, the MIPs were used to construct a calibration curve using spiked cotinine in human urine to determine if matrix effects cause changes to uptake levels or calibration. Cotinine was used as the template instead of the pseudo-templates due to the lack of specific interactions when pseudo-templates were used. It was determined that the template bleeding effect issue would be minimal in comparison to a lack of specific binding sites in the polymer.

4.1. Comparison of MIPs and NIPs

After optimization, the next step towards developing a working MIP for the analysis of any analyte is to determine how selective the interactions are. This is most commonly done by making a non-imprinted polymer that is the same as an MIP except no template is added to the pre-polymerization solution. This provides a measure of the amount of specific interactions present in the imprinted polymer. Ideally the NIP should experience little to no binding of the target analyte, although in reality it is impossible to fully eliminate all non-selective interactions. Using the optimized conditions determined in the previous section, 4 MIPs and 4 NIPs were used for the uptake of 1.00 mg/L of cotinine for 1.5 hrs. The extracts were analyzed by GC-MS and the results are shown in Fig 4.1.

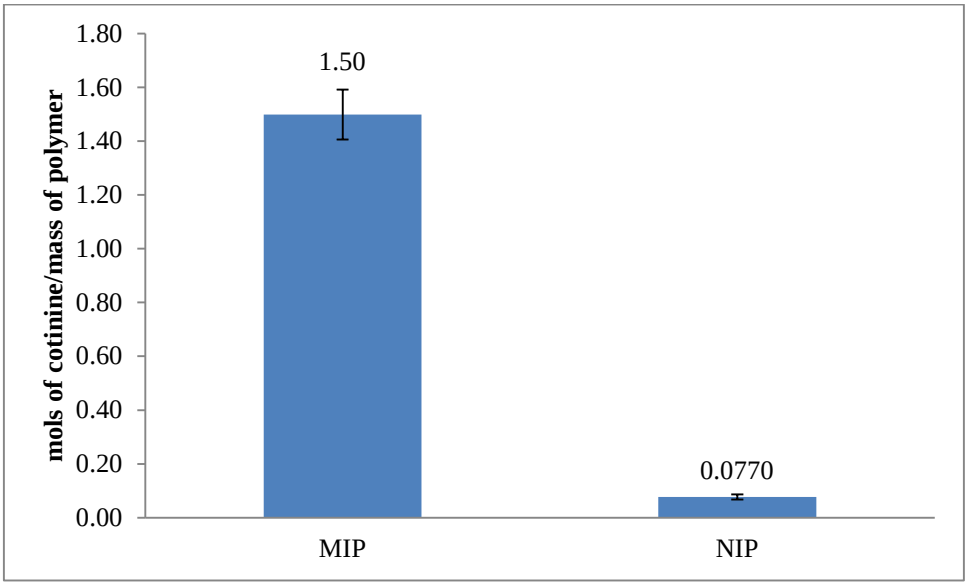

Figure 4.1. MIP vs NIP selectivity

The amount of cotinine being taken up by the MIP is significantly higher than that by the NIP. The NIP had a slight amount of cotinine taken up due to non-specific interactions within the polymer matrix. This result shows that MIPs made with cotinine are very selective for cotinine. For these cotinine MIPs, the imprinting factor was determined to be 19.5, which is very high as the neutral value would be 1.

4.2. Scanning electron microscopy of cotinine MIPs

As with most MIPs, scanning electron microscopy (SEM) is widely used to characterize the morphology of the polymers. Another common technique to assess the pore size within the polymer would be the Brunauer, Emmett and Teller (BET) Theory which the adsorption of gas molecules onto solid surfaces. This study does not focus on porosity or surface area therefore we will focus on SEM to obtain an image of the pores in cotinine MIPs.[54] SEM is a quick and easy technique to use to determine if a molecularly imprinted polymer is highly porous. As shown in a study by Sergeyeva et al., SEM images of the polymer before and after template removal, show how the pores open as template is removed.[55] The analysis was done on a FEI-MLA 650F Scanning Electron Microscope with an accelerator voltage of 15 kV, magnification of 59680x. Figures 4.2 and 4.3 below show the porosity difference between cotinine MIP and NIP.

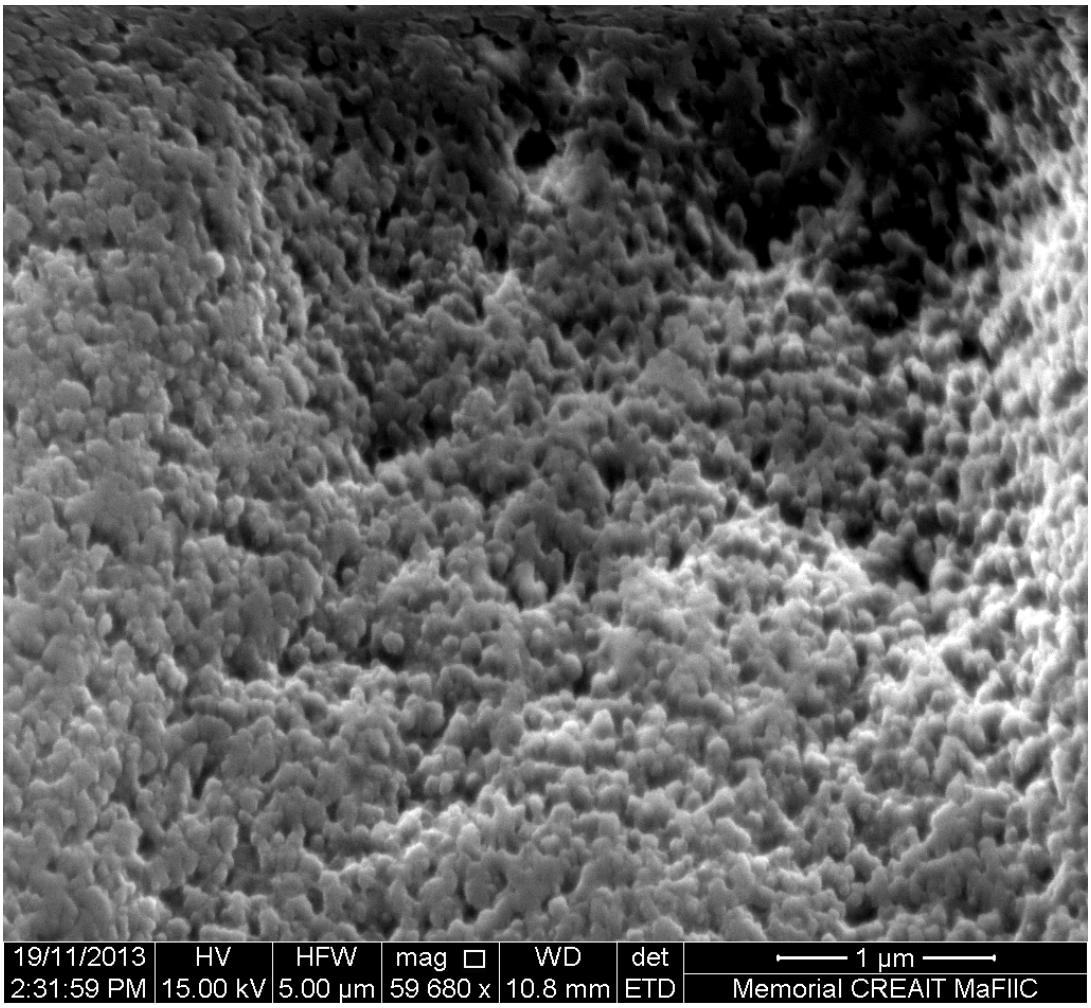

Figure 4.2. SEM image of cotinine MIP showing pores

From the inspection of the SEM images (Figure 4.2), pores of up to approximately 0.05 µm in diameter were observed. This confirms that these opaque polymers are porous, which develops during synthesis. To contrast, NIP images (Figure 4.3) were also

obtained which do not show any significant porosity at the same magnification of 59680. This shows that the template influences the phase separation process leading to pore formation, higher surface area and that differences between MIPs and NIP films is not limited to the molecular level.

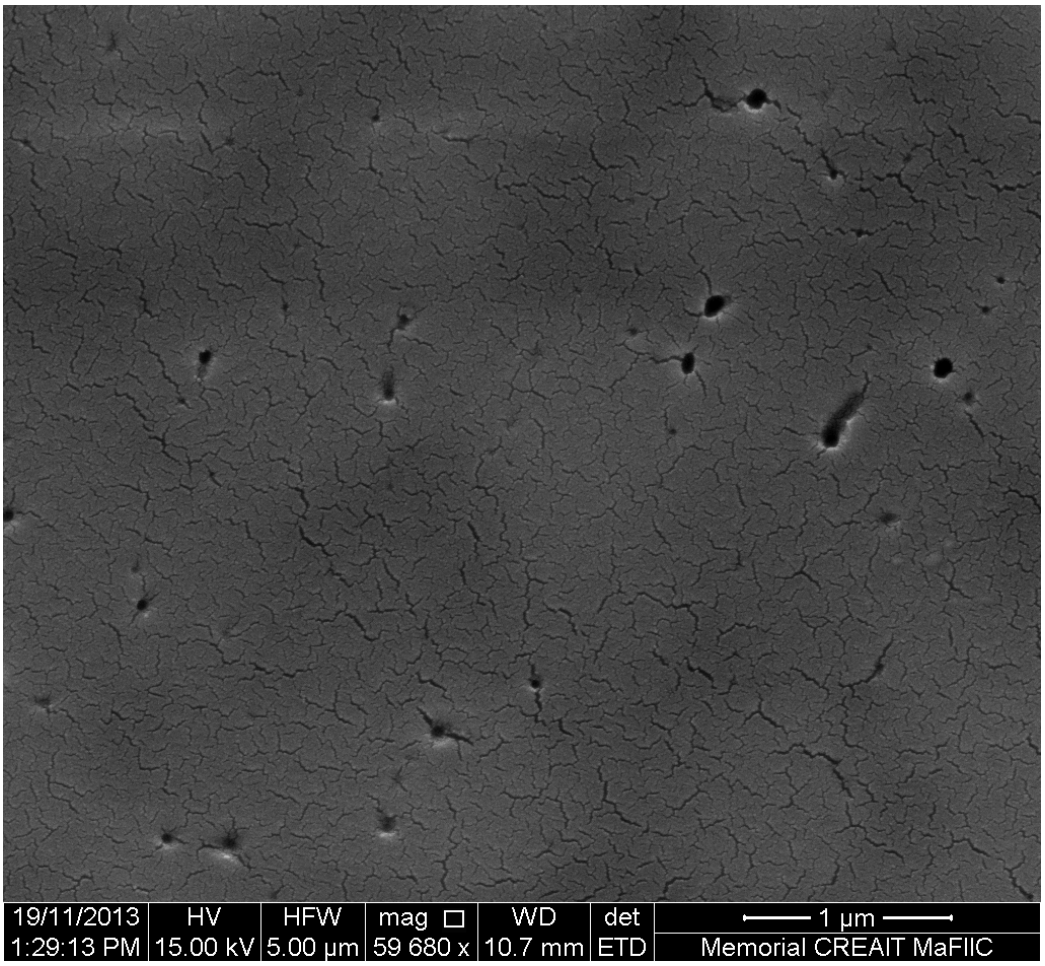

Figure 4.3. SEM image of NIP showing lack of pores.

4.3. Analysis of cotinine in human urine using thin-film MIPs

As a preliminary test to determine if our thin-film cotinine MIPs can be used to accurately determine the amount of cotinine in human urine, a calibration curve was constructed using a urine matrix spiked with cotinine. The urine samples were collected from a single male volunteer. Note that urine samples were not collected midstream which may cause discrepancies. Also, variations in the composition of each urine sample can be present due to the influence of diet and lifestyle. Each 100-mL sample of urine was spiked with increasing concentrations of cotinine ranging from 0 to 2 mg/L. All other steps were identical to previous studies.

Figure 4.4 shows a calibration curve which can be used to analyze the concentration of cotinine in human urine with moderately good linearity. Improvements can be made on the speed and reproducibility of the analysis by using another detector, the DESI-MS. The graph also shows that the Y intercept is not at zero, which it is for most calibration curves. In this case it is primarily due to template bleeding which is a major limitation of the method and would need to be corrected by using a deuterated template or an analogous compound if quantitative analysis is to be done.

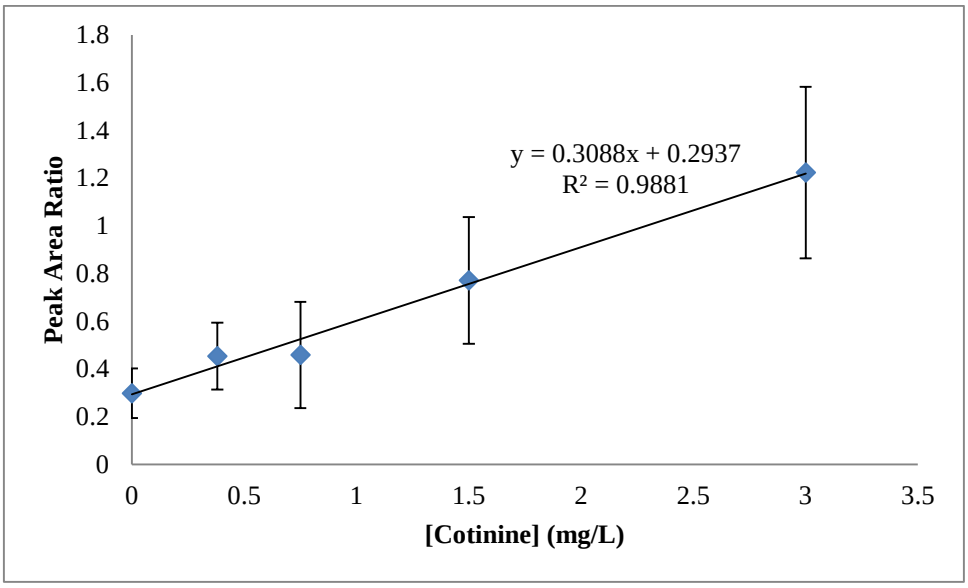

Figure 4.4. Calibration curve for the analysis of cotinine in human urine using MIPs (n=2).

4.4. Analysis of cotinine in human saliva using thin-film MIPs

Since saliva is a less invasive way to obtain bodily fluids it was also studied as a matrix to determine exposure to nicotine. Instead of using 100 mL that was used in the analysis of urine, 1 mL of saliva was used and pipetted directly onto the MIP on a glass slide, left for 1.5 hrs with no stirring possible then rinsed off with deionized water. Various samples of saliva were collected from the same male volunteer and then spiked with cotinine before it was introduced to the polymers. As it is difficult to collect large volumes of saliva, only a small volume was used. Dilution up to 100 mL with buffer

would have allowed for a more comparable study, but the concentration of cotinine in saliva is low compared to urine.[4]

Results from the analysis of cotinine using saliva as a matrix were inconclusive. No cotinine was found by GC-MS analysis. The saliva matrix may limit the uptake because of the viscosity and the slightly basic and complex composition that may interfere with uptake sites. This shows that stirring plays a huge role in the uptake step as it replenishes the concentration of the analyte at the polymer surface and will always be used when uptake is carried out.

A new approach was adapted using a larger volume of 2 mL of spiked saliva with cotinine at 1 mg/L. A tiny (~1 cm) stir bar was used in a 5 mL vial for this experiment. Stirring was used in this study to determine the effect stirring has on the uptake of cotinine. Unfortunately, satisfactory results were not obtained. Previous studies show that saliva has the lowest concentration of cotinine in available fluids and saliva is also the most complex and inconsistent matrix from the human body as its compounds can vary greatly depending on diet and lifestyle. Since normally 100 mL of uptake solution were used and 2 mL is 50 times less it would be desirable to adjust the scale of the experiment, e.g. mass of MIP, volume of extract and final volume of concentrated extract. An artificial saliva matrix would be the next logical step, but was not studied due to time constraints.

4.5. Analysis of cotinine in human samples using desorption electrospray ionization-mass spectrometry, DESI-MS

With promising results in hand for analysis of cotinine in a urine sample using gas chromatography, DESI-MS was used to analyze MIPs directly after being exposed to urine samples spiked with cotinine. The main reasoning for using DESI-MS over GC is to decrease the experiment time with the removal of the final step: a single hr of template removal before evaporation and dissolving the residue in DCM. Elimination of this single step removal may help reduce errors and provide better results. To the best of our knowledge this is the first time that cotinine has been analyzed using thin-film MIPs and DESI-MS together.

As before, 100 mL samples of urine were spiked with varying concentrations of cotinine and left to stir for 1.5 hrs. The samples were rinsed with water and were then blown dry with N_2. Any flaking polymer was also removed to prevent contamination of the DESI with unnecessary polymer. DESI-MS parameters can be found in Section 2.5 of this thesis.

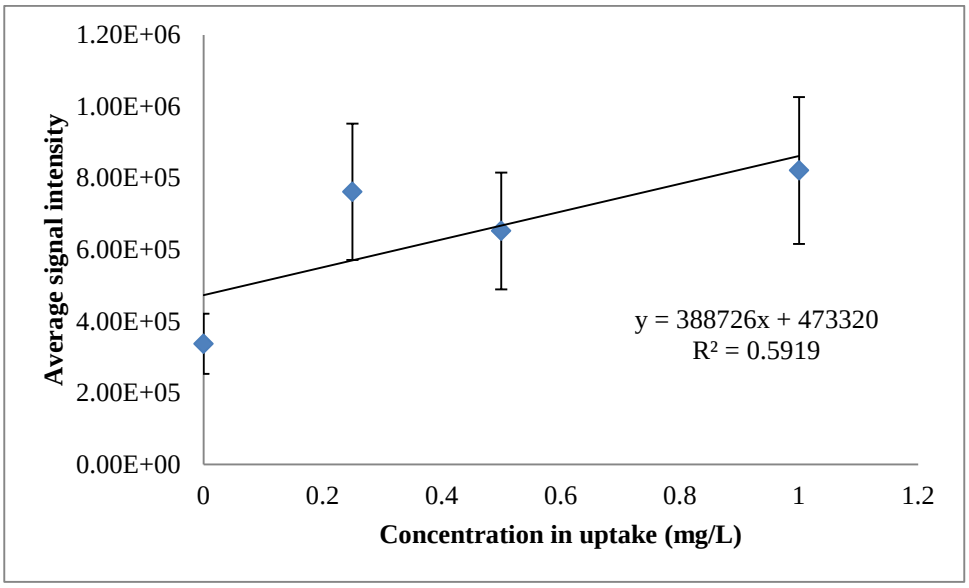

Figure 4.5. Analysis of cotinine in human urine by way of DESI-MS (n=2)

Unfortunately, our preliminary results show poor reproducibility and poor linearity which may be somewhat due to only two samples being used per data point. Attempts at using internal standards have failed to date, therefore giving us a much lower reproducibility. Lower concentrations of cotinine seem to be more problematic which may be caused by template bleeding issues. Work is required to optimize the DESI-MS method before improved results can be obtained. The polymers are also not sufficiently robust to tolerate the impact of the DESI spray and thus film flaking may be the cause of some of the reproducibility issues experienced.

4.6. Robustness vs. Selectivity

To improve the DESI-MS data, cotinine MIPs need to be more robust to prevent flaking during the analysis. This also raises concerns that if the robustness is improved, how this will affect the selectivity. To minimize the loss of the good imprinting factors obtained, new cross-linkers and porogen systems were selected to be similar to the EDGMA and 4:1 MeOH:H_2O used previously (see Figure 4.1). Triethylene glycol dimethacrylate (TEDGMA) was chosen as one of the cross-linkers as it is essentially a longer chained analogue of EGDMA. The longer linkage might improve the robustness of the polymers and it is easily dissolved in water compared to other cross-linkers.[42] Trimethylolpropane trimethacrylate (TRIM) was also chosen as a potential cross-linker for comparison since it is much larger than either of the previous compounds (see Fig 4.6 for the structures of cross-linkers used). The solubility is important especially in the phase separation process. As for the porogen composition, the systems with higher and lower water to methanol ratio were compared. Octanol was considered to create a three-solvent system because it makes a less polar system than using methanol and water alone. MIPs using these pre-polymer compositions were prepared and uploaded in 1 mg/L cotinine in 100 mL of buffer solution. The extracts were analyzed by GC-MS and the results are shown in Table 4.1.

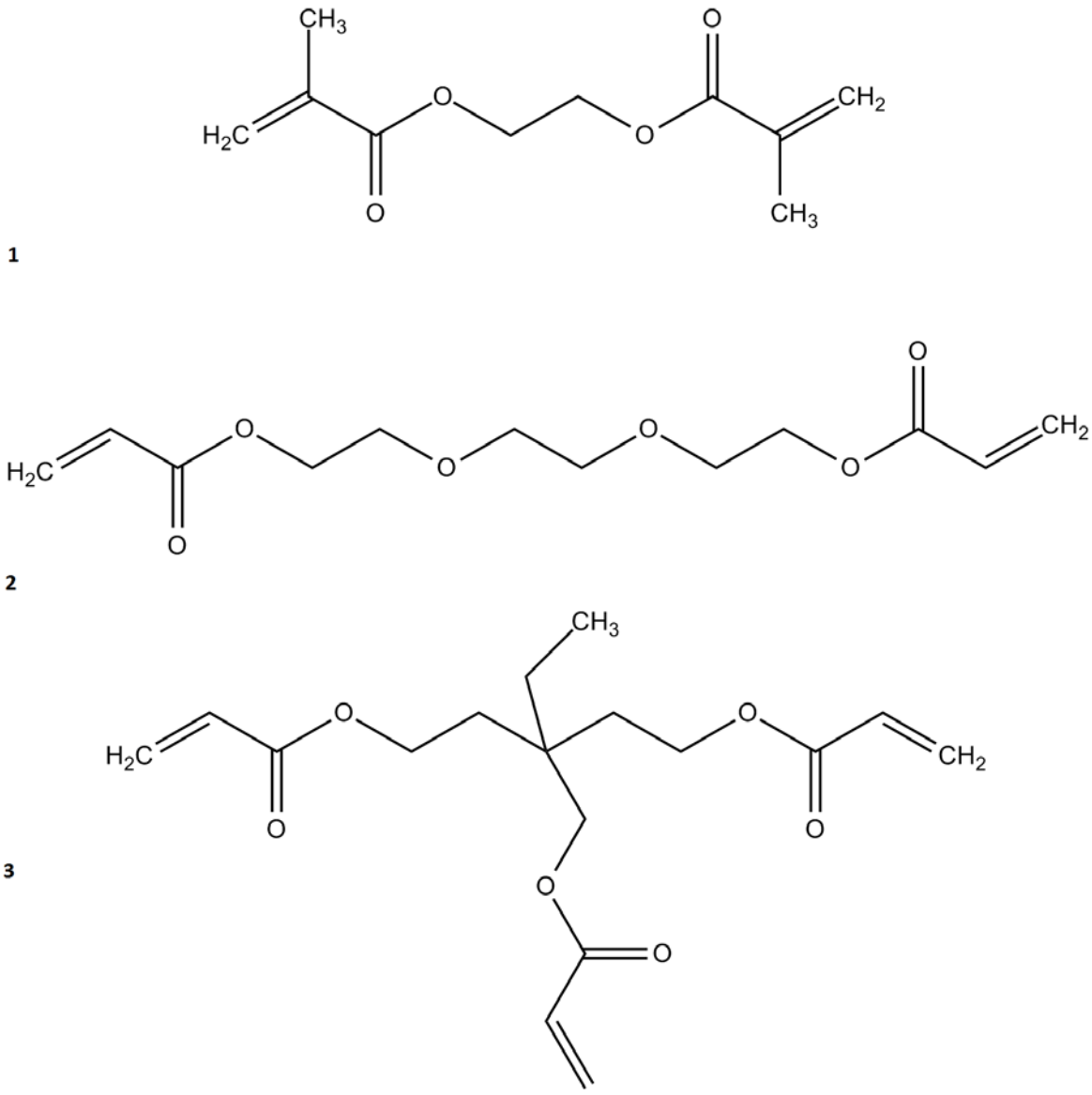

Figure 4.6. Graphical representation of cross-linkers tested(1:EGDMA, 2:TEGDMA, 3:TRIM).

Table 4.1. Imprinting factors for various pre-polymer compositions

Cross-Linker	Porogen	Imprinting Factor	Robustness
EGDMA	4:1 MeOH:H_2O	19.5	Average
TRIM	13:1 MeOH:H_2O	0.985	Poor
TRIM	MeOH	0.677	Average
EGDMA	10:30:2 OctOH:MeOH:H_2O	1.33	Good
TEGDMA	10:30:2 OctOH:MeOH:H_2O	0.981	Average
EGDMA	7:7:2 OctOH:MeOH:H_2O	5.19	Average
TEGDMA	7:7:2 OctOH:MeOH:H_2O	1.86	Average
TEGDMA	7:2 MeOH:H_2O	1.02	Poor
TEDGMA	6:2 MeOH:H_2O	0.874	Average
TEDGMA	5:2 MeOH:H_2O	5.87	Poor
TEDGMA	4:2 MeOH:H_2O	1.33	Good
TEDGMA	4:1 MeOH:H_2O	4.58	Good

Most of the compositions did not produce acceptable results, with imprinting factors less than 1. Imprinting factors less than 1 show that the selectivity of the MIP compared to the NIP is poor and would not be acceptable for use in quantitative analysis. Three compositions did however produce good results. The first composition used EDGMA with the addition of octanol to give an IF of 5.19. The change to this composition resulted in a doubling of the amount of organic solvent with the addition of octanol. This gives a good mix of lowering the polarity slightly while maintaining the aqueous portion, which seems to play a key role in uptake from aqueous matrices. The polymer also became more robust with the porogen change and no longer flaked off which should improve DESI-MS results.

The next composition that gave a high IF (4.6) used TEGDMA in a mixture of 4:1 (MeOH:H_2O). While TEGDMA maintained the same IF when compared to EGDMA with the same porogen composition, the robustness improved greatly. A ratio of 4:2 was also tested for TEDGMA and gave a good imprinting factor but had poor robustness. Therefore, a larger cross-linker can give more robust polymers for cotinine MIPs. It can also be suggested that the porogen plays a bigger role in the selectivity of the MIPs compared to the cross-linker which makes sense due to the porogens role in solvation of the pre-polymerization complex, porosity and structure of the polymers.

Chapter 5. Conclusions and future work

In conclusion, it has been shown that thin-film molecularly imprinted polymers can be used for the analysis of cotinine in human samples using GC-MS and to some extent, DESI-MS as well. After rigorous optimization studies, it was determined that only certain compositions could be used to yield acceptable results, namely when EGDMA and TEGDMA was used as cross-linkers in conjunction with a porogen containing more than half organic solvent. Comparing our imprinting factor of 5.9 to the original study by Yang et al., which yielded a factor of 6.2, they are comparable considering they are made using a different polymerization method. It is possible that the monolith MIPs have a better imprinting factor due to their increased surface area in comparison to thin-film coatings we have developed. Polymers which were synthesized in large amounts of organic solvent were found to flake off the glass slide very easily and pose problems with subsequent analyses. The optimized polymers had a sufficient degree of robustness to withstand the harsh acidic environment used for template removal. For GC-MS analysis, it was not important if the polymers were robust during the final template removal since the cotinine would be collected regardless. Though flaking during the uptake step would cause error compared to other steps due to the potential loss of analyte, it was not a concern as the extraction solvent is more forgiving than the acid wash used in template removal. For DESI-MS analysis the polymers could require a much higher degree of

robustness to provide acceptable reproducibility and initial studies towards meeting this goal have been achieved by varying the cross-linker and porogen used.

Improvements on the current work might be possible through the use of a deuterated version of cotinine as an internal standard to provide more accurate GC-MS analysis (non-deuterated nicotine would be impossible to use due to its potential presence in human samples from second hand smoke exposure). This will eliminate the template bleeding problem and allow for better quantitative analysis but at a cost. No acceptable pseudo-template has been found in this work but should be possible with more analogous templates being tested for MIP work. Perhaps a computational study would allow for both steric and electronic (e.g. hydrogen-bonding ability) similarities between potential pseudo-templates to be assessed. Using the current polymers, GC-MS is better suited for the analysis of cotinine in thin-film MIPs, compared to DESI-MS. This is due to the fact that the current polymer system can break down and flake off of the glass slide which is extremely needed for DESI measurements.

Due to the large amount of acidic wash needed to remove the initial template from the polymer, this work becomes very wasteful and cost ineffective. If cheaper and more effective methods of template removal were discovered, then the cost and time required for the MIP analysis would be reduced. This is especially problematic if the polymers were to become commercialized, and new methods would have to be discovered first.

Future work will include testing the ability for these polymers to be reused after the initial analysis which will cut down on experimental time greatly. Ideally for an MIP to be reused it must be robust enough that it can endure multiple acidic washes without the pores inside of the polymer collapsing. DESI-MS analysis should also be tested on the more robust MIP compositions discussed in Section 5.3. The MIPs prepared during this work have the potential to be produced in large quantities as thin-film polymers are suitable for easy integration into existing work flows by chemists and scientists alike, even those with little experience using MIPs. Studies into the shelf life and stability of the MIPs are still needed. The commercial potential for MIPs exists, but they must be fine-tuned to become worthwhile. There is value in pursuing this further as there are few MIPs that are robust and selective enough to give excellent results from extractions.

Chapter 6. References

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