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USING MOLECULARLY IMPRINTED POLYMERS FOR THE SELECTIVE EXTRACTION OF ANALYTES

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By

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Abbreviations

MIP	Molecularly Imprinted Polymers
NIP	Non-Imprinted Polymer
LLE	Liquid-Liquid Extraction
SPE	Solid Phase Extraction
SPME	Solid Phase Microextraction
MISPE	Molecularly Imprinted Solid Phase Extraction
MAA	Methacrylic Acid
EGDMA	Ethylene Glycol Dimethacrylate
DVB	Divinylbenzene
HPLC	High Performance Liquid Chromatography
PDA	Photo Diode Array
MS	Mass Spectrometry
ESI	Electrospray Ionization
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
MRM	Multiple Reaction Monitoring
LOQ	Limit of Quantitation
LOD	Limit of Detection
% RSD	Percent Residual Standard Deviation
ACN	Acetonitrile
MeOH	Methanol
DCM	Dichloromethane
NH_4OH	Ammonium Hydroxide
NH_4Ac	Ammonium Acetate
NaOH	Sodium Hydroxide
HLB	Hydrophilic-Lipophilic Balance
MCX	Mix Mode Cation Exchange
NAB	N-nitrosoanabasine
NAT	N-nitrosoanatabine
NNN	N-nitrosonornicotine
NNK	4-(methlynitrosamino)-1-(3-pyridyl)-1-butanone
NNAL	4-(methlynitrosamino)-1-(3-pyridyl)-1-butanol
COT	Cotinine
NIC	Nicotine
T3HCOT	Trans-3'-hydroxycotinine
NorNIC	Nornicotine
Gluc	Glucuronide
SEM	Scanning Electron Microscopy
FT-IR	Fourier Transform Infrared Spectroscopy
TGA	Thermogravimetric Analysis
FDA	Food and Drug Administration

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Abstract

Sample preparation is one of the most critical steps in bioanalysis. Traditional methods such as solid-phase extraction and liquid-liquid extraction are laborious and ultimately not selective enough to properly separate target analytes from matrix and interferents. The lack of proper sample preparation interferes with the analytical instrument's ability to properly detect, identify, and quantitate analytes. Molecularly imprinted polymers (MIPs) are synthetic polymers that have been "imprinted" with a template analyte in a co-polymer system. MIPs are capable of selectively extracting analytes from complex biological matrices and can be employed in off-line, benchtop extractions or for on/in-line instrument extractions. MIPs offer faster and more selective sample preparation, leading to high throughput sample analysis with minimal matrix effects.

The overall goal of this study was to create a molecularly imprinted polymer system for the direct analysis of target analytes in biological matrices with minimal sample preparation. A commercial MIP designed for tobacco specific nitrosamines (TSNAs) was characterized for the extent of cross-selectivity with other tobacco analytes, and the extraction of the urinary metabolite cotinine was developed utilizing the cross-selectivity of the commercial MIP. A MIP-based HPLC column was then developed by packing the commercial TSNA MIP material into an empty stainless, steel HPLC column under slurry packing conditions. A method for the quantitation of TSNAs in nicotine and tobacco products was developed and validated. The MIP-HPLC column was characterized for its chromatographic properties and packing uniformity and applied to real world samples. Finally, an in-house polymer specific for cotinine was developed and characterized. The results of this study can be utilized to develop MIPs for other matrices and apply them towards the direct analysis of target analytes in biological matrices with minimal benchtop preparation prior to instrumental analysis.

Chapter 1: Efficient and Selective Extraction of Compounds in Complex Matrices using Molecularly Imprinted Polymers (MIPs) and their Relevance to Bioanalysis

This chapter has been drawn from the article published in Journal of Analytical Toxicology

(Mulder and Halquist; 2020, DOI: 10.1093/jat/bkaa079)

1.1 Introduction

The role of a bioanalytical laboratory is to identify and quantify drugs and biomarkers of exposure in biological matrices. Such matrices include blood, plasma, urine, and tissue, which may contain high concentrations of fats, carbohydrates, proteins and salts. The presence of these extraneous compounds, known as matrix effects, can interfere with an analytical instrument's ability to accurately detect and quantify the target analyte (1). To combat this, extensive sample preparation is often required to isolate the analytes of interest from the biological matrix prior to instrumental analysis (2).

Common methods to isolate the analyte of interest are liquid-liquid extraction (LLE) and solid phase extraction (SPE). LLE is often viewed as labor intensive and difficult to automate while consuming large volumes of hazardous solvents with limited selectivity for target compounds. LLE is frequently complicated by the presence of emulsions (3). SPE is typically the preferred method for extraction due to: 1) facilitates cleaner, more selective sample extracts, 2) can be applied to a wide array of compounds, and 3) is capable of being automated. SPE requires less sample and solvent volumes to be used for extraction compared to LLE (4). SPE has further advantage over LLE through the various types of sorbent beds that allow for numerous mechanisms of action for sample extraction. Depending on the chemical properties of the target compound, SPE sorbent beds can allow for extraction of the target from the biological matrix through either normal or reverse phase interactions, weak cation or anion exchange, or a mixed mode of interactions to optimize the extraction of the target compound(s) (3). However, the

interactions between the analyte and the SPE sorbent beds are still considered to be non-specific, which can lead to co-extraction of interfering matrix compounds (5, 6). Further, high potency drugs, such as novel psychoactive substances and synthetic fentanyl analogues, or biomarkers of exposures are present at low concentrations in biological matrices. This requires efficient extraction and pre-concentration of samples prior to analysis.

Molecularly imprinted polymers (MIP) are synthetic polymers that have been "imprinted" with a template analyte in a co-polymer system (7). The co-polymer system consists of a template analyte, a functional monomer, and a cross-linking monomer. The template is either the analyte of interest, or an analyte that has a similar size, shape, and functional group chemistry to the analyte intended to be measured. The cross-linking monomer creates the porous backbone of the polymer and anchors the functional monomer in place (8). The target analyte is extracted post polymerization and the 3D cavity left behind allows the same analyte, or compounds of a size, shape, and functional group chemistry to selectively rebind to the functional monomer inside the imprinted cavity (6, 9). MIPs are often compared to antibodies for immunoassays because the phenomena of selective recognition is similar to antibody-compound interaction (10, 11). However, unlike antibodies, MIPs are synthetic and require no animal components, which reduces the cost and time of production. Furthermore, compared to immunoassays, MIPs are more robust in their ability to withstand extreme pH and temperature ranges (12).

The concept of molecularly imprinted polymers was first published in the 1970's by Wulff et al., for the separation of enantiomers with a covalently-bonded MIP (13). Mosbach et al. reported the creation of the first non-covalently bonded MIP for L-phenylamine derivatives in 1988 (14, 15). Today, MIPs are used in a wide array of applications, including as sorbent materials for SPE cartridges, solid phase microextraction (SPME) fibers, and HPLC columns. Unlike SPE

columns, which have a one-time use, studies have shown that MIP materials can be reused for multiple extractions. To reuse the MIP materials, a washing or conditioning step is performed after the final elution step to ensure that no analyte is retained and that the column is ready for reuse. Some papers have reported repeat extractions with a single MIP cartridge/column for upwards of 70-100 extractions with no significant changes in extraction efficiency (16–18). Mullet and Lai reported that their molecularly imprinted solid phase extraction (MISPE) column for theophylline still had molecular recognition capabilities for greater than one year (19).

1.2 MIP Synthesis

The imprint inside a molecularly imprinted polymer is created from a template molecule, which is either the analyte of interest, or an analyte of similar size, shape, and functional group chemistry. The template, functional monomer, and cross-linking monomer are dissolved in a solvent, also known as the porogen. The polymerization process is created through a free-radical formation, which is aided by a free-radical initiator that is activated by either heat or ultraviolet (UV) light (6). Increased polymer stability and binding capacity is best achieved when the pre-polymerization complex has a low kinetic energy. This is often achieved by light initiated polymerization at a low temperature, but the literature reports the use of both mechanisms equally (9). The template interacts with the functional monomer, either through covalent or non-covalent interactions, creating specific chemical binding sites with the functional groups of the template molecule. The cross-linking monomer creates the backbone of the polymer, staging the functional monomer in place. Once the polymerization process is complete, the template is removed either via chemical cleavage or a washing extraction. The final result is a hardened polymer with a 3-D cavity in the shape of the template molecule (Figure 1.1).

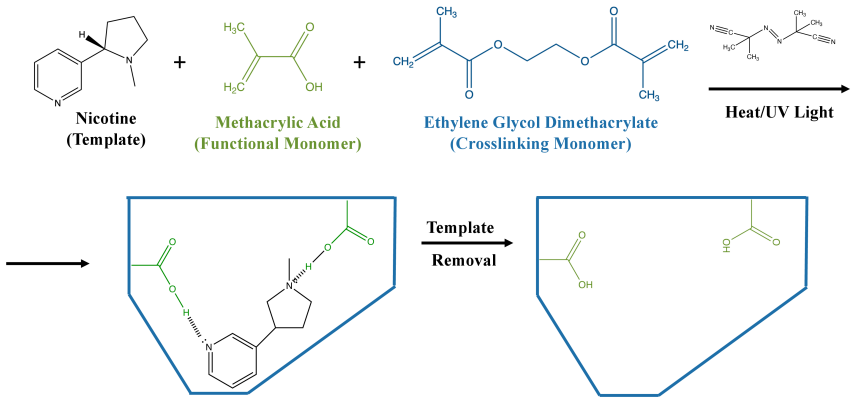


Figure 1.1: Formation of a molecularly imprinted polymer by non-covalent interactions. The polymer formation is initiated with the help of an free-radical initiator and heat or UV light. The template and functional monomer interact in this scenario through electrostatic interactions and hydrogen bonding. The cross-linking monomer forms the backbone of the polymer, holding the functional monomer in place. Post-polymerization, the template is removed and an imprinted cavity in the size and shape of the template analyte is left behind.

The analyte of interest can later re-enter the 3-D cavity and selectively re-bind with the functional monomer. The type of interaction between the functional monomer and the template/analyte depends on if the polymer was created through covalent or non-covalent interactions.

1.2.1.1 Covalent Imprinting

Covalent imprinting was the first interaction created for MIP synthesis by Wulf et al in 1973 (13). The template/analyte binds to the functional monomer through covalently binding interactions. After the polymerization process is complete, the template is removed by disrupting the covalent interactions through chemical cleavage of the covalent bond. The advantage of covalent imprinting is that the required ratio of monomer to template is 1:1. This homogenous interaction promotes specific binding sites within the MIP and greatly reduces the chance of non-

specific binding from occurring (6, 9, 20). The primary disadvantage to covalent imprinting is that there is a limited number of reliable template/analyte and monomer interactions that can also be reversed for template extraction. Those that do have reversible interactions have slow binding kinetics between the template and the monomer in comparison with non-covalent MIPs. Depending on the application, the slow binding kinetics may be undesirable (6).

Semi-covalent imprinting can overcome the disadvantages of covalent imprinting. In semi-covalent imprinting, the MIP is synthesized by covalent interactions, but the rebinding kinetics are established through non-covalent interactions. The advantage of this method is the homogenous binding sites through covalent imprinting with the faster re-binding kinetics of the non-covalent imprinting (20, 21). However, the use of semi-covalent imprinting is still limited because of the limited number of template/analyte to functional monomer complexes that are capable of forming reversible non-covalent bonds (20).

1.2.1.2. Non-Covalent Imprinting

Non-covalent imprinting was first described by Mosbach et al in 1988 and is currently the popular choice for synthesis of MIPs. The use of non-covalent interactions significantly broadens the choice of functional monomers that can be used to interact with the template molecule (13, 15). Non-covalent MIPs also have faster binding kinetics in comparison to covalent imprinted MIPs, which is advantageous in cases where high-throughput extractions are needed.

Non-covalent interactions between the template and monomer are primarily from hydrogen bonding, electrostatic and Van der Waal forces, and hydrophobic interactions. After polymerization, the non-covalent interactions are disrupted through a chemical extraction and the template is removed. The wider range of chemical interactions involved in the binding process

also allows for molecules of similar shape/structure to the template molecule to interact with the MIP. This is a favorable interaction if the goal of the MIP is to extract a class of compounds that are of a similar shape. Amphetamines, benzodiazepines, cannabinoids, and tobacco specific nitrosamines (TSNAs) are among the class of compounds that have been assessed in their ability for a MIP to extract numerous analogues from a biological matrix (16, 17, 22–32).

Unlike the covalent imprinting, where the 1:1 monomer to template/analyte ratio can create homogenous binding sites, non-covalent imprinting requires a 4:1 monomer to template/analyte ratio to ensure non-covalent interactions will occur (5, 9). This creates an excess of functional monomers that will not interact with the template/analyte monomer. The chance of non-specific binding on the surface of the polymer is increased (9). The non-specific binding can potentially allow other analytes to non-selectively bind with the surface of the analyte, but a washing step employed during extraction process can help remove the unwanted analytes prior to elution (33).

1.2.2.1 Ingredients for a MIP

The template molecule is typically the target analyte that is attempted to be extracted from the matrix. However, a common issue faced with MIPs is that, despite extensive extraction, about 1% of template molecule is retained within the MIP. Overtime, the remaining template molecule may be leached out of the polymer complex (34, 35). A strategy to circumvent this potential issue is to create a non-covalent MIPs with a compound that is of a similar, chemical structure to the main target analyte, which is sometimes referred to as a "dummy" template. So long as the "dummy" template has a similar chemical structure to the analyte of interest, the target analyte should be able to fit into the 3-D cavity in the resulting MIP (32, 36). Regardless of whether the target analyte or a "dummy" template is used, the template molecule should have functional groups

that are capable of interacting with the functional monomer (20, 34). Despite the knowledge that the template compound can risk bleeding during analysis, few papers used a “dummy” template for the extraction of another compound (32, 35–40)

The functional monomer forms the binding sites with the template monomer in the pre-polymerization complex of the MIP. The functional monomer interacts with the template by forming donor-receptor interactions with the functional groups of the template (20, 41). Functional monomers are required to have two factors that will make them desirable for MIP synthesis; One, the recognition unit that can interact with the template molecule and two, the polymerization unit (12). With non-covalent imprinting, there is a wide range of functional monomers that can be used for synthesis (Figure 1.2), the most popular being methacrylic acid (MAA), because of its ability to act as a reliable hydrogen bond donor-acceptor (12, 41). Further, Zhang et al demonstrated that because of MAA’s dimerization complex, there was a reduction of non-specific binding sites being formed within the MIP despite the 4:1 ratio of the monomer and template (42).

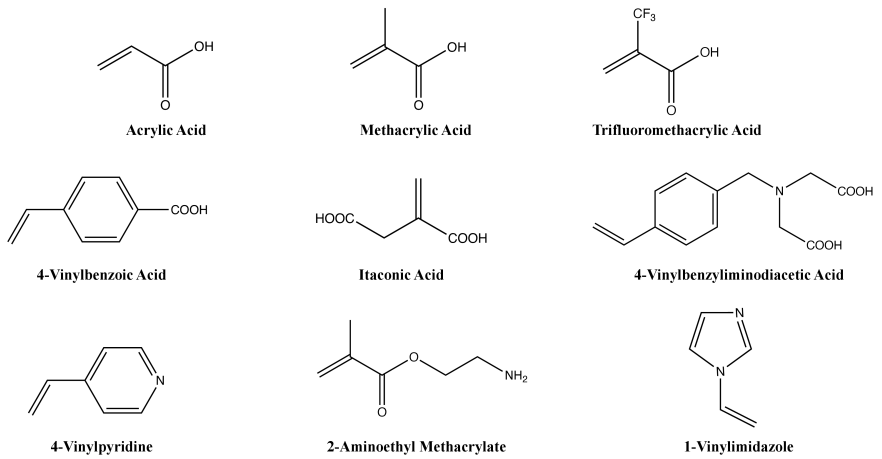


Figure 1.2: Common functional monomers that are used in non-covalent imprinting (43)

The cross-linking monomer creates a cross-linked, hardened polymer which stages the functional monomer in place. The cross-linking monomer also ensures that after the removal of the template, the polymer structure remains intact (12). The amount of cross-linker added to the polymerization matrix is important. Too little cross-linker will not create a stable enough polymer shell and lead to breakdown of the polymer (12, 41). Too much cross-linker will reduce the number of binding sites that can be created in the MIP. Today, the most popular proportion of template to functional monomer to cross-linking monomer complex is typically a ratio of 1:4:20. This ratio, with only a small amount of adjustment depending on the MIP being synthesized, has been demonstrated to create the greatest number of reliable binding sites (44, 45). There are a limited number of cross-linking monomers available to create the needed polymer complex (Figure 1.3), but the cross-linker most widely used is ethylene glycol dimethacrylate (EGDMA) (12).

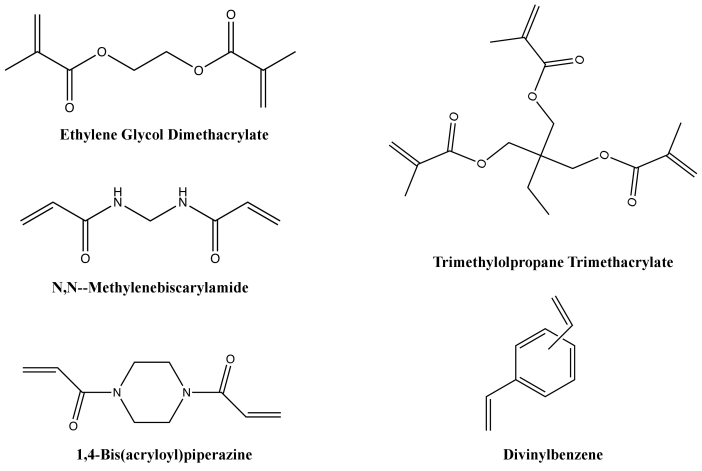


Figure 1.3: Common cross-linking monomers used in non-covalent imprinting (43)

The porogen is the solvent medium where the polymerization process takes place. The porogen must be chosen carefully as it will influence the strength of interaction between the template and functional monomer (12). In non-covalently imprinted polymers, aprotic, low polarity organic solvents are most desirable for MIP synthesis. A porogen should have low hydrogen bond donor-acceptor interactions. Polar solvents, which have very strong interactions, can promote the creation of non-specific binding sites outside the template and monomer interactions (9). This is most problematic when aqueous samples containing the target analyte are introduced to the MIP material. The more polar water molecules will compete with the target analyte for binding sites along the surface of the polymer, reducing the binding capacity of the polymer for the target analyte (3). Popular porogens used for non-covalently imprinted MIPs are acetonitrile, chloroform, dichloromethane, and toluene (20).

1.2.2.2. Bulk Polymerization

Bulk polymerization was the first polymerization strategy to be employed and is still one of the most popular polymerization techniques today (6, 20). The template, functional monomer, and cross-linking monomer are combined in the ratio described previously in a small volume of porogen. The mixture is then polymerized either through ultraviolet (UV) light or heat initiation for 24 hours, which results in a hardened polymer complex. Once the polymerization process is complete, the hardened material is grounded using a mortar and pestle and sieved for a desired particle size range, which is typically 25-38 um in size (22, 23, 38, 46, 47). After sieving, the polymer is left to dry overnight either in open air or an oven before the template is extracted.

A common issue with bulk polymerization is the grinding and sieving of the polymer post-polymerization. Grinding can destroy the binding sites, reducing the binding capacity of the

polymer when the entire process is complete (5, 11). Further, the grinding of the bulk polymer creates irregularly shaped polymer particles (Figure 1.4). The irregularly shaped particles are unsuitable for HPLC columns, which require uniformly sized and shaped polymer particles to create reproducible and reliable extractions (6). Despite these limitations, bulk polymerization remains to be one of the most popular methods employed because it requires a small amount of materials and is simple to execute compared to other polymerization methods.

1.2.2.3 Precipitation Polymerization

Precipitation polymerization is a favorable method that avoids the issues of bulk polymerization. The same ratio of template: monomer: cross-linker used in bulk polymerization is instead dissolved in an excess of porogen. In this case, the functional monomer is less than five percent (w/v) of the porogen. Similar to bulk polymerization, the template, functional monomer, cross-linking monomer, and initiator are all dissolved in a large (> 100 mL) amount of porogen. As the polymer begins to form, the growing polymer will eventually become insoluble in the solvent and precipitate out of the solution (6). Post polymerization, the polymer is filtered from the porogen and the template is extracted with a solvent wash. The resulting particles are small and spherical in shape (Figure 1.4), typically less than 10 μm (48). Unlike bulk polymerization, no grinding of the polymer is required post polymerization. The small, uniform particles are more desirable for SPE and HPLC columns, and when carried out properly can have improved binding site recognition (49).

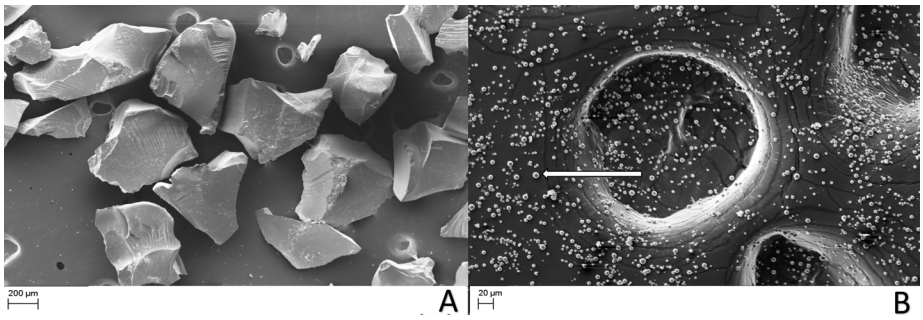


Figure 1.4: Scanning electron microscopy (SEM) of polymers formed by A) bulk polymerization and B) precipitation polymerization

Precipitation polymerization is not without its challenges. The biggest drawback for precipitation polymerization is that the process cannot be universally employed for all analytes. First, the MIP materials (Template, monomers, and initiator) must be able to dissolve into solution, and then later precipitate out as the forming polymer becomes insoluble in the solvent. There are few porogen solvents that the template and MIP reagents are dissolvable in and can also properly interact within the mixture. If the template is not compatible with the porogen, it will result in a low polymerization yield. This will be most noticeable when a non-molecularly imprinted polymer is created and creates a higher polymerization yield (6). Purification of reagents used in the polymerization process can improve the polymer yield. (48). Other types of polymerization methods that can create monodisperse particles include suspension and multi-step swelling polymerization. Suspension polymerization requires the MIP components to be suspended in an immiscible solvent that is stirred to induce particle precipitation and multi-step swelling utilizes seed particles to create the spherical, uniformly sized particles (48). While both techniques are able to create uniformly sized particles comparable to precipitation polymerization, their reputation for being laborious and time-consuming results in few papers utilizing these methods for polymerization (5, 50, 51).

1.2.3 Non-Molecularly Imprinted Polymers

Non-Molecularly Imprinted Polymers (NIPs) are polymers that are created in the absence of the template molecule. NIPs are polymerized using the same amounts of functional and crosslinking monomers, porogen, and initiator as MIPs. The purpose of the NIP is that it specifically lacks the 3D cavity formed by the presence of the template. Instead of having specific binding sites like the MIP, the NIP is composed exclusively by non-specific interactions with the available functional monomers. The NIP is useful in demonstrating how the 3D cavity formed by the template molecule is crucial in creating a specific binding site with the functional monomer. Further, the NIP can be used as a control during the extraction optimization process. When both the NIP and MIP undergo the same extraction conditions, the amount of analyte present in both the wash and elution steps can determine if the extraction conditions promote specific or non-specific interactions on the MIP (47).

1.3. MIP Applications

In bioanalysis, MIP technologies are primarily used in the place of sorbent materials for SPE or the stationary phase for liquid chromatographic columns. Depending on the type of analysis desired, the application is denoted as off-line, on-line, or in-line. Off-line mode follows the traditional SPE workflow at the bench prior to extract an analyte from a biological matrix, and is often referred to molecularly imprinted solid phase extraction (MISPE). The first group to use MIP material for off-line SPE was Andersson et al (52) for the extraction of sameridine in human plasma. On-line extraction uses the MISPE column as a pre-column for sample extraction and enrichment on the instrument prior to introduction to the analytical chromatographic column (53). Finally, in-line extraction is where the MIP material is used in place of the packing material of an

HPLC column. Both sample pre-treatment and separation happen simultaneously, which Sellergren achieved in the extraction of pentamidine from urine (21).

1.3.1. Off-line MISPE Mode

In off-line MISPE, the MIP material is used as a SPE cartridge, and the entire extraction process is performed at the bench. The extraction process is similar to traditional SPE, where the conditioning, loading, washing, and elution steps must be optimized for the analyte of interest. The loading conditions must promote the interactions between the analyte and the functional monomers within the 3D cavity. Selectivity can be greatest when loaded in the same solvent used for polymerization, which are low polarity, organic solvents (5). This can prove difficult for analytes in aqueous biological samples, as the hydrogen bonding of water can actively compete for binding site along the surface of the MIP. In cases of aqueous loading conditions, the MIP can be operated like a reverse phase SPE sorbent, and washing steps can be included (33). The washing steps will disrupt matrix components and other interferents that are retained non-specifically on the surface of the polymer (Figure 1.5). Multiple washing solvents can be used to fully remove interferents without disrupting the target analyte. Once the cartridge has been sufficiently cleaned, the elution step will disrupt the interaction between the analyte and the functional monomer (36).

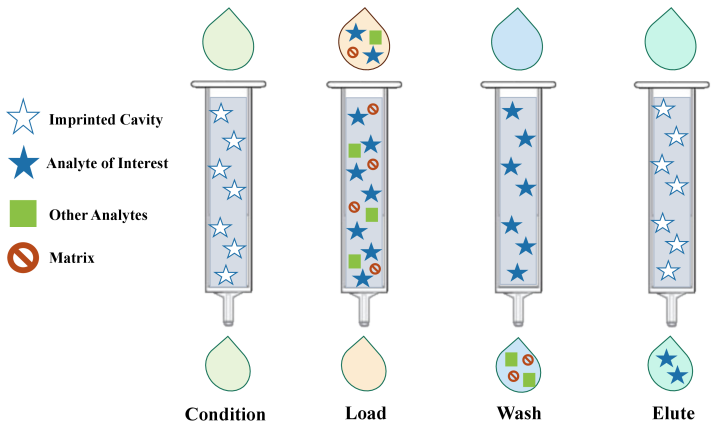


Figure 1.5: Molecularly imprinted solid phase extraction (MISPE). Conditioning the cartridge wets the sorbent bed and readies the binding sites. Upon loading, the analyte of interest will fit into the imprinted cavity and other analytes and matrix components will be retained on the surface non-specifically, and are displaced during the washing steps. During elution, the chemical interaction between the functional monomer and analyte will be disrupted and the analyte will elute off the column.

It is during the washing and elution steps that the NIP can be best utilized to determine the extent of specific and non-specific binding of the MIP. By monitoring the amount of analyte recovered in the washing and elution steps between the MIP and the NIP, the selectivity for the analyte and the binding capacity can be determined (10). Under optimal conditions, the MIP will have low to disruption for the target analyte, and high recoveries in the elution step, due to the selective retention of the analyte in the imprinted binding sites. In comparison to the non-imprinted polymer, which only has non-selective, surface interactions, the recovery of the analyte in the washing step is expected to be higher.

1.3.2 On-line and In-Line MIP modes

Where off-line MISPE is performed at the bench prior to introduction to the analytical instrument, on-line MISPE is performed on the instrument. The traditional on-line MISPE cartridge is used as a pre-column that is placed before the chromatographic column (53). With in-line MIP systems, the MIP polymer is used as the chromatographic column (5). In-line and on-line MISPE columns can be created under two conditions discussed in this review. One method is to create the traditional particle material either through bulk or precipitation polymerization. The particles are then mixed with a solvent to create a polymer slurry, that is then packed into the column under pressure, which will be discussed further in later chapters. The other method is to add the MIP materials directly to the column and polymerize *in-situ*, following a bulk polymerization method. This avoids the necessary grinding and sieving process, and creates a monolithic rod instead. The monolithic rod method has advantages similar to precipitation polymerization, as it does not require grinding and sieving and therefore does not destroy the binding sites or create particles that are irregular and of variable sizes (5, 9, 10).

The mobile phases of the analytical instrument are used as the conditioning, washing, loading, and elution solvents. The starting mobile phase acts as the conditioning and loading solvents used off-line, and a second and/or third mobile phase line are used to wash the column and elute the analyte (16). When used in place of a traditional HPLC column, the switching valve is key. As the sample is being loaded and washed, the switching valve can direct the solvents carrying the matrix and interferents to waste. When the system elutes the analyte off the column, the switching valve is changed to direct the solvent carrying the analyte to the detector (Figure 1.6). For on-line and in-line MISPE protocols, the entire extraction takes place on the analytical

instrument, and the analytes are sent directly to the detector. In chromatographic systems, this would typically be a photodiode array detector (PDA) or as mass spectrometer (MS) (16, 47).

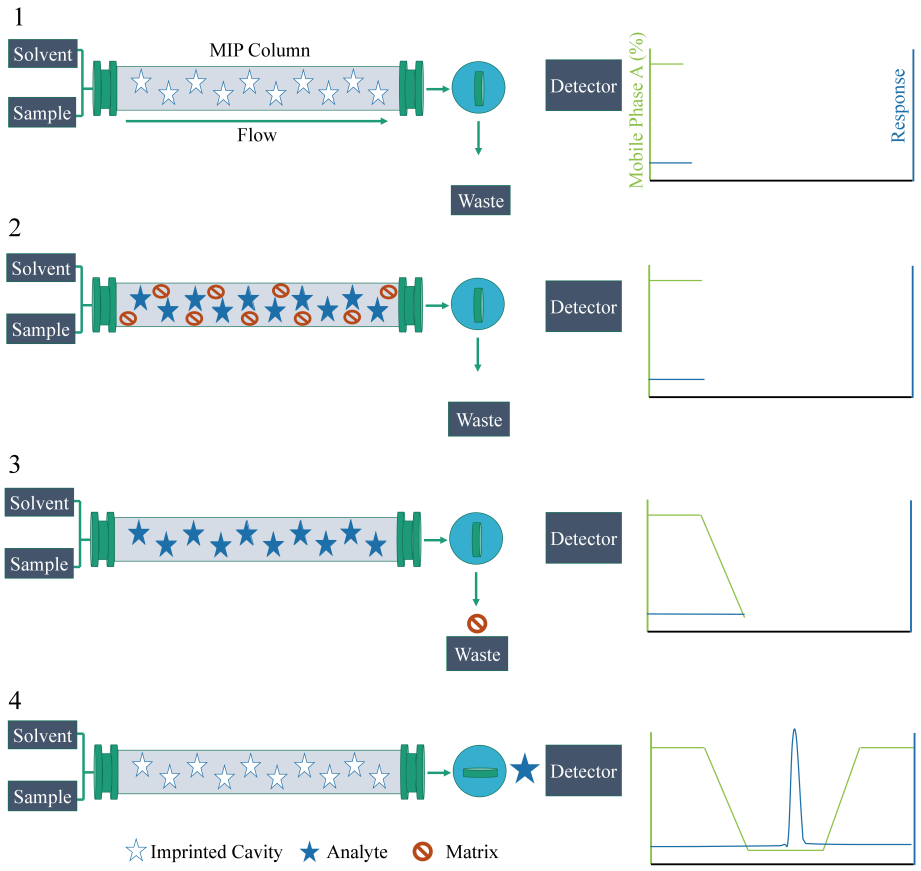


Figure 1.6: Schematic of an online MIP system. When the sample is 1) injected onto the column, the starting mobile phase promotes the interactions between the imprinted cavity and the analyte, and 2) the analyte binds to the imprinted cavity while matrix and interferents are retained on the surface. When 3), the mobile phase is changed to disrupt the surface, non-specific interactions, the matrix is sent to waste. Once the column is cleaned up, the second or third mobile phase will 4) disrupt the non-covalent interaction and elute the analyte out of the imprinted cavity and carry it to the detector.

The greatest challenge to the on/in-line systems is the limitations with the analytical instrument (50). Chromatographic instruments have binary or quaternary pumps, which limits how many solvents can used with the analytical system. Furthermore, solvent compatibility can lead to limitations with properly washing and eluting the analyte from the column. In off-line protocols, solvent-instrument compatibility is not an issue, and the sample can be dried down and reconstituted in a suitable solvent. However, if the entire extraction process is taking place on an analytical instrument, the mobile phases must be compatible with both the extraction protocol, and the detector. Other classic parameters with chromatographic systems such as flow rate and mobile phase gradient will also affect the kinetics of the interaction between the analyte and the MIP stationary phase. Despite these limitations, the advantages of on/in-line MISPE systems is that sample extraction, separation, detection, and quantitation can all be performed directly on the instrument (33, 50). This reduces the risk of sample contamination and loss that can occur during benchtop preparations (5).

1.3.3 Sample Analysis

The use of MIPs for the analysis of drugs in biological matrices, was first demonstrated by Sellergren in 1994 with an in-line MIP for the extraction of pentamidine in urine (21) Since then, there have been over a hundred publications detailing the synthesis and application of MIP materials to extract drugs from biological matrices. Tables 1.1 and 1.2 lists a select number of studies published that used MIP technology for the extraction of analytes in biological matrices. All polymers reviewed in this section were synthesized with non-covalent interactions, with MAA and EGDMA being the most popular functional and cross-linking monomers used. The polymerization strategy was primarily bulk polymerization, but a handful of papers stated that they

used precipitation polymerization. One paper used suspension polymerization(4). Plasma and urine were the most popular biological matrices applied to the MIP materials, but other papers reported successful extractions in other matrices such as hair and oral fluid (22, 23, 28, 31, 40, 46, 47, 54). Few papers have reported the use of homogenized liver samples, but the research has been limited to non-human liver samples such as bovine and pork. Muldoon et al. reported successful extraction of atrazine from bovine liver samples and showed an improved accuracy and recovery via HPLC when compared to crude extracts that did not undergo MISPE. Atrazine recovery with MISPE extracts had limits of detection in the 0.005 ppm range (55). Further research into MIP extractions for other solid, human tissues such as kidney, brain, heart, and vitreous humor is still needed.

Off-line MIP application continues to be the most popular technique. The majority were MISPE applications, but seven papers reported other MIP applications (Table 1.1). Magnetic MIP material was synthesized in three papers by applying the polymer to magnetite (Fe_3O_4), citing that the magnetic application allows for a more selective extraction process than traditional MIP sorbent materials. Rahmani et al applied magnetic MIP material for SPE extraction of morphine in plasma and urine and reported extraction recoveries of 84.9-105.5% and 94.9-102.8% in plasma and urine, respectively (56). Abrao & Figueirdo created Restricted Access Molecularly Imprinted (RAMIP) fibers for the solid-phase micro-extraction (SPME) of benzodiazepines in plasma (57). Coupled with a simple HPLC-DAD analysis, the authors were able to achieve linear ranges for five benzodiazepines within the reported therapeutic ranges and have comparable results to traditional SPE methods.

The use of commercial MIPs have also been reported. Supelco has the SupelMIP™ line of molecularly imprinted polymer SPE cartridges for the extraction of class specific compounds in

biological matrices: beta-blockers/beta agonists, clenbuterol, tobacco specific nitrosamines (TSNAs), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). The TSNA and NNAL cartridges have been extensively characterized for their recovery and urine and cross-selectivity, to be discussed in later chapters (32, 36, 58, 59). Widstrand et al and Kumazawa et al used the SupelMIP™ for amphetamines by SupelCo for the extraction of amphetamines (24, 25). Kumazwa et al was able to successfully extract amphetamine, methamphetamine, and five designer amphetamines from whole blood with the SupelMIP™ cartridges. The use of whole blood was also novel as it has more matrix components than plasma and urine, but the researchers were able to achieve recoveries of 88.5% and greater with the amphetamine based MISPE and did not have to perform a protein precipitation step prior (25). Widstrand et al extracted five amphetamine derivatives from urine and reported lower limits of quantitation (LLOQ) a whole order of magnitude less than the LLOQs achieved with traditional hydrophilic polymer SPE cartridges (24). Murakami et al used AFFINILUTE MIP-Amphetamine cartridges from Biotage to extract 11 synthetic cathinone's from urine and whole blood. The inter-day recoveries for urine was between 79.8% and 81.2% and for whole blood was between 68.5% and 74.8%, demonstrating that the MIP material can be applied to an entire class of compounds, depending on the desired application (30). Despite these commercial products being shown to be capable of extracting a number of class related drugs from complex biological matrices, Biotage removed their amphetamine MISPE cartridges from market in 2019 and the SupelMIP™ cartridges for amphetamines are no longer available.

Table 1.1: Molecularly imprinted polymers synthesized for the extraction of drug compounds from various biological matrices for off-line extraction protocols.

Template	Polymerization Strategy	Matrix	Analyte	Comments	Reference
Sameridine analogue	Bulk	Plasma	Sameridine	Dummy template Cleaner baseline with MIP extraction	(52)
Pentycaine	Bulk	Plasma	Bupivacaine	65-75% recovery with cleaner extracts	(37)
Amobarbital	Suspension	Urine	Barbiturates	81-86% recoveries	(4)
Hyoscyamine	Bulk	Serum & urine	Scopolamine	46-59% recovery in urine 60-79% in serum	(35)
Naproxen	Bulk	Urine	Naproxen	60% recovery	(60)
Ciprofloxacin	Bulk	Urine	Ciprofloxacin	Reduced analysis time 80-87% recovery	(61)
Propranolol	Bulk	Plasma	Clenbuterol	Leaching of template caused interferences at low levels	(38)
NNAL[a]	Bulk	Urine	NNAL	LOD of 1.7 pg/mL	(62)
Enrofloxacin	Bulk	Urine	Enrofloxacin	Two-step SPE with MIP LOD 30 µg/kg	(63)
Cotinine	Bulk	Urine	Cotinine	Recovery > 80%	(64)
Diazepam	Bulk	Hair	Diazepam	93% recovery LOD 0.09 ng/mL LOQ 0.14 ng/mL	(22)
Carbamazepine	Bulk	Urine	Carbamazepine	65% recovery	(65)
SupelMIP™ for TSNAs[b]		Urine	TSNAs	Commercial polymer 98-107% recovery Matrix effects < 15%	(36)
Nicotine	Bulk	Hair	Nicotine	Cleaner extracts compared to traditional SPE	(66)
Diazepam	Bulk	Hair	Benzodiazepines	62-103% recovery Achieved lower LOD and LOQ for some benzodiazepines over SPE	(23)
Amoxicillin	Bulk	Urine	Amoxicillin + Cephalexin	63-65% recovery with cleaner extracts	(67)

Clomiphene	Bulk	Urine	Tamoxifen	85% recovery	(39)
Carbamazepine	Precipitation	Urine	Carbamazepine & Oxcarbazepine	90 and 83% recovery	(48)
Ergonovine Maleate	Bulk	Hair & urine	LSD	83% recovery LOQ of 0.2 pg/mL	(40)
SupelMIP™ for TSNAs		Urine	TSNAs & NNAL	Commercial polymer Cross-selective for NNAL	(58)
Ephedrine	Bulk	Plasma	Ephedrine	68% recovery Cross-selective for catecholamine neurotransmitters	(68)
Tramadol	Bulk	Plasma & Urine	Tramadol	Recovery > 80%	(69)
ATCA[c]	Other	Urine	ATCA	MIP coated stir bar Binding capacity 35 ng	(70)
SupelMIP™ for Amphetamines		Urine	Amphetamines	Commercial Polymer (Discontinued) LLOQ 1 order of magnitude lower than traditional SPE methods	(24)
Methamphetamine	Precipitation	Urine	Amphetamines	81-93% recoveries	(17)
Methamphetamine	Precipitation	Urine	Methamphetamine and ecstasy	80-88% recovieres	(26)
SupelMIP™ for Amphetamines		Whole Blood	Amphetamines	Commercial polymer (Discontinued) Recovery 85%+ without prior benchtop preparation (protein precipitation)	(25)
Cocaine	Bulk	Hair	Cocaine	Recoveries close to 80% Specific capacity 8.96 µmol/g	(46)
THC-OH[d]	Bulk	Urine	Cannabinoids	71-78% recoveries	(27)
SupelMIP™ for NNAL		Urine	NNAL & TSNAs	Commercial polymer Cross-selectivity of NNAL MIP for TSNAs	(32)
Testosterone	Bulk	Synthetic Urine	Testosterone	LOD 10 ng/mL for mixed sample Cleaner extracts	(71)

Indapamide	Bulk	Urine	Indapamide	80-81% recovery LOD 0.025 µg/mL LOQ 0.075 µg/mL	(72)
Catechin	Other	Oral Fluid & Urine	Cannabinoids	Water-compatible MIP pills 50-111% recoveries	(73)
Cocaine	Precipitation	Plasma	Cocaine	Porous-membrane protected MIP LOD < 1 ng/mL	(74)
Cocaine	Other	Urine	Cocaine + Metabolites	Magnetic MIP 79-106% recoveries	(75)
THC-COOH[e]	Precipitation	Plasma & Urine	Cannabinoids	Plasma LOQ 0.36-0.49 ng/L Urine LOQ 0.47-0.57 ng/L	(29)
6-Mercaptopurine	Precipitation	Plasma	6-Mercaptopurine	Magnetic MIP 85-97% recoveries	(76)
Affinilute MIP for Amphetamines		Blood and urine	Synthetic Cathinones	Commercial Polymer (Discontinued) Cross-selectivity with > 65% recovery in urine and whole blood	(30)
Morphine	Precipitation	Plasma & urine	Morphine	Magnetic MIP 85-106% recoveries (plasma) 95-103% recoveries (urine)	(56)
Diazepam	Other	Plasma	Benzodiazepines	Restricted access MIP fiber	(77)
Cocaine	Bulk	Oral Fluid	Amphetamines + Synthetic Cathinones	81-115% recoveries	(31)

[a]4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol, [b]Tobacco Specific Nitrosamine, [c]2-aminothiazoline-4-carboxylic acid, , [d]11-Hydroxy-Δ^9-tetrahydrocannabinol, [e]11-Carboxy-Δ^9-tetrahydrocannbinol

As noted in other reviews, publications detailing the use of MIP technology for on-line and in-line extraction protocols is low (Table 1.2). Mullett and Lai (19) were among the earliest to use on-line MISPE as a pre-column for the extraction of theophylline in serum, utilizing pulsed elution to create a quick extraction procedure that could flow directly onto the analytical column. A protein precipitation step was performed prior to sample loading, but the rest of the extraction was carried

out on the instrument and the authors were able to achieve a limit of detection (LOD) of 12 ng/mL and a linear dynamic range of 0.25-1000 µg/mL. The authors also stated that the pre-column used for the method development showed no performance deterioration over the course of the experiments (19). A more recent publication by Bouveral et al created miniaturized, monolithic columns for the on-line extraction of cocaine and its main urinary metabolite, benzoylecgonine, in plasma, saliva, and urine. The authors reported the ability to extract and detect cocaine and benzoylecgonine at trace levels in all three biological matrices (54).

For in-line extraction protocols, the use of traditional bulk polymerization followed by slurry packing of HPLC columns was the most common method reported. Santos et al used the in-line HPLC method for the extraction of five tricyclic antidepressants in plasma. This method paired the MIP material with restricted access media (RAM), preventing the need for any protein precipitation of the samples prior to their analysis on the instrument. The use of protein precipitation of plasma samples prior to instrument introduction for on-line and in-line MISPE extraction was reported for the majority of the papers reviewed (16, 19, 54, 78) With the addition of the RAM material incorporated with the MIP, the only off-line pretreatment required was a dilution step. The authors were able to report low limits of quantitation (15 µg/L) for all five compounds in plasma without the need for off-line protein precipitation or chromatographic separation (79).

Table 1.2: Molecularly imprinted polymers synthesized for the extraction of drug compounds from various biological matrices for on-line or in-line extraction protocols

Template	Polymerization Strategy	Matrix	Analyte	Comments	References
Pentamidine	Bulk	Urine	Pentamadine	In-line, HPLC-UV[a]	(80)
Theophylline	Bulk	Serum	Theophylline	On-line	(19)
Ibuprofen + Naproxen	Mult-Step Swelling	Plasma	Ibuprofen + Naproxen	On-line, HPLC-UV	(81)
Verapamil	Bulk	Plasma & Urine	Verapamil + metabolites	On-line, GC-MS[b]	(82)
Cephalexin	Bulk	Serum	Cephalexin	In-line HPLC-UV	(83)
Diazepam	Bulk	Plasma	Benzodiazepines	In-line LC-ESI-MS[c]	(16)
SupelMIP™ for NNAL		Urine	NNAL	In-line LC-MS/MS[d]	
Amitriptyline	Bulk	Plasma	Tricyclic Antidepressants	In-line LC-MS/MS	(84)
Cocaine	Bulk	Plasma, urine, & saliva	Cocaine	In-line Nano-LC-UV	(54)

[a]high-performance liquid chromatography- ultraviolet, [b]gas chromatograph-mass spectrometry, [c]liquid chromatography- electrospray ionization-mass spectrometry, [d]liquid chromatography-tandem mass spectrometry

1.4. Conclusions

Molecularly imprinted polymer technology has a wide range of capabilities that have been published in the literature since its' conception in the 1970's. Since 1994, there has been an evolution of using this technology for sample preparation for a wide variety of specimens, including complex biological matrices. The advantages of MIP technology are that they have the

same inherent mechanism and selectivity of immuno-technologies, but their ability to withstand fluctuations in temperature and pH changes makes them advantageous and robust in comparison. Further, MIP materials can be employed in a range of different extraction techniques that can both improve the extraction recovery of drug compounds and fit the needs of the laboratory. Trends in MIP synthesis and application are improving to overcome the limitations of traditional extraction protocols, but the use of MIPs to extract solid tissues such as liver, brain, kidney, heart, etc. needs to be explored. Bulk precipitation was one of the first polymerization methods reported and is still among the most popular methods to be employed for MIP synthesis. However, precipitation polymerization is emerging as another popular method that does not require bulk polymerization's grinding and sieving step, and creates small, uniform particles that are ideal for chromatographic packing material. Off-line MISPE currently offers a more selective and sensitive extraction protocol when compared to traditional SPE protocols. The advancement of using molecularly imprinted polymers as the packing material for chromatographic columns will create fast, efficient, and selective extraction protocols to improve the analysis of biological specimens in bioanalytical laboratories.

Chapter 2: Specific Aims

Aim 1: Characterize a commercial Molecularly Imprinted Polymer for the analysis of tobacco alkaloids in urine.

A commercialized MIP for urinary extraction of tobacco specific nitrosamines (TSNAs) has been shown to be cross-selective for tobacco alkaloids and metabolites. The extent of cross-selectivity with the TSNA MIP is first through recovery studies in water. Characterization of the polymer performance under various extraction conditions, such as sample pH and elution solvent strengths are determined with nicotine and its urinary metabolite, cotinine. The performance of the MIP and a non-imprinted polymer (NIP) in comparison of traditional SPE methods, such as hydrophilic-lipophilic balance and mixed mode cation exchanged cartridges are assessed. N-Nitrosonornicotine (NNN) is used as a positive control, and morphine acts as a non-tobacco alkaloid to determine the full extent of MIP cross-selectivity. The selectivity of cotinine over clinically relevant levels of TSNAs, cotinine metabolites, such as trans-3-hydroxycotinine, and nicotine are explored. Recovery studies of cotinine in matrix are conducted in urine at three concentrations (Chapter 3).

Aim 2: Design MIP HPLC columns for direct analysis of TSNAs in tobacco products via LC-MS/MS.

A slurry packing method for the commercial polymers in a stainless steel HPLC column is developed. An LC-MS/MS method for the analysis of NNN and 4-(methylnitrosamino)-1-)3-pyridyl)-1-butanone (NNK) in nicotine and tobacco products and electronic cigarette e-liquids is developed on a SCIEX UPLC with a Sciex 6500+ Q-Trap mass spectrometer. Parameters such as injection volume, mobile phase, flow rate, and column temperature are optimized. The method is validated following the US Department of Health and Human Services Center for Tobacco

Products Guidance for Industry. Column uniformity of the packing method is determined from making three columns and assessing retention time, peak area, calculated concentration, and accuracy. Column characterization is assessed by asymmetry, tailing factor, and theoretical plate number. Real world samples are analyzed with this method. The cross-selectivity of other tobacco alkaloids and metabolites such as nicotine and cotinine are also assessed (Chapter 4).

Aim 3: Develop a polymerization technique for Molecularly Imprinted Polymers (MIP) using cotinine as a model.

A MIP specific for cotinine was created. The polymerization technique utilized was chosen based on the time, effort, yield, and stability of the polymer. Molecular modeling was used to characterize the potential strength of interaction between the analyte and functional monomer. A non-imprinted polymer (NIP) was created under similar conditions, and the performance of the MIP vs NIP was assessed. Criteria to determine the best polymer combination will be carried out recovery studies outlined in Aim 1 and are compared with the results in Aim 1. Characterization of the polymer morphology and surface chemistry are carried out with scanning electron microscopy (SEM), Fourier Transform infrared spectroscopy (FTIR), and Thermogravimetric Analysis (TGA) (Chapter 5).

Chapter 3: Characterization of Molecularly Imprinted Polymers for the Extraction of Tobacco Alkaloids and their Metabolites in Human Urine

This chapter has been drawn from the article published in Biomedical Chromatography

(Mulder, Pearcy, and Halquist; 2022, doi: 10.1002/bmc.5361)

3.1 Introduction

For molecularly imprinted polymers (MIPs) created by non-covalent polymerization techniques, specificity and selectivity of the MIP is governed by the analyte's size, shape, functional group chemistry, and its interactions with the functional monomer. Therefore, analytes with similar structures and chemical properties to the target analyte can be cross-selective with the MIP (85, 86). This has been reflected in the literature with MIP extraction strategies that are selective for a class of compounds, such as with amphetamines, benzodiazepines, cannabinoids, and tobacco specific nitrosamines (16, 23, 24, 28, 29, 32, 36, 57, 58, 77, 87). Murakami et al demonstrated the advantages of the MIP cross selectivity for a larger class of compounds. Using a commercial MIP SPE cartridge designed for amphetamine, methamphetamine, and 3,4-methylnedioxymethamphetamine, 11 synthetic cathinones were successfully extracted from urine and whole blood. Through adjusting extraction parameters, such as the loading pH, the authors achieved recoveries between 60-89% for the synthetic cathinones in urine. The favorable recoveries with the amphetamine-MIP were compared to recoveries of 12-90% with hydrophilic based SPE and to recoveries of 8-92% with liquid-liquid extractions. Further, the authors reported lower matrix effects with the amphetamine-MIP cartridges compared to the traditional SPE method. The authors did note that as the analyte structure changed, specifically with the increase in an alkyl side chain, the recovery of the analyte diminished, indicating that shape and size of the analyte does have an effect on the analyte's ability to interact with the imprinted cavity (30).

Tobacco specific nitrosamines (TSNAs) are a group of tobacco by-products formed during the curing, harvesting, and fermentation process of tobacco leaves (88) (Figure 3.1). Two TSNAs, N'nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are highly carcinogenic, contributing to mouth, throat, and lung cancer in association with tobacco use (Hecht, 1998). TSNA levels are used as biomarkers of exposure to tobacco products in both smokers and non-smokers, with concentrations below 10 pg/mL in the urine of non-smokers. Due to their low concentrations in urine, sensitive and reliable extraction techniques are required for accurate detection on analytical instruments (36). Current methods commonly describe using upwards of two solid phase extractions (SPE) to clean up patient samples for suitable detectability on the instrument. In response to these challenges, Supelco (Bellefonte, PA) has designed the commercial SupelMIP™ TSNA SPE cartridge that is "class selective" for four TSNAs: NNN, NNK, N'nitrosonoranabasine (NAB), and N'nitrosonoranatabine (NAT). The manufacturer reports that using the MISPE cartridge results in limits of detection as low as 4 pg/mL in urine, and no extra SPE extractions are required (36, 89).

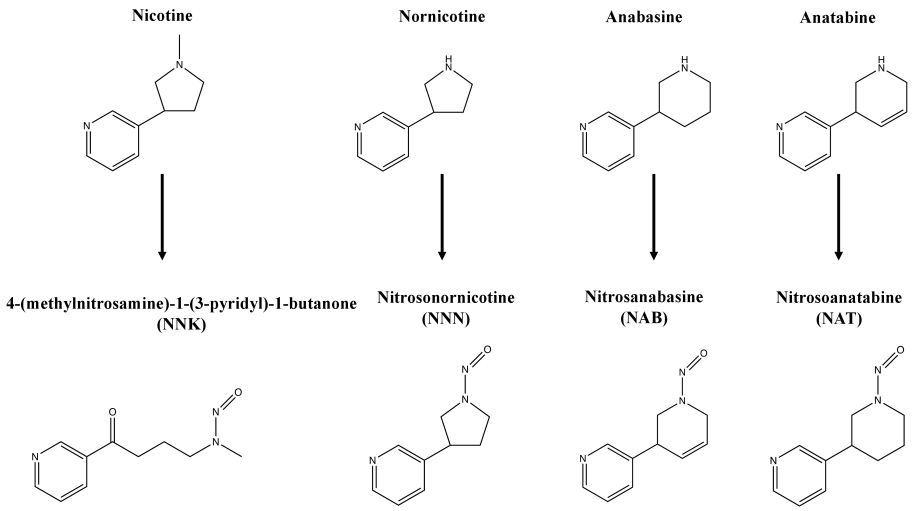


Figure 3.1: Tobacco specific nitrosamines (TSNAs) and their precursors. TSNAs are formed by Nitrosation of the precursors in the fermenting and curing process of tobacco leaves in fertilized soil.

Due to the MIPs class-selective nature, previous literature has demonstrated that NNK's metabolite, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) can also be extracted with the SupelMIP™ TSNA cartridge (32, 90). A SupelMIP™ NNAL SPE cartridge was also shown to be cross-selective for the 4 TSNAs (32, 91). This is most likely due to the fact that all the analytes of interest share a pyridine ring that have similar dissociation constants (pKa) that interact with the functional monomer. Previous research within the laboratory has also demonstrated that there is a degree of selectivity with nicotine, cotinine, and other tobacco alkaloids such as nornicotine (92). Current literature typically splits the tobacco exposure analysis to focus on nicotine, cotinine, and trans-3-hydroxycotinine, or TSNAs and NNAL (93). Few papers have developed methods for the simultaneous detection of NNAL and cotinine in urine, but highlight the need for effective sample preparation methods to detect non-smoker levels of these analytes without matrix interference (94, 95). Since the TSNA MIP cartridge is designed to be class selective for four TSNA analytes, and

has demonstrated cross-selectivity with NNAL, it is possible that the cross-selectivity of the MIP can be expanded to include other tobacco alkaloids and their metabolites. This would allow for the measurement of total nicotine equivalence in one, simple extraction step.

This study sought to characterize the cross-selectivity of the TSNA MIP with other tobacco alkaloids: the TSNA precursors nicotine and nornicotine, nicotine's urinary metabolite, cotinine, and cotinine's urinary metabolite trans-3-'hydroxycotinine. The extraction performance with the MIP for NNN, cotinine, and nicotine was assessed when changing the extraction conditions, such as loading pH and elution solvent. The performance of the MIP in regards to the recovery of NNN, nicotine, and cotinine were compared with their recoveries with a non-imprinted polymer (NIP), and two traditional SPE cartridges. Finally, morphine, an analyte that is of a different class of compounds than the tobacco alkaloids, was extracted with the four extraction techniques to determine the full extent of cross-selectivity with the imprinted polymer.

3.2. Materials and Methods

3.2.1 Materials and Chemical Reagents

SupelMIP™ TSNA (50 mg/3 mL) SPE cartridges were purchased from Sigma Aldrich (St. Louis, MO). Oasis hydrophilic-lipophilic balance (HLB, 30 mg/1-mL) and mixed-mode cation exchange (MCX, 30 mg/1-mL) SPE cartridges were purchased from Waters (Milford, MA). NIP material was bought from Biotage (Uppsala, Sweden).

HPLC grade acetonitrile (ACN), dichloromethane (DCM), heptane, methanol (MeOH), tetrahydrofuran (THF) and water (H_2O) were purchased from VWR International (Radnor, PA). Ammonium formate (97%) and Orthophosphoric acid (OPA, 85% wt.) were purchased from Sigma Aldrich (St. Louis, MO), and ammonium acetate and triethyl amine (TEA) were purchased

from Fisher Scientific (Waltham, MA). Methyl tert-butyrl ether (MtBE) was purchased from Honeywell (Charlotte, NC). Cotinine (99.7%), cotinine-d3 (99.8%), morphine (99.7%), nicotine (99.6%), nornicotine (98.4%), n-nitrosonornicotine (NNN, 99,8%), and trans-3'-hydroxycotinine (T3HCOT, 98.2%) were purchased from Cerilliant (Round Rock, TX).

3.2.2 Preparation of Reagents

Standard stock solutions of cotinine, morphine, nicotine, and NNN were prepared individually at 100 mg/mL concentrations in MeOH and stored at -20 ºC. Standard working solutions were prepared fresh daily by diluting the analytes down to their appropriate concentrations in 10 mM ammonium acetate, at pH 5.5 or pH 9.2. The 10 mM ammonium acetate solutions were adjusted to their appropriate pH using acetic acid or ammonium hydroxide and verified with a pH-meter. Human urine was obtained from laboratory donations and stored at -20 ºC. Acidic levels of urine were verified using litmus paper. Samples were prepared fresh daily by diluting cotinine down to its appropriate concentrations in human urine.

3.2.3. Physical Characteristics of The Commercial Polymer

A Hitachi (Schaumburg, IL) SU-70 Scanning Electron Microscope (SEM) was used to characterize the polymer morphology. Samples were mounted onto a 25 x 6 mm aluminum disk coated with carbon tape and coated with gold particles for 90 seconds at 10mA and 10pA. An accelerating voltage of 3-5 keV at 300-5000x magnification was used at a working distance of 15 mm. Functional groups of the polymer were identified using a Thermo Scientific Nicolet iS50 FT-IR instrument with a diamond crystal (Waltham, MA). Sample spectra were a composite of 64

sample scans, with background subtraction. Scans were acquired from 500 nm to 4000 cm^{-1} with a resolution of 4 cm^{-1}. Samples were analyzed using Omnic Spectroscopy Software.

3.2.4 SupelMIP™ TSNA MIP and NIP Extraction Protocol

Samples were extracted following a modified method recommended by the manufacturer (89). The MIP cartridges were primed with 1 mL of methanol followed by 1 mL of water. The solvents were pulled through the cartridges by centrifugation. The protocol, including the revolutions per minute (RPM) and time, are listed in Table 3.1. Samples were loaded onto the cartridge in 1 mL aliquots at 1 µg/mL concentrations in 10 mM ammonium acetate buffered to a pH of 5.5 or 9.2. The loading fraction was passed through the cartridge gravimetrically, and the cartridge was dried for 10 minutes to remove any residual aqueous solvent. A 1 mL aliquot of heptane was used to further remove any aqueous solvent and disrupt hydrophobic interactions. The cartridge was dried again, and the analytes were eluted with two 1 mL aliquots of 9:1 (v/v) DCM:MeOH. The wash and elution steps were evaporated at 55 ºC and reconstituted in 200 µL mobile phase.

Table 3.1: Extraction protocol for TSNA MIP SPE cartridges

Step	Solvent	RPM	Minutes
1. Prime	1 mL MeOH	200	3
	1 mL H_2O	200	3
2. Load	1 mL Sample in 10 mM Ammonium Acetate	Gravimetric	
3. Dry		1500	10
4.Wash	1 mL Heptane	700	6
5. Dry		1500	2
6. Elute	1 mL 9:1 (v/v) DCM:MeOH	350	10
	1 mL 9:1 (v/v) DCM:MeOH	200	5

Non-imprinted polymer cartridges were created with non-imprinted polymer material purchased from Biotage. A frit was placed at the bottom of an emptied 3 mL SPE cartridge and 50 mg of the NIP material was slurry packed in 1 mL aliquots of methanol. A second frit was placed above the NIP material and the same extraction protocol used for the MIP cartridges above was followed.

Cotinine in human urine was extracted with the SupelMIP™ TSNA cartridge. Cotinine at 10, 100, and 1000 ng/mL concentrations were prepared in urine prior to the extraction. Cotinine-d3 at 68 ng/mL was added to each sample and the sample underwent the SupelMIP™ TSNA SPE extraction outlined above.

3.2.5 Waters Oasis HLB and MCX Extraction Protocols

Samples were extracted following previously published methods for the extraction of NNN with traditional SPE cartridge. In brief, the cartridges were primed with 1 mL aliquots of methanol followed by water. The same centrifuge parameters used for the MIP and NIP cartridges were used on the SPE cartridges. Samples were loaded onto the cartridge in 1 mL aliquots at 1 µg/mL. The samples extracted with the HLB cartridges were loaded under acidic conditions. NNN and cotinine were prepared in 10 mM ammonium formate, pH 2.73 and nicotine and morphine were prepared in 10 mM ammonium acetate, pH 5.5. The cartridges were washed with 1 mL of 95:5 (v/v) H_2O:MeOH and eluted with two 1 mL fractions of methanol (96). The sample was evaporated at 55 ºC and concentrated in mobile phase. The samples extracted with the MCX cartridges were loaded under basic conditions at 10 mM ammonium acetate, pH 10, washed with 1 mL methanol, and eluted with two 1 mL fractions of 9:1 (v/v) MeOH:25% NH_4OH. Elution fractions were evaporated at 55 ºC and concentrated in mobile phase (87).

3.2.6 HPLC Apparatus

All experiments were conducted on a Waters Acquity H Class UPLC and a Waters Acquity PDA detector (Milford, MA). For NNN and cotinine, chromatographic separation was carried out on a Phenomenex C18 column (50 mm x 2.1 mm, 2.6 µm) at 25 ºC. The aqueous mobile phase A was 10 mM ammonium acetate, pH 10 and the organic mobile phase B was acetonitrile under 90:10 aqueous:organic isocratic conditions. The flow rate was 0.3 mL/min and the PDA was set to monitor NNN at 238 nm and cotinine at 260 nm.

For Nicotine, chromatographic separation was carried out on an Xterra RP18 column (150 mm x 4.6 mm, 5 µm) at 25 °C. Mobile phase A was 0.1% (v/v) TEA in water pH adjusted to 7.6 ± 0.05 by OPA (85%) and 1N NaOH. Mobile phases B and C were 0.1% (v/v) TEA in methanol and acetonitrile respectively. Mobile phase D and diluent were 80% (v/v) methanol in water. The chromatographic conditions were operated under the following method: The quaternary pump used a gradient method with the initial mobile phase composition at 60:26:14 for mobile phases A, B, and C, respectively at a flow rate of 0.8 mL/min. At 6 minutes, the gradient was changed to 100% mobile phase D and held for 2 minutes before returning to initial conditions, which were held until the end of the run (15 minutes). The PDA was set to monitor nicotine at 260 nm (97)

For morphine, chromatographic separation was carried out on the Xterra RP 18 column (150 mm x 4.6 mm, 5 µm) at room temperature. Mobile phase A was 2 mM ammonium formate and mobile phase B was ACN at 50:50 isocratic conditions. The flow rate was 0.8 mL/min and the PDA was set to monitor morphine at 210 nm (98).

3.2.7 LC-MS/MS Apparatus

Recovery experiments were carried out on a Waters Alliance e2695 HPLC and a Waters Quattro Micro API mass spectrometer (Milford, MA). Chromatographic separation was carried out with a Phenomenex synergi-4µ-Polar RP column (150 x 4.6 mm, 4 µm) kept at 40 ºC. Aqueous mobile phase A was 10 mM Ammonium Acetate with 0.1% acetic acid in water and mobile phase B was 10 mM Ammonium Acetate with 0.1% acetic acid in methanol. The HPLC was kept at isocratic conditions of 60% mobile phase A at a flow rate of 0.65 mL/min.

The mass spectrometer was operated in positive ion mode using multiple reaction monitoring (MRM). The source block temperature was set to 150 ºC and the desolvation temperature was set to 450 ºC and the desolvation gas flow at 450 L/hr. The capillary voltage, cone voltage, extractor, and RF lens were set to 2.85 kV, 30 V, 2 V, and 0.4 V, respectively. The entrance and exit lens were both set to 30 and the ion energy 0.6. The multiplier was set to 650 and the nebulizer gas flow was 150 L/hr. Cotinine and cotinine-d3 were quantified using the MRM transition of $[M+H]^+$ ion of cotinine at m/z 177.4 → 79.82 and cotinine-d3 at m/z 180.4 → 79.93. Limit of detection (LOD) and limit of quantitation (LOQ) were established based on signal-to-noise ratio of the lowest concentration injected. Limit of detection was determined to be 3 ng/mL and limit of quantitation was 10 ng/mL.

3.3 Results

3.3.1. Physical Characteristics of the Commercial Polymer

SEM analysis of the polymer revealed large, spherically shaped polymer particles that were less than 100 µm in size (Figure 3.2). There were no morphological differences between the MIP and NIP particles. FT-IR results of the MIP and NIP polymer also showed no functional group

differences (Figure 3.3). A band from 2900-3000 cm^{-1} was characteristic of a bonded alcohol from a carboxylic acid, and a carboxylic acid band was present at 1700 cm^{-1}. Bands between 1140-1252 cm^{-1} were esters and aliphatic ethers.

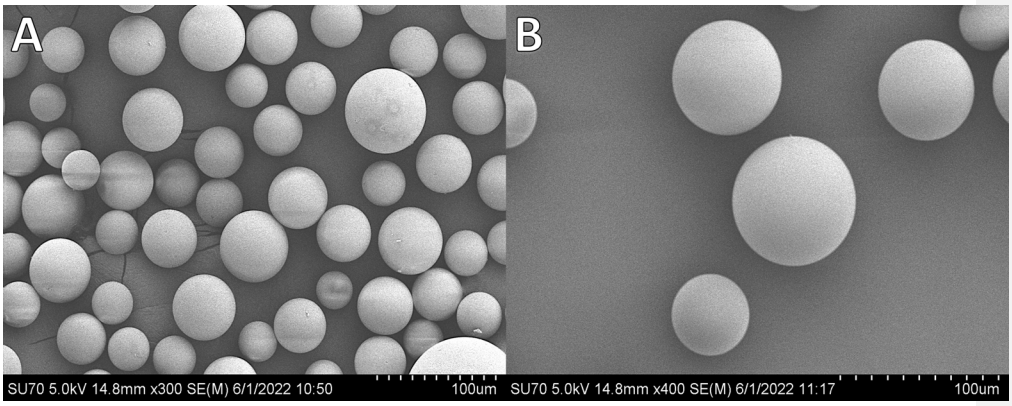


Figure 3.2: Scanning Electron Microscopy (SEM) of A) Commercial TSNA MIP material and B) Commercial NIP material

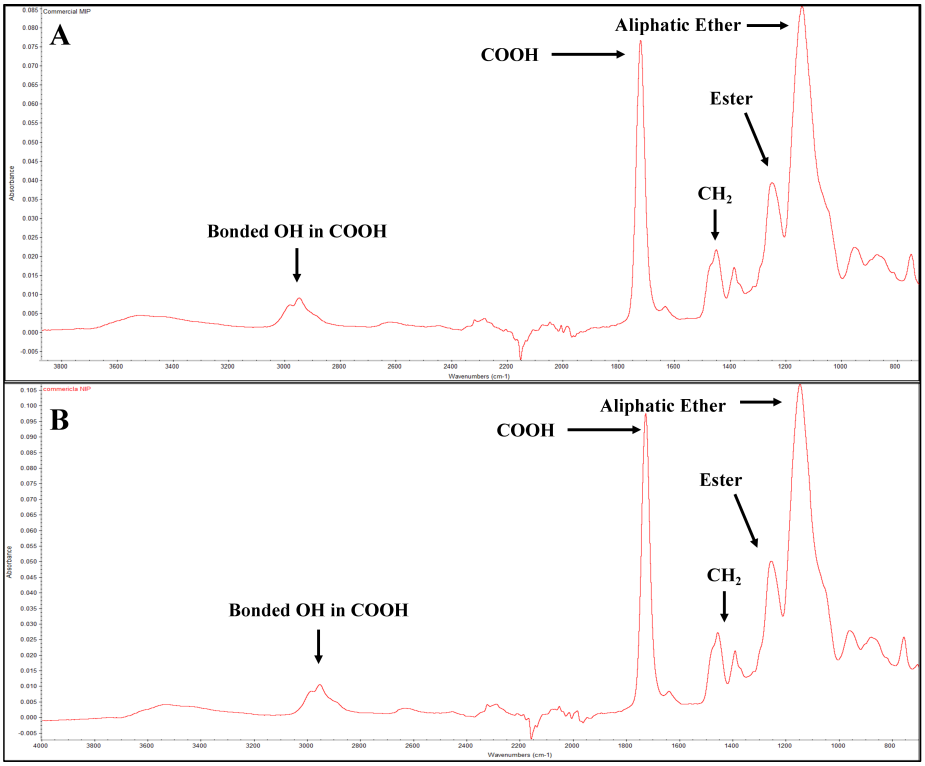


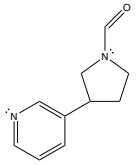
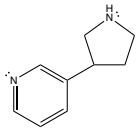
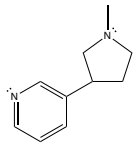
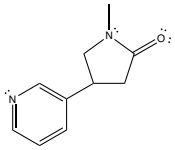
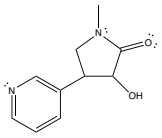
Figure 3.3: FT-IR Spectroscopy of A) Commercial TSNA MIP and B) Commercial NIP

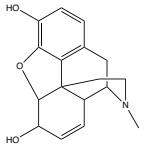
3.3.2 Cross-Selectivity of the TSNA MIP Cartridge

Table 3.2 lists the chemical information of the analytes extracted with the MIP TSNA cartridge. The protocol for the SupelMIP™ TSNA cartridges recommends that the samples are loaded at a pH of 5.5. At this stage, the four TSNAs (NNN, NAT, NAB, and NNK) are about 3-16% ionized. The positive control, NNN, had a recovery of 93 ± 4% under these extraction conditions (Figure 3.2). TSNA precursors, nicotine and nornicotine, which are between 3-16% ionized at a pH of 9.2 and 10 respectively, had recoveries of 55 ± 17% and 66 ± 4%. For nicotine's

metabolite, cotinine, and cotinine's metabolite, trans-3-hydroxycotinine, the analytes are 16% ionized at a pH of 5.5 and had recoveries of 85.6 ± 0.3% and 37.0 ± 0.6%, respectively.

Table 3.2: Chemical information of NNN, nornicotine, nicotine, cotinine, trans-3'-hydroxycotinine, and morphine

Analyte	Structure	Molecular Weight (g/mol)	pKa	Log P
NNN (Positive Control)		177.20	4.79	-0.08
Nornicotine		148.20	9.92	0.20
Nicotine		163.26	8.58	0.72
Cotinine		176.21	4.79	-0.30
Trans-3'-Hydroxycotinine		192.21	4.79	-1.48

Morphine		285.338	8.21	0.80

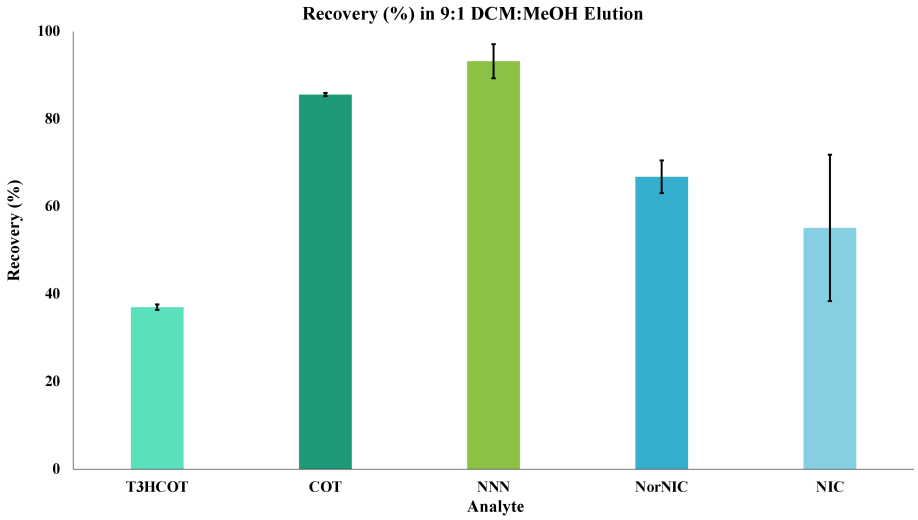


Figure 3.4: Mean recovery (mean ± SD, N = 3) of trans-3'-hydroxycotinine (T3HCOT), cotinine (COT), NNN, nornicotine (NorNIC), and nicotine (NIC) following the extraction method in Table 3.1. Samples were loaded onto cartridge at an ionization state of 3-16%.

Cross-selectivity of the imprinted polymer for each analyte was calculated using equation 3.1,

$$\frac{Recovery\ (\%)\ of\ Analyte}{Recovery\ (\%)\ of\ NNN} \times\ 100 \qquad (3.1)$$

Where the recovery (%) of the analyte is the non-TSNA tobacco alkaloid or metabolite extracted with the MIP cartridge, and the recovery (%) of NNN extracted with the MIP cartridge, which the

MIP is designed for. The cross-selectivity of the analytes nicotine, nornicotine, cotinine, and trans-3'-hydroxycotinine were: 59%, 71%, 98%, and 40%.

Cross selectivity of cotinine was further characterized by the recovery of cotinine in the presence of other interferents. Recovery of 1 µg/mL of cotinine in the presence of 1 µg/mL per interferent in 10 mM ammonium acetate, pH 5.5 was measured with NNN, NNN and nicotine, trans-3'hydroxycotinine, and vitamin C. The presence of the other interferents at equal concentrations did not significantly interfere with the recovery of cotinine ($P > 0.05$). The presence of vitamin C, however, did increase the variability (% RSD > 15%) in cotinine recovery (Figure 3.3)

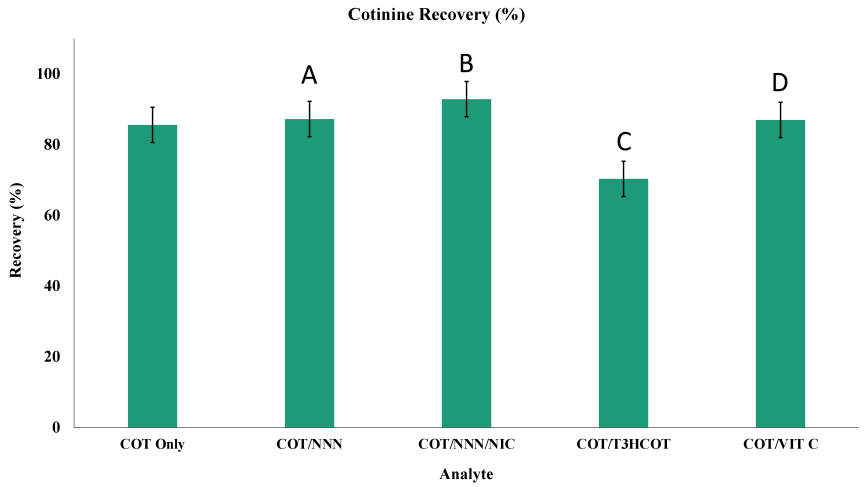


Figure 3.5: Average recovery (mean ± SD, N = 3) of cotinine in the presence of A) NNN, B) NNN and nicotine, C) trans-3'-hydroxycotinine, D) vitamin C. Cotinine was extracted at 1 µg/mL in the presence of 1 µg/mL per interferent.

3.3.3 MIP performance under different extraction conditions

When varying the loading pH of the MIP cartridge, the recovery of the analytes were affected. Analytes that had pKa values less than 5 (Table 3.2), such as NNN, cotinine, and trans-3'-hydroxycotinine, had recoveries less than 60% when the pH of the loading solution was increased from pH 5.5 to pH 10. For analytes that had pKa values greater than 5, such as nicotine and nornicotine, the recovery was less than 70% when the pH was lowered to pH 5.5 (Figure 3.4).

Table 3.2: Chemical information of NNN, nicotine, cotinine, and morphine

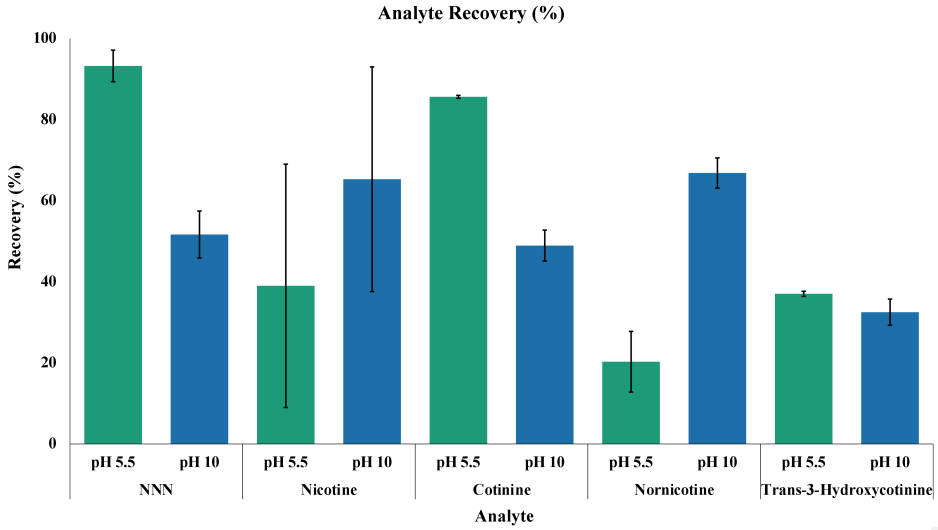


Figure 3.6: Average recovery (mean ± SD, N = 3) of analytes when loading pH was 10 mM ammonium acetate adjusted to pH 5.5 or pH 10

When adjusting the loading pH to be either 100% ionized (pH 2.79) or 0% ionized (pH 10), a drop in recovery was observed with NNN recoveries as low as 28 ± 7% and 52 ± 4%, respectively (Figure 3.5). When cotinine is 100% ionized (pH 2.79) and 0% ionized (pH 10), cotinine recovery was 7.2 ± 1.1% and 49 ± 4%, respectively. There was a significant difference ($P < 0.05$) in the recovery at 0% ionization and 100% ionization in comparison with the recovery at pH 5.5 for both

NNN and cotinine. Ionization states of NNN, cotinine, and the functional monomer, methacrylic acid, are listed in Appendix 1. At a loading pH where nicotine is 100% ionized (pH 5.5), recovery was 39 ± 30%, and when 0% ionized (pH 10), recovery was 65 ± 28%.

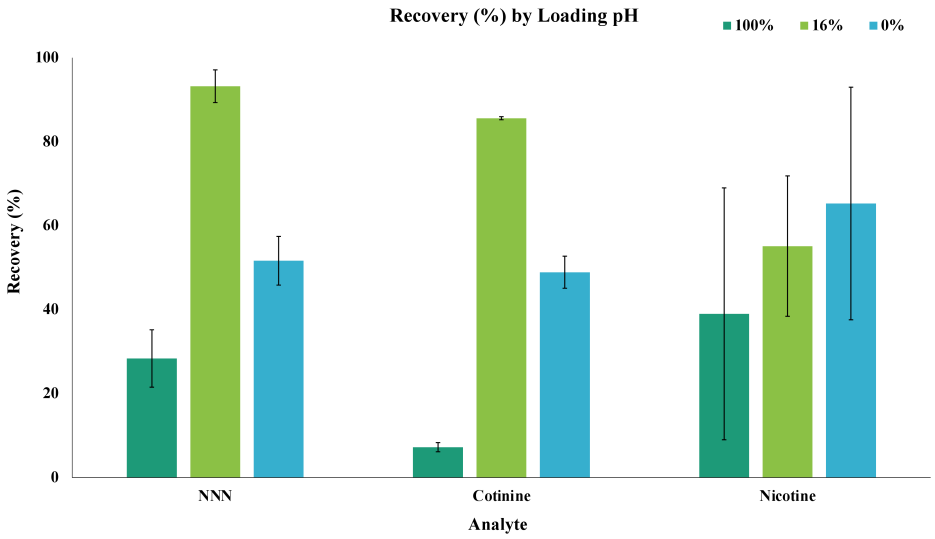


Figure 3.7: Effect of pH/ionization on average recovery (mean ± SD, N = 3) of (A) NNN, (B) nicotine, and (C) cotinine when the pH of the loading solvent was adjusted. NNN and cotinine were 100% ionized at a pH of 2.79, 16% ionized at a pH of 5.5, and 0% ionized at a pH of 10. Nicotine was 100% ionized at pH of 5.5, 16% ionized at a pH of 9.2, and 100% ionized at a pH of 10.

The high variability (% RSD > 15%) of nicotine recovery with the MIP cartridge was improved when the elution solvent was adjusted to a more lipophilic solvent, consisting of 9:1 MtBE:THF. Recovery of nicotine at pH 5.5 under these conditions was 42.6 ± 1.7% (% RSD < 15%) (Appendix 2, Figure A.1).

3.3.4 Recovery with MIP in comparison with non-imprinted polymer and traditional SPE methods

With the NIP SPE cartridge, the recovery of NNN at a pH of 5.5 was 48 ± 26%. There was a statistical difference ($p = 0.0092$) in the average recoveries of NNN at pH 5.5 with the MIP and NIP cartridges. Compared with the traditional SPE methods, the average recovery of NNN with the hydrophilic-lipophilic based (HLB) SPE cartridge was 7.8 ± 0.2% (Figure 3.6). With the mixed-mode cation exchange cartridge (MCX), NNN was prematurely eluted in the washing stage with average recovery 27 ± 6%, and was not present in the elution fraction. Recovery of NNN with the MIP cartridge compared to the traditional SPE cartridges was significantly different ($p < 0.05$). For cotinine, the NIP SPE cartridge had an average recovery of 66 ± 23%, and there was no statistical difference in the average recoveries of the MIP and NIP cartridges ($p = 0.2316$). With the traditional SPE cartridges, cotinine's average recovery with the HLB SPE cartridge was 4.87 ± 0.09% (Figure 3.6). Similar to NNN, cotinine was prematurely eluted in the washing step of the MCX cartridge, with average recovery being 33 ± 14%. There was a statistical difference ($p < 0.05$) between the recoveries of cotinine with the MIP cartridge and the traditional SPE cartridges. For nicotine, average recovery with the non-imprinted polymer was 87 ± 5%. There was a statistical difference ($p = 0.0448$) in the average recoveries of nicotine with the MIP cartridge and the NIP cartridge. With the traditional SPE cartridges, nicotine's average recovery was 27 ± 15% for the HLB cartridge and 24.0 ± 0.6% for the MCX cartridge (Figure 3.6). A portion of nicotine was prematurely eluted during the washing step of the MCX cartridge.

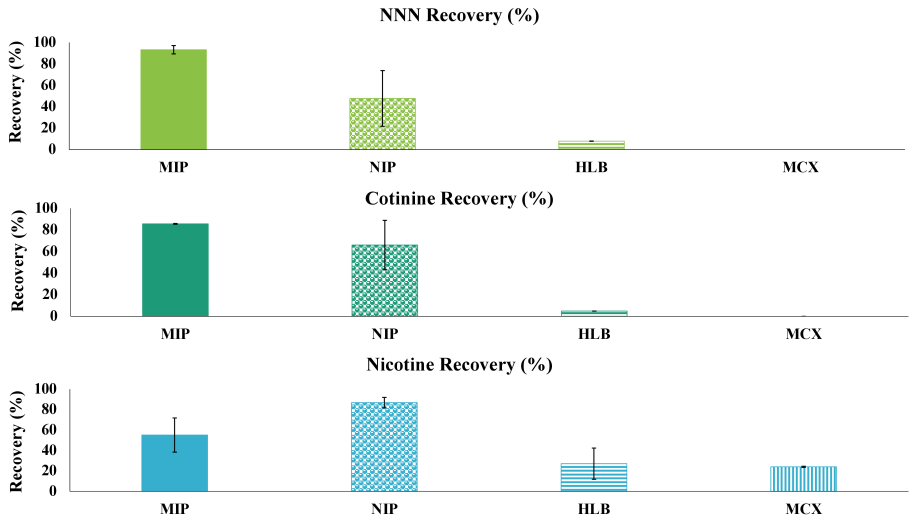


Figure 3.8: Average recovery (mean ± SD, N = 3) of analytes with A) the TSNA MIP cartridge, B) the non-imprinted polymer (NIP) cartridge, C) the hydrophilic-lipophilic balance SPE cartridge, and D) the mix mode cation exchange SPE cartridge.

3.3.5 Recovery of morphine with the TSNA MIP cartridge

At a pH of 9.2, morphine is 16% ionized and had an average recovery of 82 ± 5% with the TSNA MIP (Figure 3.7). Using equation 3.1, the cross reactivity of morphine with the TSNA MIP cartridge was 87.9%. Average recovery with the NIP cartridge at the same pH was 91.93%. There was no statistical difference between the morphine recoveries with the MIP and NIP cartridges ($p = 0.6216$). With the traditional SPE cartridges, morphine had an average recovery of 88 ± 4% with the HLB cartridge and 112 ± 2% with the MCX cartridges. Only MIP cartridge and the MCX cartridge had a statistically different recovery ($p = 0.0380$).

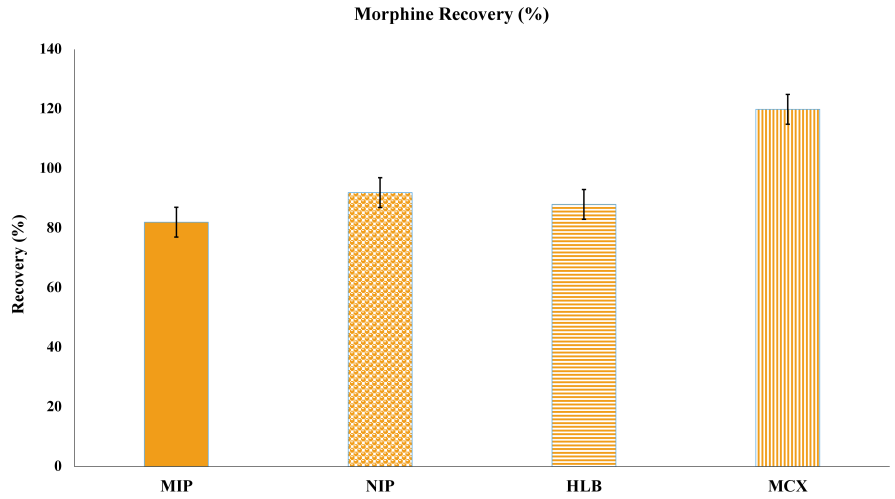


Figure 3.9: Average recovery (mean ± SD, N = 3) of morphine with A) the TSNA MIP cartridge, B) the non-imprinted polymer (NIP) cartridge, C) the hydrophilic-lipophilic balance SPE cartridge, and D) the mix mode cation exchange SPE cartridge.

3.3.6 Recovery of cotinine in urine with TSNA MIP Cartridge

The addition of an aqueous wash step consisting of 1 mL of 10 mM ammonium acetate, pH 5.5 caused a loss of recovery of cotinine in the extraction. Prior to the aqueous wash, cotinine had an extraction recovery of 85.6 ± 0.3%. With the addition of the wash step, the extraction recovery of cotinine dropped to 43.8 ± 1.4%, with 41.8 ± 9.6% recovered in the aqueous wash fraction (Figure 3.8). Therefore, an aqueous wash to further remove matrix effects was not included in the extraction.

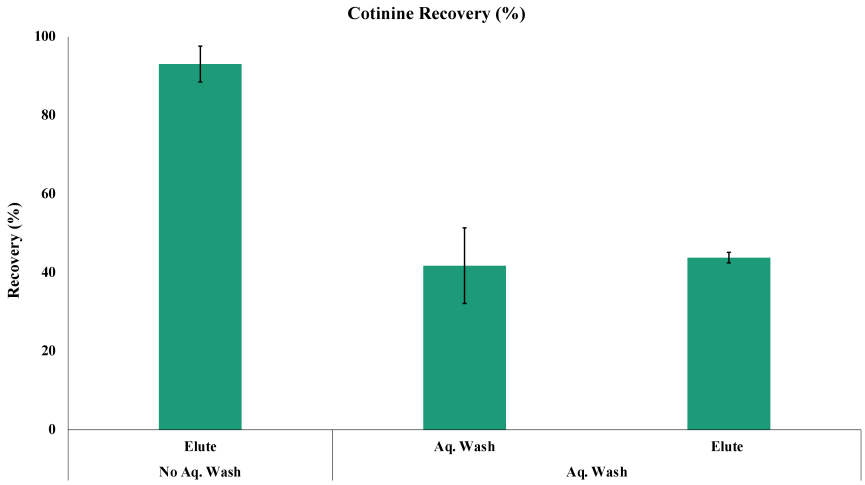


Figure 3.10: Average recovery (mean ± SD, N = 3) of cotinine with and without an aqueous (aq.) washing step with 10 mM ammonium acetate, pH 5.5.

Absolute recovery and matrix effects of cotinine in urine were calculated. Absolute recovery was determined by the measured response of the cotinine extract to an unextracted cotinine solution of the same concentration. Matrix effects were calculated by the measured response of a blank extract that was infused with cotinine prior to injection to a neat, unextracted cotinine standard of the same concentration. Absolute recovery of cotinine in urine was greater than 85% for all three concentrations. Matrix effects measured at the middle concentration, 100 ng/mL, was -7.84% (Table 3.3).

Table 3.3: Average recovery (mean ± SD, N = 3) of cotinine at 10, 100, and 1000 ng/mL in urine with matrix effects for middle concentration (range).

Concentration (ng/mL)	Recovery (%)	Matrix Effects (%)
10	97.1 ± 2.5	
100	107.8 ± 2.1	5.4 – 9.3
1000	89.3 ± 8.5	

3.4. Discussion

3.4.1. Physical Characteristics of the Commercial Polymer

While the preparation of the commercial polymer is ultimately proprietary, some information about the polymer and its characteristics could be determined by SEM and FT-IR. The SEM analysis showed that the MIP and NIP particles are spherical in nature, and are smaller than 100 μm in size. The certificate of analysis that is accompanied with the cartridges state that the particles are about 30-90 μm in size, and the average particle size is 50 μm.

The FT-IR analysis allowed for the determination of functional groups present on the polymer. The bands at 2900-3000 cm^{-1} that represent the bonded alcohol with the carboxylic acid could potentially be from methacrylic acid, or a MAA-like molecule that is used as the functional monomer. As discussed in Chapter 1, methacrylic acid is one of the most popular choices for functional monomers due to its proton donating and proton receiving capabilities with other analytes (12, 41). The aliphatic ether and ester bands from 1140-1252 cm^{-1} could be from methacrylate- or polystyrene-based crosslinking groups. If the polymers were made using MAA and methacrylate- or styrene-based crosslinking groups, they were most likely made by free-radical polymerization and for non-covalent bonding.

3.4.2 Characterizing the Cross-Selectivity of the TSNA MIP

The theory behind molecularly imprinted polymers is that the selective interaction is governed by the size, shape, and functional group chemistry of the target analyte with the imprinted cavity and functional monomer (7). Following this theory, analytes with similar structures and chemistries as the target analyte should be able to interact with imprinted cavity under similar conditions, allowing for imprinted polymers that are class selective for a group of analytes. Murakami et al (30) demonstrated that a commercial MIP for amphetamines could successfully be

applied to synthetic cathinones with specific adjustments made to the extraction method. The manufacturer's recommendation for the Affinilute MIP for amphetamines recommends that the sample loading conditions are buffered at a pH of 8, which is when methamphetamine and amphetamine are in their ionized state (24). The authors demonstrated that when the pH of the loading solvent was lowered to a pH of 6, which is when synthetic cathinones are in their ionized state, the recovery of the synthetic cathinones improved (30). Synthetic cathinones and amphetamines have partition coefficient (logP) values within one order of magnitude, and adjustments to the elution solvent to disrupt the non-covalent interactions between the analyte and functional monomer was not required.

The imprinted polymer used in this study was designed for the extraction of four TSNAs in urine, making it a class selective MIP. Further, the cross-selectivity of NNK's urinary metabolite, NNAL (32, 36), suggests that it could be class selective for more tobacco alkaloids. This is due to the fact that all the tobacco alkaloids in this study share the common functional group: A pyridine ring. The alkaloids also have a pyrrole functional group that has either a secondary or tertiary amine, a carbonyl group, and/or a hydroxyl group.

With the tobacco alkaloids extracted with the TSNA MIP, the effect of loading pH and elution solvent of the extraction in relation to the recovery of the analyte was observed. The loading pH is going to promote the non-covalent interactions between the analyte and the imprinted cavity, and the elution solvent is going to disrupt the non-covalent interaction. As previously stated, the optimized extraction conditions for TSNAs required that the analytes be in a state of 3-16% ionized (pH 5.5) when loaded onto the cartridge, and eluted with a 9:1 (v/v) DCM:methanol solution (Table 3.1). As NNN was the TSNA used as the positive control for this study, it was unsurprising that it had the highest recovery of the tobacco alkaloids in this study. The analyte that had the

second highest recovery was cotinine, which had the same pKa (4.79) as NNN, and had a logP value that was within one order of magnitude of NNN. Trans-3'hydroxycotinine, however, while having the same pKa as NNN, had a logP value that was nearly two orders of magnitude lower than NNN, resulting in low (< 50%) recoveries with the current elution solvent (Figure 3.3).

When adjusting the loading pH to either pH 5.5 or pH 10 (Figure 3.4), a change in recovery for NNN, cotinine, and nornicotine was observed. When further characterizing the effect of loading pH on analyte recovery when NNN, cotinine, and nicotine were at ionization states of 0, 16, or 100% ionized (Figure 3.6), a change in recovery was observed for NNN and cotinine. The drop in recovery of NNN and cotinine when 0% ionized (pH 10) is most likely due to the fact that NNN and cotinine are no longer ionized and cannot interact with the functional monomer, methacrylic acid, through electrostatic interactions (Appendix 1, Figure A.1). However, when NNN and cotinine are 100% ionized (pH 2.79), methacrylic acid, a weak acid, is no longer in its ionized state, and again, electrostatic interactions between the analyte and functional monomer, cannot occur. At a pH of 5.5, NNN and cotinine are 16% ionized, and methacrylic acid is 86% ionized, allowing for electrostatic interactions to occur, and to promote the selective binding inside the imprinted cavity.

Under the manufacturer conditions, the cross-selectivity of the MIP for the tobacco alkaloids was not only driven by the pH of the of the analyte, but also by the functional group chemistry and elution solvent. This was best reflected in nicotine, where there was no change in average recovery by pH due to the high variability (% RSD >15%). While NNN is partially derived from nicotine, the functional group chemistry of nicotine is different from that of NNN. The first sight of ionization of nicotine is the tertiary amine on the pyrrole, which also lacks the nitrosamine group that may be a site of interaction for NNN. Further, the partition coefficient of nicotine is

more lipophilic than NNN. When the elution solvent was adjusted to more lipophilic solvents, such as the 9:1 MTBE:THF, the variability of nicotine's recovery decreased with % RSD values below 5%. Trans-3'hydroxycotinine also showed no change in recovery when the pH of the loading solvent was changed, suggesting that a more hydrophilic loading solvent may have further improved its recovery with the TSNA MIP cartridge.

3.4.3 TSNA MIP Performance in Comparison with Other Extraction Techniques

The non-imprinted polymer (NIP) serves as a control for the MIP. The NIP is created in the absence of a template, preventing the formation of imprinted cavities (8). Therefore, all interactions with the NIP are considered non-specific. The recovery of NNN with the NIP cartridges resulted in less than 50% recovery and high variability (RSD > 15%). The low recovery and high variability with the NIP in comparison with the TSNA MIP cartridge suggests that specific interactions with the imprinted region of the MIP aid in the retention and recovery of the TSNAs. Cotinine followed a similar trend with the NIP cartridge (Figure 3.6). Nicotine, however, had greater than 80% recovery with low variability (RSD < 10%) with the NIP cartridge, without having to make changes to the elution solvent. This suggests that the imprinted cavity has an effect on the retention and recovery of nicotine with the TSNA MIP cartridge.

Extraction of the analytes using traditional SPE cartridge methods designed for TSNAs were compared to the recovery of the analytes with the TSNA MIP cartridges. The hydrophilic-lipid balance (HLB) cartridges are designed with hydrophilic and lipophilic copolymers, acting as a reverse phase SPE cartridge and allowing for a wide range of analytes to be extracted with the cartridge (99). The mix mode cation exchange (MCX) cartridge is a reverse phase sorbent derived

from the HLB copolymers and is designed for base analytes. The extraction protocols used in this study were optimized for the extraction of TSNAs (87, 96)

Previous literature stated that TSNAs have low recoveries with traditional SPE cartridges and require multiple extractions to efficiently remove matrix interferents. (36, 96). Using previously published methods for the extraction of NNN the HLB SPE recoveries for both NNN and cotinine were less than 10%. Nicotine had recoveries less than 30% with the same method. With the MCX cartridges, NNN and cotinine eluted during the methanolic washing step. The early elution with the MCX cartridge could be due to the fact that the analytes were loaded in an unionized state. Nicotine had recoveries less than 30% with the MCX cartridges, and a fraction of nicotine also eluted during the washing step. The low recoveries of nicotine with the traditional SPE cartridges is likely due to the fact that the extraction methods were designed for the extraction and recovery of TSNAs. Previous literature using SPE and micro-solid phase extraction techniques for the recovery of nicotine in urine yielded greater than 80% recoveries (100, 101).

3.4.4 Recovery of a Non-Tobacco Alkaloid with the TSNA MIP cartridge

The extraction of morphine with the TSNA MIP cartridge was used to assess the recovery of an analyte that was not a tobacco alkaloid and was structurally different. Morphine was selected because of its similar pKa and logP values to that of nicotine (Table 3.8), and it was initially theorized that morphine would exhibit the same variability observed with nicotine. However, morphine had greater than 80% recovery with the TSNA MIP and low variability (RSD < 15%). While morphine most likely does not fit into the imprinted cavities, it could interact with the MIP cartridge through surface, non-specific interactions. Non-specific interactions are formed during the polymerization of the imprinted polymer, due to the presence of excess functional monomers

that do not form bonds with the template. (9). Morphine's interactions with the excess functional would lead to the high recoveries. The similarly high recoveries of morphine with the non-imprinted polymer and two SPE cartridges further suggest that the interactions with the MIP cartridge were due to non-specific surface interactions rather than selective, specific interactions with the imprinted cavity. The addition of a second washing steps could potentially disrupt morphine's non-specific interaction with the polymer. In future studies, it would also be beneficial to determine if morphine's presence would affect the extraction recovery of the TSNAs.

3.4.5 Extraction of Cotinine in Urine with the TSNA MIP

In a biological matrix such as urine, cotinine would not be the only analyte present in the sample. Potential analyte interferents, such as NNN, nicotine, trans-3'hydroxycotinine, and vitamin C may compete with cotinine for binding sites and inhibit its recovery with the imprinted polymer. However, when assessing cotinine's recovery in the presence of these potential interferents, cotinine's recovery was not significantly affected (Figure 3.4). Coupled with the high recoveries (> 80%) of cotinine in water, the extraction of cotinine in a biological matrix with the MIP TSNA was possible.

The addition of an aqueous wash step was intended to remove salts and matrix interferents from the polymer. Cotinine being a urinary metabolite, however, is hydrophilic, and therefore was able to be displaced by the polymer when an aqueous washing step was added to the protocol. This also suggests that there is a degree of specific and non-specific interactions between cotinine and the imprinted polymer. With the omission of the aqueous washing step, however, cotinine's successful recovery in urine (> 85%) for all three concentrations indicates that cotinine can be reliably extracted from biological samples with the TSNA MIP. Coupled with the previously

reported successful co-extraction of NNAL with the TSNA MIP, the simultaneous extraction of cotinine with the TSNAs and their metabolites warrants further exploration. Urinary cotinine levels are often correlated with NNN and the urinary metabolite of NNK, NNAL, for total nicotine equivalence (91, 93). The TSNA MIP cartridges could potentially simultaneously extract cotinine and TSNAs while avoiding matrix challenges that have been encountered in other simultaneous extractions (95, 102).

3.5 Conclusions

An extensive characterization of the cross-selectivity of the TSNA MIP cartridges was performed in this study. NNN, which the TSNA MIP was designed for, had the highest recoveries of all the analytes extracted. The TSNA MIP's superior performance for the extraction of NNN compared with traditional SPE cartridges suggests that MIPs can improve the extraction recovery. Cross-selectivity of the TSNA MIP cartridge with other tobacco alkaloids was dependent upon the analyte's structure and functional group chemistries. Cotinine had similar recoveries with NNN, most likely due to its similar chemical properties with the TSNAs. Nicotine, despite being a precursor to TSNAs, had highly variable recoveries, possible due to the differing structural and chemical properties between the precursor and the TSNAs. This was remedied with the change in elution solvent, but ultimately led to lower recoveries than traditional SPE methods. Non-tobacco alkaloids, such as morphine, initially had high (> 80%) recoveries with the MIP, but also displayed high recoveries with the other extraction methods, suggesting that the interactions with the polymer were non-specific surface interactions that could be disrupted with more comprehensive washing steps. This study showed that the MIP's cross-selectivity can be utilized for the selective extraction of cotinine in urine. The experiments carried out in this study better defined the

interactions between the analyte and the imprinted polymer. Further the utilization of the TSNA's cross-selectivity with other tobacco biomarkers can allow for simultaneous extraction and identification of multiple biomarkers with one, simple sample preparation technique.

Chapter 4: Direct Analysis of Tobacco Specific Nitrosamines in Tobacco Products Using a Molecularly Imprinted Polymer-Packed Column

This chapter has been drawn from an article Under Review for Publication in Journal of Separation Science

4.1 Introduction

The presence of TSNAs, specifically in N-nitrosonornicotine (NNN) in nicotine and tobacco products, are of great interest to the Food and Drug Administration (FDA). NNN and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are considered highly carcinogenic, contributing to mouth, throat, and lung cancer in association with cigarette use, and are listed on the FDA's Harmful and Potentially Harmful Constituents list (103–105). In smokeless tobacco, the FDA has proposed recommendations that NNN levels in such products do not exceed 1 μg/g. (106). As of 2020, however, these limits have not been met in current US tobacco products, with 2.5-5.0 μg/g NNN detected in smokeless tobacco products (107). Electronic cigarettes (E-cigarettes), while marketed as a safer alternative to cigarettes, have no TSNA regulations proposed by the FDA. TSNAs have been detected in e-cigarette e-liquids with reported levels from 0.22-9.84 ng/mL for NNN and 0.11-1.11 ng/mL for NNK (108–110). While the TSNA content in e-cigarettes is lower than the levels found in traditional cigarettes, the possible consumption of TSNAs is not properly labelled on e-liquid bottles (110, 111).

Because TSNAs are found at low levels inside nicotine and tobacco products, and at even lower concentrations in e-cigarette products, sensitive and selective methods are required for TSNA analysis. Kim and Shin (2013), reported using solid phase extraction (SPE) methods for the extraction of TSNAs from the propylene glycol in e-liquid refills. They reported a less than 30% recovery with three different SPE based methods (112). With molecularly imprinted polymers (MIPs), however, the imprinted cavity for TSNAs can lead to more selective extraction in complex

matrices (86). The specific selectivity of the imprinted polymer for the analyte of interest, allows for extensive washing to remove matrix components, and leads to cleaner extracts. The cleaner extracts aid in the better detection and higher recoveries in analytical instruments. Commercial MIPs for the extraction of TSNAs in urine have been previously reported on with recoveries greater than 30% (87).

As discussed in Chapter 1, the most common application for MIPs is in the offline, benchtop extraction as the sorbent for SPE cartridges. MIPs, however, can be used in place of traditional HPLC columns, allowing for the sample to be simultaneously extracted and detected with the MIP directly on the analytical instrument (16, 21, 53, 84, 113). With in-line MIP extractions, the analyte is retained inside the imprinted cavity, and through careful solvent selection, matrix components can be washed off the polymer and directed to waste before the analyte is eluted and directed to the detector. In-line MIPs are primarily achieved by polymerizing monolithic stationary phases inside the analytical column, or creating the polymer under bulk polymerization and packing the polymer into the column under slurry conditions (21, 114, 115). The first known in-line MIP was reported by Sellergren in 1994, which demonstrated the enrichment of pentamidine in urine. In-line MIPs have been used for a limited number of analytes, primarily in urine and plasma. In urine, no sample preparation is needed, but for plasma, a protein precipitation or liquid-liquid extraction is used to remove proteins prior to introduction on the analytical column (16, 19).

Presented is the development of a molecularly imprinted high performance liquid chromatography (HPLC) column using the commercial TSNA polymer by a slurry packing method for the direct analysis of TSNAs in nicotine and tobacco products. A non-imprinted polymer (NIP) column was first created and used to develop the initial chromatographic

parameters. A TSNA MIP-packed HPLC column was then created and an optimized method for the analysis of NNN and NNK was validated following the proposed guidelines from the US Department of Health and Human Services Center for Tobacco Products Guidance for Industry (116). The MIP-packed column was also characterized for chromatographic properties and column uniformity of the packing method. Nicotine and tobacco products consisting of moist oral snuff (SNUS), oral nicotine products, pipe tobacco, and e-cigarette e-liquids were analyzed using the MIP-HPLC column. Finally, other tobacco biomarkers, such as cotinine, were assessed with the MIP HPLC column.

4.2. Materials and Methods

4.2.1 Materials and Chemical Reagents

TSNA molecularly imprinted polymer powder (particle size 30-90 μm) and non-imprinted polymer made under the same conditions were purchased from Biotage (Uppsala. Sweden). Acetic acid, ammonium hydroxide (28-30%), HPLC grade acetonitrile, chloroform, isopropyl alcohol (IPA), methanol, toluene, and water were purchased from VWR International (Radnor, PA). Ethanol (EtOH) and formic acid were purchased from Sigma Aldrich (St. Louis, MO). N-nitrosonirnicotine (NNN), NNN-d4, 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) were purchased from Toronto Research Chemicals (Toronto, ON). Propylene glycol (PG) was purchased from Amresco LLC, VWR, USA. USP grade vegetable glycerin (VG) was purchased from JT Baker, USA. Cotinine (99.7%) and nicotine (99.6%) were purchased from Cerilliant (Round Rock, TX).

4.2.2 Preparation of NIP and MIP columns

The NIP column and TSNA MIP column were prepared following a modified method (117) under slurry conditions using a Teledyne (Thousand Oaks, CA) packing pressure system. Three HPLC columns were packed from the TSNA MIP material from the same lot. The end fitting and 0.2 µm frit from one end of a Restek (Bellefonte, PA) column assembly kit (50 mm x 2.1 mm ID, ¼" OD) was removed and attached to a reservoir with a glass frit (Figure 4.1). One gram of NIP or TSNA MIP material from Biotage was suspended in 20 mL of a 1:1 (v/v) chloroform:ethanol solution to create a slurry, which was sonicated for 15 seconds. The mixture was poured into the reservoir and the system was capped and secured. Pushing solvent consisting of 1:1:1 (v/v/v) ethanol:isopropyl alcohol:toluene was used to push the slurry mixture through the column. Helium gas was used to degas the solvent during the packing process. The pushing solvent was slowly increased from 0.5 mL/min to 10 mL/min with a total system pressure around 2000 psi. The flow was kept at 10 mL/min for one hour before the system was disassembled and the frit and end fitting were reassembled on the column. The freshly packed column was washed with 100% acetonitrile at a flow rate of 0.5 mL/min for 60 minutes using an external Shimadzu LC-10AD HPLC pump (Kyoto, Japan). A non-imprinted polymer column was created for initial method development using nicotine as a model.

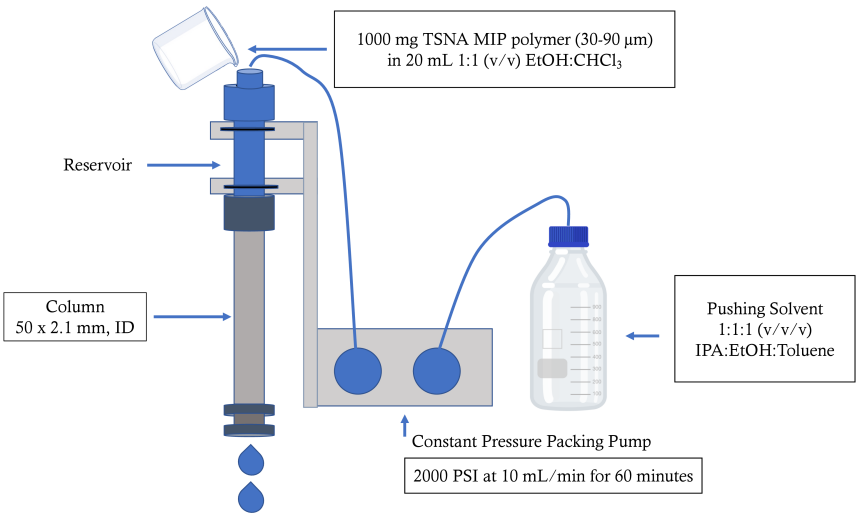


Figure 4.1: Preparation of TSNA MIP HPLC packed column. An empty 50 x 2.1 mm, ID Restek column was attached to a reservoir on a Teledyne Constant Pressure Packing Pump. A slurry mixture consisting of 1000 mg MIP mixed with 20 mL of 1:1 (v/v) ethanol:chloroform was poured into the reservoir. The slurry was pushed through the reservoir and into the column with 1:1:1 (v/v/v) ethanol:isopropyl alcohol:toluene operated a 10 mL/min (2000 PSI) for one hour.

4.2.3 Preparation of Reagents

Standard stock solutions of cotinine (1 mg/mL), nicotine (1 mg/mL), NNN (1 mg/mL), NNK (1 mg/mL) and NNN-d4 (100 µg/mL) in MeOH was used for analysis. A working stock solution of 100 µg/mL cotinine and nicotine and working stock concentrations of 1000 and 100 ng/mL of NNN/NNK in MeOH were stored at -20 ºC. Working stock concentration of 10 µg/mL NNN-d4 was prepared in MeOH and stored at -20 ºC. Nicotine standards prepared at 5 µg/mL in 10 mM ammonium acetate; pH 9.2 was used for all method development experiments.

Calibrators were prepared fresh daily in 10 mM ammonium acetate, pH 5.5. Internal standard at 25 ng/mL NNN-d4 was prepared fresh daily in 10 mM ammonium acetate, pH 5.5. QC samples in a matrix to match the SNUS, oral nicotine products, and pipe tobacco were prepared in

10 mM ammonium acerate, pH 5.5. Quality control (QC) samples in a matrix to match electronic cigarettes e-liquids were prepared by dissolving NNN/NNK working standards in a mixture of propylene glycol and vegetable glycerin (70:30 v/v). Prior to analysis, e-liquid QCs were diluted 1:10 in 10 mM ammonium acetate, pH 5.5.

4.2.4 Method development and optimization

Initial method development was conducted with a non-imprinted polymer packed column on a Waters Acquity H-Class UPLC with an Acquity Photodiode Array Detector. Nicotine at a concentration of 5 μg/mL in 10 mM ammonium acetate, pH 5.5 was injected in 10 μL aliquots onto the system. Method development studies consisted of mobile phase composition, gradient conditions, flow rate, and column temperature for optimal chromatographic results. Void volume (V_0) of the column was determined injecting a 7:3 (v/v) ACN:MeOH mixture onto the column. Retention time of the solvent peak was used to determine the void volume at the flow rate of the analytical method. The void volume was later used to calculate retention factor (k'). Method optimization for the TSNA MIP column was conducted on a SCIEX ExionLC 2.0 Binary Pump UPLC equipped with a SCIEX SelexION 6500+ Q-Trap. The chromatographic quality of other tobacco biomarkers, such as cotinine, were also assessed with the TSNA MIP column under the developed chromatographic conditions.

4.2.5 Method Validation

Method validation for the analysis of NNN and NNK with the TSNA MIP column was carried out following the US Department of Health and Human Services Center for Tobacco Products Guidance for Industry for linearity, limit of quantitation (LOQ), and accuracy and

precision (116). Column uniformity and autosampler stability were also assessed during this method validation. Six calibration standards having concentrations of 0.10, 0.25, 0.50, 1.00, 5.00, and 10.0 ng/mL (Range of 0.04-10 μg/g) of NNN and NNK were prepared in 10 mM ammonium acetate, pH 5.5 in triplicate. Linearity was evaluated through linear regression with a weighting of 1/x and coefficient of variation (r^2). Standards were back-calculated from the generated linear regression and the residual concentrations were required to be no more than 15% deviation from the nominal value, also known as amount deviation from normal (% DFN). The lower limit of quantitation (LLOQ) was the lowest calibration standard concentration of NNN and NNK. Accuracy and precision were determined from quality control (QC) samples injected in triplicate for three different validation runs (N=9) QC samples prepared in 10 mM ammonium acetate, pH 5.5 were prepared at four concentrations: A limit of quantitation-QC (LLQC, 0.1 ng/mL), a low-QC (LQC, 0.3 ng/mL), a medium-QC (MQC, 3.0 ng/mL), and a high-QC (HQC, 7.5 ng/mL). QC samples prepared in 70:30 (v/v) PG:VG were diluted 1:10 to three concentrations: A low-QC (LQC, 0.3 ng/mL), a medium-QC (MQC, 3.0 ng/mL), and a high-QC (HQC, 7.5 ng/mL). The acceptable criteria for accuracy were calculated concentrations within ± 15% of the nominal concentrations (% DFN). Precision was expressed as a percent relative standard deviation (% RSD) and should not exceed 15%.

4.2.6 Autosampler Stability

Autosampler stability was assessed by reinjecting one set of controls left in the autosampler for 24 hours, 48 hours, and 72 hours, in addition to a fresh set of controls with each injection set. The QC injections from each day were calculated using the original calibration curve. Acceptable

criteria for autosampler stability were accuracy values within ± 15% of the nominal concentrations (% DFN) and precision levels calculated as % RSD not exceeding 15%.

4.2.7 Column Uniformity

Three HPLC columns were packed on separate occasions following the methods outlined in section 2.3. Low, medium, and high QC concentrations in 10 mM ammonium acetate and 70:30 PG:VG were injected in triplicate on each column. Retention time, retention factor (k'), peak area, calculated concentration, accuracy, asymmetry, peak tailing, and theoretical plate number (N) were assessed. Retention factor (k') was calculated by,

$$k' = \frac{t_r - t_0}{t_r} \quad (1)$$

where t_0 is the void volume and t_r is the retention time in minutes. Tailing factor (T_f) was calculated as,

$$T_f = \frac{a+b}{2a} \quad (2)$$

where a is the front half of the peak at 5% of the peak height and b is the back half of the peak at 5% peak height. Theoretical plate number (N) was calculated as

$$N = 5.54\left(\frac{t_R}{W_{50}}\right)^2 \quad (3)$$

where t_r is the retention time and W_{50} is the width of the peak at 50% peak height. Acceptable criteria for column uniformity were precision (% RSD) values < 15% for each parameter and calculated concentrations within ± 15% of the nominal value (% DFN).

4.2.8 Preparation of Samples

Fourteen electronic cigarette e-liquids were analyzed for this study (Figure 2). Ten e-liquids were purchased from various shops in the United States prior to 2016 and stored at room temperature away from light. Four e-liquids were purchased from shops in Europe after 2016 and stored in the refrigerator. E-liquid samples were prepared in a 1:5 dilution in 10 mM ammonium acetate, pH 5.5 prior to analysis.

Figure 4.2: Nicotine and Tobacco products analyzed in this study: A) 14 e-liquids; B) 8 oral nicotine pouches and 3 Camel SNUS products; C) one pipe tobacco product labelled as "dohka".

Twelve nicotine/tobacco products described as either oral nicotine pouches, smokeless tobacco (SNUS), or pipe tobacco labelled as dohka were purchased from various vendors. Samples were stored in their original containers at room temperature away from light. Samples were prepared following the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) method. In brief, 250 mg of sample removed from the product pouch were vortexed

with 10 mL of 10 mM ammonium acetate, pH 5.5 for 60 minutes. The samples were filtered through a Whatman™ 0.45 μm polyether sulfone membrane syringe (Maidstone, United Kingdom). The SNUS and pipe tobacco products were further diluted in a 1:100 or 1:1000 dilution in 10 mM ammonium acetate, pH 5.5.

4.3. Results

4.2.1 Chromatographic Conditions

Results of the method development with the NIP column with nicotine as a model analyte are listed in Appendix 2. Mobile phase A was 10 mM ammonium acetate and mobile phase B was 100% acetonitrile, and the injection volume was 10 μL. The non-imprinted polymer column (50 mm x 2.1 mm, 30-90 μm) was operated under the conditions listed in Table 4.1, and operated at 50 °C. The PDA monitored nicotine at a wavelength of 260 nm, and the entire run was 10 minutes. Under 100% mobile phase A conditions, nicotine was retained on the column. When the mobile phase conditions were changed to an organic mobile phase, nicotine had a retention time of 4.58 minutes (Figure 4.3). The chromatographic peak had asymmetry and tailing factors of 1.10 and 1.09, respectively (% RSD < 2, N=3). Theoretical plate number was 737 (% RSD 3.16, N=3).

Table 4.1 HPLC gradient for nicotine analysis on NIP column on Acquity UPLC-PDA

Time (min)	Flow (mL/min)	% Mobile Phase A	% Mobile Phase B
Initial	0.1	100	0
2.0	0.1	100	0
3.1	0.1	0	100
4.0	0.2	0	100
7.0	0.1	0	100
7.1	0.1	100	0
10.0	0.1	100	0

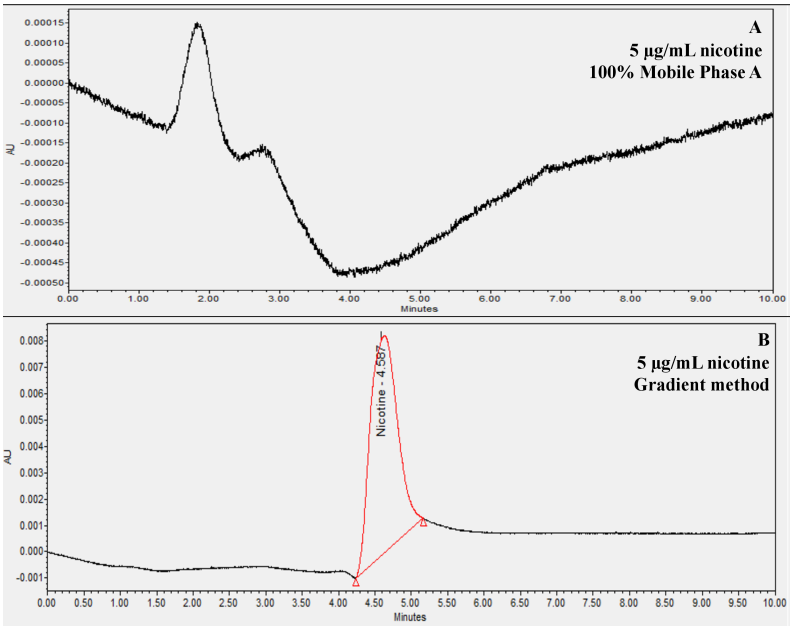


Figure 4.3: Representative chromatograph of nicotine on NIP column with final conditions. A) Nicotine injected under isocratic method of 100% mobile phase A is retained on column. B) When injected with a gradient that changes to 100% mobile phase B, nicotine is eluted from column.

The method for the analysis of TSNAs with the TSNA MIP column was optimized and validated using a SCIEX ExionLC 2.0 Binary Pump UPLC equipped with a SCIEX SelexION 6500+ Q-Trap. Mobile phase A was 10 mM ammonium acetate, pH 5.5 and mobile phase B was 0.1% formic acid in methanol. The in-house prepared MIP-packed column (50 x 2.1 mm ID, 30-90 µm) was operated at a flow rate of 0.45 mL/min at a temperature of 40 ºC. The autosampler was kept at a temperature of 5 ºC and the injection volume was 10 µL. A gradient method was developed and outlined in Table 4.2, and the entire run time was five minutes (Figure 4.4). The ESI source of the mass spectrometer was operated in positive mode. The declustering potential

was set to 20 eV and the temperature of the source was set to 550 °C and ion source gases 1 and 2 were set to 60 and 25 mL/min respectively. The mass spectrometer was operated in multiple reaction monitoring mode (MRM) for the following ions with collision energy in parentheses: NNN, 178 > 120 *m/z* (27 V) and 178 > 148 *m/z* (14 V); NNK, 208 > 122 *m/z* (16 V) and 208 > 148 *m/z* (18 V); and NNN-d4, 182 > 124 *m/z* (27 V) and 182 > 152 *m/z* (14 V). Void volume of the column under these analytical conditions was 0.1608 ± 0.0096 mL.

Table 4.2: Gradient conditions for the SCIEX SelexIon LC 2.0 HPLC Pumps

Time	Mobile Phase A (%)	Mobile Phase B (%)
0.0	100	0
1.0	40	60
2.0	40	60
3.0	0	100
3.1	0	100
4.0	0	100
4.1	100	0
5.0	100	0

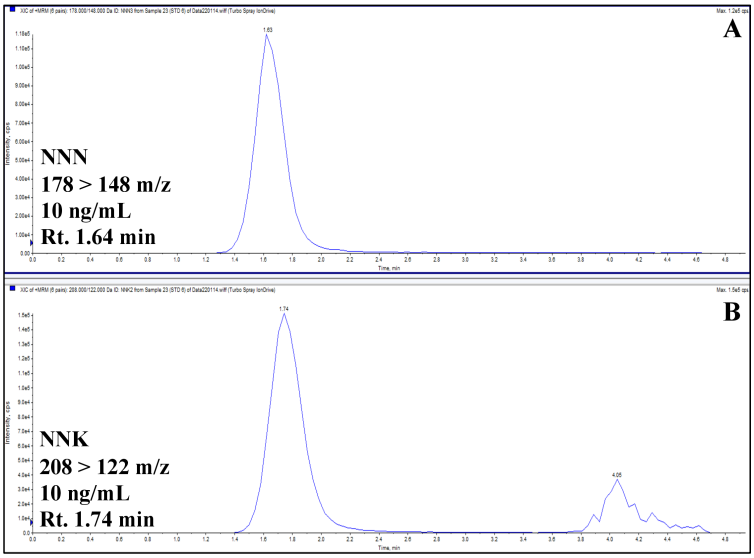


Figure 4.4: Representative chromatographs of (A), 10 ng/mL NNN (Rt. 1.64 min) and NNK (Rt. 1.74 min).

4.3.2 Method Validation

The method for NNN and NNK was validated following US Department of Health and Human Services Center for Tobacco Products Guidance for Industry. The method developed with the MIP-packed HPLC column had linearity ($r^2 > 0.9985$) over six non-zero concentration points from 0.1 ng/mL to 10 ng/mL (0.04 μg/g to 10 μg/g). Accuracy for the 6 points was between 92-110% and precision was between 2.5-8.2%. The limit of quantitation (LOQ) was 0.1 ng/mL and the limit of detection (LOD) was 0.03 ng/mL. Table 4.3 shows the results of the accuracy and precision studies for the quality control (QC) samples. Accuracy for the four QC samples prepared in 10 mM ammonium acetate, pH 5.5 to represent SNUS, oral nicotine products, and pipe tobacco were between 91.8-97.8% and 86.5-99.2% for NNN and NNK respectively. Accuracy for the three QC samples that represent e-liquid formulations were between 100.5-103.9% and 100.6-109.7% for NNN and NNK respectively. Precision expressed as % RSD were < 15% for all concentrations for NNN and NNK for all tobacco products. Autosampler stability for NNN and NNK in both solutions indicated stability up to 72 hours (Figures A. X and A.Y).

Table 4.3: Accuracy and precision of quality control calibrators (N = 9) in nicotine and tobacco products (10 mM ammonium acetate, pH 5.5) and e-cigarette e-liquids (70:30 PG:VG). Calculated concentration (ng/mL) is expressed as average ± standard deviation.

10 mM ammonium acetate, pH 5.5 (N=9)						
	NNN			**NNK**		
Calibrator	**Calculated Concentration (ng/mL)**	**Accuracy (%)**	**Precision (%)**	**Calculated Concentration (ng/mL)**	**Accuracy (%)**	**Precision (%)**
LLQC (0.1 ng/mL)	0.09 ± 0.01	95.8	6.2	0.10 ± 0.01	99.2	6.0
LQC (0.3 ng/mL)	0.27 ± 0.01	91.8	2.1	0.27 ± 0.01	86.5	4.2
MQC (3.0 ng/mL)	2.87 ± 0.05	96.5	1.8	2.70 ± 0.07	88.1	2.4
HQC (7.5 ng/mL)	7.38 ± 0.12	97.8	1.6	7.05 ± 0.03	86.4	0.5
70:30 PG:VG (N=9)						
	NNN			**NNK**		
Calibrator	**Calculated Concentration (ng/mL)**	**Accuracy (%)**	**Precision (%)**	**Calculated Concentration (ng/mL)**	**Accuracy (%)**	**Precision (%)**
LQC (0.3 ng/mL)	0.31 ± 0.04	102.6	14.1	0.31 ± 0.01	100.6	3.7
MQC (3.0 ng/mL)	3.01 ± 0.14	100.5	4.9	3.08 ± 0.24	101.3	7.8
HQC (7.5 ng/mL)	7.80 ± 0.20	103.9	2.5	8.40 ± 0.19	109.7	2.3

4.3.3 Column Uniformity and Characterization

Three MIP-packed HPLC columns were packed over the course of this study. Column uniformity and characterization was achieved by injecting the quality control calibrators for NNN and NNK in both 10 mM ammonium acetate, pH 5.5 and 70:30 PG:VG in triplicate on each of the three columns (N=9). Column uniformity was determined by assessing retention time (min), retention factor, peak area, calculated concentration (ng/mL), and accuracy (%), expressed in Table 4.4. The columns were considered uniform if each parameter had a % RSD value less than 15%.

Table 4.4: Column uniformity and characterization results for NNN and NNK with the three MIP-packed HPLC columns expressed as average (% RSD) for each parameter (N = 9).

	10 mM ammonium acetate, pH 5.5 (N=9)					
	Calibrator	**Retention Time (min)**	**Retention Factor**	**Peak Area**	**Calculated Concentration (ng/mL)**	**Accuracy (%)**
NNN	LLQC (0.1 ng/mL)	1.64 (1.2%)	3.66 (1.60%)	19028 (8.7%)	0.11 (5.6%)	106 (5.4%)
	LQC (0.3 ng/mL)	1.64 (1.0%)	3.64 (1.28%)	48559 (5.1%)	0.27 (6.4%)	92 (6.4%)
	MQC (3.0 ng/mL)	1.64 (1.1%)	3.64 (1.40%)	475267 (4.1%)	2.74 (2.4%)	91 (2.4%)
	HQC (7.5 ng/mL)	1.64 (1.1%)	3.64 (1.40%)	1397370 (3.6%)	7.62 (7.9%)	101 (7.9%)
NNK	LLQC (0.1 ng/mL)	1.75 (0.8%)	3.97 (0.95%)	23425 (8.2%)	0.10 (13.3%)	95 (13.3%)
	LQC (0.3 ng/mL)	1.76 (0.8%)	3.98 (0.94%)	53407 (4.0%)	0.26 (9.5%)	87 (9.6%)
	MQC (3.0 ng/mL)	1.76 (0.8%)	3.98 (0.94%)	515287 (2.3%)	2.74 (3.3%)	91 (3.3%)
	HQC (7.5 ng/mL)	1.76 (0.8%)	3.98 (0.97%)	1526294 (1.4%)	8.35 (2.1%)	111 (2.1%)
	70:30 PG:VG (N=9)					
	Calibrator	**Retention Time (min)**	**Retention Factor**	**Peak Area**	**Calculated Concentration (ng/mL)**	**Accuracy (%)**
NNN	LQC (0.3 ng/mL)	1.64 (0.7%)	3.64 (0.76%)	60515 (11.9%)	0.32 (4.9%)	107 (4.9%)
	MQC (3.0 ng/mL)	1.63 (0.5%)	3.62 (0.94%)	614715 (6.0%)	2.89 (3.2%)	96 (3.2%)
	HQC (7.5 ng/mL)	1.64 (0.5%)	3.64 (0.61%)	1417081 (9.5%)	7.49 (2.1%)	99 (2.1%)
NNK	LQC (0.3 ng/mL)	1.76 (0.7%)	3.99 (0.87%)	74677 (18.4%)	0.30 (5.5%)	119 (5.5%)
	MQC (3.0 ng/mL)	1.75 (0.5%)	3.97 (0.62%)	673063 (8.1%)	3.01 (4.5%)	100 (4.5%)
	HQC (7.5 ng/mL)	1.76 (0.5%)	3.99 (0.62%)	1631529 (8.1%)	7.91 (5.5%)	105 (5.5%)

Column characterization was expressed as asymmetry, tailing factor, and theoretical plate number (N) in Table 4.5. Asymmetry values for NNN and NNK were between 0.9-2.2 in both

sample mediums and tailing factor values for NNN and NNK were between 1.0-1.6, with the NNK in PG:VG having a % RSD greater than 15% . Theoretical plate number (N) for NNN and NNK were between 91-317 and were highly variable between columns (% RSD > 15%).

Table 4.5: Column uniformity for the three MIP-packed HPLC columns expressed as average (% RSD) in 10 mM ammonium acetate, pH 5.5 (N=36) and 70:30 PG:VG (N=27).

10 mM ammonium acetate, pH 5.5 (N=9)				
	Calibrator	**Asymmetry**	**Tailing Factor**	**Theoretical Plate Number**
NNN	LLQC (0.1 ng/mL)	1.34 (17.0%)	1.24 (10.5%)	231 (21.1%)
	LQC (0.3 ng/mL)	1.27 (6.9%)	1.34 (11.9%)	211 (35.7%)
	MQC (3.0 ng/mL)	1.13 (9.1%)	1.09 (5.1%)	226 (34.3%)
	HQC (7.5 ng/mL)	1.12 (9.2%)	1.08 (4.8%)	237 (24.8%)
NNK	LLQC (0.1 ng/mL)	1.43 (15.5%)	1.30 (10.4%)	200 (36.9%)
	LQC (0.3 ng/mL)	1.30 (6.5%)	1.23 (4.6%)	202 (28.2%)
	MQC (3.0 ng/mL)	1.22 (8.7%)	1.15 (4.6%)	208 (26.8%)
	HQC (7.5 ng/mL)	1.22 (8.2%)	1.15 (4.3%)	210 (25.2%)
70:30 PG:VG (N=9)				
	Calibrator	**Asymmetry**	**Tailing Factor**	**Theoretical Plate Number**
NNN	LQC (0.3 ng/mL)	1.09 (13.2%)	1.08 (7.4%)	231 (33.7%)
	MQC (3.0 ng/mL)	1.12 (12.5%)	1.09 (6.1%)	225 (29.6%)
	HQC (7.5 ng/mL)	1.18 (14.0%)	1.18 (14.0%)	217 (27.4%)
NNK	LQC (0.3 ng/mL)	1.68 (17.6%)	1.41 (4.3%)	193 (34.7%)
	MQC (3.0 ng/mL)	1.22 (8.1%)	1.14 (4.0%)	217 (33.2%)
	HQC (7.5 ng/mL)	1.23 (8.9%)	1.13 (4.1%)	229 (31.4%)

4.3.4 TSNA Content in Nicotine E-liquids and Tobacco Products

The quantitated content of NNN and NNK are tabulated in Table 4.6. The Camel SNUS products had between 1.3-1.6 μg/g NNN and 0.44-0.45 μg/g NNK present in the three products. The pipe tobacco sample had low levels of TSNAs present in the sample, with an average concentration of 1.87 ± 0.04 μg/g NNN and no NNK detected in the sample. The oral nicotine samples contained no presence of TSNAs. The electronic cigarette e-liquids had NNN levels from 2.1-202.6 ng/mL and NNK levels from 1.5-52.8 ng/mL. A representative chromatogram of an e-liquid and a SNUS product are shown in Figure 4.5.

Table 4.6: TSNA levels in nicotine and tobacco products (N=26).

Sample Results			
Sample	**Type**	**NNN (ng/mL or μg/g)**	**NNK (ng/mL or μg/g)**
Avail Captain's Cut	E-liquid	28 ± 2	53 ± 5
Avail Continental Breakfast	E-liquid	BDL	1.8 ± 0.07
Avail Sapphire Morning	E-liquid	203 ± 6	35.7 ± 0.9
Avail Seduction	E-liquid	94 ± 33	17.1 ± 5.3
Cedar Reserve American Red	E-liquid	BDL	BDL
German Liquids Golden Blend	E-liquid	BDL	BDL
High Voltage Melatonin	E-liquid	BDL	BDL
Nirvana Citrus OD	E-liquid	BDL	1.6 ± 0.2
Nirvana Headrush	E-liquid	BDL	38.2 ± 2.3
Palm Strawberry Flavor	E-liquid	29 ± 2	BDL
Supreme Nicotine 258 Rally Squirrel	E-liquid	5.9 ± 0.4	21.3 ± 1.6
Top Vapor Honeydew	E-liquid	21 ± 1	19.3 ± 0.5
Virginia White USA Mix	E-liquid	2.1 ± 0.2	BDL
Virginia White Tobacco	E-liquid	1.5 ± 0.1	BDL
Camel Mellow	SNUS	1.61 ± 0.02	0.454 ± 0.080
Camel Mint	SNUS	1.37 ± 0.05	0.441 ± 0.040
Camel SNUS Mint	SNUS	1.49 ± 0.04	0.455 ± 0.028
Nirvana Skull Control	Pipe Tobacco	1.87 ± 0.04	BDL

N = 3 for each sample injection
BDL = Below Detection Limit
No TSNAs were detected in the eight oral nicotine pouches

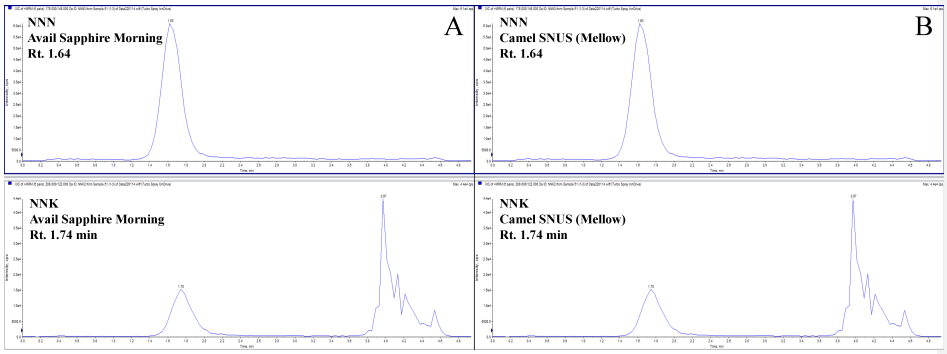


Figure 4.5 Representative chromatographs of A), an e-liquid sample and B) a SNUS product

4.3.5. Analysis of Other Tobacco Biomarkers with TSNA MIP Column

Attempts to measure cotinine with the TSNA MIP column were unsuccessful. Cotinine was first assessed on the Waters Acquity UPLC-PDA with the TSNA MIP column under the conditions developed for the NIP column. Under these conditions, cotinine eluted in under two minutes, when the loading mobile phase was still the dominant mobile phase on the column. (Figure 4.6). The retention factor of cotinine was 0.10, compared with TSNA NNN, which had a retention factor of 1.61 under the same analytical conditions. When attempting to measure cotinine with the LC-MS/MS conditions, ammonium acetate was unable to properly analyze cotinine, and no signal was observed. Changing the mobile phase to a pH gradient of 10 mM ammonium acetate from a pH of 10 to a pH of 5.5 did not improve cotinine's signal.

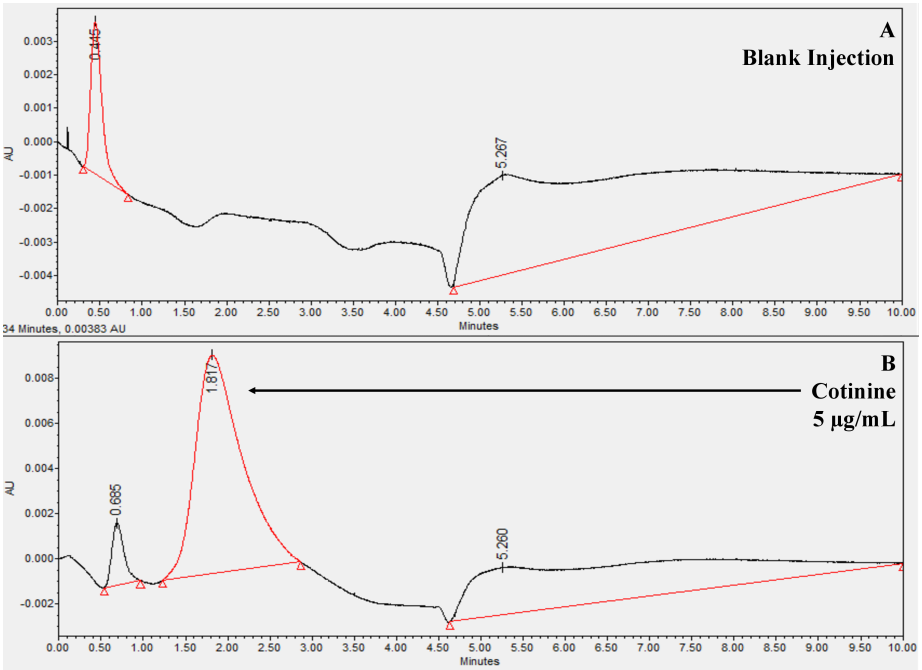


Figure 4.6: Representative chromatograms of A) a blank injection of 10 mM ammonium acetate, pH 5.5 and B) 5 µg/mL cotinine in 10 mM ammonium acetate, pH 5.5.

4.4. Discussion

4.4.1 Method Development and Characterization of MIP-Packed HPLC Column

The theory behind an in-line molecularly imprinted polymer column is similar to the interactions with a MIP-based SPE cartridge. In solid phase extraction, the analyte of interest is bound to the stationary phase on the cartridge and interferents and matrix components are removed through a series of solvent washes (Figure 1.6) (118). For an in-line MIP-based HPLC column, the selection of mobile phase solvents is critical for both retaining the analyte, and later removing it from the polymer once it has been successfully cleared of interferents. The starting mobile phase

acts as the loading condition in the solid phase extraction, and the subsequent mobile phases are used as washing and/or eluting solvents. It is critical that the starting mobile phase should not prematurely elute the analyte from the column

Following the manufacturer's recommendations for the SupelMIP TSNA cartridges, which states that samples should be adjusted to a pH of 5.5 prior to loading, the starting mobile phase was 10 mM ammonium acetate, buffered to a pH of 5.5 (89). Nicotine with the non-imprinted polymer was used for initial method development parameters, particularly for the mobile phase composition, gradient, flow rate, and column temperature. As shown in Figure 4.3, nicotine was fully retained on the column when the mobile phase was operated in isocratic mode with 100% 10 mM ammonium acetate, pH 5.5. When acetonitrile was used as the elution solvent, mobile phase B, the interaction between nicotine and the surface functional monomers was interrupted and nicotine was eluted.

For the TSNA MIP column, the developed method continued to use 10 mM ammonium acetate, pH 5.5 as the starting mobile phase (mobile phase A). The elution solvent was adjusted to 0.1% formic acid in methanol to improve the analyte signal in the detector. It was determined that at 60% mobile phase B, the non-covalent interaction between the analytes and the functional monomer were disrupted. After the analytes were displaced from the column, the gradient was increased to 100% mobile phase B to ensure full removal of the analytes and wash the column prior to the next sample. Increasing the temperature of the column from room temperature to 45 °C further improved the peak signal. Under these conditions, the entire chromatographic run time was five minutes, and NNN eluted at 1.64 minutes and NNK eluted at 1.76 minutes (Figure 4.4). The method was successfully validated.

As the MIP-packed HPLC columns were made in-house, the reproducibility of the packing method and the performance of the MIP polymer was assessed. Column uniformity was determined by assessing the retention time (min), peak area, calculated concentration (ng/mL), and accuracy of NNN and NNK QC samples in both mediums. The columns were considered uniform if each parameter had precision values (Expressed as % RSD) less than 15%. Based on these results for retention time, peak area, calculated concentration, and accuracy (Tables 4.4), the column packing procedure used in this study was able to successfully create MIP-packed HPLC columns for the purpose of TSNA analysis.

Asymmetry and peak tailing are used to describe the chromatographic peak shape of an analyte. The United States Pharmacopeia (USP) recommends that chromatographic peaks have asymmetry values between 0.9-1.2, and peak tailing less than two (119). The MIP-based HPLC column was slightly asymmetrical with peak tailing (Figure 4.4, Table 4.5), however, the peak tailing was within acceptable limits. The slightly peak tailing is most likely from the slow kinetics of the non-covalent interactions between the analyte and functional monomer, which has been previously described in literature (120).

Theoretical plate number is used to describe the efficiency of the column in regards to resolution. USP guidance typically recommends that the theoretical plate number be greater than 2000 (119). The theoretical plate number with the MIP columns used in this study was between 200-237 theoretical plates and had % RSD values greater than 15% (Table 4.5). This is $1/10^{th}$ the recommended theoretical plate number by USP standards. The low theoretical plate number is a result of the large polymer size of the MIP material used in the HPLC column, as theoretical plate number is inversely proportional to particle size. The smaller the particle size, the larger the number of theoretical plates, and therefore the greater the efficiency of the column (121).The

polymers used in this study were initially designed for solid phase extraction (SPE), and ranged from 30-90 μm in size, with an average polymer size of 50 μm. This is much larger than the traditional RP HPLC polymer size, which are typically 5 μm or smaller. Due to the large particle size of the MIP material, NNN and NNK could not be separated chromatographically. This is a common issue observed with in-line MIPs. The large, irregular particle sizes formed by bulk polymerization also create large, broad peaks with little chromatographic resolution between analytes (16, 54, 78, 79). Analytes are primarily separated within the detector. Pulsed elution methods have been used to improve separation between analytes (19).

Despite the fact that theoretical plate number did not meet the acceptance criteria for column uniformity, all other parameters in this study met the acceptance criteria and the columns were uniformly packed. Further, column efficiency was not a significant parameter for the analysis of TSNAs with this particular polymer, as they all have different transition ions, allowing for separation within the detector.

4.4.2 TSNA Content in Nicotine E-liquids and Tobacco Products

The federal registrar has proposed recommendations that oral tobacco products, such as SNUS, have a limit of 1 μg/g of NNN present in products (106). Using the developed, validated method, it was established that the TSNAs present in the SNUS and tobacco samples were within the same range as TSNA levels reported in literature (122). Furthermore, the three Camel SNUS products showed little variation (% RSD < 10%) in the amount of NNN and NNK present. Similar to the Camel SNUS products, the pipe tobacco sample had low levels of TSNAs present in the sample, with an average concentration of 1.87 ± 0.04 μg/g NNN and no NNK detected in the sample. The oral nicotine samples contained no presence of TSNAs. The description of the On!

and Zyn oral nicotine pouches specifically state that the nicotine in the products are tobacco-free. Therefore, no TSNAs should be present in any of the oral nicotine samples that claim to be tobacco-free.

Compared to the tobacco products, the electronic cigarette e-liquids had a wide distribution of NNN and NNK levels (Table 4.6). While e-cigarettes have been marketed as a safer alternative to traditional cigarettes, TSNAs are still present in the e-liquids and capable of being inhaled by e-cigarette users (108, 110, 123). Both NNN and NNK are listed on the FDA's Harmful and Potentially Harmful constituents list and are noted to be the leading causes of mouth, throat, and lung cancer in association with cigarette use (105). Most e-liquid labels do not disclose if the nicotine content in the e-liquids are derived from tobacco, and there are no warning labels about the potential of exposure to harmful products other than nicotine. E-liquids did not come under FDA regulation until 2015, and there are no current recommendations on the maximum concentration of NNN or NNK that should be present in e-liquids. Therefore, it is unsurprising that there is a wider distribution of TSNA levels in e-liquids in comparison to the tobacco products, which are heavily regulated. It should be noted, however, the majority of the e-liquids tested in this study were purchased prior to the 2015 FDA regulation. The four e-liquids that were bought after 2015 (Cedar Resesrve American Red, German Liquids Gold Blend, Virginia White Tobacco, and Virginia White USA Mix), had lower levels (less than 3 ng/mL) of TSNAs present, but it would be beneficial to use the developed method for e-liquids made in 2020 or later.

4.4.3 Analysis of Other Tobacco Biomarkers with TSNA MIP Column

The inability to retain cotinine on the TSNA MIP column can be linked back to the results shown in Chapter 3. In Chapter 3, cotinine was retained on the TSNA MIP cartridge until

the introduction of an aqueous wash step consisting of 10 mM ammonium acetate, pH 5.5 The loss of cotinine during the washing step was due to the partition coefficient (logP) of cotinine (logP -0.3), which is more hydrophilic than the TSNAs (logP -0.08 to 0.98). The partial loss of cotinine also indicated that cotinine was retained through a degree of specific interaction with the imprinted cavity and non-specific interactions on the surface of the polymer. In a closed, high pressure system such as a HPLC column, cotinine's affinity for the mobile phase limited its retention on the TSNA MIP column. A polymer designed more specifically for cotinine would possibly avoid the lack of interaction between cotinine and the imprinted cavity, and create better retention on the HPLC column.

4.5 Conclusions

An analytical method for detecting TSNAs NNN and NNK using a molecularly imprinted polymer packed-HPLC column was established and validated. The MIP-packed columns were uniform with regards to retention time, peak area, calculated concentration, and accuracy. Column characterization showed slight asymmetry and peak tailing, and a wide distribution of theoretical plated with high variability (% RSD > 20%). The non-uniformity of the theoretical plates is most likely due to the large particle size and wide particle size distribution of the MIP polymer. The developed analytical method, however, exceeded the current recommendations for analytical methods and was able to successfully detect NNN and NNK in multiple nicotine and tobacco products and e-liquids. Cotinine was unable to be retained on the MIP HPLC column due to lack of affinity for the stationary phase under chromatographic conditions. MIP-packed columns can allow for the direct, targeted analysis of analytes beyond consumer products (i.e. in waste water, biological samples, etc) with little benchtop preparation prior to instrumental analysis.

Chapter 5: Development and Characterization of a Molecularly Imprinted Polymer for the Selective Extraction of Cotinine in Human Urine

This chapter has been drawn from an article Under Review for Publication in Analytical and Bioanalytical Chromatography

5.1 Introduction

When developing a molecularly imprinted polymer, there are multiple factors that must be considered to ensure that the MIP is selective for a target analyte(s). The first is the template analyte, that is used to form the imprint. The template analyte is the compound that is intended to be measured, or a compound that has a similar chemical characteristics to the target analyte (7, 41). The size and shape of the template will create the size and shape of the imprinted cavity, and the functional groups of the template analyte will interact non-covalently with the functional monomer. The functional monomer therefore, needs to have complementary functional groups that promote the non-covalent interactions (i.e. electrostatic interactions, hydrogen bonding, Van der Waals forces, etc) between the template and the functional monomer (7, 44). The cross-linking monomer stages the functional monomer in place and creates the backbone of the polymer, so that the polymer is not destroyed when the template is removed (43). The porogen is the solvent used to dissolve the individual polymer components into solution, and promote the polymerization of the components (41). It is important that the resulting polymer is insoluble in the porogen as it is formed, creating either a monolith or precipitating out of solution.

In Chapters 3 and 4 a commercial polymer developed for tobacco specific nitrosamines (TSNAS) was characterized and packed into a HPLC column for direct analysis of tobacco products with LC-MS/MS. It was observed that the TSNA MIP had a high degree of cross-selectivity with nicotine's urinary metabolite, cotinine (124). In bioanalysis, cotinine is typically the analyte most routinely measured in regards to nicotine exposure. This is primarily due to the

fact that cotinine is metabolized directly from nicotine, unlike other tobacco biomarkers such as carbon monoxide and carboxyhemoglobin, which can be formed from other types of environmental exposure (125). Nearly 80% of nicotine is metabolized into cotinine via enzyme CYP2D6, can be detected in blood, urine, saliva, hair, breastmilk, and meconium, and has a half-life of up to 18 hours (93, 125–129). Despite cotinine's successful recoveries with the commercial TSNA MIP, there were limitations with the TSNA MIP in the extraction of cotinine. The largest issue was that the recovery of cotinine was reduced when an aqueous washing step was introduced to the extraction protocol. The affinity for water further complicated cotinine's interaction with the MIP-packed HPLC column, resulting in early elution with little retention on the column. The creation of a MIP specifically designed for cotinine would most likely reduce or eliminate the challenges observed with the TSNA MIP.

In literature, protocols for the successful creation MIPs for the extraction of cotinine in urine and hair have been reported (64, 130). The polymerization method employed in both methodologies was bulk polymerization. As discussed in Chapter 1, bulk polymerization, while regarded for its straightforward pre-polymerization process, has a undesirable caveats in the post-polymerization processing. These issues include the grinding of the monolithic polymer into a fine powder, typically through mechanical grinding or by hand with a mortar and pestle. The process is regarded as laborious and time consuming, resulting in a jagged, irregularly shaped particle morphology, which is undesirable for HPLC column packing (5). The grinding also risks destroying binding sites, which can reduce the polymers overall binding capacity (5, 11).

Precipitation polymerization was developed in response to the post-polymerization processing issues commonly found with bulk polymerization. In precipitation polymerization, the reaction is carried out in a dilute solvent/porogen where the monomers are at 4-6% (w/v) of the

solution. Once the polymer reaches a critical mass, the polymer becomes insoluble in the solvent and precipitates out of the solution (7, 114). The resulting polymer is a powder form and has a morphology of uniformly shaped spheres. There is no risk of destroying binding sites as there is no need to grind the polymer, thus potentially increasing the binding capacity of the precipitated polymer (48, 49). There are currently no published methods for the extraction of cotinine using a MIP formed by precipitation polymerization.

The purpose of this study was to develop and characterize an in-house molecularly imprinted polymer for the selective extraction of cotinine in urine. A polymerization method was developed and the polymer was characterized for functional groups, morphology, and mechanical stability. Nicotine was used as the template analyte to create the imprinted cavity within the polymer. The performance of the MIP in terms of selectivity, adsorption capacity, and recovery of cotinine under various conditions was studied and compared with the commercial TSNA polymer from Chapter 3. Finally, the extraction of cotinine in a urine matrix at low, medium, and high concentrations was assessed.

5.2 Materials and Methods

5.2.1 Materials and Chemical Reagents

Ammonium formate (97%), 2,2'Azobis(2-methylpropionitrile) (AIBN), divinylbenzene (DVB), ethylene glycol dimethacrylate (EGDMA), methacrylic acid (MAA), (S)- nicotine, nicotine hydrogen tartrate, orthophosphoric acid (OPA, 85% wt.), and trifluoro acetic acid (TFA) were purchased from Sigma Aldrich (St. Louis, MO). Acetic acid (HAc), ammonium hydroxide (NH_4OH), HPLC grade acetonitrile (ACN), dichloromethane (DCM), heptane, methanol (MeOH), tetrahydrofuran (THF) and water (H_2O) were purchased from VWR International (Radnor, PA).

Ammonium acetate, 750 µL centrifuge cartridge with a 0.2 µm nylon filter, and triethyl amine (TEA) were purchased from Fisher Scientific (Waltham, MA). Cotinine (99.7%), cotinine-d3 (99.8%), nicotine (99.6%), and trans3'-hydroxycotinine (T3HCOT, 98.2%) were purchased from Cerilliant (Round Rock, TX). Polyetheretherketone (PEEK) capillary tubing (500 µm id, 1/6 in OD) and stainless steel (0.5 µm) frit was purchased from Upchurch Scientific (Oak Harbor, WA). Stainless steel external column end-fittings and ferrules were purchased from Valco Instrument Co. Inc. (Houston, TX).

5.2.2. MIP Polymerization Process

Bulk polymerization with nicotine as a template was adapted from Yang et al (47). One mmol (160.6 µL) of (S)- nicotine, the template, was mixed with 4 mmol (339 µL) of MAA, the functional monomer, in 5.6 mL of DCM inside a 20 mL scintillation vial. The pre-polymerization complex sat for 5 minutes to promote the non-covalent bonding between the template and functional monomer. After 5 minutes, 20 mmol (3,772 µL) of EGDMA, the cross-linker, and 0.24 mmol (39.4 mg) of AIBN, the initiator, were added. The solvent was purged of oxygen under a N_2 stream for 5 minutes and the vial was sealed and placed in a 60 °C water bath for 24 hours. Post polymerization, the polymer was hand-ground into a fine powder with a mortar and pestle and water sieved for particles between 25 and 38 µm in size. To remove the template, the polymer material was washed and filtered with a glass, vacuum apparatus. One wash cycle consisted of 100 mL of 9:1 (v/v) MeOH:HAc followed by 100 mL of methanol. The contents were filtered using a 47 mm diameter Nylon filter membrane, 0.45 µm pore, and the samples were washed in this cycle until nicotine was no longer detected in the methanolic wash. A non-imprinted polymer (NIP) was created following the same steps as above, but omitting nicotine as the template.

Precipitation polymerization with nicotine as a template was adapted from Sambe et al (114). The pre-polymerization complex consisted of 1.5 mmol (241 µL) of (S)-nicotine was mixed with 6 mmol (509 µL) of MAA in 128 mL of 3:1 (v/v) ACN: toluene in a large vial. The mixture was sonicated for 15 minutes to promote the non-covalent bonding between the template and functional monomer. Then, 28.8 mmol (4,025 µL) DVB, the crosslinker, and 1.9 mmol (312 mg) of AIBN, the initiator, were added. The solvent was purged of oxygen under a N_2 stream for 15 minutes and the vial was sealed and placed in a 60 °C water bath for 24 hours. Post polymerization, the template was dispersed in 200 mL of tetrahydrofuran for 15 minutes and then filtered with a glass, vacuum filtration apparatus. This process was repeated two more times before the polymer was washed with 200 mL of water, followed by two cycles of 200 mL methanol. The polymer was dried at room temperature. A NIP was created following the same steps as above, but omitting nicotine as the template.

5.2.3. Sample Preparation

Samples were extracted using a 0.2 µm frit spin cartridge in a 750 µL microcentrifuge tube. The polymer was slurry packed into the cartridge by mixing 25 mg of MIP or non-imprinted polymer (NIP) in 500 µL of methanol and pipetting the mixture into the cartridge. The extraction was carried out in a microcentrifuge under the initial conditions outlined in Table 5.2. The MIP cartridges were primed with 500 µL of methanol followed by 500 µL of water. Samples were loaded onto the cartridge in 500 µL aliquots at 1 µg/mL concentrations in 10 mM ammonium acetate buffered to a pH of 5.5. The loading fraction was added to the cartridge and mixed with the polymer for 5 minutes before being spun through. A 500 µL aliquot of 10 mM ammonium acetate, pH 5.5 was used to wash the polymer, and then the cartridge was dried for 10 minutes to

remove any aqueous solvent. A 500 µL aliquot of heptane was used to further remove any aqueous solvent and disrupt hydrophobic interactions. The analytes were initially eluted with two 500 µL aliquots of 9:1 (v/v) DCM:MeOH. All fractions were collected and evaporated to dryness under N_2 at 50 °C and reconstituted in 500 µL of mobile phase.

Cotinine in human urine was extracted with the SupelMIP™ TSNA cartridge. Cotinine at 10, 100, and 1000 ng/mL concentrations were prepared in urine prior to the extraction. Cotinine-d3 at 68 ng/mL was added to each sample and the sample underwent the SupelMIP™ TSNA SPE extraction outlined above.

Table 5.1: Extraction Protocol of In-House MIP

Step	Solvent	RPM	Minutes
1. Prime	500 µL Methanol	5,000	5
	500 µL DI H_2O	5,000	5
2. Load	500 µL Sample*	3,000	10
3. Wash 1	500 µL 10 mM ammonium acetate, pH 5.5	5,000	5
4. Dry		15,000	10
5. Wash 2	500 µL Heptane	7,000	6
6. Elute**	500 µL Methanol	5,000	10
	500 µL Methanol	5,000	5

* Vortex and allow sample and polymer to mix for 5 minutes before spinning down
** Evaporate to dryness under N_2 at 50 °C and evaporate in 500 µL mobile phase

5.2.4 Chromatographic Conditions

A Waters Acquity H Class UPLC and a Waters Acquity PDA detector (Milford, MA) was used for polymer characterization. The method for cotinine is described in Chapter 3 and repeated here. In brief, cotinine chromatographic separation was carried out on a Phenomenex C18 column (50 mm x 2.1 mm, 2.6 µm) at 25 ºC. The aqueous mobile phase A was 10 mM ammonium acetate,

pH 10 and the organic mobile phase B was acetonitrile under 90:10 aqueous:organic isocratic conditions. The flow rate was 0.3 mL/min and the PDA was set to monitor cotinine at 260 nm.

Quantitation of cotinine in urine was carried out on a Waters Alliance e2695 HPLC and a Waters Quattro Micro API mass spectrometer (Milford, MA). Chromatographic separation was carried out with a Varian Polaris SI-A (3.0 x 50 mm, 5 µm) column kept at 35 °C. Mobile phase A consisted of 10 mM ammonium formate in 0.05% formic acid and mobile phase B was 0.05% formic acid in 1:1 (v/v) methanol:acetonitrile. The LC was operated at a flow rate of 0.4 mL/min with the gradient conditions listed in Table 5.1. The mass spectrometer was operated in positive ion mode. The cone voltage was set to 20 V and the capillary voltage was set to 2.5 kV. The source block temperature and desolvation temperature were 150 °C and 350 °C, respectively. Nebulizer gas flow and desolvation gas flow were set to 68 L/hr and 490 L/hr. The mass spectrometer was operated in multiple reaction monitoring mode (MRM) for the following ions with collision energy in parentheses: cotinine, 176 > 80 m/z (20 V); cotinine-d3, 179 > 101 m/z (20 V).

Table 5.2: Chromatographic conditions of Waters eAlliance LC

Time (min)	A%	B%
1.00	0	100
3.00	25	75
4.00	0	100
4.50	0	100

5.2.5 Characterization of In-House Polymer

5.2.5.1. Characterization of Physical Properties

A Hitachi (Schaumburg, IL) SU-70 Scanning Electron Microscope (SEM) was used to characterize the polymer morphology. Samples were mounted onto a 25 x 6 mm aluminum disk coated with carbon tape and coated with gold particles for 90 seconds at 10mA and 10pA. An

accelerating voltage of 3-5 keV at 300-5000x magnification was used at a working distance of 15 mm. Functional groups of the polymer were identified using a Thermo Scientific Nicolet iS50 FT-IR instrument with a diamond crystal (Waltham, MA). Sample spectra were a composite of 64 sample scans, with background subtraction. Scans were acquired from 500 nm to 4000 cm^{-1} with a resolution of 4 cm^{-1}. Samples were analyzed using Omnic Spectroscopy Software. For thermogravimetric analysis (TGA), a TGA 5500 by TA Instruments (New Castle, DE) was used. About 2 mg of sample was added to the high aluminum pans and heated from 40 to 400 °C under ramp conditions of 10 °C/min. Samples were analyzed using TRIOS software (TA instruments, New Castle, DE). In-house MIP samples were compared with non-imprinted polymer and commercial SupelMIP™ TSNA MIP material.

5.2.5.2. Characterization of Polymer Extraction Performance

The in-house MIP was characterized for its recovery of cotinine under various conditions (N = 3). The extraction protocol listed in Section 5.2.3 was used for all recovery studies. Extraction optimization was achieved by adjusting the ph/ionization state of cotinine under loading conditions to promote the non-covalent interaction between cotinine and the imprinted polymer. Optimization of the elution conditions were studied to determine the solvent that would best disrupt the non-covalent interaction between cotinine and the imprinted polymer and lead to the highest recoveries. The recovery of cotinine in the presence of common interferents were also carried out. Recovery was calculated using equation 5.1

$$Recovery = \frac{Extracted}{Unextracted} \times 100 \quad (5.1)$$

where the extracted is a known concentration of cotinine that has undergone an extraction with the imprinted polymer. The unextracted is a sample of cotinine at the same concentration as the

extracted, but was prepared in the final reconstitution solvent and not taken through the extraction. Throughout these experiments, a concentration of 1 µg/mL cotinine in 10 mM ammonium acetate, pH 5.5 was used. The optimized extraction protocol and the interferent studies were also performed with the non-imprinted polymer (NIP).

Cotinine recovery in the presence of a urine matrix was assessed at low (10 ng/mL cotinine), medium (100 ng/mL cotinine), and high (1000 ng/mL cotinine) concentrations in human urine (N = 3). Extraction recovery was determined using equation 5.1, and matrix effects were calculated using equation 5.2

$$Matrix\ Effects = \left(\frac{PostSpike}{Unextracted} - 1\right) \times 100 \quad (5.2)$$

where the PostSpike is a blank sample in matrix that has been taken through the extraction and infused with a known concentration of cotinine after the extraction has been carried out. Samples were analyzed via the LC-MS/MS method outlined in Section 5.2.4.

Adsorption capacity studies were carried with the MIP and NIP material at four concentrations: 1, 5, 15, and 25 µg/mL prepared in 10 mM ammonium acetate, pH 5.5 (N = 3). Samples were prepared by weighing 25 mg of polymer material in a test-tube and adding 500 µL of sample to the tube. The samples were vortexed for 60 minutes and then the solvent was filtered with a Whatman™ 0.45 µm polyether sulfone membrane syringe (Maidstone, United Kingdom). An aliquot of the sample was taken and the sample was analyzed via the HPLC-PDA method described in Section 5.2.4. Adsorption capacity was determined using Equation 5.3,

$$Q = \frac{C_0 - C_e}{W} \times V \quad (5.3)$$

Where Q is the adsorption capacity (mg/g), C_0 and C_e are the initial and final concentrations (mg/L) of cotinine in the solvent, V is the amount of solvent (L) mixed with the sample, and W is the weight of the polymer (g). Extraction Percentage (E) was calculated using Equation 5.4,

$$E = \frac{C_0 - C_e}{C_e} \times 100 \quad (5.4)$$

Adsorption capacity and Extraction Percentage of the non-imprinted polymer was also assessed.

Student T-tests or Analysis of Variance (ANOVA) with Tukey's Honest Significance Test (HSD) were used for all statistical analyses using JMP v.14.

5.2.6 Preparation of MIP HPLC Columns

A cotinine MIP column was prepared following the column preparations outlined in Chapter 4 (117). Repeated here, the MIP column was prepared under slurry conditions using a Teledyne (Thousand Oaks, CA) packing pressure system. The end fitting and 0.2 µm frit from one end of a Restek (Bellefonte, PA) column assembly kit (50 mm x 2.1 mm ID, ¼" OD) was removed and attached to a reservoir with a glass frit (Figure 4.1). One gram in-house MIP material was suspended in 20 mL of a 1:1 (v/v) chloroform:ethanol solution or 1:1 (v/v) methanole:isopropyl alcohol to create a slurry, which was sonicated for 15 seconds. The mixture was poured into the reservoir and the system was capped and secured. Pushing solvent consisting of either 1:1:1 (v/v/v) ethanol:isopropyl alcohol:toluene or 100% methanol was used to push the slurry mixture through the column. Helium gas was used to degas the solvent during the packing process. The pushing solvent was slowly increased at a rate of 0.5 mL/min until a total system pressure around 8000 psi was reached. The final flow rate was kept steady for one hour before the system was disassembled and the frit and end fitting were reassembled on the column. The freshly packed column was washed with 100% acetonitrile at a flow rate of 0.5 mL/min for 60 minutes using an external Shimadzu LC-10AD HPLC pump (Kyoto, Japan).

A second packing method was attempted using a micro-column design (53). Polyetheretherketone (PEEK) tubing of 500 µm inner diameter and a 1/16 outer diameter, and a

stainless steel 0.5 μm frit in a polymer ring was purchased from Upchurch Scientific (Oak Habor, WA). Stainless steel end-fittings and ferrules were purchased from Valco Instrument Co. Inc. (Houston, TX). The PEEK tubing was cut to 20 mm in length with a PEEK tubing cutter, and the frit, ferrule, and fittings were wrench tightened onto the PEEK tubing. One end of the fittings and frits were removed, and the system was slurry packed under negative pressure (Approximately 500 psi) using a vacuum pump. The slurry consisted of a 10 mg/mL suspension of the in-house polymer suspended in methanol. The suspension was added drop wise in 5 μL intervals using a micropipette and the suspension was mixed frequently to maintain homogeneity. The procedure was continued until the polymer was seen at the top of the column. Post packing, the column was reassembled and placed on the external Shimadzu LC-10AD HPLC pump at a flow rate of 0.05 mL/min to further compress the packing material. The column was disassembled and the process was repeated to fill any drops in packing caused by the pump compression.

5.3 Results

5.3.1 Characterization of MIP Physical Properties

For the bulk polymerization method (47), the resulting polymer after 24 hours of polymerization was a monolithic resin (Appendix 4, Figure A.4). The MIP, imprinted with liquid, free-base nicotine, was yellow in color due to the yellow coloring of nicotine. The NIP, which did not contain any nicotine in the mixture, was a white/translucent color. The monolithic resin, pre-ground, yielded three grams of material each for MIP and NIP. Extensive grinding and sieving were required to generate polymer particles in the 25-38 μm range. The morphology of ground MIP and NIP particles that were ground to a size of less than or greater than 106 μm was analyzed via scanning electron microscopy (SEM). Images showed that the ground bulk polymer was jagged

and irregularly shaped and sized. There were no visible morphology differences for MIP vs NIP material (Figure 5.1). Due to the jagged and irregularly shaped polymer particles, and the extensive polymer preparation in the grinding and sieving step, further method development with the bulk polymerization method was not carried out with cotinine.

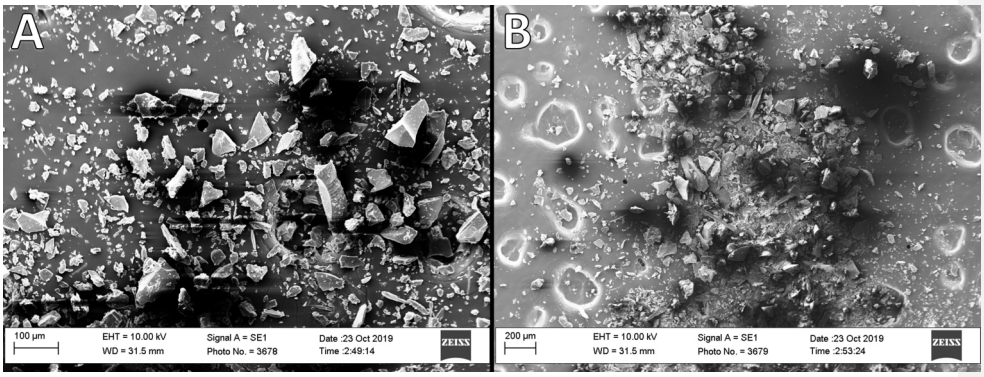


Figure 5.1: Scanning Electron Microscopy (SEM) images of A) MIP and B) NIP material created by bulk polymerization method.

For precipitation polymerization, after 24 hours the solution went from a clear mixture to an opaque, cloudy mixture, and sedimentation of the polymer was evident at the bottom of the bottle (Appendix 4, Figure A.5). The solvent was filtered off with a glass, vacuum filtration apparatus, and the polymer was washed with successive cycles of tetrahydrofuran, water, and methanol. Post washing, the resulting polymer was a fine, powdery substance when dry. SEM analysis of the polymer revealed small, spherically shaped polymer particles that were less than 10 µm in size (Figure 5.2). There was greater heterogeneity in the size of the MIP particles compared to the NIP particles. FT-IR results of the MIP and NIP polymer also showed no functional group differences (Figure 5.3). A band from 2900-3000 cm^{-1} was characteristic of a bonded alcohol from a carboxylic acid, and a carboxylic acid band was present at 1700 cm^{-1}. Bands

between 700-800 cm^{-1} were evidence of substituted benzene rings. Thermogravimetric analysis (TGA) showed little decomposition of the polymer, with 5-6% (0.2-0.3 mg) sample loss over 40 to 400 °C (Figure 5.4). There was no difference in the TGA results between the MIP and the NIP particles. When compared with the commercial SupelMIP™ TSNA polymer characterized in Chapter 3, the commercial polymer showed greater decomposition, with greater than 75% (2.04 mg) sample loss over 40 to 400 °C (Figure 5.4).

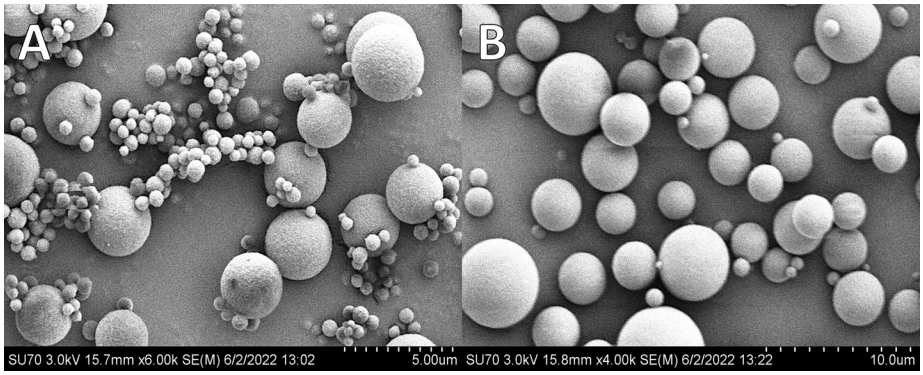


Figure 5.2: SEM images of A) MIP material and B) NIP material. The MIP material had a greater abundance of smaller particles present compared to the NIP material.

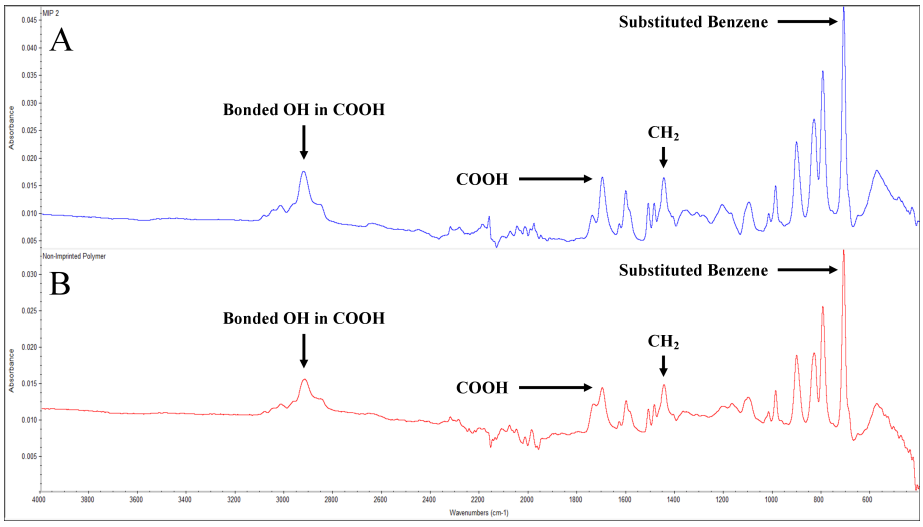


Figure 5.3: FT-IR spectra of A) MIP and B) NIP

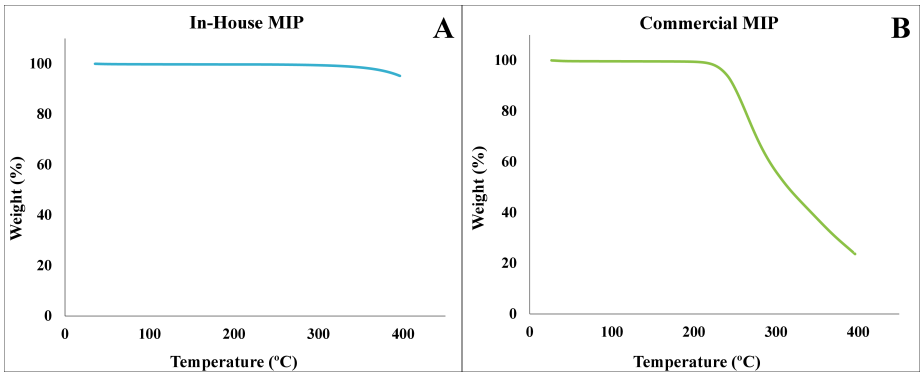


Figure 5.4: TGA analysis of A) The in-house MIP and B) The commercial SupelMIP™ TSNA polymer.

5.3.2 Characterization of Polymer Extraction Performance

5.3.2.1. Optimization of Extraction Conditions

When using the extraction method optimized for the SupelMIP™ TSNA MIP cartridges, cotinine was fully bound to the polymer during the loading and heptane washes. This was evidenced by the lack of cotinine observed in the loading and heptane wash fractions that were analyzed by the HPLC-PDA. With the 9:1 (v/v) DCM:MeOH elution solvent, cotinine had a recover of 83%, but had high variability (% RSD > 15%). When the elution solvent was adjusted to 100% methanol, the elution was 99 ± 2% with a % RSD less than 5% (Figure 5.5). Unlike the commercial SupelMIP™ TSNA polymer discussed in Chapter 3, the addition of an aqueous washing step (10 mM ammonium acetate, pH 5.5) did not prematurely disrupt cotinine. The recovery of cotinine with the in-house polymer was statistically different (P = 0.0090) than the recovery of cotinine with the commercial SupelMIP™ TSNA cartridge.

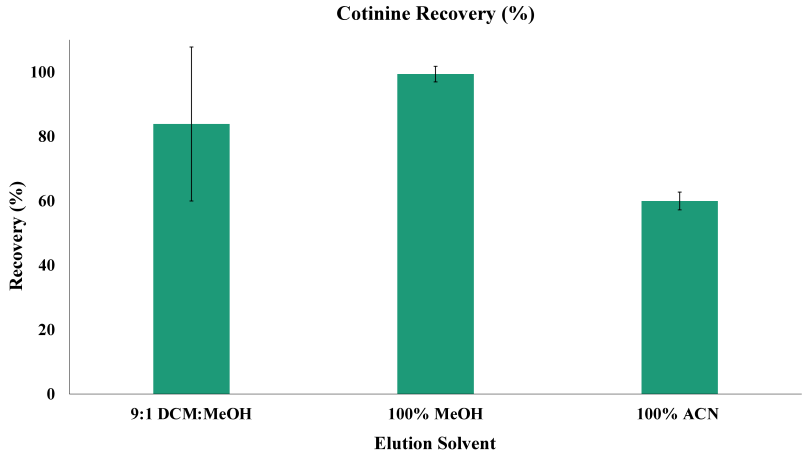


Figure 5.5: Effect of elution solvent on average recovery of cotinine (N = 3).

When adjusting the pH to be 100% ionized (pH 2.79) or 0% ionized (pH 10), changes in cotinine recovery were observed. Under both conditions, the recovery of cotinine dropped to 19 ±

5% and 54 ± 10% at 100% and 0% ionized, respectively, with high (% RSD > 15%) variability (Figure 5.6). Premature elution of cotinine was observed in the loading and aqueous washing steps. The difference in recovery based on pH of the loading solvent was significant different ($p < 0.001$).

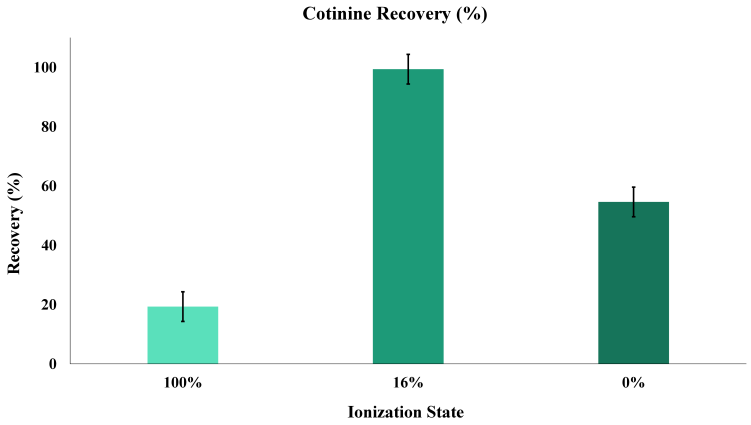


Figure 5.6: Effect of loading pH/ionization state on average recovery of cotinine (N = 3). Cotinine is 100% ionized at a pH of 2.79, 16% ionized at a pH of 5.5, and 0% ionized at a pH of 10.

5.3.2.2. Adsorption Capacity Studies

Adsorption capacity studies were conducted at four concentrations: 1, 5, 15, and 25 µg/mL cotinine in 10 mM ammonium acetate, pH 5.5. After one hour of mixing, cotinine was not detected in the filtered solvent at 1 µg/mL and 5 µg/mL, suggesting 100% uptake of cotinine with the polymer. After 15 µg/mL, 0.62 ± 0.04 µg/mL cotinine was detected in the filtered solvent. At 25 µg/mL, 2.52 ± 0.02 µg/mL cotinine was detected in the filtered solvent. A plateau was not established for absorption capacity, as at 15 and 25 µg/mL, the adsorption capacity continued to increase with a final adsorption capacity at 25 µg/mL of 448.2 ± 2.1 µg/mg (Figure 5.7). The non-imprinted polymer (NIP), followed a similar trend. After one hour of mixing, cotinine was not detected in the filtered solvent at 1 µg/mL and 5 µg/mL, suggesting 100% uptake of cotinine with

the polymer. After 15 µg/mL and 25 µg/mL, 0.67 ± 0.01 µg/mL and 2.98 ± 0.10 µg/mL cotinine were detected in the filtered solvent, respectively. The adsorption capacity at 25 µg/mL was 448.2 ± 2.1 µg/mg. At the final concentration of 25 µg/mL, there was a statistically significant difference between the MIP and NIP polymer's adsorption capacity ($p = 0.0031$). Extraction percentage at 25 µg/mL for the MIP and NIP material were statistically different ($P = 0.0104$) with average extraction percentage of 90 ± 0.06% and 88 ± 0.41% for the MIP and NIP respectively.

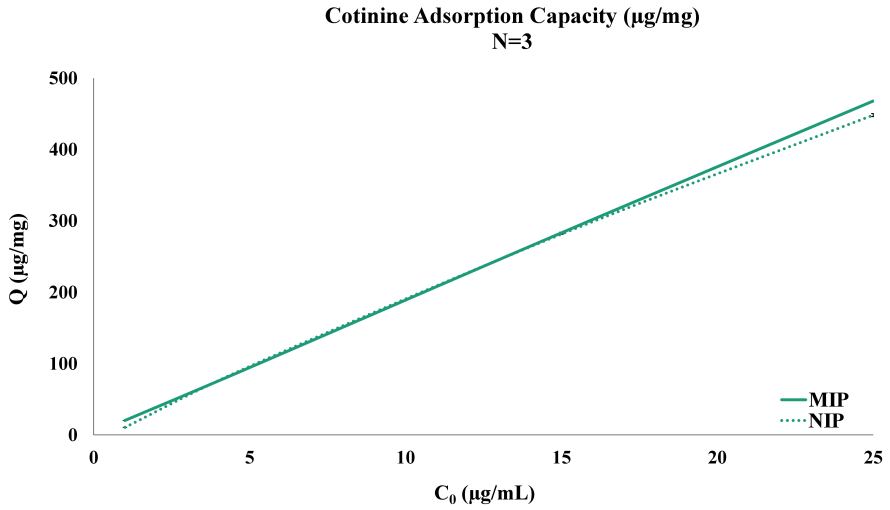


Figure 5.7: Average (N = 3) adsorption capacity (Q) for MIP and NIP with 25 mg of polymer and 500 µL of sample. C_0 is initial concentration of sample mixed with the polymer.

5.3.2.3. Effect of Cotinine Recovery in the Presence of Other Compounds

Recovery of cotinine in the presence of other potential compound interferents was assessed. Recovery of 1 µg/mL of cotinine in the presence of 1 µg/mL per interferent in 10 mM ammonium acetate, pH 5.5 was measured with nicotine, trans-3'-hydroxycotininine, cotinine-glucuronide, and a mixture of nicotine and trans-3'hydrooxycotinine (Figure 5.8). The presence of other interferents

at equal concentrations did not significantly interfere with the recovery of cotinine alone ($P > 0.05$). The recovery of cotinine in the presence of trans-3'-hydroxycotinine with the in-house polymer was not statistically different ($P = 0.0880$) from the recovery of cotinine in the presence of trans-3'hydroxycotinine with the commercial SupelMIP™ TSNA polymer.

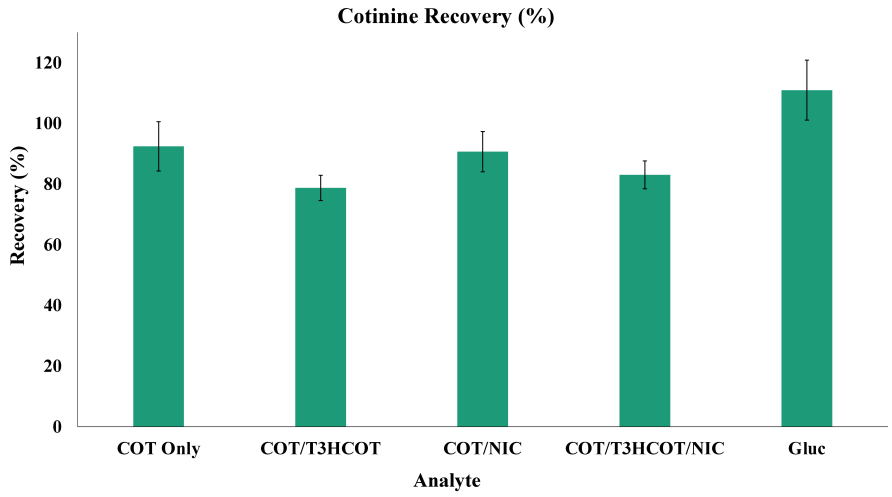


Figure 5.8: Average recovery (mean ± SD, N = 3) of cotinine in the presence of A) trans-3'-hydroxycotinine (T3HCOT), B) Nicotine (NIC), C) trans-3'-hydroxycotinine and nicotine, D) cotinine glucuronide. Cotinine was extracted at 1 µg/mL in the presence of 1 µg/mL per interferent.

When the effect of recovery of cotinine with the non-imprinted polymer was tested in the presence of nicotine as an interferent, there was a drop in recovery experienced. In samples that contained only 1 µg/mL of cotinine in the sample, the recovery with the non-imprinted polymer was 85.3 ± 3.4%. The recovery of cotinine alone with the non-imprinted polymer was not statistically different ($P = 0.2636$). However, in the presence of 1 µg/mL of nicotine, the recovery of cotinine dropped to 59.4 ± 3.2% (Figure 5.9) Cotinine and nicotine were detected in the loading and aqueous wash fractions of the extraction of the NIP. The drop in cotinine recovery in the

presence of nicotine with the non-imprinted polymer was statistically different in comparison with cotinine alone on the NIP ($P < 0.007$). The recovery of cotinine in the presence of nicotine with the NIP was also statistically different ($P = 0.0059$) in comparison with cotinine in the presence of cotinine with the MIP.

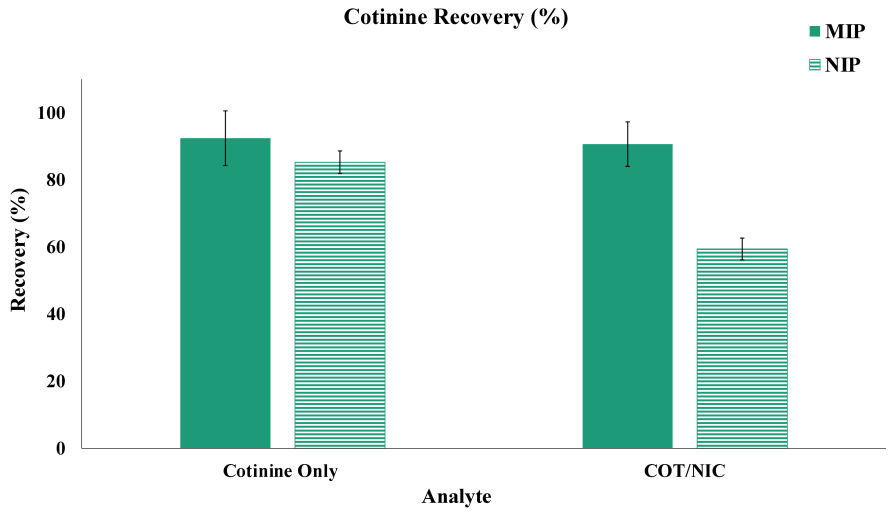


Figure 5.9: Comparison of average recovery of cotinine (N = 3) with the MIP vs NIP A) with cotinine only and B) with 1 µg/mL cotinine in the presence of 1 µg/mL nicotine. All samples prepared in 10 mM ammonium acetate, pH 5.5.

5.3.2.4. Cotinine Recovery in a Urine Matrix

Absolute recovery and matrix effects of cotinine in urine were calculated (Table 5.3). Absolute recovery of cotinine in urine was between 77-103% at all three concentrations. Matrix effects were -10 to 20%. Compared with the recovery of cotinine in urine with the commercial SupelMIP™ TSNA cartridge, the recovery of cotinine in urine was not statistically different ($P = 0.2473, 0.3403$) for 10 and 1000 ng/mL, respectively. The recovery of cotinine at 100 ng/mL in

urine was statistically different (P = 0.0095) for the in-house polymer from the commercial polymer.

Table 5.3: Average recovery (mean ± SD, N = 3) of cotinine at 10, 100, and 1000 ng/mL in urine with matrix effects (range).

Concentration (ng/mL)	Recovery (%) ± Standard Deviation	Matrix Effects (%)
10	86 ± 12	-1 – 9
100	77 ± 6	0 – 16
1000	103 ± 19	-10 – 20

5.3.3. Preparation of MIP Packed Columns

When using the Teledyne constant pressure packing system, the backpressure of the in-house MIP column rapidly increased to above 8000 psi, reaching the systems maximum pressure of 10,000 psi. Attempts to change the pushing solvent from the 1:1:1 (v/v) ethanol:isopropyl alchol:toluene pushing solvent to 100% methanol did not reduce the high back pressure. The polymer packed into the column was extruded, and SEM images of the polymer did not show any morphology changes from the MIP material that was not packed into a HPLC column (Appendix 6, Figure A.6).

Using the micro-column design, approximately 2.4 mg of polymer was packed into the PEEK tubing before polymer was observed at the top of the column. When placed on the external packing pump, back pressure of approximately 2,300 PSI was observed at a flow rate of 0.05 mL/min. A new batch of polymer was created, polymerizing for 48 hours instead of 24 to reduce the number of < 1 μm sized particles was packed into a new microcolumn using the same method. Approximately 0.9 mg of polymer was packed, and the back pressure at 0.05 mL/min was approximately 2,400 PSI. Attempts to pack a MIP column was halted at the conclusion of this experiment.

5.4 Discussion

5.4.1. Developing a Polymerization Method

When developing a polymerization method, determining the template and functional monomer interactions is a critical step to making a successful molecularly imprinted polymer. The template must make the imprinted cavity that the analyte of interest can fit into, and the interaction with the functional monomer must be strong enough that washing steps to remove matrix don't disrupt the template-monomer interaction (36). A common critique of MIPs is that a portion of the template remains bound in the polymer, leading to potential leaching (86). If the template is also the analyte of interest, this leaching can lead to falsely elevated analyte levels. It is common for the template to instead be an analyte that has a similar size, shape, and functional group chemistry as the analyte of interest. This can either be an alkaloid of the analyte, or can be a molecule that has been developed to have the similar physical and chemical properties of the analyte (28, 35, 37, 40, 52, 91). With the polymerization methods utilized for this study, extensive template leakage was observed with the bulk polymerization method despite successive washes. It was then determined that nicotine would act as a "dummy" template, and cotinine, nicotine's urinary metabolite, would be used as the analyte of interest instead. The two compounds share similar functional group chemistries such as the pyridine ring and a pyrrole group, with a tertiary amine.

Bulk polymerization was a further issue with the goals of this study due to the post-polymerization processing and polymer yield post processing. The monolithic resin needed to be extensively grounded into smaller particle sizes, which under SEM were jagged and irregularly shaped (Figure 5.1). While this morphology would not be a critical issue in solid phase extraction techniques, if packed inside a HPLC column, the non-uniformity of the polymer particles would cause issues with the analyte chromatography (131). Precipitation polymerization instead created

small, spherical and mostly uniformly sized particles that would be more desirable for HPLC analysis.

The results of the precipitation polymerization particles can be better compared to the commercial SupelMIP™ TSNA material, which was a polymer coated spherical material, albeit at a much larger (30-90 μm) particle size range (Figure 5.2). Using FT-IR, carboxylic acid functional groups could be identified, suggesting that while the polymers have different imprints (A NNK derivative for the commercial polymer, nicotine for the in-house polymer), they have similar mechanisms of action holding the analyte inside the imprinted cavity (Figure 5.3). The most interesting comparison with the commercial and in-house polymer were the TGA results. From 40 to 400 °C, the in-house polymer (And the in-house NIP) did not have substantial degradation, with only 5-6% sample loss occurring. The commercial polymer, however, had about 75% sample loss, which began just above 200 °C, suggesting that the in-house polymer is more stable at higher temperatures than the commercial polymer (Figure 5.4). The stability of the in-house polymer over the commercial polymer may be due to its polymerization technique. It was noted in the SEM data that the commercial polymer appeared to have a surface coating around a spherical bead (Appendix 6, Figure A.7). The surface coating vs the fully formed particle in the in-house MIP may have led to less stability at higher temperatures.

The non-imprinted polymer, created under the same conditions as the imprinted polymer, but in the absence of the template, had the same functional groups and TGA results. The polymer size, however, was slightly bigger and more uniform than the imprinted polymer. Possibly due to the presence of the template affecting the size of the final polymer.

5.4.2 Characterization of MIP Performance

5.4.2.1. Optimization of Extraction Conditions

As noted in Chapter 3, MIP extraction performance can be affected by the conditions and solvents used in the sample extraction. When initially using the same extraction conditions for the SupelMIP™ TSNA cartridges for the extraction of cotinine with the in-house polymer, cotinine had > 80% recovery. This was similar to the recovery values (> 80%) of cotinine with the commercial polymer, however, with the in-house polymer, cotinine had highly variable (% RSD > 15%) recoveries with 9:1 (v/v) DCM:MeOH as the elution solvent (Figure 5.5). This high variability observed with nicotine in Chapter 3 was resolved by changing the elution solvent to a solvent that was more suitable for disrupting the non-covalent interactions between the analyte and the functional monomer. In this case, changing the elution solvent from the non-polar dichloromethane to 100% methanol not only reduced the high variability, but improved the recovery to almost 100%. The recovery of cotinine under these conditions was statistically significant.

When determining the effect of the pH of the loading solvent on cotinine recovery, the best recovery (99.3 ± 2.4%) was observed when the pH of the loading sample was buffered to a pH of 5.5. Under these conditions, cotinine is cotinine is 16% ionized (Figure 5.6). Both the functional monomer and the analyte need to be in a state of ionization where they can interact through electrostatic interactions. Because of cotinine's pKa (4.79), when cotinine is 100% ionized (pH 2.79), methacrylic acid (pKa 4.6), a weak acid, is in its unionized state and cannot interact through electrostatic interactions. Under the condition of pH of 5.5, cotinine's recovery in buffered water was almost 100%.

In Chapter 3 it was concluded that an aqueous wash consisting of 10 mM ammonium acetate at a pH 5.5 prematurely disrupted the cotinine from the commercial TSNA MIP, causing about a 50% recovery in the washing step. This was theorized to possibly be due to the extent in which cotinine fit into the imprinted cavity and due to cotinine's affinity for water based on its partition coefficient. With the in-house polymer that had an imprinted cavity in the shape of nicotine, cotinine may have a stronger interaction with the functional monomer due to its spatial arrangement within the imprinted cavity, and is not so easily disrupted by the aqueous wash. Changing the pH of the buffered water to either more acidic or more basic could potentially disrupt the non-covalent interactions.

5.4.2.2 Adsorption Capacity of Cotinine

The adsorption capacity determines how many micrograms of an analyte can be retained on one milligram of polymer. To try and replicate the commercial SupelMIP™ TSNA cartridges, which used 25 mg of polymer, 25 mg of the in-house polymer was used for this study. The 500 µL of sample at 1, 5, 15, and 25 µg/mL of cotinine in 10 mM ammonium acetate, pH 5.5 was chosen to replicate the extraction conditions developed with the in-house polymer. Adsorption capacity is determined when the plot of adsorption capacity over staring concentration (Q vs. C_0) reaches a plateau, where the adsorption capacity of the polymer no longer changes with increasing concentration (132, 133). A plateau was not observed with the four concentrations used in this study, suggesting that the adsorption capacity of the polymer had not been reached (Figure 5.7). However, the polymer was shown to be able to reach the high end of the clinical range of cotinine detected in heavy smokers (10-1,300 ng/mL) which means that the polymer is capable of being used for clinical samples of smokers and non-smokers (134, 135). The adsorption capacity of the

MIP is also compared to the adsorption capacity of the non-imprinted polymer, which lacks the imprinted binding sites. The lower adsorption capacity value, Q, for the non-imprinted polymer was smaller, and statistically different than the adsorption capacity of the MIP, which suggests the MIP has more available binding sites, most likely due to the presence of the imprinted cavities.

In comparison to the adsorption capacity recorded with cotinine specific MIPs in literature, the in-house precipitation polymerization MIP had higher adsorption capacity than the polymer formed by bulk polymerization. Larpant et al (130) determined that from 62.5 – 4,000 μg/mL cotinine, the adsorption capacity for the bulk polymerization polymer was 125.41 μg/g (0.125 μg/mg). The in-house polymer formed by precipitation polymerization had an adsorption capacity of 94.4 μg/mg at 5,000 μg/mL. This is most likely due to the fact that the precipitation polymerization does not have a risk in losing binding sites from the grinding and sieving required in bulk polymerization, and the spherical particles formed have more surface area available for binding with the polymer (5, 11)

5.4.2.3. Effect of Cotinine Recovery in the Presence of Other Compounds

In clinical urine samples, other analytes beside cotinine will be present. Nicotine, cotinine's precursors, will be present at lower concentrations than cotinine, and trans-3'hydryoxycotinine, cotinine's urinary metabolite, will be present in higher concentrations. Glucuronide metabolites of cotinine will also be present within urine samples. The presence of these analytes, which have the similar size, shape, and functional group chemistry as cotinine, can potentially compete with cotinine for binding sites, ultimately reducing cotinine's recovery. Similar to the trends observed in Chapter 3, the presence of potential interferants did not significantly affect the recovery of cotinine in buffered water ($P = 0.2636$). Further, the recovery of cotinine in the presence of trans-

3'hydroxycotinine with the in-house polymer was not statistically different from the recovery of cotinine in the presence of trans-3'hydroxycotinine with the commercial SupelMIP™ TSNA cartridge.

The adsorption capacity study discussed in Section 5.4.2.2 determined that at 25 μg/mL, cotinine's adsorption capacity was 448.2 ± 2.1 μg/mg, which suggests that there are enough binding sites for cotinine that the presence of interferents at equal concentrations would not inhibit cotinine's extraction. As discussed in Chapter 3, concentration dependent studies of the interferents, especially for trans-3'hydroxycotinine and glucuronides that will be present in higher concentrations in urine than cotinine, may eventually change cotinine's recovery in the presence of interferents.

With the non-imprinted polymer, cotinine saw a loss in recovery in the presence of equal amounts of nicotine. While the non-imprinted polymer also had an adsorption capacity for cotinine that was also above 400 μg/mg, the NIP lacks the imprinted binding sites and all interactions are surface based. The presence of two different analytes, while similar in size, shape, and functional group structure, on the surface of the polymer may inhibit the overall adsorption capacity due to the spatial arrangements around the surface functional monomers.

5.4.2.4. Cotinine Recovery in a Urine Matrix

After extensively characterizing the polymer in the absence of matrix, the successful recovery of cotinine in a urine matrix was demonstrated. With the commercial polymer in Chapter 3, the recovery of cotinine in urine from 10-1000 ng/mL was between 89-107%, with less than 10% ion enhancement observed at the middle concentration, 100 ng/mL (Table 3.3). With the in-house polymer, the recovery of cotinine in urine from 10-1000 ng/mL was between 77-103%, with

some ion enhancement and suppression observed at each concentration (Table 5.3). The recovery of cotinine at 100 ng/mL with the in-house polymer was lower and statistically different ($P < 0.05$) than the recovery of cotinine with the commercial MIP. However, the low and high concentrations of cotinine with the in-house polymer was not statistically different ($P > 0.05$) than the recoveries observed with the commercial polymer. This suggests that the in-house polymer is just as capable as the commercial polymer in extraction cotinine from urine. Further, the ability to use an aqueous washing step with the in-house polymer allows for adjustments to the extraction procedure to reduce the variability in matrix effects observed with the in-house polymer.

5.4.3. In-House MIP Packed Columns

Attempts to pack HPLC or microcolumns with the in-house MIP material were ultimately unsuccessful. This is despite the polymers being less than 5 μm in size, which is suitable for HPLC columns. When using the Teledyne packing system, it was first theorized that the toluene present in the packing solvent was causing the polymer to swell, leading to the high back pressures first observed. Toluene was used in the polymerization mixture, to promote the growth of the polymer (114, 136). However, when using 100% methanol, as described in Sambe et al., the high back pressure continued to be an issue with the stainless steel column. High back pressures were also observed with the microcolumn design, which was also packed in 100% methanol.

Breakdown of the polymer was not observed in the SEM images of the extruded material, but it is possible that the 0.2 μm frits were clogged by the < 1 μm sized particles that were observed in the SEM images (Figure 5.2). However, when the polymerization was carried out for 48 hours instead of 24 hours, in an attempt to allow the small particles to grow larger, high back pressure was still observed with the microcolumn design. Sieving of the MIP material to create a more

homogenous particle size distribution and larger frit sizes may help reduce the chances of high back pressure.

5.5 Conclusions

Using precipitation polymerization and nicotine as the template analyte, a molecularly imprinted polymer capable of selectively extracting cotinine from a urine matrix was developed. The use of precipitation polymerization allowed for the creation of small, spherical beads compared with the jagged, irregularly shaped polymer particles that were created with bulk polymerization. Further, the creation of spherical MIP particles suggests that the polymer could be packed into a HPLC or capillary column for direct analysis on an analytical instrument. The SEM and FTIR used to characterize the spherical morphology and the polymer functional groups showed few differences between the imprinted and non-imprinted polymer. Thermogravimetric analysis showed that the in-house polymer had less decomposition from 40-400 °C when compared with the commercial TSNA polymer that was characterized in Chapter 3. Characterization of the extraction performance determined that cotinine had an adsorption capacity was greater than 400 μg/mg of polymer, and had a greater adsorption capacity than the NIP. The presence of potential analyte interferents did not affect the recovery of cotinine with the in-house polymer, and the recovery in the presence of trans-3'hydroxycotinine with the in-house polymer was not statistically different from the commercial polymer. The developed extraction protocol yielded close to 100% recovery of cotinine in buffered water and between 77-103% recovery of cotinine in urine. The successful development of a cotinine selective polymer for the extraction of cotinine in urine can be used for routine, clinical samples.

Chapter 6: Research Challenges, General Conclusions, and Future Directions

6.1 Research Challenges and Pitfalls

Limitations with the fully characterizing the commercial TSNA polymer stemmed from the fact that the polymerization information is proprietary. The results obtained from FT-IR and SEM helped to partially identify possible functional groups, such as carboxylic acid and the presence of substituted benzene rings. However, the polymerization technique, including the template, functional monomer, and crosslinking monomers is unknown, which makes it difficult to fully compare the commercial polymer with the in-house polymer ultimately created in Chapter 5, especially in regards to the difference in polymer stability with thermogravimetric analysis. Initial extractions of nicotine with the commercial polymer in Chapter 3 lead to recoveries with high variability that was not resolved by adjusting the pH to match the ionization state (16%) of the positive control, NNN. While the recovery variability was resolved by changing the elution solvent to solvents more compatible with nicotine's, the low recovery in comparison with traditional SPE extractions resulted in halting future recoveries of nicotine with the commercial polymer.

Cotinine, while having the most favorable recovery with the commercial TSNA polymer, was prematurely eluted from the cartridge if an aqueous washing step was introduced. This is due to cotinine's general affinity for water over TSNAs. The use of an aqueous washing step in the TSNA protocol was to remove potential interferents, both matrix and potentially competing analytes. The inability to use an aqueous washing step in the extraction protocol could prevent the sample from being properly removed of matrix and other interferents. This was apparent during a post-column infusion that showed ion suppression was present, and the analytical method for cotinine had to be adjusted to chromatographically separate cotinine from the matrix effect. The

high affinity of cotinine to water continued to be an issue in Chapter 4. Cotinine was not retained on the MIP-HPLC column, and therefore could not be used for direct analysis.

In Chapter 4, the large polymer particle size reduced the separation efficiency for NNN and NNK, causing them to co-elute chromatographically. This is due to the fact that the particles were designed for SPE purposes, and there are no currently available commercial MIPs that are within the recommended polymer size range for HPLC packing material. While this was ultimately not an issue TSNAs because of their differing transition ions, attempts to use MIPs for compounds sharing similar transition ions, such as cannabinoids, will require efficient chromatographic separation for proper identification. The successful use of the MIP-HPLC column in this research project was limited to product samples that were not in matrix. The use of the MIP-HPLC column with samples in biological matrices will need to be carefully designed to properly clean and extract the sample without sending matrix to the analytical detector.

In Chapter 5, extensive leaching of nicotine from the polymer prevented the use of the polymer for nicotine extractions. Instead, nicotine was used as a "dummy" template and the measurement of cotinine was used instead. For the purposes of this study, nicotine was a reasonable choice as the template analyte as it has a similar size, shape, and functional group chemistry to cotinine. However, for the incorporation of MIPs for the analysis of routine, clinical samples, it would be more advantageous to use a template analyte that has little clinical relevance, such as myosmine or anatabine. In the case of template leaching, the presence of either compound in a urine sample would not interfere with clinical interpretations.

Methods to fully characterize the thermophysical property of MIPs beyond particle size need to be further explored. MIPs are constructed under conditions where the template, co-polymers, solvent, temperature, and duration of polymerization are carefully considered.

Molecular modelling and small batch experiments can offer methods for determining the best polymerization conditions, but do not take into account later variables, such as extraction solvents, solvent pH, and temperature conditions that could affect the polymer integrity and overall MIP performance. This was most notable in Chapter 5 when attempting to pack the in-house polymer into a HPLC column. The packing solvent, toluene, was also used as the polymerization solvent, which could lead to further swelling of the polymer that would ultimately affect MIP performance. This potential swelling issue required selecting another solvent for packing purposes. Experiments to characterize the thermophysical properties of the polymer under different extraction conditions would help to further enhance not only the performance of the polymer, but the stability of the polymer. Thermogravimetric analysis, which helps show the thermal stability of the polymer, does little to determine the stability of the polymer under routine extraction conditions with real samples.

Further understanding of the thermophysical properties of the MIP can assist in properly defining the physical properties of the imprinted cavity. The threshold for defining a molecule has been "molecularly imprinted" is decided by comparing MIP performance to the performance of a non-imprinted polymer (NIP). The NIP has been created in the same co-polymer system under the same conditions as the MIP, but lacks the presence of the template analyte. The polymer is considered "imprinted" if the MIP has a higher adsorption capacity over the NIP, which can be used to calculate the theoretical number of binding sites for each polymer. These in-direct methods, while demonstrate the advantages of the MIP over the NIP, do not actually define that an imprinted cavity is physically present. Future literature should look beyond MIP performance that is limited to the desired outcome, but to also properly define the physical properties of the polymer in comparison with other extraction techniques.

The in-house polymer generated in Chapter 5 had polymer particles that were less than 5 μm in size, which is a favorable size for HPLC packing material. However, attempts to pack the in-house polymer in a stainless steel column or in a microcolumn resulted in high back pressures that prevented the polymer from being incorporated into an online MIP system. The high backpressure may be due to the swelling of the polymer in organic materials or the presence of sub-micron particles causing the column frits to clog. Designing polymerization conditions to create more uniformly sized particles could help in preventing the sub-micron particles that caused the frit clogging, and lead to better packing efficiency. The issues stemming from the inability to pack the MIP material into a HPLC column needs to be further investigated.

6.2 General Conclusions and Future Directions

The present research in this study aimed to develop a molecularly imprinted polymer (MIP) that is capable of extracting a target analyte from a biological matrix. In literature, MIPs are often described as creating a more selective extraction for the target analyte while reducing non-selective extraction of matrix. The improved extraction leads to cleaner sample extracts that allow analysts to detect samples down to an instrument's analytical limits, but without needing to extensively prepare the sample to remove interferents. Chapter 1 describes the mechanism of action of a MIP and the various polymerization processes for MIPs with non-covalent interactions. The applications of MIPs in off-line, bench top sample preparations, and for on-line/in-line, direct analysis methods with analytical instruments were discussed and select literature was reviewed to demonstrate the advantages of MIP technology in regards to bioanalysis. Limitations of MIPs and proposed advancements to overcome these limitations were also discussed.

In Chapter 3, a commercial MIP polymer was first used to extensively characterize and understand how the MIP extraction process worked. The extraction performance of the commercial polymer was compared with traditional sample extraction methods, and for cross-selectivity with a class of compounds. Through these experiments, the mechanism of action between the analyte and the imprinted polymer was better understood. The SupelMIP™ SPE cartridges for tobacco specific nitrosamines (TSNAs) were chosen as a proof of concept for how the MIP works. The commercial TSNA selective MIP showed greater than 90% recoveries with low variability (% RSD < 15%) for TSNAs in comparison with traditional TSNA SPE extraction protocols.

This further confirms that the MIPs can lead to cleaner, more selective extracts. The cross-selectivity of the TSNA MIP for other tobacco alkaloids and metabolites was dependent upon the size, shape, and functional group chemistry of the analytes being extracted. It was determined that the loading conditions and elution conditions are critical for successful binding between the analyte and the imprinted cavity and the later disruption of interaction for elution. Cotinine, nicotine's urinary metabolite and a reliable biomarker for nicotine exposure had greater than 80% recoveries in both water and urine.

In Chapter 4, the commercial TSNA MIP material was used to create a MIP-packed HPLC column for the detection and quantitation of NNN and NNK using a LC-MS/MS system. The developed method was validated following the US Department of Health and Human Services Center for Tobacco Products Guidance for Industry, and twenty-six tobacco products and e-liquids were successfully analyzed for NNN and NNK. Three MIP-packed HPLC columns were created and were determined to be uniform in regards to retention time, peak area, calculated concentration, and accuracy. The HPLC columns were characterized and showed slight asymmetry and tailing, with low theoretical plate numbers due to the large (30-90 μm) particle size of the

polymer, which were initially developed for SPE extraction. The MIP-HPLC columns demonstrated that the MIP material can be used in place of the silica stationary phase of traditional reverse phase HPLC columns in LC-MS/MS systems, and samples require little benchtop preparation prior to instrumental analysis

Chapter 5 used the experience gained in Chapters 3 and 4 to develop an in-house polymer that had the spherical morphology that would be desired for eventual HPLC column packing. Bulk polymerization, a traditional and popular method, created small, jagged, and irregularly shaped particles that would not be appropriate for later HPLC use. Precipitation polymerization, however, created small, spherical particles that were less than 5 μm in size, though the MIP material did show some heterogeneity in the particle size distribution. This small particle size of the MIP, however, is more desirable with HPLC columns compared with the particle size of the commercial polymer used in Chapter 4. Further characterization of the polymer with thermogravimetric analysis showed that the in-house polymer had less decomposition from 40-400 °C when compared with the commercial polymer characterized in Chapters 3 and 4, which began to decompose rapidly above 200 °C. Nicotine was used as the template, but cotinine was used as the analyte of interest to avoid any interferences from potential template leaching. Cotinine was chosen to compare the extraction performance of the in-house MIP to the extraction performance of cotinine with the commercial TSNA MIP. The successful extraction of cotinine in urine was developed at 10, 100, and 1000 ng/mL, and was comparable with the recoveries observed with the commercial polymer in Chapter 3. Further, with the in-house polymer, cotinine was not prematurely displaced in the presence of an aqueous washing step. Upon successfully packing the in-house MIP material into a HPLC column, the retention of cotinine during an aqueous washing step suggests that cotinine could be selectively retained with the in-house MIP material under aqueous conditions. The

adsorption capacity of cotinine with the precipitation polymerization method was higher than the adsorption capacity of cotinine reported in literature with polymers formed by bulk polymerization techniques.

This study has demonstrated that molecularly imprinted polymers can provide improved, reliable extractions of analytes in complex, biological matrices. Through first using a commercial MIP, the chemistry behind MIP selectivity and retention of the target analyte was better understood. Further, it was shown that a MIP-packed HPLC column could be used for direct analysis with little to no benchtop preparation before introduction to the analytical instrument. The knowledge gained in these experiments ultimately lead to the successful development of an in-house MIP for the targeted extraction of an analyte in a biological matrix, and could overcome the limitations observed with the commercial polymer. Further development of this polymer can allow for a MIP-packed HPLC column with the in-house material. The MIP polymerization method developed in this study can be applied to analytes outside of the tobacco alkaloid class, allowing for the direct, selective extraction of a wide arrange of target analytes in bioanalysis without the need for extensive sample preparation. The improvements in the sample extraction technique leads to cleaner extracts, more reliable detection and quantitation, and therefore, more reliable reporting for high throughput analyses.

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Appendix

Appendix 1: Effect of Sample pH on Ionization State during Sample Loading

Table A.1: Ionization State of methacrylic acid, NNN, and Cotinine under various pH conditions

	pKa	% Ionized at pH 2.7	% Ionized at pH 5.5	% Ionized at pH 10
Methacrylic Acid	4.65 (Weak acid)	1.36%	88%	100%
NNN	4.79 (Weak base)	100%	16%	0%
Cotinine	4.79 (Weak base)	100%	16%	0%

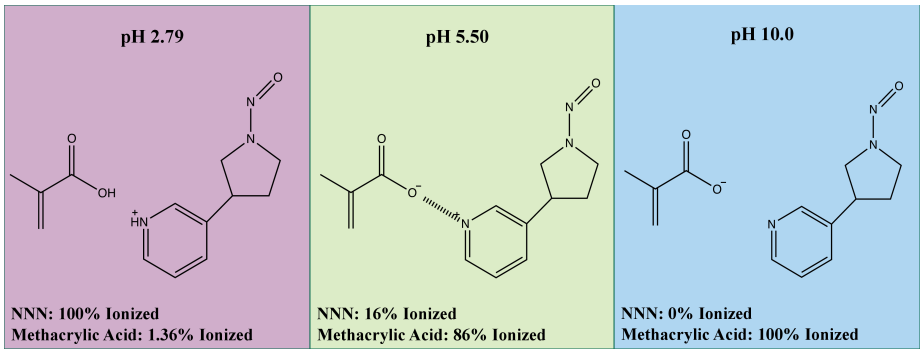

Figure A.1: Ionization state of NNN and methacrylic acid at pH 2.79, pH 5.50, and pH 10.0. The analyte and functional monomer must both be in their ionized states in order to non-covalently interact through electrostatic interactions. When either is in a state of un-ionization, the non-covalent interactions cannot occur and the MIP performance suffers.

Appendix 2: Effect of Nicotine Recovery by Elution Solvent

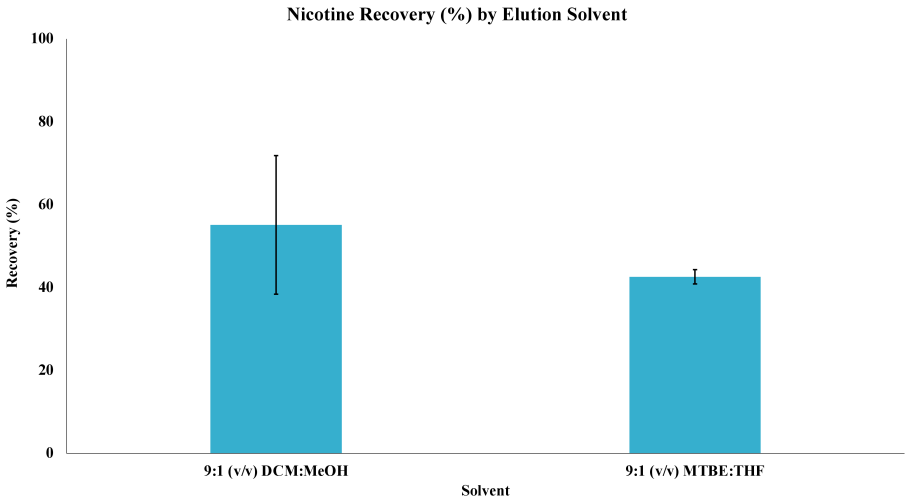

Figure A.2. Effect of average (N = 3) recovery of nicotine when changing elution solvent.

Commented [HM1]: This figure has been changed per Dr. Halquist's suggestions

Appendix 3: Nicotine Method Development with NIP Packed Column

Table A.2. Nicotine Method Development with NIP Packed Column

Gradient Change					
Gradient (min)	**Peak Area**	**Peak Height**	**Asymmetry**	**Tailing Factor**	**USP Plate Number**
2-3	3528291 (0.37)	7718 (0.33)	1.01 (2.07)	1.02 (0.93)	437 (0.93)
2-4	337238 (9.80)	7263 (5.43)	1.04 (10.48)	1.03 (4.60)	430 (8.33)
2-5	341297 (9.36)	7400 (4.46)	1.04 (3.47)	1.03 (1.12)	416 (14.50)
2-6	283740 (2.22)	5994 (2.73)	0.98 (0.64)	0.99 (0.59)	294 (1.36)
Column Temperature (°C)					
Temperature (°C)	**Peak Area**	**Peak Height**	**Asymmetry**	**Tailing Factor**	**USP Plate Number**
30	308084 (9.09)	6794 (3.60)	0.96 (9.67)	0.99 (3.61)	457 (10.89)
40	295748 (0.82)	7040 (0.33)	0.88 (14.11)	0.94 (5.29)	515 (1.85)
45	297008 (1.31)	7331 (0.81)	0.97 (6.27)	0.99 (3.33)	576 (2.11)
50	300292 (1.10)	7616 (1.00)	0.96 (3.07)	0.98 (1.58)	600 (2.22)
Gradient Flow 1*					
Flow (mL/min)	**Peak Area**	**Peak Height**	**Asymmetry**	**Tailing Factor**	**USP Plate Number**
0.100	356565 (0.04)	8400 (0.21)	0.82 (4.31)	0.92 (2.13)	508 (3.19)
0.125	349062 (1.20)	8882 (0.33)	0.80 (2.82)	0.92 (1.23)	461 (3.90)
0.150	276907 (3.05)	8510 (2.49)	0.79 (20.44)	0.88 (3.85)	513 (2.15)
0.200	267316 (1.19)	9577 (0.52)	0.93 (4.57)	1.03 (2.96)	544 (6.38)
Gradient Flow 2*					
Flow (mL/min)	**Peak Area**	**Peak Height**	**Asymmetry**	**Tailing Factor**	**USP Plate Number**
0.20	246588 (1.39)	6818 (0.76)	0.87 (8.58)	0.96 (3.66)	631 (1.65)
0.25	203479	8604	0.94	1.00	821

	(0.36)	(0.07)	(1.63)	(0.52)	(1.46)
0.30	173444	8619	1.12	1.10	1087
	(0.27)	(0.33)	(9.28)	(4.97)	(2.47)

Average (N = 3) (% RSD)
Acceptance criteria: % RSD < 2% for Peak Area; Lowest % RSD for all other parameters
Accepted parameter highlighted in green
* Flow from 0-4 minutes; 5-10 minutes
** Flow from 4-5 minutes

Appendix 4: Protocol for TSNA Preparation with MIP HPLC-Column

Tobacco Specific Nitrosamines (TSNA) preparation with MIP HPLC-Column

Reagents

Mobile Phase A: 10 mM ammonium acetate, pH 5.5 ± 0.05
1. Weigh 0.7708 g ammonium acetate
2. Mix with 1000 mL H_2O
3. Adjust pH to 5.5 ± 0.05 with acetic acid or ammonium hydroxide

Mobile Phase B: 0.1% formic acid in methanol
1. Measure out 1000 mL MeOH in a graduated cylinder
2. Remove 1 mL MeOH
3. Add 1 mL formic acid. Mix well.

E-Liquid Matrix: 70:30 (v/v) propylene glycol: vegetable glycerin (PG:VG)
1. Measure out 70 mL of propylene glycol in a graduated cylinder
2. Measure out 30 mL of vegetable glycerin in a graduated cylinder
3. Combine and mix well

Seal Wash: 50:50 MeOH:H_2O

Transport: 10 mM ammonium acetate, pH 5.5 ± 0.05
Diluent: 10 mM ammonium acetate, pH 5.5 ± 0.05

Stock Preparations
- Working stock (Calibrators): 1000 ng/mL NNN/NNK in MeOH
- Working stock 1 (Controls): 1000 ng/mL NNN/NNK in MeOH (WS1)
- Working stock 2 (Controls): 100 ng/mL NNN/NNK in MeOH (WS2)
- Working stock 3 (Controls): 1 ng/mL NNN/NNK in MeOH (WS3)
- Internal standard stock: 1000 ng/mL NNN-d4 in methanol
- Working internal standard stock: 250 ng/mL NNN-d4 in methanol

The standard curve, internal standard, and controls are prepared in 5 mL volumetric flasks, using 10 mM ammonium acetate pH 5.5 as the diluent unless otherwise specified.

Calibration Curve

Level	Conc. (ng/mL)	Spike Vol (mL)	Source	Final Vol (mL)
1	0.10	0.500	STD 4	5
2	0.25	0.125	STD 6	5
3	0.50	0.250	STD 6	5
4	1.00	0.500	STD 6	5
5	5.00	0.025	STOCK	5
6	10.00	0.050	STOCK	5

Internal Standard

Level	Conc. (ng/mL)	Spike Vol (mL)	Source	Final Vol. (mL)
ISTD	25	0.500	Working ISTD STOCK	5

Controls

10 mM ammonium acetate, pH 5.5				
Level	**Conc. (ng/mL)**	**Spike Vol (mL)**	**Source**	**Final Vol. (mL)**
LLOQ	0.1	0.500	WS3	5
LOW	0.3	0.500	MID	5
MID	3.0	0.150	WS2	5
HIGH	7.5	0.375	WS2	5

70:30 PG:VG*				
Level	**Conc. (ng/mL)**	**Spike Vol (mL)**	**Source**	**Final Vol. (mL)**
LOW	3.0	0.500	MID	5
MID	30.0	0.150	WS1	5
HIGH	75.0	0.375	WS1	5

* Prepared in 70:30 PG:VG and stored in refrigerator. At time of run, dilute 1:10 to 0.3, 3.0, 7.5 ng/mL (see below)

Sample Preparation

Calibration Curve and Controls (10 mM ammonium acetate, pH 5.5)

All standards are prepared fresh for each analytical run

1. Prepare standards and internal standards following the tables above
2. In a HPLC vial, using a Biohit pipette, add 0.250 mL ISTD and 0.750 mL of standard (1.000 mL total)
3. Mix well

70:30 PG:VG controls

1. Using a 5 mL volumetric flask, add ~ 1 mL of diluent
2. Using a microman CP1000 pipette, add 0.500 mL to the volumetric flask (1:10 dilution)
3. Fill to volume with diluent. Mix well.
4. In a HPLC vial, using a Biohit pipette, add 0.250 mL ISTD and 0.750 mL of standard (1.000 mL total)
5. Mix well

E-liquids

1. Using a 5 mL volumetric flask, add ~ 1 mL of diluent
2. Using a microman CP1000 pipette, add 1.000 mL sample to the volumetric flask (1:5 dilution).
3. Fill to volume with diluent. Mix well.
4. In a HPLC vial, using a Biohit pipette, add 0.250 mL ISTD and 0.750 mL of sample (1.000 mL total)
5. Mix well

SNUS, pipe tobacco, oral nicotine pouches

1. Check balance with calibrated weights and print
2. Open pouch and weigh out 250 mg sample. Transfer to a 16 x 125 mm test tube.
3. Add 10 mL of 10 mM ammonium acetate, pH 5.5 to test tube
4. Cover and vortex for 60 minutes
5. Using a 5 mL volumetric flask, add ~ 1 mL of diluent
6. Using a Biohit P1000 pipette, add 0.500 mL sample to the volumetric flask (1:10 dilution)
7. Fill to volume with diluent. Mix well.
8. In a HPLC vial, using a Biohit pipette, add 0.250 mL ISTD and 0.750 mL sample (1.000 mL total)
9. Mix well.

HPLC Parameters (SelexION LC.2 HPLC)

Column Conditions

Column: TSNA MIP column, 50 x 2.1 mm, ID (Biotage TSNA MIP material 30-90 µm)
Column Temperature: 40 °C

Autosampler Conditions

Temperature: 5 °C
Injection volume: 10 µL

Gradient

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0.01	100	0
1.00	40	60
2.00	40	60
3.00	0	100
3.10	0	100
4.00	0	100
4.10	100	0
5.00	100	0

Mass Spectrometer Parameters (SCIEX SelecION 6500+ Q-Trap/MS)

Regression Model

Linear, weighted 1/x

MS File

Ionization Mode: MRM+ Mode
Acquisition time: 5.0 minutes

Analyte	Precursor (m/z)	Product (m/z)	Dwell (msec)	CE (volts)
NNN 1	178	120	400	27
NNN 2	178	148	400	14
NNK 1	208	122	400	16
NNK 2	208	148	400	18
NNN-d4 1	182	124	400	27
NNN-d4 2	182	152	400	14

Tune Parameters

Declustering Potential (eV)	20
Source Temperature (°C)	550
Ion Source Gas 1 (mL/min)	60
Ion Source Gas 2 (mL/min)	25
Curtain Gas (mL/min)	40
Collison Exit (V)	15
Period Summary	
Duration (min)	5
Cycles	123
Cycle (sec)	2.43

Appendix 5: Autosampler Stability of NNN and NNK

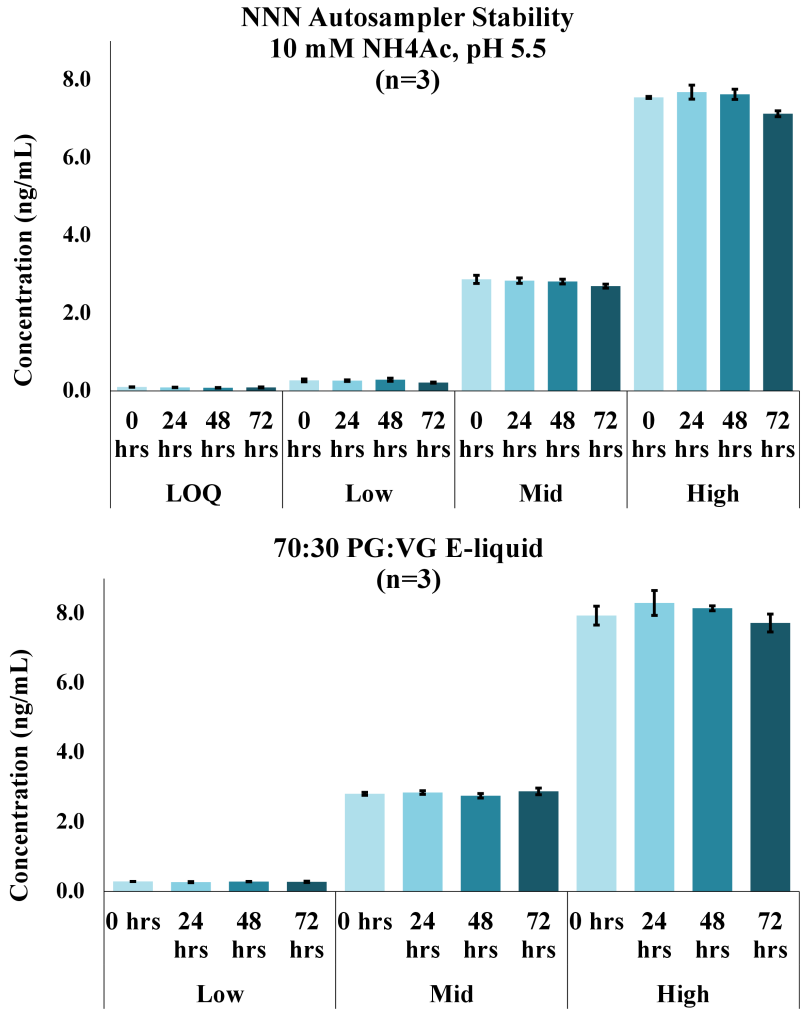

Figure A.3: Average autosampler stability for NNN (N = 3). Accuracy over the three days was between 71-102% for QCs in 10 mM ammonium acetate, and between 90-111% for QCs in 70:30 PG:VG

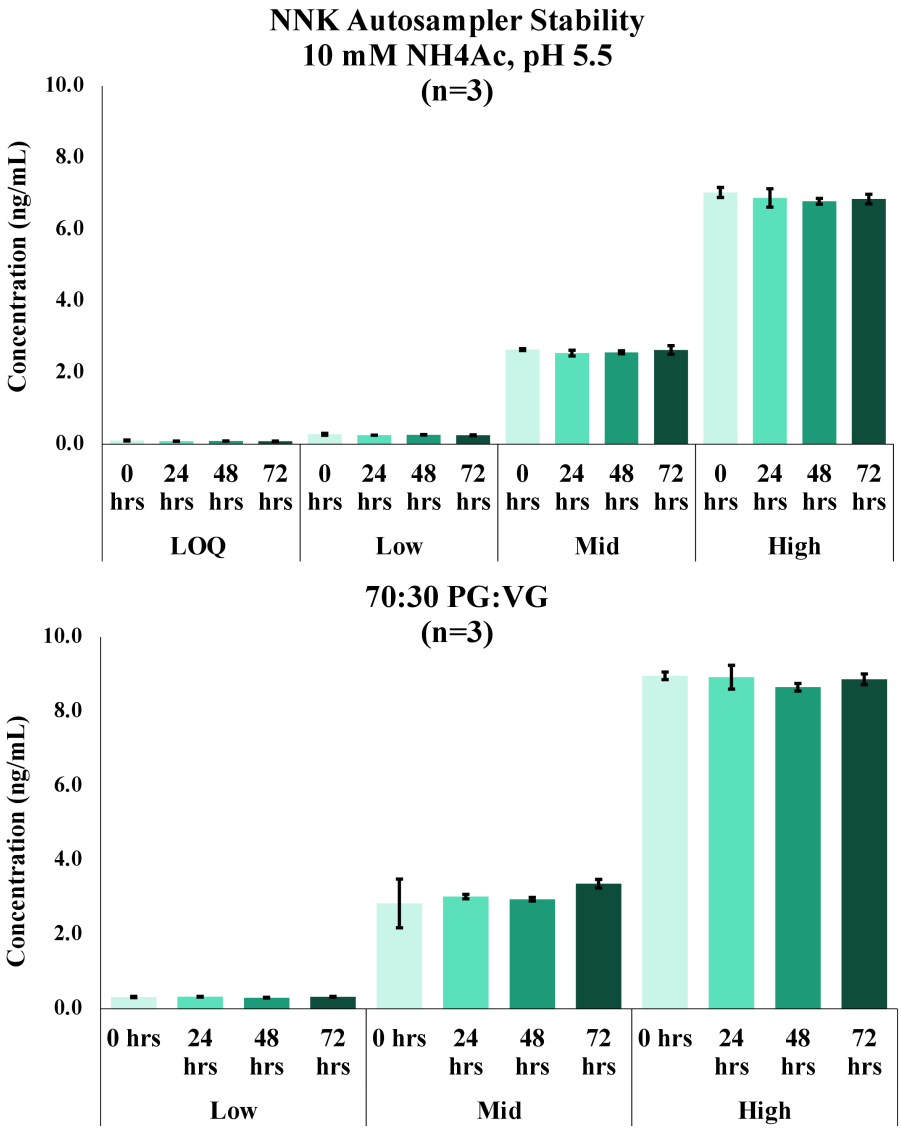

Figure A.4: Average autosampler stability for NNK (N = 3). Accuracy over the three days was between 83-103% for QCs in 10 mM ammonium acetate, pH 5.5, and between 98-119% for QCs in 70:30 PG:VG

Appendix 6: Cotinine MIP Preparation

Cotinine MIP Preparation

Materials

Template: 1.5 mmol Nicotine (241 μL)
Functional Monomer: 6 mmol methacrylic acid (MAA) (508 μL)
Crosslinking Monomer: 28.8 Divinylbenzene (DVB) (4,025 μL)
Initiator: 1.9 mmol AIBN (312 mg)
Porogen: 128 mL of 3:1 (v/v) Acetonitrile: Toluene (96 mL ACN, 32 mL toluene)
One (1) 250 mL HPLC bottle
Nitrogen gas (N_2)

Methods

Pre-polymerization Mixture

1. Combine 1.5 mmol nicotine with 6 mmol MAA in 128 mL of 3:1 (v/v) ACN:Toluene in a 250 mL HPLC bottle.
2. Sonicate in water bath for 15 minutes.
3. Add 28.8 mmol DVN and 1.9 mmol AIBN to mixture
4. Degass mixture under N_2 for 15 minutes.
5. Seal mixture with bottle cap and parafilm bottle cap
6. Place polymer mixture into a 60 °C water bath for 48 hours
7. Create a non-imprinted polymer following above steps, but omit nicotine

Post-Polymerization

1. Using a glass vacuum filtration apparatus and a 0.45 μm Nylon Membrane Filter, filter off excess solvent from polymer.
2. Disperse polymer into 200 mL tetrahydrofuran. Sonicate for 30 minutes.
3. Centrifuge sample at 3000 RPM for 10 minutes. Pour off solvent and fine particles.
4. Repeat steps 2-3 2x (3 Tetrahydrofuran washes total)
5. Place polymer in 200 mL H_2O
6. Using a glass vacuum filtration apparatus and a 0.45 μm Nylon Membrane Filter, filter off excess solvent from polymer.
7. Place polymer in 200 mL MeOH. Sonicate for 15 minutes
8. Using a glass vacuum filtration apparatus and a 0.45 μm Nylon Membrane Filter, filter off excess solvent from polymer.
9. Repeat steps 7-8 (2 MeOH washes total)
10. Allow polymer to dry out overnight at room temperature, or in 70 °C oven for 1 hour.

Appendix 6: Bulk and Precipitation Polymerization Photos

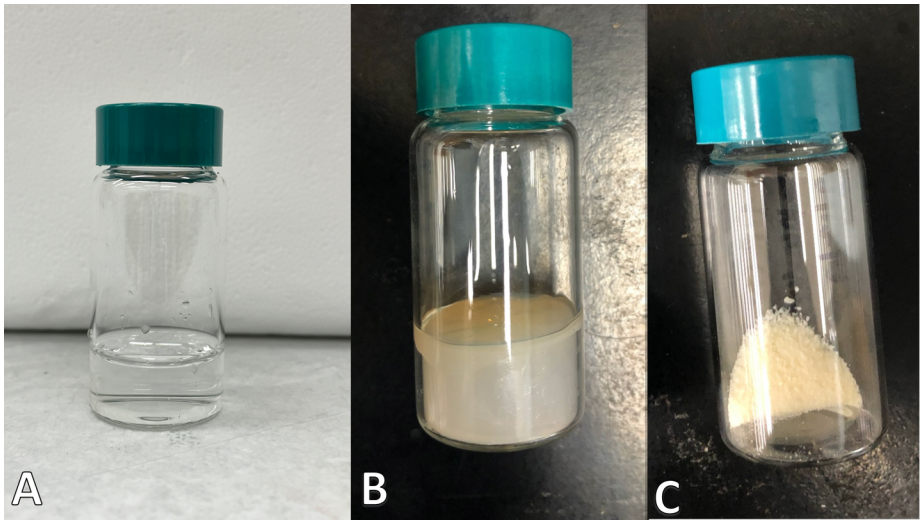

Figure A.5: Results of bulk polymerization. The pre-polymerization complex (A) is a clear solution as the template, functional monomer, crosslinking monomer, and initiator are soluble in the porogen. After 24 hours (B) the solution has become a hard, monolithic resin. After grinding and sieving the polymer to its desired polymer size (C), a hard, grainy polymer is the final result of the polymer.

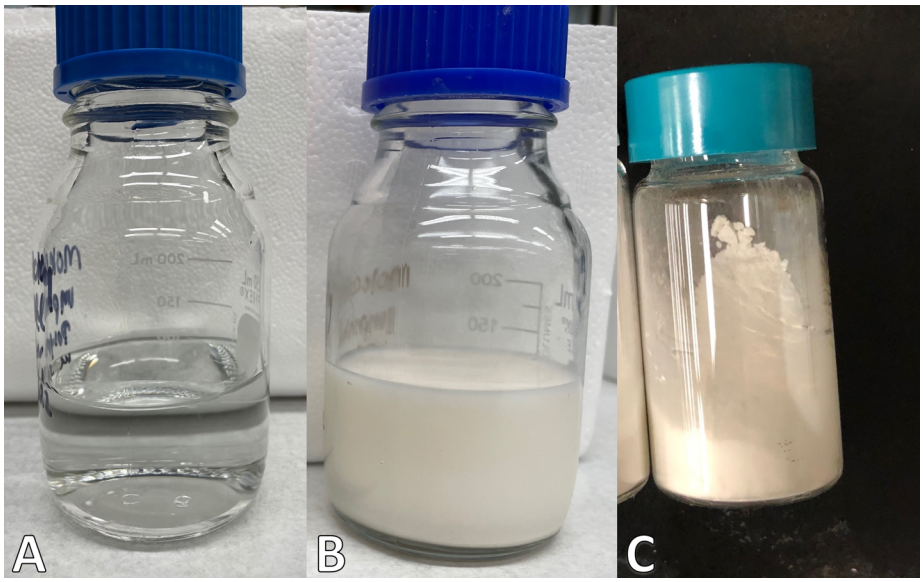

Figure A.6: Results of precipitation polymerization. The pre-polymerization complex (A) is a clear solution as the template, functional monomer, crosslinking monomer, and initiator are soluble in the porogen. After 24 hours (B) the solution turns cloudy as the formed polymer becomes insoluble in the porogen and precipitates out. After filtering off the solvent, a fine, powdery material (C) is the final result of the polymer.

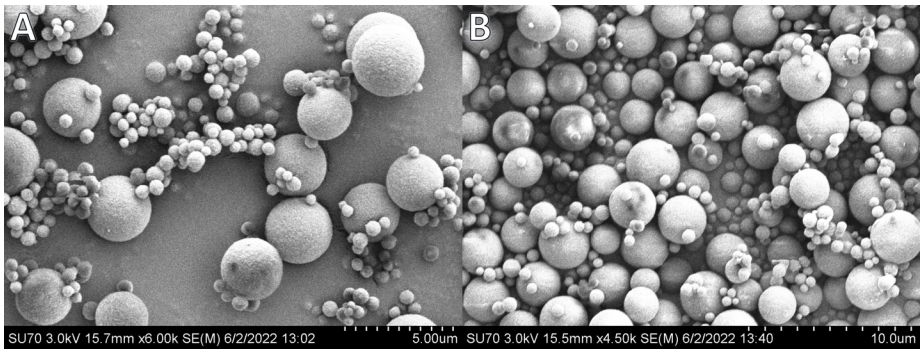

Figure A.7. SEM Images of in-house polymer A) pre-packed into the HPLC column and B) after being extruded from the column post-packing.

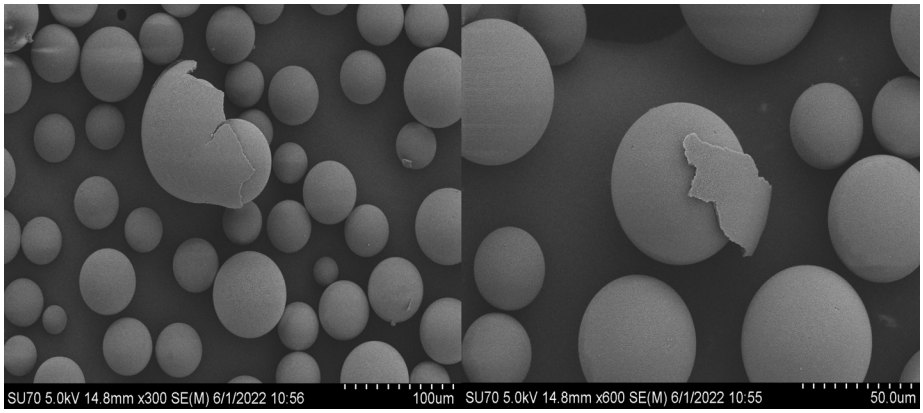

Figure A.8: SEM images of commercial TSNA polymer. Evidence of polymer encapsulated beads on the surface.

Appendix 8: Extraction of Cotinine with In-House MIP

Extraction of Cotinine with In-House MIP

Reagents

Mobile Phase A: 10 mM ammonium formate in 0.05% formic acid

1. Measure out 1000 mL H_2O in a graduated cylinder
2. Remove 0.500 mL H_2O
3. Add 0.500 mL formic acid
4. Dilute 0.6306 g ammonium formate with mixture
5. Mix well and store at room temperature for up to 2 weeks

Mobile Phase B: 0.05% formic acid in 1:1 Methanol:Acetonitrile

1. Combine 500 mL of methanol and 500 mL of acetonitrile in a HPLC bottle
2. Remove 0.500 mL of the mixture
3. Add 0.500 mL formic acid.
4. Mix well and store at room temperature up to two weeks

Wash 1: 10 mM ammonium acetate, pH 5.5 (100 mL)

4. Weigh 77.1 mg ammonium acetate
5. Mix with 100 mL H_2O
6. Adjust pH to 5.5 ± 0.05 with acetic acid or ammonium hydroxide
7. Store in refrigerator up to two weeks

Reconstitution Solution: 1% Formic Acid in Acetonitrile

1. Measure out 200 mL of acetonitrile in a graduated cylinder
2. Remove 2 mL
3. Add 2 mL of formic acid
4. Mix well and store at room temperature up to two weeks

Stock Preparations

Standard Stock: 100 µg/mL cotinine in methanol (5 mL)
Working Standard Stock: 10 µg/mL cotinine in methanol (5 mL)
Internal Standard Stock: 10 µg/mL cotinine-d3 in methanol (5 mL)
Working Internal Standard Stock: 0.250 µg/mL cotinine-d3 in methanol (5 mL)

Extraction Procedure

1. Weigh out 25 mg of polymer and mix with 0.500 mL methanol
2. Pipette the 0.500 mL mixture into a ThermoScientific 0.750 mL centrifuge filter with 0.2 µm nylon spin cartridge filter
3. Follow extraction procedure outlined in the table below. Extractions are carried out using Eppendorf 5424R microcentrifuge

Step	Solvent	RPM	Minutes
1. Prime	500 µL Methanol	5,000	5
	500 µL DI H_2O	5,000	5
2. Load	500 µL Sample	3,000	10
3. Wash 1	500 µL 10 mM ammonium acetate, pH 5.5	5,000	5
4. Dry		15,000	10
5. Wash 2	500 µL Heptane	7,000	6
6. Elute	500 µL Methanol	5,000	10
	500 µL Methanol	5,000	5

4. Collect the elution fraction in a 10 mL Kimble centrifuge tube and evaporate to dryness under N_2 at 50 °C.
5. Reconstitute with 0.500 mL of reconstitution solution. Vortex, and transfer 200 µL to a HPLC vial with inserts.

HPLC Parameters (Waters eAlliance HPLC)

Column Conditions

Column: Varian Polaris Si-A, 3.0 x 50 mm, 5 µm
Column Temperature: 35 °C

Autosampler Conditions

Injection Volume: 10 µL
Autosampler Temperature: 5 °C

Gradient

Time (min)	A%	B%	Flow (mL/min)	Curve
1.00	0	100	0.400	6
3.00	25	75	0.400	6
4.00	0	100	0.400	6
4.50	0	100	0.400	6

Mass Spectrometer Parameters (Waters QuattroMicro API MS)

MS File

Ionization Mode: MRM+
Acquisition Time: 4.5 minutes

Analyte	Precursor (m/z)	Product (m/z)	Dwell (Seconds)	Collision Energy (eV)	Cone (V)
Cotinine	176	80	0.400	20	20
Cotinine-d3	179	101	0.400	20	20

Tune Parameters

Source Page (ESI)	
Capillary (kV)	2.5
Cone (V)	20.0
Extractor (V)	2.0
RF Lens (V)	0.2
Source Block Temperature (°C)	150
Desolvation Temperature (°C)	350
MS 1	
Entrance (V)	1.0
Exit (V)	1.0
Ion Energy	0.5
LM Resolution	15.0
HM Resolution	15.0
MS 2	
Ion Energy	1.0
LM Resolution	13.0
HM Resolution	13.0
Multiplier	650
Nebulizer Gas Flow (L/hr)	68
Desolvation Gas Flow (L/hr)	490

Vita

Haley Ann Mulder was born on May 18, 1993 on Long Island, NY. She received a BS in Forensic Science with a focus in Chemistry from Virginia Commonwealth University in 2016. She went onto receive her Master's of Science in Forensic Science on the Drug Analysis and Toxicology track from Virginia Commonwealth University in 2018, under Dr. Michelle Peace. Her masters research focused on characterizing the aerosol output from electronic cigarettes adulterated with methadone and particle size of electronic cigarette aerosol adulterated with methadone or methamphetamine. Her masters work yielded five publications and nine presentations at local, national, and international conferences. She was the recipient of the Educational Research Award for her masters thesis in 2018 from the Society of Forensic Toxicologists.

Haley began her PhD with Dr. Matthew Halquist in August of 2018, focusing on extraction techniques of small molecules in biological matrices. Her dissertation has yielded two first author publications and two poster presentations. She currently has two first author manuscripts related to her dissertation under review. During her dissertation work, she was the recipient of the June K Jones Scholarship from the American Academy of Forensic Sciences (2020), and the VCU Graduate School Dissertation Assistantship Award (2022). In 2022, she was the runner up for the Charles Rector and Rorrer Dean's Award from the VCU School of Pharmacy.