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DEVELOPMENT OF MOLECULARLY IMPRINTED  
POLYMERS FOR FORENSIC APPLICATIONS

by

SARA GILMAR MARTINEZ  
B.S. University of Central Florida, 2012

A thesis submitted in partial fulfillment of the requirements  
for the degree of Master of Science  
in the Department of Chemistry  
in the College of Sciences  
at the University of Central Florida  
Orlando, Florida

Fall Term  
2016

Major Professor: Michael E. Sigman

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## ABSTRACT

In some forensic disciplines various methods of extraction are used to perform analysis. Among these methods, solid phase extraction (SPE) and solid phase microextraction (SPME) are used in fields such as toxicology and explosives analysis. To enhance extraction efficiency in SPE and SPME, molecularly imprinted polymers (MIPs), which are designer polymers, can be more selective for the binding of an analyte or group of analytes that are similarly structured. Separation of analytes from complex mixtures is possible by utilizing these polymers. This may be especially useful in forensic applications where sample sizes may be small and composition may be complex.

In this work, MIP solid phase microextraction fibers (MIP-SPME) were fabricated and caffeine was selectively sampled in the presence of theophylline and theobromine. Calibration studies were performed using the MIP-SPME to quantitate the concentration of caffeine in teas and coffees. MIP-SPME fibers were also prepared with 2,4-dinitrotoluene and deuterated 2,6-dinitrotoluene. Less selectivity was obtained for extraction of 2,4-DNT and 2,6-DNT in the presence of other DNT isomers. Fabricated blank polymers extracted analytes at the same response as templated polymers for both caffeine and DNT, despite expected results. MIP-SPE columns were also fabricated using deuterated 2,6-DNT to determine if changing the extraction procedure would increase extraction selectivity. Using different solvents in the extraction

procedure changed the extraction performance efficiency of the MIPs due to the change in solvent polarity. All samples were analyzed using gas chromatography mass spectrometry.

*This work is dedicated to my family who always show immense support in my times of need.*

*Love you guys.*

## **ACKNOWLEDGMENTS**

I would like to express my appreciation to all those that helped while writing this paper. First, I would like to thank my advisor and chair, Dr. Sigman, for guiding me during the course of this project. He was very patient and provided great mentorship during the thesis process. I would also like to thank Dr. Bridge and Dr. Yestrebsky for being part of my committee.

Second, I would like to thank my fellow graduate students, some who have come and gone, and those still performing research, at the National Center for Forensic Science. Thanks for your unending support, especially Jessica and Shanna. You helped me keep my sanity and kept it fun. Additionally, I would also like to thank Mary Williams, who provided guidance and support in the lab, and Krystal my undergraduate assistant, who helped me with this experiment.

Lastly, I would especially like to thank my family, to whom this document is dedicated. I love you all immensely. Thank you mami and daddy for being my pillars of support when I got too overwhelmed. Thank you Jorge for being a great older brother, you really helped me understand what it is like to be a graduate student, and will always be my role model. I would also like to thank Audry, you are an awesome sister, and I appreciate all your help whenever I needed it. And to my fur babies Mango and Henry, you helped by being your silly selves and keeping me company.

*“Do or do not. There is no try.”*

*-Yoda*



## TABLE OF CONTENTS

LIST OF FIGURES .....	xi
LIST OF TABLES .....	xv
LIST OF ABBREVIATIONS.....	xvii
CHAPTER 1: INTRODUCTION .....	1
CHAPTER 2: BACKGROUND .....	7
Molecularly Imprinted Polymers .....	7
Theory.....	7
Applications.....	15
Advantages and Disadvantages .....	16
Solid Phase Microextraction .....	18
Theory.....	18
Applications.....	22
Advantages and Disadvantages .....	23
MIP-SPME .....	24
Instrumentation.....	24
Sample Introduction .....	24
Gas Chromatography-Mass Spectrometry .....	25

Quantitation .....	25
CHAPTER 3: EXPERIMENTAL.....	28
Polymerization Procedure .....	28
Pre-polymer solution .....	28
Polymerization.....	29
GC Inlet Desorption .....	30
Furnace Extraction.....	33
Soxhlet Extraction .....	34
Sample Solution Preparation and Procedure .....	39
Calibration and Quantitation .....	44
Teas and Coffee .....	45
Selectivity .....	46
Conventional SPME versus MIP-SPME.....	47
MIP-SPE versus MIP-SPME .....	48
CHAPTER 4: RESULTS AND DISCUSSION.....	51
Calibration and Quantitation .....	51
Selectivity: Caffeine .....	54
Selectivity: DNT .....	64

Conventional SPME versus MIP-SPME.....	77
MIP-SPE versus MIP-SPME .....	80
CHAPTER 5: CONCLUSIONS .....	87
Conclusion.....	87
Future Work .....	90
APPENDIX A: CAFFEINE CONCENTRATION CALCULATION .....	91
APPENDIX B: DNT ISOMERS CALIBRATION CURVES.....	93
APPENDIX C: STATISTICAL ANALYSIS FOR SELECTIVITY TABLES .....	98
REFERENCES .....	103

## LIST OF FIGURES

Figure 1: <i>Simple MIP diagram</i> .....	8
Figure 2: <i>Initiation of polymerization scheme. I.) Cleavage of AIBN through photo and thermoinitiation. II.) Attachment of cleaved AIBN radical to EGDMA to create new radical and continue linking process.</i> .....	9
Figure 3: <i>Active site polymerization scheme</i> .....	11
Figure 4: <i>Molecular structure of caffeine</i> .....	14
Figure 5: <i>DNT template isomer molecules</i> .....	14
Figure 6: <i>Non-templated fiber first desorption for 30 secs at 200°C</i> .....	31
Figure 7: <i>Non-template fiber 40<sup>th</sup> desorption for 30 secs at 200°C</i> .....	32
Figure 8: <i>Caffeine from templated fiber desorption for 10 mins at 300°C</i> .....	33
Figure 9: <i>Caffeine templated fiber desorption for 60 mins at 300°C</i> .....	34
Figure 10: <i>Templated polymer before extraction</i> .....	36
Figure 11: <i>Templated polymer after Soxhlet extraction</i> .....	37
Figure 12: <i>MIP monolith attachment to SPME needle</i> .....	38
Figure 13: <i>MIP-SPME manual injection assembly</i> .....	39
Figure 14: <i>Isotopic caffeine molecular structure</i> .....	41
Figure 15: <i>Molecular structures of caffeine analogues theobromine and theophylline</i> .....	41
Figure 16: <i>Molecular structures of DNT isomers</i> .....	42
Figure 17: <i>MIP-SPME preparation and use cycle</i> .....	43

Figure 18: <i>MIP-SPE procedure</i> .....	49
Figure 19: <i>Caffeine standard calibration curve; solutions prepared at 10, 50, 100, and 200 ng/μL concentrations from a caffeine stock solution (50:50 (v/v) methanol/Epure water)</i> .....	52
Figure 20: <i>Caffeine standard mass spectrum with molecular ion and base peak at 194 m/z</i> .....	55
Figure 21: <i>Theobromine standard mass spectrum with molecular ion and base peak at 180 m/z</i>	56
Figure 22: <i>Theophylline standard mass spectrum with molecular ion and base peak at 180 m/z</i>	57
Figure 23: <i>TIC of caffeine derivatives mix in methanol using caffeine templated fiber</i> .....	59
Figure 24: <i>TIC of caffeine derivatives mix in 50:50 water/methanol solution using caffeine templated fiber</i> .....	60
Figure 25: <i>TIC of caffeine derivatives mix in methylene chloride using caffeine templated fiber</i>	61
Figure 26: <i>Preliminary blank polymer sampling in caffeine derivatives mix; peaks from degradation of polymer</i> .....	62
Figure 27: <i>Blank polymer sampled in Earl Grey Crème tea</i> .....	63
Figure 28: <i>Caffeine Template Polymer in Earl Grey Crème tea</i> .....	64
Figure 29: <i>2,3-DNT standard mass spectrum; unique ion at 135 m/z used to differentiate between 2,4-DNT isomer</i> .....	65
Figure 30: <i>2,4-DNT standard mass spectrum; unique ion at 119 m/z used to differentiate between 2,3-DNT isomer</i> .....	66
Figure 31: <i>2,6-DNT standard mass spectrum</i> .....	67
Figure 32: <i>3,4-DNT standard mass spectrum</i> .....	68

Figure 33: 2,4-DNT templated fiber sampled in DNT isomers mix; 2,3-DNT and 2,4-DNT coelute at approximately 9.3 minutes, while 2,6-DNT elutes at approximately 8.8 minutes and 3,4-DNT elutes at approximately 9.7 minutes.....	69
Figure 34: 2,6-DNT- $\alpha,\alpha,\alpha\text{-d}_3$ templated fiber sampled in DNT isomers mix .....	70
Figure 35: Blank polymer sampled in DNT isomers mix .....	71
Figure 36: Calibration curves for DNT isomers. (Process for calculating values in selectivity tables. 2,3-DNT/2,4 DNT = 0.4607 (first sampling). Process repeated for each sampling. Final value seen in table: ( $1^{\text{st}}$ sampling + $2^{\text{nd}}$ sampling + $3^{\text{rd}}$ sampling)/3; (DNT isomer row/DNT isomer column) = 0.49) .....	72
Figure 37: Comparison of the average selectivity ratios of isomers 2,4-DNT versus 2,3-DNT for each fiber .....	74
Figure 38: CW/DVB fiber sampled in solution of caffeine, theobromine, and theophylline .....	78
Figure 39: CW/DVB fiber sampled in solution of caffeine and theophylline.....	79
Figure 40: Methylene chloride elution with templated 2,6-DNT- $\alpha,\alpha,\alpha\text{-d}_3$ SPE.....	81
Figure 41: Methanol wash with templated 2,6-DNT- $\alpha,\alpha,\alpha\text{-d}_3$ SPE .....	82
Figure 42: DNT isomers solution in pentane before loading onto MIP-SPE .....	83
Figure 43: Pentane sample solution after loading onto MIP-SPE .....	84
Figure 44: Methanol elution step dried with nitrogen gas and reconstituted using pentane .....	85
Figure 45: Caffeine Level estimation chart taken from manufacturer's (Teavana <sup>®</sup> ) website.....	92
Figure 46: 2,3- DNT Isomer calibration curve used to calculate ratios for determining selectivity in Tables 10-13 .....	94

Figure 47: <i>2,4-DNT Isomer calibration curve used to calculate ratios for determining selectivity in Tables 10-13</i> .....	95
Figure 48: <i>2,6-DNT Isomer calibration curve used to calculate ratios for determining selectivity in Tables 10-13</i> .....	96
Figure 49: <i>3,4-DNT Isomer calibration curve used to calculate ratios for determining selectivity in Tables 10-13</i> .....	97
Figure 50: <i>Comparison of the average selectivity of isomers 2,4-DNT versus 2,3-DNT for each fiber</i> .....	102

## LIST OF TABLES

Table 1: SPE Common Sorbent Bed Types .....	2
Table 2: SPME Fiber Coatings .....	4
Table 3: Agitation Methods .....	21
Table 4: Pre-polymer Solution.....	28
Table 5: Sample Solutions .....	40
Table 6: Teas and Coffee Preparation.....	46
Table 7: Significant Values for Each Compound Analyzed .....	47
Table 8: Concentrations of Real World Samples.....	53
Table 9: Data used to determine significance of selectivity across the templated and blank fibers .....	73
Table 10: Selectivity Ratios using a 2,4-DNT Templated Fiber .....	75
Table 11: Selectivity Ratios using a 2,6-DNT- $\alpha,\alpha,\alpha$ -d <sub>3</sub> Templated Fiber .....	76
Table 12: Selectivity Ratios using a Blank Fiber.....	76
Table 13: Selectivity Ratios using a 2,4-DNT Templated Fiber in Methylene Chloride .....	77
Table 14: Summary of Caffeine Extractions .....	88
Table 15: Data used to determine significance of selectivity across the templated and blank fibers .....	99
Table 16: Data used to determine significance of selectivity across the templated and blank fibers .....	99



Table 17: Data used to determine significance of selectivity across the templated and blank fibers .....	100
Table 18: Dixon's Q test performed on second sampling of blank fiber to determine if its values are considered outliers .....	101

## LIST OF ABBREVIATIONS

AIBN	2,2'-azobisisobutyronitrile
BPA	Bisphenol A
CW	Carbowax
DES	Diethylstilbestrol
DVB	Divinylbenzene
EGDMA	Ethylene glycol dimethacrylate
EIC	Extracted ion chromatogram
HEX	Hexestrol
GC	Gas Chromatography
LC	Liquid Chromatography
LLE	Liquid-liquid extraction
MAA	Methacrylic acid
MIP	Molecularly imprinted polymer
MIP-SPE	Molecularly imprinted polymer-solid phase extraction
MIP-SPME	Molecularly imprinted polymer-Solid phase microextraction
MISPE	Molecularly imprinted solid phase extraction
mL	milliliter
MS	Mass spectroscopy
<i>m/z</i>	mass to charge ratio

NCFS	National Center for Forensic Science
PDMS	Polydimethylsiloxane
SPME	Solid phase microextraction
TIC	Total ion chromatogram

## CHAPTER 1: INTRODUCTION

In today's forensic laboratories there is always a need for rapid and non-destructive methods of analysis. The current methods in place are often not efficient enough to handle the caseload and turnover required. This is problematic due to the time consuming nature of the techniques and processes in place. Unknown samples obtained from crime scenes are generally analyzed through these techniques and processes. The techniques and processes are generally destructive and, as previously mentioned, time-consuming. Destructive analysis is detrimental for subsequent testing. This is especially problematic if there is only a small quantity of sample collected. Examples of common methods in use for analysis for drugs and explosives include solid phase extraction (SPE)<sup>1</sup>, liquid-liquid extraction (LLE)<sup>2</sup>, and solid phase microextraction (SPME).<sup>3</sup> These methods allow for limited selectivity in extraction of analytes of interest from the sample.<sup>4,5</sup> This section will discuss these current methods, and why it would be beneficial to have a more selective extraction method.

SPE is the most common method of sample extraction and preparation in use in analytical laboratories, including forensic crime labs.<sup>6</sup> This technique can use small volumes of sample, such as 1 mL, but can also use large volumes such as 10 mLs.<sup>7</sup> These large volumes are not practical because of the possibility of limited sample quantities collected from a crime scene. SPE uses a sorbent bed packed between two fritted disks located at the bottom of a cartridge.<sup>6</sup> This sorbent bed has an affinity for interaction with an analyte or analytes of interest based on

certain parameters, such as changes in pH and solvent solubility or polarity. Through this affinity, the analyte is retained onto the sorbent bed. The analyte elutes from the sorbent bed when a solvent, for which it has a greater affinity, washes the sorbent bed. The interactions between the sorbent bed and analytes are dependent upon the solvent chosen for elution. This solvent can be acidic or basic, which helps in selective extraction of acidic or basic molecules. The sorbents can consist of a variety of materials, the most common being silica-based.<sup>8</sup> Examples of some sorbent beds and the groups of analytes to which they show affinity are listed in Table 1.<sup>6,8</sup> The samples and solvents can be passed through the sorbent bed by a variety of methods such as vacuum suction or positive pressure.<sup>6</sup> In order to begin the extraction process, the sorbent bed must go through an activation step to promote retention of the target analyte.

**Table 1: SPE Common Sorbent Bed Types**

<b>Sorbent Bed Type</b>	<b>Group</b>	<b>Application</b>
Silica gel	Inorganic Oxides	Isolation of polar pesticides from fats and oils, class fractionation of lipids, isolation of quaternary, etc.
Alumina		
Florisil (synthetic magnesium silicate)		
Diatomaceous earth		
Chemically-bonded silicas (Octadecylsilane – C <sub>18</sub> : Nonpolar; Silica – SI: Polar; Benzenesulfonylpropylsilane – SCX: ion exchange)	Non-specific	Isolation of contaminants from aqueous solution
Porous polymers		
Carbon		
Mixed-mode (combination of any of the above)	Compound- and class-specific	Isolation of various types of compounds

In preparing the sample for analysis, SPE is most commonly used to isolate a target analyte(s) or interfering compounds. The most common use is pre-concentration of the sample for

quantitative analysis. Another isolation method is the collection of different fractions, such as acidic and basic fractions through the use of different sorbent bed-solvent combinations.<sup>9</sup> The SPE cartridges can be used as off-line or online methods. The method where the SPE cartridge is not attached to a LC or GC instrument is called off-line. The online method is when the SPE cartridge is coupled to an instrument before the chromatographic column.<sup>6</sup> This is useful when automation is necessary to reduce analysis time.<sup>9</sup> The extracted sample is collected to undergo analysis on an analytical instrument, such as GC or LC.

The second most common sample preparation method used in forensic laboratories is LLE.<sup>10</sup> In LLE the separation of the target analyte from the sample matrix is performed through the use of organic solvents, which are not miscible with the sample matrix. For example, a target analyte present in the aqueous layer may move into the organic layer if it has a greater affinity or solubility. This greater affinity can be controlled by changes in solvent polarity and pH.<sup>10</sup> The main disadvantage of the LLE method is the volumes of sample and organic solvent required. This method can also be automated like the SPE method, using a 96-well extraction plate, but is performed off-line.<sup>11</sup> After LLE, whichever phase contains the target analyte(s) is collected and analyzed on the instrument of choice.

The final sample preparation method mentioned above is SPME. Much like the previous methods, it extracts the analyte(s) present in a sample solution based on affinity. In this case, the analyte(s) are bound to the polymer coating present on the SPME needle. Table 2 lists the

coatings and thicknesses available for SPME fibers.<sup>3</sup> The advantage of this method is the option of fiber introduction, which can be performed through headspace or direct immersion analysis.<sup>3</sup> This particular method will be discussed in more detail in Chapter 2.

**Table 2: SPME Fiber Coatings**

<b>SPME Coating</b>	<b>Thickness (μm)</b>	<b>Analytes</b>
Polydimethylsiloxane (PDMS)	7, 30, 100	Nonpolar and polar
Polyacrylate (PA)	85	Polar
Polydimethylsiloxane/polydivinylbenzene (PDMS/DVB)	65	Volatile
Polyethylene glycol/polydivinylbenzene (Carbowax/DVB)	65	Polar
Polyethylene glycol/template polydivinylbenzene resin (Carbowax/TR)	65	Molecular weight discrimination

Each of these methods is used in a variety of applications like medical, pharmaceutical, industrial, and forensic. Though each method has its merits, there are also downsides associated with each. A common downside of these methods is that they tend to be time-consuming. These methods are not selective when extracting the analytes of interest. Since their methods of extraction are based on affinity, they do not extract just the target analyte. Perhaps a more selective extraction can be achieved by utilizing molecularly imprinted polymers (MIP).

Molecularly imprinted polymers have been studied in medical (extraction of clinical drugs from biological samples), environmental (contaminants in water sources), and analytical (contaminants in food) fields.<sup>12</sup> Their versatile nature comes from our ability to design or template the polymers with a target analyte for specific analyses. These polymers can be formulated as SPE, SPME coatings and monoliths, sensors, and packed column beads.<sup>13</sup> In this

study, SPME and MIP were combined to create MIP-SPME polymer monoliths that use the same principles and theories as SPME, but are more selective and stable. Added benefits of these materials are their potential utility in the extraction of the target analyte from complex solutions, and less time consuming analyses when compared to conventional analytical methods. Selective extraction of caffeine in the presence of theophylline and theobromine was studied, along with the competitive extraction of selected dinitrotoluene isomers from a mixture of isomers. The MIP-SPME method is demonstrated for the extraction and quantitation of unknown concentrations of caffeine from teas and coffees, using external calibration curves for caffeine. Through the use of MIP-SPME, analysis times can be decreased, sample can be preserved, and backlogs in forensic laboratories can be decreased.

Backlogs and turn around time are important issues in forensic laboratories.<sup>14</sup> Backlog refers to the number of cases not yet analyzed. Delays from case backlog can span days or weeks. Turn around time refers to the time lapse from the submission of the evidence to when the report of analysis is approved and submitted to the requesting agency; which can be negatively influenced by backlog.<sup>15</sup> These two issues place varying constraints on laboratory efficiency depending on the laboratory. These issues become worse when the number of qualified personnel is decreased, especially with the emergence of new types of evidence for which protocols must be developed (like designer drugs); or when time spent on testimony increases, etc.<sup>15</sup> Various reports state these issues increase as the amount of evidence and number of cases exceed what can be handled in a timely manner by laboratory sections.<sup>15,16</sup> One suggestion, besides hiring more personnel, is



the utilization of new faster methods of analysis. With shorter analysis time, the backlogs and turn around times could decrease. This is where the use of the MIP-SPME method could aid in the reduction of time spent overall, especially if it becomes automated. The MIP-SPME method is versatile enough that it can be expanded to other areas of application besides the forensic field. Some of these applications will be discussed in the next chapter.

## CHAPTER 2: BACKGROUND

### Molecularly Imprinted Polymers

#### *Theory*

Molecularly imprinted polymers (MIPs) are designer polymers created for specific analyses. The first foray of molecular imprinting was reported in 1931, but the first modern appearance occurred in 1972 with Wulff and Sarhan.<sup>17</sup> Molecularly imprinted polymers can be templated with any analyte or group of analytes to aid in extraction of these analytes from known or unknown solutions. These polymers are highly selective and stable for the templated analyte. The polymers are also stable in a variety of temperatures and pHs. This stability makes them robust and versatile for different applications. These applications are mentioned later in this chapter. The polymers are prepared by creating a solution containing a functional monomer, cross-linker, initiator, porogen, and template molecule. The functional monomer and template molecule form a complex, which forms a rigid matrix with the cross-linker. An active site or cavity is then formed from the template molecule. This cavity is complementary in size and shape to the template molecule. Upon removal of the template molecule, as demonstrated in Figure 1, the target analyte can be re-absorbed into the cavity during the sampling process. The porogenic solvent gives the polymer its macroporous structure, which allows the molecules access to the active sites created by the imprinting process.<sup>18</sup>

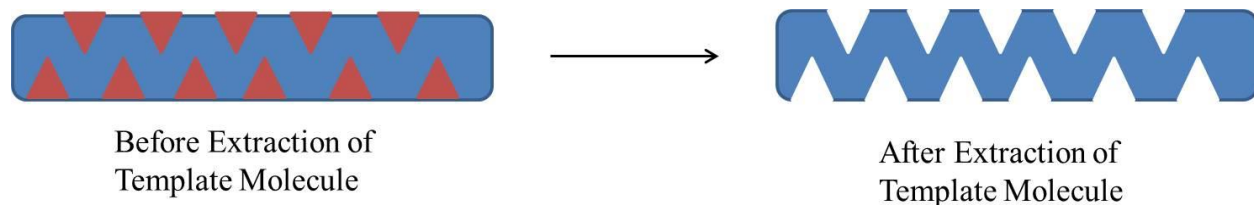


Figure 1: *Simple MIP diagram*

The polymerization process consists of three main chain reaction steps: initiation, propagation, and termination. There are different forms of polymerization, but the one used in this study is free radical initiated polymerization. The free radical polymerization begins when the initiator comes into contact with light at a particular wavelength (photoinitiation) or heat (thermoinitiation). The initiator cleaves its homolytic covalent bond to form a free radical, which can freely add to the cross-linker and functional monomer vinyl groups. This product is also a free radical, which allows it to add to other cross-linker molecules and keeps the chain reaction going until termination.<sup>19</sup> An example of this process is shown below in Figure 2.

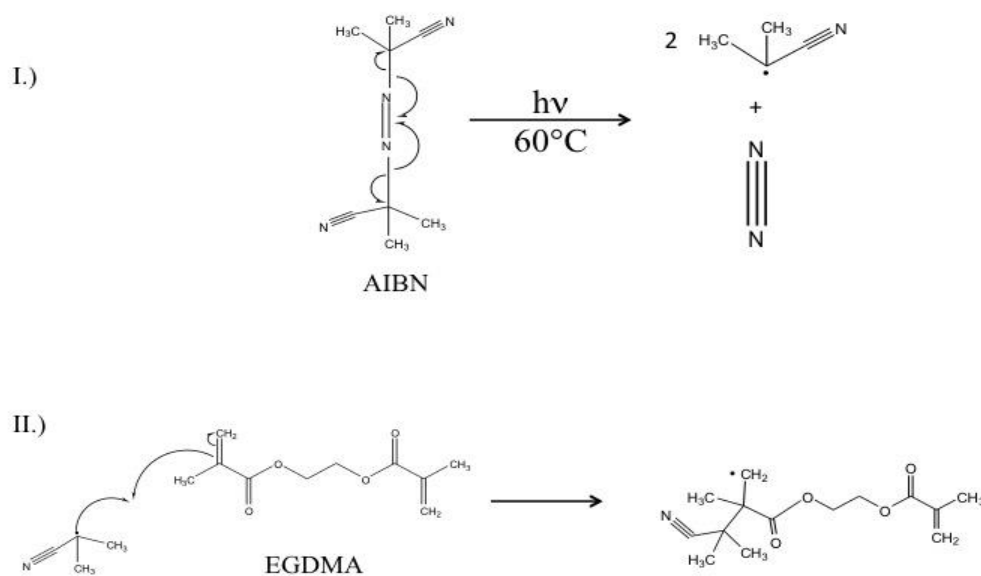


Figure 2: *Initiation of polymerization scheme. I.) Cleavage of AIBN through photo and thermoinitiation. II.) Attachment of cleaved AIBN radical to EGDMA to create new radical and continue linking process.*

A process named chain transfer causes branching and additional chain extension to occur, if the transfer occurs intramolecularly or intermolecularly at a distance from the chain end. An active site is formed, which holds the template molecule.<sup>19</sup> The chain transfer process also helps with cross-linking of the cross-linker and functional monomer. The cross-linking allows for additional rigidity to the polymer, which helps in selectivity and stability of the active site. Rigidity helps keep the shape of the active site by preventing the site from becoming malleable and accepting any molecule that can bind. The active site formed from cross-linking is the basis of MIPs. Termination of polymerization generally occurs through the coupling of two macroradicals to create a polymer chain. Another method of termination is through

disproportionation, which occurs when a hydrogen atom from another free radical chain transfers to the end of another growing chain.<sup>19</sup> This creates a polymer similar to the coupling process, only there is an unsaturated site formed in the disproportionation process.

Figure 3 demonstrates a basic formation of how an active site is created during polymerization. The materials in the pre-polymer solution shown in the figure are used in noncovalent imprinting. Noncovalent imprinting uses noncovalent bonding through interactions such as hydrogen bonding, electrostatic interactions, or metal ion coordination. The noncovalent binding demonstrated in Figure 3 is hydrogen bonding. The functional monomer, methacrylic acid (MAA), in Figure 3, is primarily used in noncovalent MIPs and is very versatile,<sup>17</sup> often being used with a variety of template molecules ranging from peptides to drugs.<sup>18</sup> The monomer's binding properties are described in more detail later in this section. Ethylene glycol dimethacrylate (EGDMA) is used as the cross-linker in the polymer.<sup>17</sup> The EGDMA molecules bind to the MAA to form the polymer backbone. The initiator used to begin the polymerization process is 2,2'-azobisisobutyronitrile (AIBN).<sup>17</sup> As the initiator, it cleaves its covalent bonds through interaction with UV light or heat, creating two radicals to begin the polymerization process. The template molecule can be any analyte desired for analysis, which can bind to the functional monomer of the polymer through two possible methods, covalent or noncovalent.<sup>17</sup>

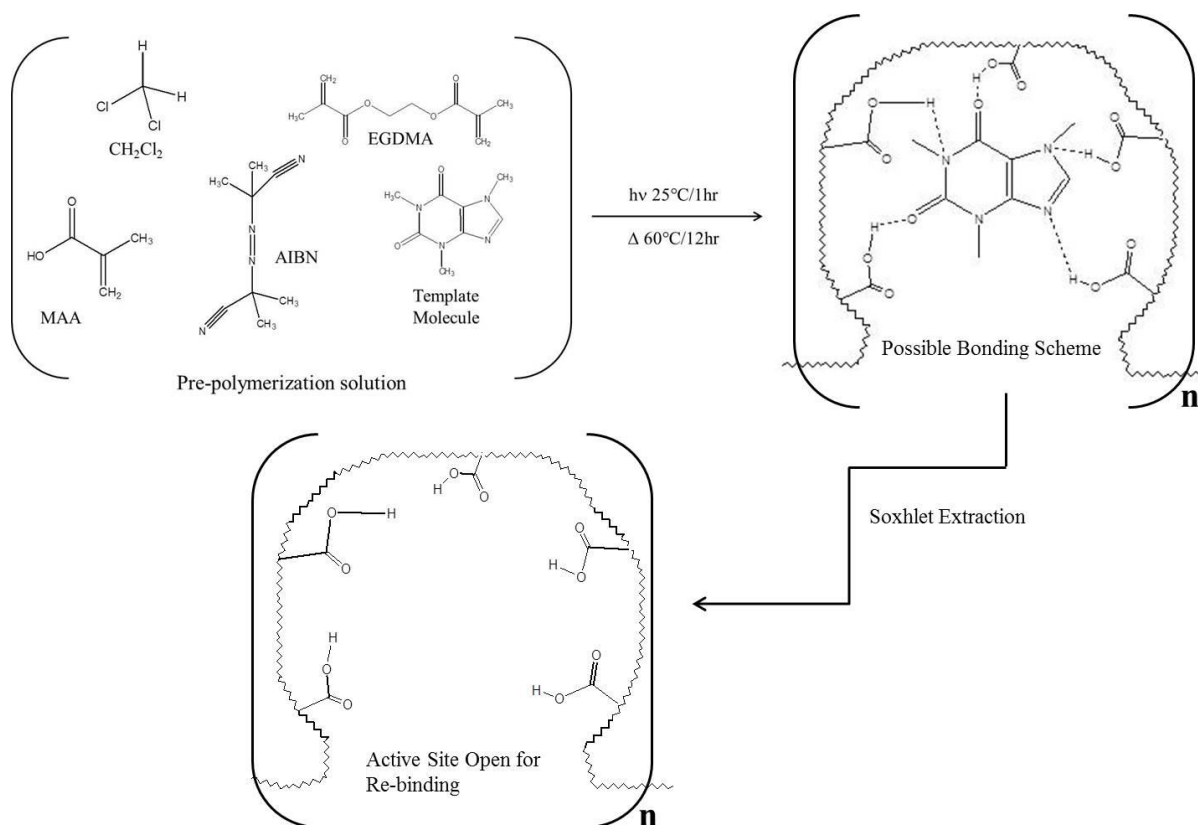


Figure 3: *Active site polymerization scheme*

Covalent imprinting uses covalent binding, which is reversible, to form linkages between a template molecule and monomer. In Wulff *et al.*, ester linkages between a sugar, as the template molecule and phenylboronic acid derivatized with a vinyl group, was as the monomer were used in the formation of a covalently imprinted polymer.<sup>18</sup> The covalent bonded complex has greater control over the imprinting process, but the polymers are slow to rebind target analytes due to the reformation of the covalent bonds. These types of polymers are better for single use rather than multiple use analyses.<sup>17</sup>

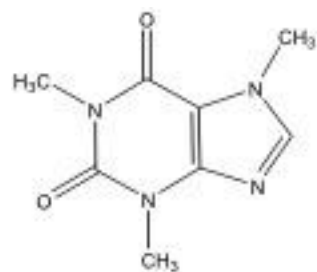
Noncovalent imprinting is the most popular method currently in use, and was developed by Arshady and Mosbach in the 1980s.<sup>17</sup> Through noncovalent imprinting, the template and monomer bind together using interactions like hydrogen bonding, electrostatic interactions, hydrophobic interactions, charge transfer, van der Waals forces, and  $\pi$ - $\pi$  interactions. The main advantages of noncovalent imprinting is its enhanced efficiency, versatility, and adaptability.<sup>17</sup> A drawback is that the interactions between the monomer and template molecule are weak, so the amount of functional monomer has to be much larger than the template molecule to overcome these weak forces. This also means there is a large amount of the functional monomer present in the polymer that does not incorporate into the active binding site for the template molecule, which leads to a large number of low affinity and low selectivity binding sites. This could result in other molecules binding onto the sites that are not templated, but share similar affinities, otherwise known as polyclonality.<sup>17, 18</sup> This has an adverse effect since the main purpose of the imprinting process is selectivity for the analyte for which the polymer has been templated.<sup>18</sup>

The most common noncovalent functional monomer is methacrylic acid. The monomer's carboxylic acid functional groups interact with the template molecule through hydrogen bonding. Molecules that bind through hydrogen bonding interact using lone pair electrons present on an electronegative atom, like oxygen and nitrogen, and molecules that are already bonded to hydrogen, like O-H and N-H. When these bonds are formed, they adopt a linear conformation where the hydrogen is positioned to bond to the oxygen or nitrogen.<sup>17</sup> Hydrogen bonding

introduces a reversibility property when the noncovalent bonds are formed. This is important for the unbinding and rebinding needed for the extraction, desorption, and resampling process. The hydrogen bonds are inherently weak, so the bonds do not require a significant amount of energy to break and lead to greater molecular recognition. The directionality of the hydrogen bonding is important to how the structure of the polymer houses the template molecule and how strongly they bind.<sup>17</sup> In this study, MIPs were imprinted with caffeine and two different isomers of dinitrotoluene.

Caffeine is a methylxanthine used in a variety of applications. The most common use is as an ingredient in beverages like soda, tea, and coffee in varying ranges of caffeine concentration.<sup>20</sup> The structure of caffeine contains points where hydrogen bonding occurs. The oxygens on the carbonyl groups and the nitrogens present on both rings of the main structure have lone pair electrons, which can bond with the hydrogens on the hydroxyl groups on the MAA. The exact formation and position of the hydrogen bonds are unknown for a given template molecule, but more bonding between the monomer and template molecule aids in enhanced molecular recognition, i.e. selectivity.<sup>21</sup> Figure 4 depicts the molecular structure of caffeine.





Caffeine

Figure 4: *Molecular structure of caffeine*

The other two molecules used for templating were isomers of DNT. These compounds can be found in soil, waste streams, and explosives.<sup>22</sup> The most common application for DNT molecules is as explosives, which is why templating these compounds are beneficial for explosives analysis.<sup>23</sup> The molecular structures for the compounds used for polymer fiber templating are depicted in Figure 5.

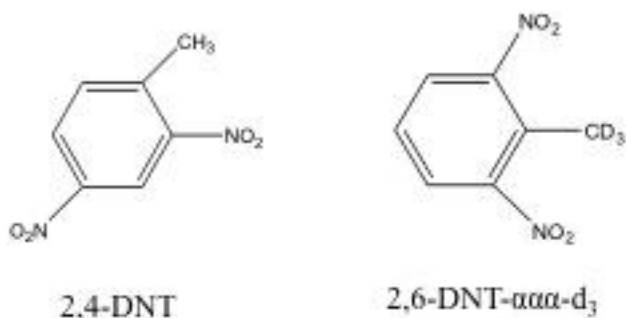


Figure 5: *DNT template isomer molecules*

A hydrogen bond is created between these explosives and MAA by using the oxygens on the nitro groups of the DNT binding to the hydrogen on the carboxyl group of the MAA, and the hydrogens of the DNT to the carbonyl oxygen on MAA.<sup>24</sup> Due to this binding, selective extraction is possible for templated polymers.

### *Applications*

MIPs are used in various applications<sup>17</sup> such as medical, environmental, analytical, and pharmaceutical fields, etc. The forms of these MIPs are numerous,<sup>13,25</sup> and some examples include beads in packed columns for liquid chromatography (LC), sorbent beds in molecularly imprinted solid phase extraction (MISPE) cartridges<sup>12</sup>, coatings for use in biosensors and solid phase microextraction (SPME) fibers<sup>26</sup>.

An example of an application in the medical and pharmaceutical fields is as a drug delivery system.<sup>27</sup> MIPs can target certain peptides or antibodies in the patient's body. Once the MIP has recognized said target (peptide or antibody) it releases the drug, bonded to the polymer through covalent or noncovalent interactions, into the body. An example of an environmental application utilizing MISPE is extracting food contaminants from "natural or anthropogenic sources."<sup>28</sup> Toxins and other compounds that cause foodborne illness can be extracted using MISPE cartridges imprinted with the toxins. If the toxins are present, even in low amounts, the contaminated foodstuffs can be disposed of properly. By utilizing MIP technology, the selectivity inherent in the technique, allows for it to bypass issues that generally plague conventional methods.<sup>28</sup> Another example of an environmental application utilizing a technique similar to the monolith MIP-SPME fiber is the MIP coating method. Djozan *et al.* fabricated a MIP coating on an aluminum wire<sup>26</sup> and it was used to extract triazines from spiked samples of rice, onion, maize, and bran. They found the polymers were highly selective for triazine compounds where they observed recoveries greater than 85%. They also performed quantitation to determine the concentration of triazine in each sample.

MIPs are also used in the forensics field, which is of great importance in the scheme of this study. Djozan *et al.* have developed a method of MIP-SPME where the polymer fibers are bulk polymers known as monoliths.<sup>29</sup> With this technique, they were able to extract atrazine and other triazines from aqueous solutions similar to those in their MIP-SPME coatings experiment. They also performed experiments with diacetylmorphine and its analogues, as well as with methamphetamine using the monolith method.<sup>30,31</sup> With this method, they found great success in extracting the templated molecules as well as their analogues from solution. None of the other molecules present in the solutions were extracted. These papers were ultimately what influenced the MIP extraction method used in this project.

#### *Advantages and Disadvantages*

There are many advantages to using MIPs versus other extraction techniques. Two major advantages of MIPs are their enhanced selectivity and stability (at high temperatures, different pressures, and various pHs).<sup>25, 32</sup> They are able to extract target analytes from solution with better efficiency due to the imprinting process. The imprinting process forces the polymer active sites to remain in a certain conformation and retain affinity through hydrogen bonding. With this conformation and retained affinity, the analyte of interest is able to be absorbed into the polymer active sites upon sampling.<sup>17</sup> Another advantage is their versatility. Since they can be formulated into a variety of formats, they have a wide appeal for analytical science.<sup>25</sup> Other advantages for MIPs are their “physical robustness, high strength, and resistance to elevated temperatures and pressures.”<sup>32</sup> With most SPME coatings, solvents need to be chosen carefully

so they do not become damaged.<sup>33</sup> Acids, bases, metal ions, and organic solvents do not majorly affect the polymers.

Despite having many advantages, there are some disadvantages associated with MIPs. Template bleeding is a major disadvantage.<sup>28,34</sup> This is caused when not all of the template molecule is removed from the polymer during desorption or cleaning process. Though the polymer proves efficient in selectively extracting the template molecule, some of the template remains, which can hinder interpretation of results. Some remedies have been suggested, the most prevalent being the use of an analogue, usually a deuterated or isotopic form of the molecule, in place of the actual molecule of interest.<sup>28,34</sup> The deuterated or isotopic molecules would be structurally similar and possess the same properties such as retention time to the target analyte. By using a deuterated or isotopic molecule, the results would demonstrate the extraction of the target analyte versus the deuterated or isotopic molecule. The isotopic analogue could be differentiated from the target analyte through the use of mass spectrometry. The only foreseeable obstacle to the use of isotopic analogues would be cost in obtaining the analogue versus the target analyte. Another disadvantage is preparing polymers that do not have a proper template to monomer ratio. If the ratio is too high or too low, the MIP will result in non-specific absorption.<sup>32</sup> This is not beneficial to analysis, since the main purpose of this technique is to selectively extract templated molecules from samples. Some other problems intrinsic to MIPs are reproducibility of certain applications and the availability of template molecules needed for imprinting.<sup>32</sup>

## Solid Phase Microextraction

### *Theory*

The SPME method uses polymer coatings, which have an affinity to any molecule of interest dependent on their desired use. These coatings can be any micrometer thickness, which can directly impact extraction time, and can be composed of various polymer formulations. Table 2 lists the SPME fiber coatings used for analysis. The formulations of the polymer coatings are manufactured depending on their particular application. The general rule, as stated by Pawliszyn, is “similar attracts similar.”<sup>3</sup> This means that the coating should be similar to the analyte(s) of interest, to extract them efficiently from the questioned sample.

Unlike MIPs, which are all imprinted for selectivity, the SPME polymer coatings are not imprinted. The selectivity and sensitivity are not based on how the active sites are formed by the template molecule, but on adsorption rates of the molecules.

Selectivity of the coating is based on the polarity and molecular weight of the molecules extracted.<sup>3</sup> This means the whole process is dependent on the type of compound undergoing analysis. The sensitivity of the method is determined by the thickness of the coating and the distribution constant, so the thicker the coating the more extraction time required. Unlike MIP, the SPME coating is able to extract various types of compounds through adsorption.

SPME coatings can be composed of a variety of polymer formulations dependent on the application. The most common polymer coating used for nonpolar applications is

polydimethylsiloxane (PDMS), and is used in a variety of experiments because of its versatility. PDMS can also be coupled with other polymers to enhance selectivity and sensitivity, like divinylbenzene (DVB).<sup>3</sup> Table 2 in the Introduction lists coatings used in SPME analysis. Each coating has its own advantages and disadvantages.<sup>3,35</sup> Sometimes SPME coatings will be custom made, which is beneficial when manufactured coatings will not extract analytes of interest or not as selectively as desired. MIPs are an example of such designer coatings.<sup>3</sup> The thickness of the coating plays an important role in SPME analysis. It determines the extraction time and the method's sensitivity, which is usually based on the distribution constant for given analytes. For larger constants, a thinner coating is preferred, while the opposite is true for smaller distribution constant values.<sup>3</sup>

To aid in selectivity and sensitivity, a derivatization reagent can be added to the sample. This is favorable when quantitation is desired, though the incorporation of a derivatizing agent can complicate the method.<sup>3</sup> Derivatizing a sample at different stages of extraction has differing results. If it occurs before or during extraction, the selectivity and sensitivity are enhanced for the extraction process and detection. If performed after extraction, only the chromatographic behavior and detection are affected.<sup>3</sup> As with the designer coatings, designer reagents can also be fabricated and implemented into the procedure.<sup>3</sup>

Selecting a mode of extraction is another important aspect of SPME, and is dependent on the state of matter of the sample collected. The three modes of extraction are headspace, immersion,

and membrane protection.<sup>3</sup> Headspace analysis is best for samples that are considered very dirty or will damage the polymer coating. The samples can be in liquid form or solid. Immersion or direct analysis is best for samples that are simple, meaning there are no components present that can damage the SPME coating. The membrane protection method is useful for very complex or dirty samples. The membrane is a barrier for the coating to protect the fiber from damage. The extraction time for a membrane protected fiber is longer, due to molecules needing to diffuse through the membrane before reaching the coating of the fiber. The choice of extraction mode is also dependent on the volatility of the analytes. Low volatility analytes are best extracted using direct or membrane protection extraction. Medium volatility analytes tend to be extracted using direct or headspace extraction. High volatility analytes are best extracted using headspace extraction.<sup>3</sup> Total extraction time must be optimized based on other factors, which are mentioned below.

Agitation of the sample aids in overall extraction, though it is not necessary. The agitation method keeps the sample mixed if in liquid form, and also aids in allowing the analytes to make contact with the surface of the polymer through either headspace or direct analysis.<sup>3</sup> There are numerous agitation methods, and each has its advantages and disadvantages. Some examples of methods are listed in Table 3.<sup>3</sup>

**Table 3: Agitation Methods**

Method	Advantages	Disadvantages
Static (no agitation)	Simple, good for gas phase	Limited to headspace extraction
Magnetic stirring	Common, good performance	Stir bar is required
Fiber movement	No need for stir bar	Stress on SPME fiber
Sonication	Short extraction time	Heats sample

Magnetic stirring agitation is commonly used by most laboratories because of its availability, and is one of the only methods that can be used with all three extraction modes.<sup>3</sup> The speed of the stirrer affects the equilibration and extraction time. Both are adversely affected by the speed, so the faster the setting the shorter the times.

Sample volume is generally optimized as well. If possible, the distribution constant should be calculated to determine which volume should be used for sampling. In forensics, volumes can be limited, and the analytes are not generally known until after analysis.<sup>36</sup> Optimization is dependent on vial size and the extraction mode, which in turn affect sensitivity, equilibration, and extraction time.<sup>3</sup> Modifying the pH, salt concentration, etc. of the sample can also influence sensitivity, equilibration, and extraction time. The pH of the solution can interact with the pKa of the molecule making it easier to leave the solution or retain concomitants. The salt concentration of the solution can also help the molecule be retained or more easily extracted.

Like the methods described in the Introduction, SPME can be automated or can be injected into a system manually. Manual injection is generally more flexible than automated. The choice of which system to use depends on the equipment and time available.<sup>3</sup> Both methods can be



coupled with a variety of instruments, the most common instrument used for separation is GC, and a common detector is a MS. Desorption time is also dependent on the type of instrument used for analysis. For instance, when SPME injection occurs in a GC there are many factors to optimize desorption conditions. Some of these factors include the inner diameter of the inlet port liner, fiber exposure depth, temperature of the inlet port, and flow rate.<sup>3</sup>

Quantitation is also possible with this method, and must be optimized. To obtain concentrations of analytes, a calibration method must be chosen. Calibration methods used in other extraction techniques may be used, but sometimes special procedures may be required.<sup>3</sup> When a calibration method is chosen and used, the linear dynamic range must be determined. This range determines if the concentrations lie within the defined range or if the sample must be concentrated or diluted.<sup>3</sup> The calibration method used in this project is discussed later in this chapter.

### *Applications*

Similar to MIP, SPME is used in a myriad of applications. Some of the applications are medical, environmental, and forensic. An environmental application using SPME was the analysis of air samples.<sup>37</sup> In this experiment, the group gathered indoor air samples to determine the quantities of volatile organic compounds present. Their method was compared to existing methods in place from the National Institute for Occupational Safety and Health. They found the SPME method performed much better in sensitivity, sampling time, and overall cost-effectiveness.<sup>37</sup> A medical and forensic application of SPME was drug analysis of biological fluids.<sup>38</sup> In this study, the researchers used SPME along with other extraction techniques to demonstrate how the different

techniques have their advantages and disadvantages in extraction of drugs from blood, urine, and saliva. Another forensic application of SPME involved extracting drugs from saliva.<sup>39</sup> In this experiment, they used two different extraction modes, headspace and direct immersion, to extract various drugs from samples. The researchers were able to validate their method and successfully extract the drugs of interest.

#### *Advantages and Disadvantages*

There are various advantages and disadvantages when using SPME. One advantage is SPME's ability to sample in a solution or gas, through headspace analysis, without much sample pretreatment, if the sample is not too complex.<sup>3</sup> A major advantage of headspace analysis is the lack of solvent present on the fiber, which allows the fiber to be used multiple times without degrading. Solvent mediated coating degradation is a disadvantage for some applications that require direct immersion. Since the fiber coating is sensitive to most solvents, great care must be taken in determining what media the sample is comprised of and solvents used.<sup>3</sup> Another advantage is SPME's wide field of use. The wide array of coatings lends to the technique's versatility.<sup>3</sup> These coatings are also a disadvantage. They are only useful in certain experiments, and are not as selective as other similar techniques, such as MIP. This lack of selectivity is a major disadvantage if extraction of a certain target analyte is desired. This is why other desirable methods of analysis are sought after.

### MIP-SPME

MIP-SPME is a technique utilizing the SPME sample extraction method and combining it with the selectivity of a MIP. In this study, the MIP-SPMEs were fabricated as polymer monoliths much like those in the Djozan papers discussed in the MIP section. Other groups have also used polymer monoliths for extracting compounds from solutions, such as Turiel *et al.*, who used monoliths to extract brombuterol with clenbuterol imprinted fibers.<sup>40</sup> Xu *et al.*, used monoliths to selectively extract bisphenol A (BPA), diethylstilbestrol (DES), and hexestrol (HEX) from tap water.<sup>41</sup> Both experiments were successful in their analyses, and concluded that MIP-SPME is a viable technique for a variety of applications.

### Instrumentation

#### *Sample Introduction*

There are two common instruments used in conjunction with SPME and MIP-SPME. One instrument is a gas chromatograph-mass spectrometer (GC-MS) and the other is liquid chromatograph-mass spectrometer (LC-MS). Both instruments have their advantages and disadvantages, but this depends on the experiment performed. In GC-MS applications, samples are extracted using the SPME or MIP-SPME by direct immersion of the fiber into the solution or by headspace extraction. The fiber is then desorbed in the inlet port of the GC. The sample is introduced to high temperatures during sample introduction, which may not be useful for thermally labile compounds.<sup>3</sup> In LC-MS, the fiber is desorbed in a chamber, which is directly connected to the injection loop of the instrument, which introduces the mobile phase into an analytical column, after the fiber's introduction to the sample. In this technique, the sample is

introduced at ambient or slightly higher temperatures, which is useful for molecules that may degrade upon contact with high temperatures.<sup>3</sup>

### *Gas Chromatography-Mass Spectrometry*

The method used in this study for analysis was GC-MS, which was used to determine the presence of the selected analytes. The instrument's inlet port desorbed the MIP-SPME fiber allowing the introduction of the analytes into the GC column. A key element used as an identifier of a molecule of interest is its retention time. The retention times for different molecules are generally different from each other. Though this is not true for enantiomers, which will have the same retention time. The mass spectrometer was used to identify the ions of interest for each molecule. The ions are separated based on mass to charge ( $m/z$ ) ratio, and appear as patterns based on how the molecule may or may not fragment.

### *Quantitation*

Quantitation is the method of determining the amount or concentration of an analyte or analytes in a sample. This type of analysis is different from qualitative analysis. Qualitative analysis is the process of identifying the presence of a compound or compounds in an unknown or known solution. Quantitation is useful in the forensic field, especially in drug toxicology cases where the concentration is necessary to determine the amount of substance present in an individual.

There are various methods of quantitation available to determine the quantity of an analyte present in a sample. The method of quantitation used in this project was external calibration.

The external calibration method is performed by preparing standard solutions of the target analyte at differing concentrations. The results from the analysis of each standard, for example their respective relative abundances, are plotted. The resulting scatter plot has a trendline added to determine the linearity of the data points measured by the coefficient of determination ( $R^2$ ). A value close to 1 demonstrates linearity of the data points to the projected trendline denoting overall variance, or spread, of measured values. From this regression analysis, the concentration can be calculated by using the analyte response obtained from analysis of an unknown sample. The most important guideline to follow is that the sample concentrations do not exceed the linear dynamic range of the method. If the concentration of the analyte is too high or low in the solution, the range needs to be expanded. This can be accomplished through dilutions or increasing the concentration.

The research that will be discussed in subsequent chapters focuses on developing and evaluating the specificity of MIPs for use in forensic applications. MIPs, in this study, use noncovalent binding (i.e. hydrogen bonds) as the process for templating the molecules of interest and extracting target molecules from solution. Quantitation of caffeine will be performed on real world samples of teas and coffee using external calibration curves at various concentrations ranging from 10 to 200 ng/ $\mu$ L in order to determine if quantitation is possible with these MIPs.

The evaluation of selectivity will be performed qualitatively on a solution containing caffeine and its analogues, as well as, quantitatively on a solution containing four DNT isomers using

calibration curves. Selectivity for the DNT isomers will be evaluated by performing statistical analysis on the concentrations calculated for each DNT isomer after extraction from solution versus non-templated polymers. Through these experiments, the development of a MIP-SPME method can be created in order to analyze small quantities of samples in complex matrices without pretreating or pre-extraction of solutions thus reducing analysis time and sample preparation.

## CHAPTER 3: EXPERIMENTAL

### Polymerization Procedure

#### *Pre-polymer solution*

A pre-polymer solution was prepared to fabricate the polymer monoliths. The pre-polymer solution recipe went through multiple changes until an optimal mixture was obtained, as given in Table 4.

**Table 4: Pre-polymer Solution**

Compound Name	Use	Quantity (mM)
Methacrylic acid (MAA)	Functional monomer	0.54
Ethylene glycol dimethacrylate (EGDMA)	Cross-linker	2.2
2,2'-Azobisisobutyronitrile (AIBN)	Initiator	0.03
Caffeine	Template	0.28
2,4-dinitrotoluene	Template	0.04
2,6-dinitrotoluene- $\alpha,\alpha,\alpha$ -d <sub>3</sub>	Template	0.04
Methylene Chloride	Porogen	---

For the DNT pre-polymer solutions the monomer to template molecule ratio was 14:1, while the final caffeine pre-polymer solution monomer to template ratio was 2:1. The different template concentrations in the pre-polymer solutions differ from the 10:1 ratio suggested in Spivak *et al.*<sup>42</sup> The suggested monomer to template ratio from Spivak *et al.* is based on the theory of MIP where the functional monomer amount is significantly larger than the amount of template molecule. The concentration of caffeine was larger to enhance the response level during analysis. The pre-polymer solution quantities were based on the Djozan *et al.* paper mentioned in Chapter 2.<sup>29</sup>

### *Polymerization*

The pre-polymer solutions were prepared from the compounds listed in Table 4. The template molecule stock solutions were prepared in methylene chloride, and then added to the other components. A non-templated pre-polymer solution was prepared using methylene chloride to evaluate the differences in extraction between a templated polymer and a non-templated polymer. The pre-polymer solution was mixed until homogeneous, and 100  $\mu$ L was transferred into a 2 mL glass vial containing multiple hand-pulled capillary tubes. These capillary tubes were pulled from the flame-heated mid-section of a 9" glass Pasteur pipette. After cooling to room temperature, the pulled section was broken into pieces roughly 2 cm in length. The inner diameters of the new capillary tubes were approximately 0.30 mm. These tubes were used as molds for the polymer monoliths.

The vials containing the pre-polymer solution and capillaries were capped and placed in a Rayonet photoreactor (Southern New England Ultraviolet Co., Branford, CT) equipped with 350 nm lamps for 60 minutes. The vials were then transferred to an oven (set to 60°C) overnight, for roughly 16-18 hours, to allow the polymer monoliths to cure. The proposed mechanism for formation of the polymer complex was shown previously in Chapter 2. After the polymer monoliths were cured, they were removed from their molds.

Removal of the template molecules from the active sites formed during the polymerization process was the next step. Various methods of removing template molecule from polymer fibers



were attempted, but the processes were time consuming and they posed the potential for polymer degradation or loss. One method consisted of multiple short-time (0.5-1 min) desorptions of the polymer fiber in the inlet port of the GC. Another method used a Carbolite (Carbolite Gero, United Kingdom) furnace to heat nitrogen gas, which was subsequently passed over the fiber. The polymer fiber was inserted into the gas flow for extended periods of time (10-60 mins) at temperatures in the range of 200-380°C). An additional hot solvent vapor extraction method similar to Soxhlet extraction was attempted wherein the MIP monolith was adhered to a SPME needle while undergoing extraction with heated solvent vapor under reflux. The heated solvent vapor caused the epoxy to lose the adhesion of the MIP to the SPME needle. These template molecule removal methods were replaced with Soxhlet extraction because it reduces the potential of polymer degradation due to continued introduction to high temperatures during analysis of samples.

#### *GC Inlet Desorption*

A non-templated polymer fiber was desorbed at 300°C first to determine how long it would require to remove unreacted monomer, cross linker, and oligomeric species prior to sampling. After forty desorptions, the non-templated polymer fiber was finally clean as demonstrated in Figures 6 and 7. This method was not optimal because of the number of desorptions and the amount of time it took for the fiber to become clean. Based on these disadvantages, GC inlet desorption was not attempted for a templated polymer.

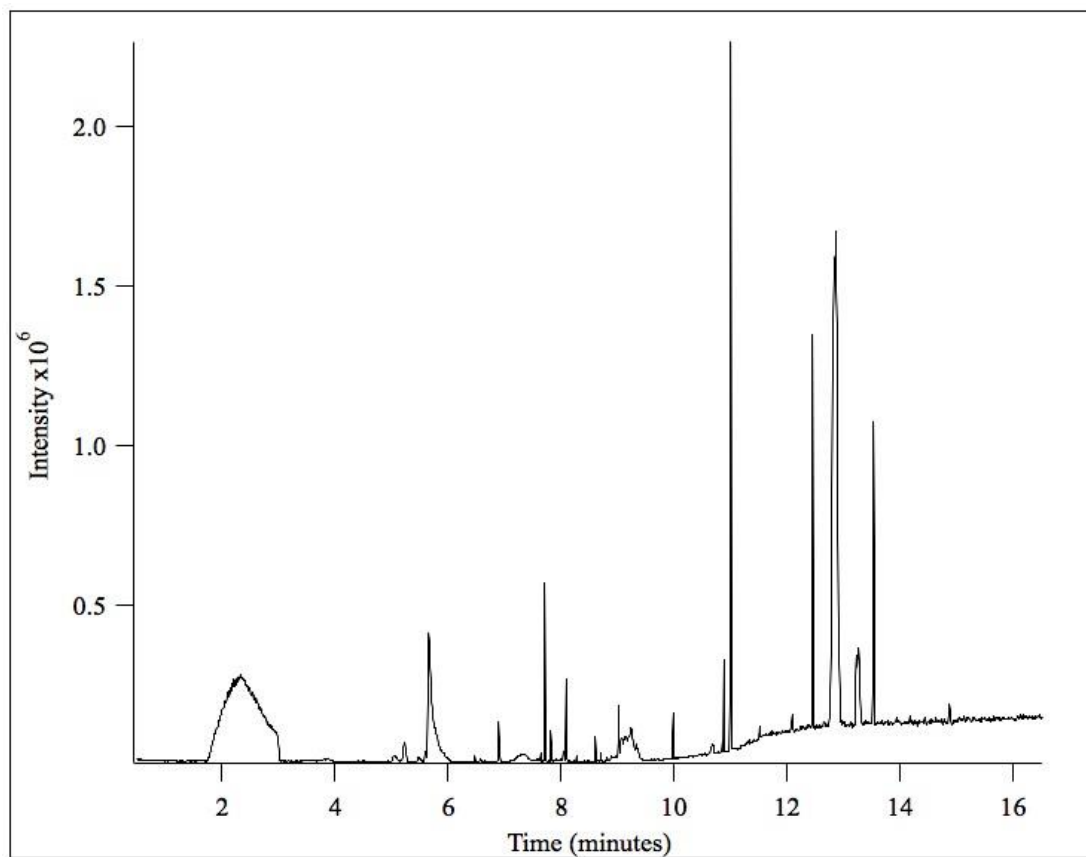


Figure 6: *Non-templated fiber first desorption for 30 secs at 200°C*

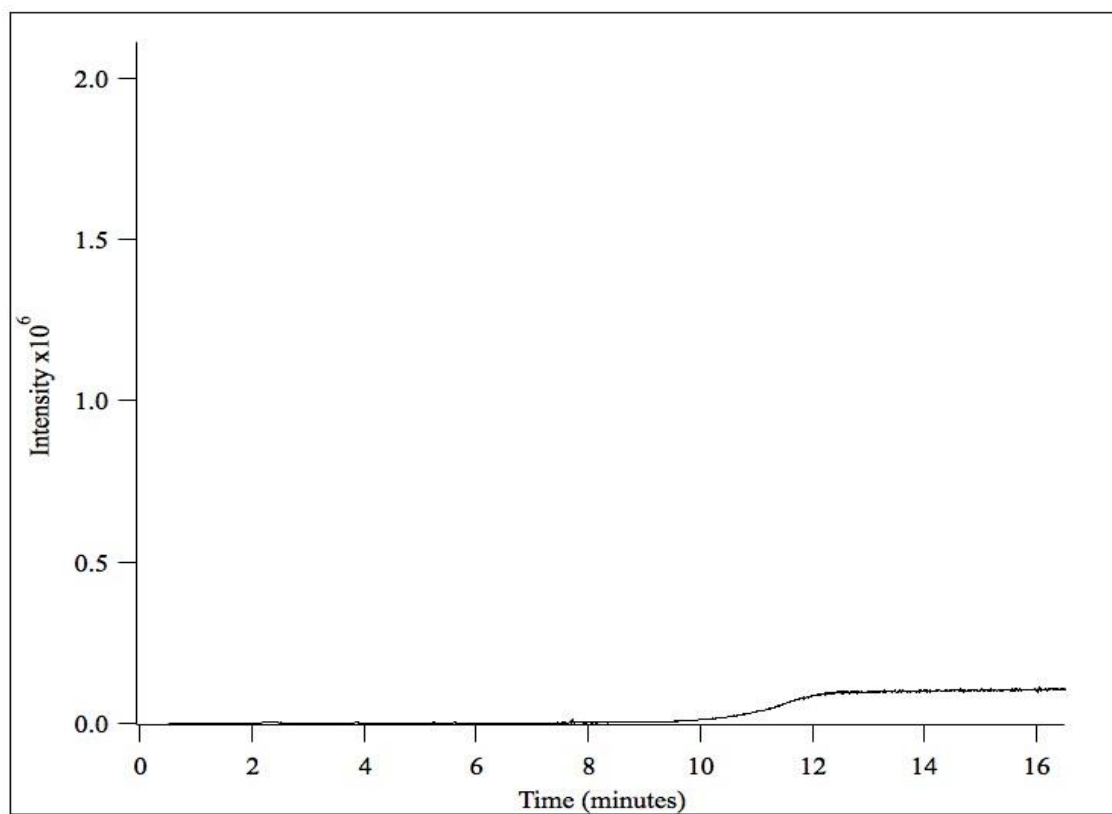


Figure 7: *Non-template fiber 40<sup>th</sup> desorption for 30 secs at 200°C*

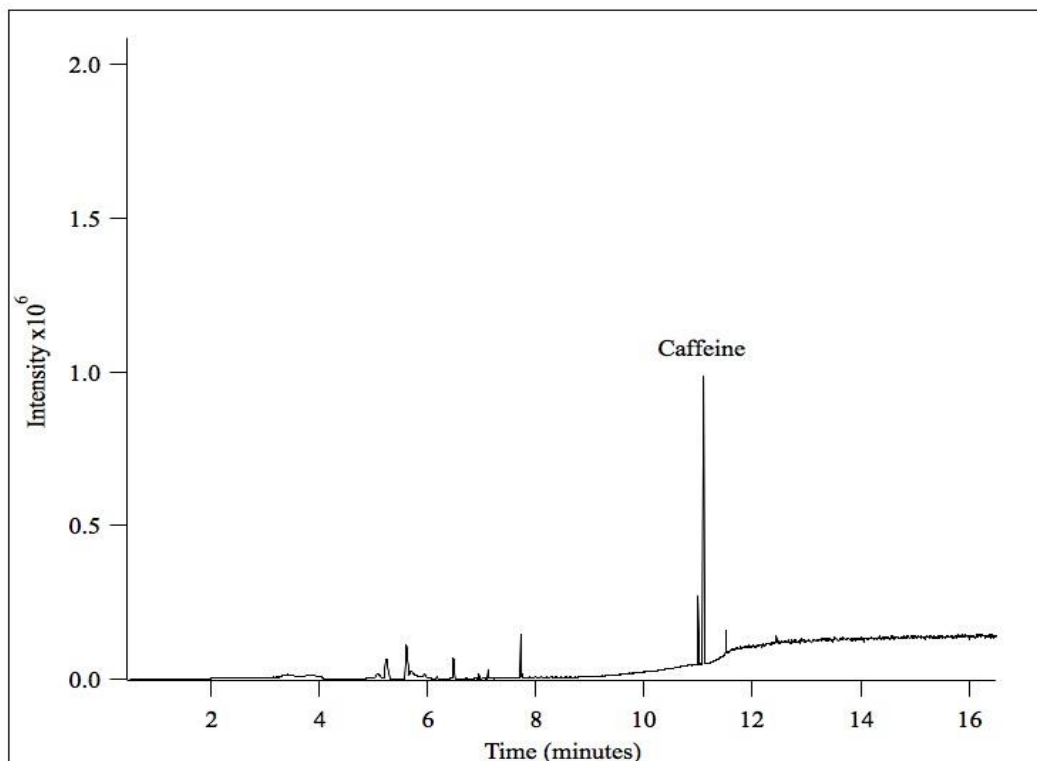


Figure 8: *Caffeine from templated fiber desorption for 10 mins at 300°C*

#### *Furnace Extraction*

A caffeine templated fiber was desorbed in the Carbolite furnace for 10 minutes at 300°C, and the chromatogram resulting from desorption is shown in Figure 8. From the chromatogram, the quantity of caffeine is observed to be significantly lower than what was seen prior to any extraction, see Figure 10. The fiber was desorbed an additional five times, each for periods of 20, 30, 40, 50 and 60 minutes at 300°C, giving a total desorption time of 210 minutes. After 60 minutes of desorption, the caffeine quantity was observed to have decreased, which is demonstrated in Figure 9. Though the Soxhlet extraction and Carbolite furnace desorptions resulted in approximately the same amount of residual template, the Soxhlet extraction proved the optimal method due to efficiencies of bulk fiber processing. With the Soxhlet extraction

method, multiple polymer fibers could be extracted at the same time versus the Carbolite furnace method where only one fiber could be desorbed at a time. In addition, fibers extracted by the Carbolite furnace method had to be attached to a SPME needle prior to extraction.

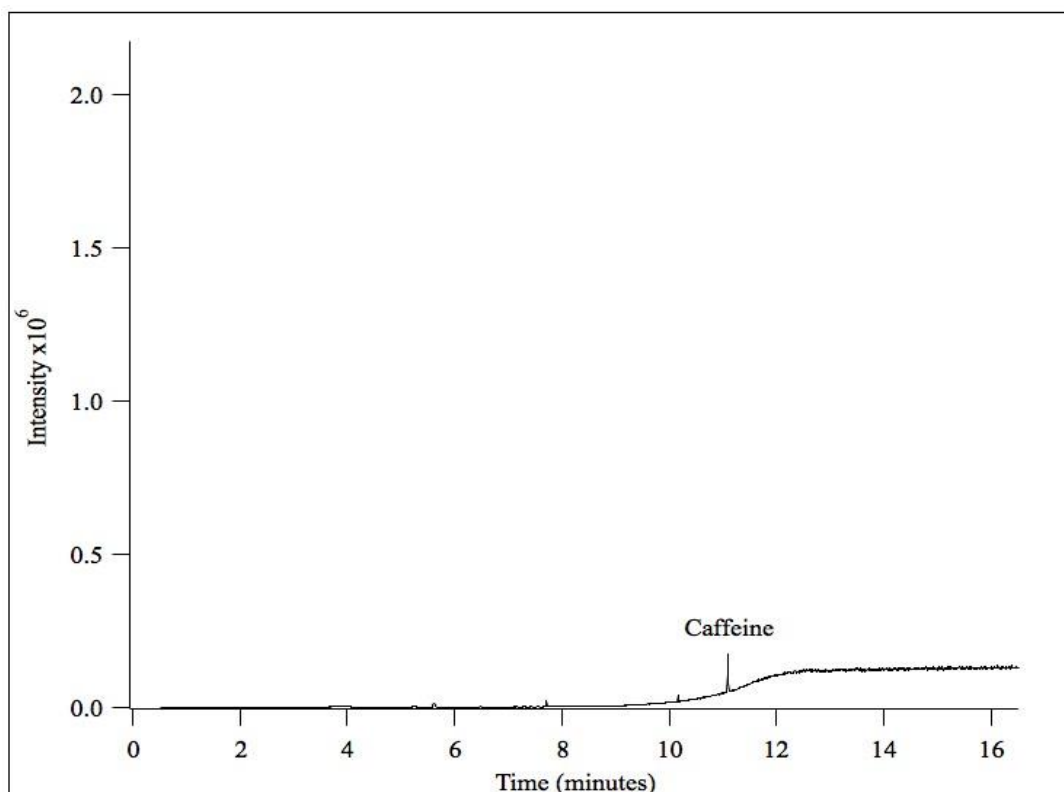


Figure 9: *Caffeine templated fiber desorption for 60 mins at 300°C*

#### *Soxhlet Extraction*

Soxhlet extraction was determined to be the best method of template extraction. This method of extraction is performed using hot solvent vapor which condenses and drips onto the polymer monoliths. The condensed solvent washes or rinses the template molecule from the active sites of the polymer through solubility of the template molecule. After the removal of the polymer monoliths from their capillary molds, they were placed in a Soxhlet extractor for 12 hours

continuous extraction. Extraction was performed to remove the template molecules from the active sites, which were formed during the polymerization process. Acetonitrile was the first solvent used for extraction of the caffeine template molecule from the caffeine templated polymers. The other solvent used was methanol. The solvents were tested to determine their ability to extract the template molecule from the polymer, at different washing time intervals. The DNT templated polymers underwent Soxhlet extraction with acetone first, then with methanol. The selection of these solvents was to determine their ability to extract the DNT molecules from the template polymers, much like the caffeine templated polymers. These solvents were selected based on the template molecules' solubilities.<sup>43</sup> After the tests were run, acetonitrile was the extraction solvent used for subsequent caffeine templated fiber extractions, while methanol was the extraction solvent for the dinitrotoluene polymer fibers. This process was conducted to ensure the templated and non-templated polymers were treated the same before testing their extraction properties.

0 hr Soxhlet Total Ion Chromatogram

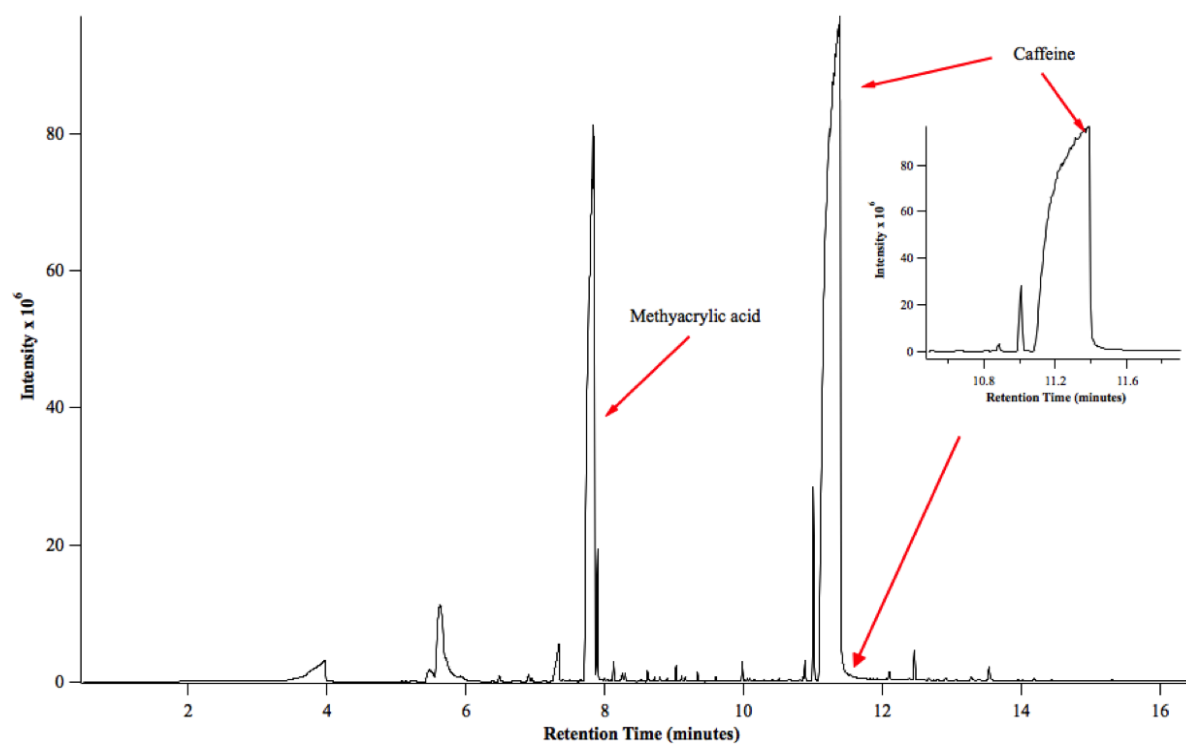


Figure 10: *Templated polymer before extraction*

### 12 hr Soxhlet Total Ion Chromatogram

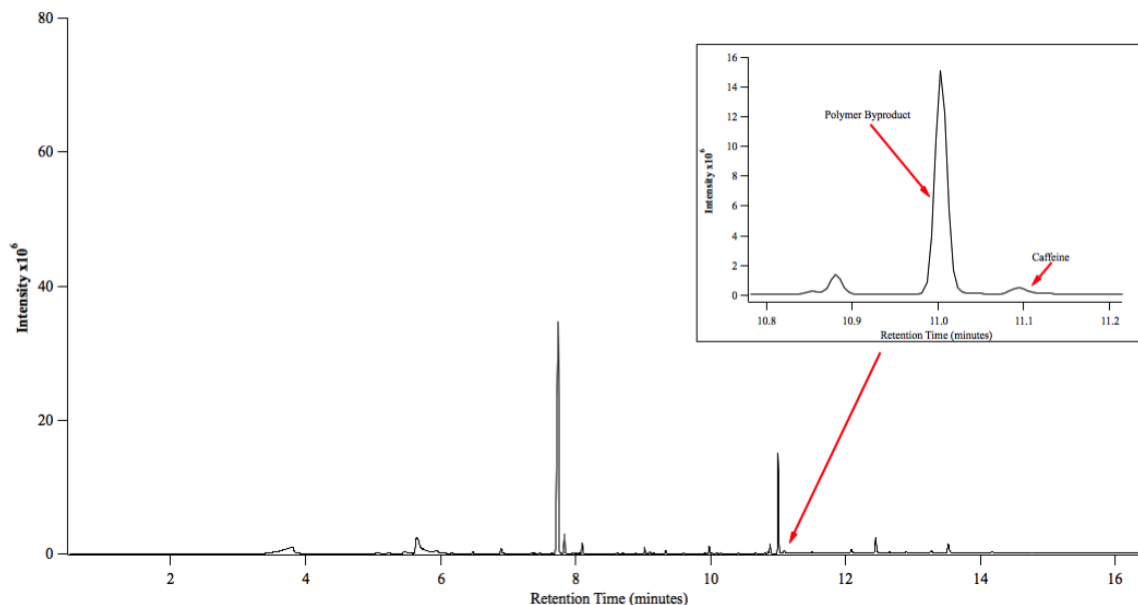


Figure 11: *Templated polymer after Soxhlet extraction*

Comparing Figures 10 and 11 demonstrates that, before Soxhlet extraction quantity of caffeine templated recovered on desorption was large, whereas after 12 hours of extraction, the quantity of recovered caffeine decreased significantly due to more of the template molecule being removed due to the solvent washes. The peak at 11 minutes seen in Figures 10 and 11, as well as in later figures, was present before any desorption took place. It seemed to decrease in response more in the methods that used heat and gas, versus the Soxhlet method. The identity of this peak could not be easily established using the instrument software, but it is likely degradation from the polymer. As it does not interfere with the determination of the target analyte it was ignored.

Following Soxhlet extraction, the monoliths were cut to size (approximately 6 mm) and attached to a SPME needle using JB Weld epoxy, at room temperature, as shown in Figure 12.



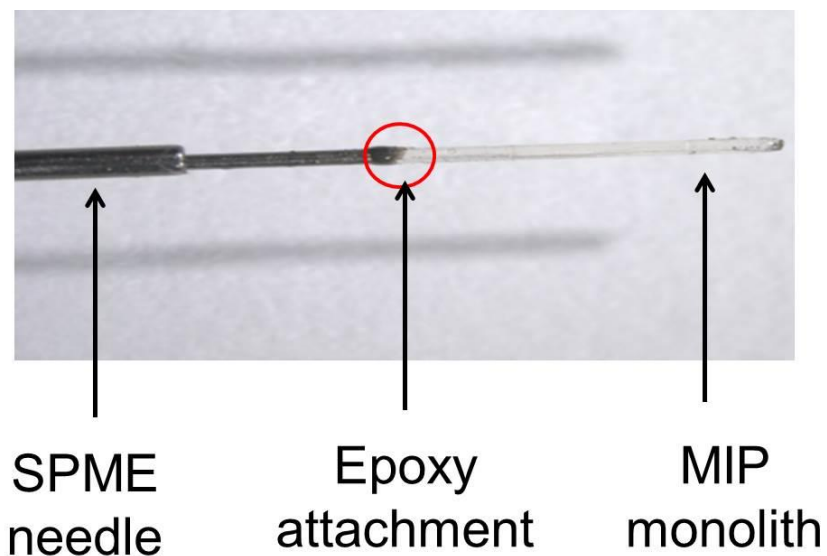


Figure 12: *MIP monolith attachment to SPME needle*

The MIP-SPME was placed in the assembly shown in Figure 13, and the monolith fiber was desorbed in the GC inlet port set to 220°C for 1 minute multiple times in order to achieve a baseline level of residual template present on the polymer. Establishing a baseline residual template level was important for quantitation and selectivity studies.

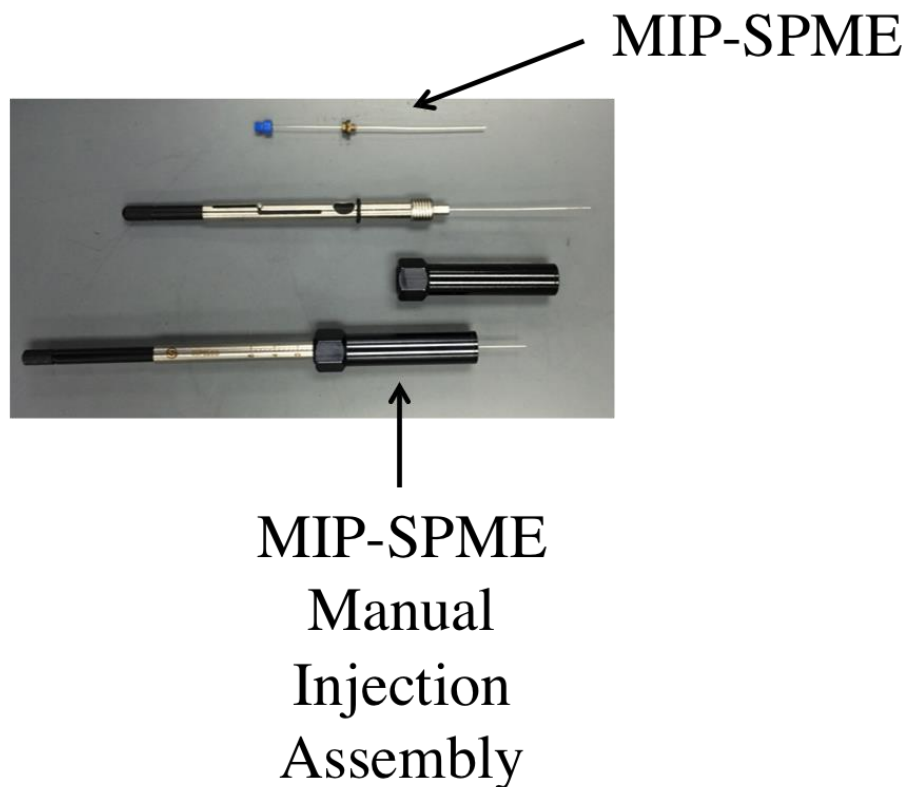


Figure 13: *MIP-SPME manual injection assembly*

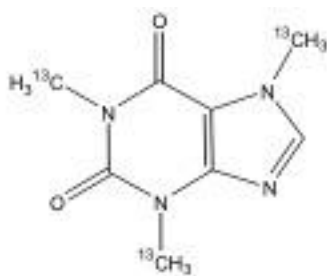
#### Sample Solution Preparation and Procedure

The caffeine solutions were prepared based on previous literature reports where three analytes of interest were dissolved in a single solution.<sup>44</sup> Theophylline and theobromine were less soluble than caffeine in water, methanol, and methylene chloride, so a 1:1 mixture of water and methanol was used to dissolve all three analytes. Stock solutions for each analyte were prepared and dilutions were performed to achieve a solution containing each analyte at a concentration of 100 ng/ $\mu$ L. Stock solutions of teas and coffee were prepared based on manufacturer directions.<sup>45,46,47</sup>

**Table 5: Sample Solutions**

<b>Solution</b>	<b>Analytes (ng/<math>\mu</math>L)</b>	<b>Solution Composition</b>
Caffeine	Caffeine (100)	1:1, Epure water and Methanol
Caffeine- $^{13}\text{C}_3$	Caffeine- $^{13}\text{C}_3$ (100)	1:1, Epure water and Methanol
Caffeine Analogues Mixture	Caffeine (100), Theobromine (100), and Theophylline (100)	1:1, Epure water and Methanol
Chamomile Tea (Badia <sup>®</sup> , Doral, FL)	None	1:1, Tap water and Methanol
Earl Grey White Tea (Teavana <sup>®</sup> , Atlanta, GA)	Caffeine (unknown)	1:1, Tap water and Methanol
Earl Grey Crème-Black Tea (Teavana <sup>®</sup> , Atlanta, GA)	Caffeine (unknown)	1:1, Tap water and Methanol
Instant Coffee Packet (Folgers <sup>®</sup> , The J.M. Smucker, Co., Orville, OH)	Caffeine (unknown)	1:1, Tap water and Methanol
2,4- dinitrotoluene	2,4- dinitrotoluene (100)	Methanol
Dinitrotoluene (DNT) Isomers Mixture	2,4-DNT (100), 2,6-DNT (100), 3,4-DNT (100), and 2,3-DNT (100)	Methanol
2,6- dinitrotoluene	2,6-DNT (100)	Methanol

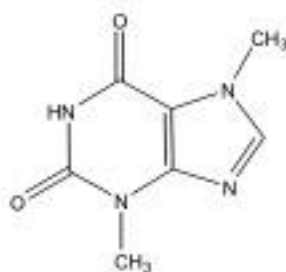
All solutions were prepared in 1 mL volumes. The majority of samples were prepared at 100 ng/ $\mu$ L for the sampling procedure, the exceptions were the tea and coffee samples. The tea and coffee samples were tested for quantitative determination, and their preparation is mentioned in the next section. A caffeine- $^{13}\text{C}_3$  standard solution, purchased from Cerilliant (Round Rock, Texas), was used to verify that the MIP-SPME monoliths ensured caffeine observed in the analysis had been extracted from solution and was not residual template. The only differences between the two caffeines are their molecular weights. The caffeine- $^{13}\text{C}_3$  weighs three more mass units than unlabeled caffeine, shown in Figure 14. This structural similarity allows the caffeine- $^{13}\text{C}_3$  to bind to the active sites in the same way unlabeled caffeine would bind.



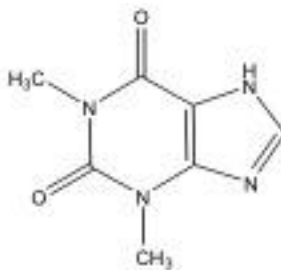
Caffeine-<sup>13</sup>C<sub>3</sub>

Figure 14: *Isotopic caffeine molecular structure*

The molecular structures for theobromine and theophylline are shown in Figure 15. Though the structures are similar to caffeine, theobromine and theophylline are each missing one methyl group, which could affect the binding strengths of the molecules to the polymer active sites.



Theobromine



Theophylline

Figure 15: *Molecular structures of caffeine analogues theobromine and theophylline*

The DNT isomers differed based on the locations of the substituent groups, shown in Figure 16.

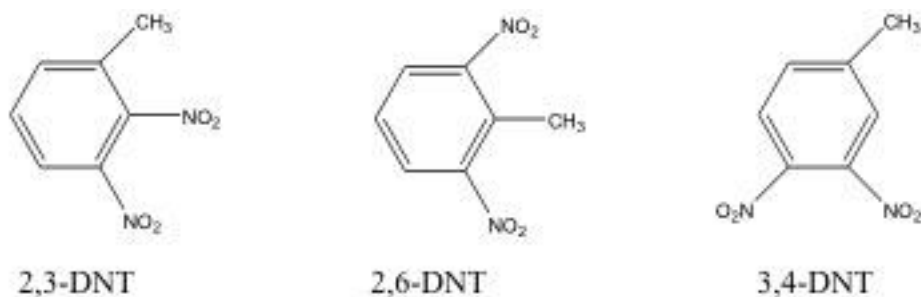


Figure 16: *Molecular structures of DNT isomers*

All solutions were sampled utilizing the same procedure. Half of the polymer monolith was immersed into the 1 mL solution with a micro stir bar to agitate the solution. The total sampling time was 20 minutes. After sampling, the fiber was placed in an oven at 60°C for 25 minutes to dry any excess moisture on the fiber. The fiber was then removed from the oven and allowed to cool to room temperature for easier handling. The fiber was then desorbed for 1 minute in the inlet port (220°C).

Blanks, or solutions absent of any analytes, were analyzed between the analysis of samples of caffeine or DNT. The blank samples were analyzed to establish a baseline level of residual template that continued to desorb from the MIP-SPME. Before sampling in a blank solution of methanol or methanol/Epure water, the fiber was desorbed for another minute to reestablish a baseline for the next sampling. Figure 17 demonstrates the process of MIP-SPME preparation and use from start to finish.

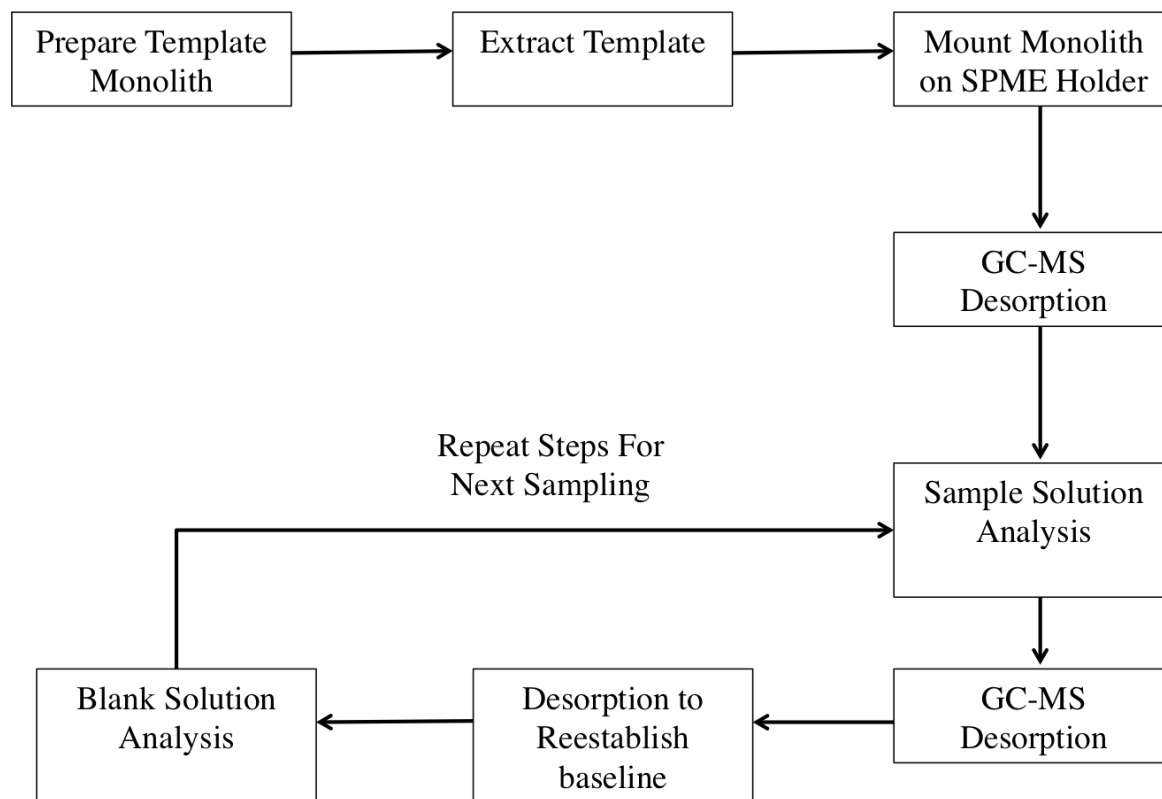


Figure 17: *MIP-SPME preparation and use cycle*

The analyses were performed on a Thermo Finnigan Trace Gas Chromatograph (GC)-Polaris Q Mass Spectrometer (MS). The inlet port was set to 220°C, with an initial oven temperature of 40°C which was held for 1 min. The oven temperature was then ramped 20°C per minute to a final temperature of 250°C and held for 5 minutes. The total run time was 16.5 minutes. The carrier gas, which was helium, was set to a constant flow rate of 1 ml/min. The ionization source used was electron ionization (EI), and the MS was set to a scan range of 50 to 225  $m/z$ . The ion source temperature was at 200°C and the MS transfer line was at 280°C. The ion source also had an electron voltage of -70eV, which was emitted by the filaments to aid in fragmentation of ions.

### Calibration and Quantitation

The quantities of caffeine present in the teas and coffee were determined by an external calibration method. A MIP-SPME fiber was used to sample five caffeine calibration standards. The calibration standards were prepared at 10, 50, 100, 150, and 200 ng/uL concentrations, using the caffeine stock solution discussed in the previous section. The peak area responses or integrated areas under the curve were plotted to form external calibration curves of the total ion chromatogram (TIC) and extracted ion chromatogram (EIC) of 193-195 *m/z* for each calibration standard. The subtracted peak areas for each range were plotted, where the subtracted peak areas were calculated by subtracting the previous blank desorption peak area response from the desorption after sample analysis. The previous blank desorption peak area before sample analysis was the baseline of caffeine on the fiber. This was subtracted from the desorption peak area after sample analysis to calculate the response level of caffeine absorbed onto the polymer. This peak area response level was used to calculate the concentration of caffeine in a sample. The external calibration curves plotted using one polymer fiber were not the best method for calculating concentration, so another set of curves were plotted using four MIP-SPME fibers.

The four MIP-SPME fibers were each assigned a caffeine standard solution from concentrations of 10, 50, 100, and 200 ng/ $\mu$ L from the previous calibration experiment. Three samplings were performed with each fiber and its respective caffeine standard solution. External calibration curves were plotted using the same method as the previous calibration curves. The subtracted peak area responses were averaged. Once the values were plotted, a linear regression was

performed to obtain the equation of the line. From the EIC linear regression equation, the concentrations for each tea and coffee sampling were calculated.

### *Teas and Coffee*

The tea and coffee sample solutions were prepared similarly to the caffeine standard solutions using tap water instead of Epure water. Tap water was used to simulate how a real cup of tea or coffee is prepared. Any compounds present in tap water could add to the complexity of the sample. Tea and coffee stock solutions were prepared following the manufacturers recommendations, and these instructions included temperature of water, quantity of product required, and steep time. For each solution, 1 cup or 237 mL of heated tap water was measured in a 500 mL graduated cylinder and transferred to 500 mL glass beakers. The tap water was heated using an Oster brand electric kettle with a digital Fahrenheit temperature read out. The loose leaf teas required the use of a tea ball, which houses the leaves used in preparing the tea, purchased from a local Publix supermarket. Table 6 lists the teas and coffee used in the experiment.



**Table 6: Teas and Coffee Preparation**

Sample	Form	Water Temperature (F)	Steep Time (mins/quantity)
Chamomile Tea	Bagged	212	5 / 1 bag
Earl Grey White Tea	Loose leaf	175	2 / 1.5 teaspoon*
Earl Grey Crème (black) Tea	Loose leaf	195	2 / 1 teaspoon*
Instant Coffee Packet	Powder	195	0 / 1 packet <sup>†</sup>

\*Quantities were approximately 2.4 g.

<sup>†</sup>Quantity is about 3 g.

The solutions were sampled three times each to determine average peak area responses of each sample and to determine reproducibility. The caffeine concentrations were determined by subtracting the previous blank peak area response from the sample desorption, and then using the EIC of 193-195  $m/z$  best-fit equation. The calculated concentrations were then averaged. A blank polymer was sampled in tea to determine if caffeine was extractable with an untemplated fiber.

### Selectivity

Selectivity of templated polymers was determined by sampling mixtures containing similarly structured compounds with polymer monoliths. To differentiate between the compounds, their respective retention times and ions were noted from standards. These values were compared to the data from the polymer desorptions. Table 7 lists the molecular weights, retention times, and ion(s) of interest for each compound.

**Table 7: Significant Values for Each Compound Analyzed**

<b>Compound</b>	<b>Molecular Weight (g/mol)</b>	<b>Retention Time (mins)</b>	<b>Ion (s) of interest (<i>m/z</i>)</b>
Caffeine	194.14	11.09	194
Caffeine- <sup>13</sup> C <sub>3</sub>	197.14	11.09	197
Theobromine	180.16	11.38	180
Theophylline	180.16	11.91	180
2,4-DNT	182.13	9.31	182, 165, 119, 89, and 63
2,6-DNT	182.13	8.84	182, 165, 119, 89, and 63
2,6-DNT- $\alpha,\alpha,\alpha$ -d <sub>3</sub>	185.13	8.84	185 and 168
2,3-DNT	182.13	9.32	181, 135, 165, 89, and 63
3,4-DNT	182.13	9.70	181, 89, and 63

The caffeine templated polymer fibers were sampled in solutions containing caffeine, theophylline, and theobromine. The 2,4-DNT and 2,6-DNT- $\alpha,\alpha,\alpha$ -d<sub>3</sub> templated polymer fibers were sampled in solutions containing 2,4-DNT, 2,3-DNT, 3,4-DNT, and 2,6-DNT. For the two DNT polymers, the selectivity ratios were calculated and compared to blank polymers sampled in the same solution. The caffeine polymers did not require any additional calculations, since the only peak observed in chromatograms was caffeine.

#### Conventional SPME versus MIP-SPME

A MIP-SPME templated with caffeine and a conventional SPME fiber with a carbowax/divinylbenzene (CW/DVB) 65  $\mu$ m coating (Supelco, Sigma-Aldrich, St. Louis, MO) were compared to determine which method was more selective in extraction ability. A CW/DVB 65  $\mu$ m coating was used based on a previous experiment comparing various coatings extracting methylxanthines from human body fluids.<sup>48</sup> Solutions containing theophylline,

theobromine, and caffeine were sampled by each method, and the results for each were compared. The sampling method for the MIP-SPME was used for the SPME after the coating was conditioned for 30 mins.

#### MIP-SPE versus MIP-SPME

A 2,6-DNT- $\alpha,\alpha,\alpha$ -d<sub>3</sub> template and blank pre-polymer solution were prepared using the same process when fabricating the MIP-SPMEs. The bulk polymer was crushed, and a Soxhlet extraction was performed for 12 hours using methanol as the solvent. After Soxhlet extraction, the crushed polymer was sieved to obtain particles 0.1 mm to 0.2 mm in size. 20 mg of crushed polymer was added to an empty 1 mL Alltech (Grace, Columbia, MD) solid phase extraction cartridge. One 2,6-DNT- $\alpha,\alpha,\alpha$ -d<sub>3</sub> template polymer SPE column and one blank polymer SPE column were prepared. The MIP-SPE procedure is demonstrated in Figure 18.

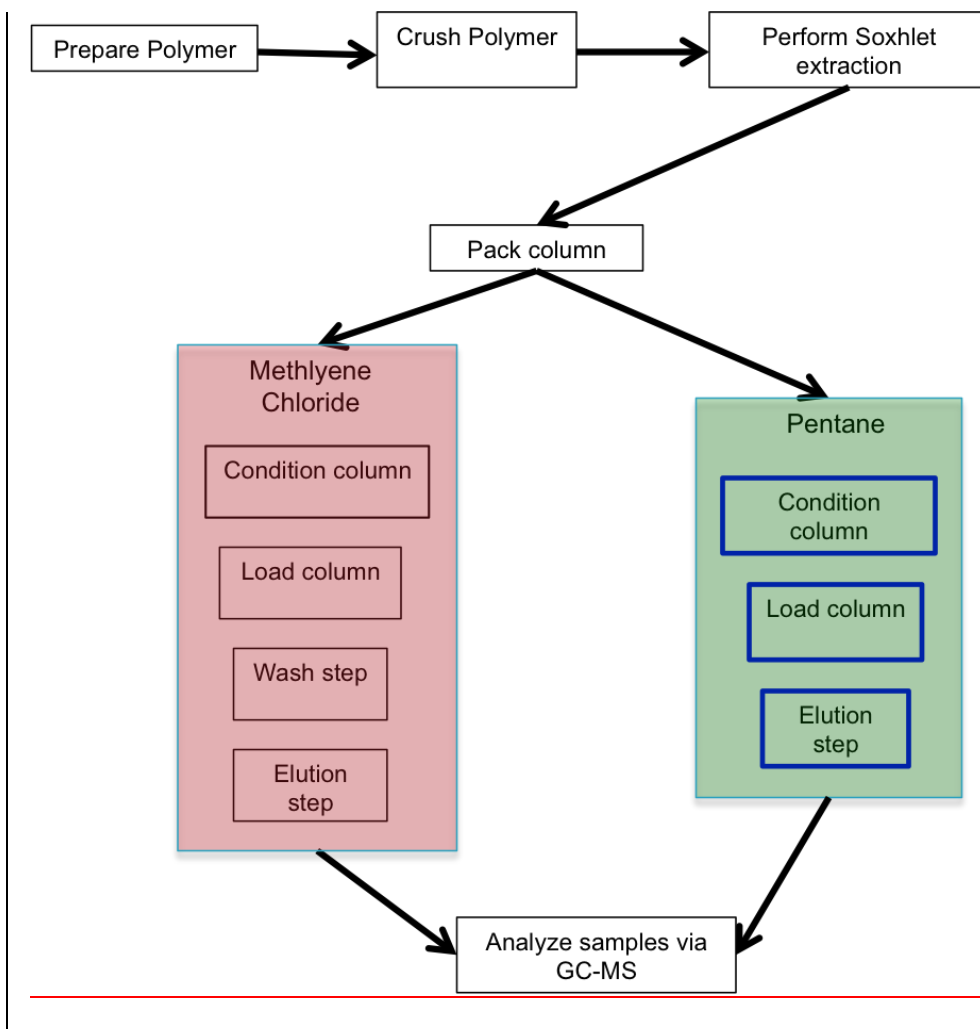


Figure 18: *MIP-SPE procedure*

The columns were conditioned with 1 mL of methylene chloride. Once the methylene chloride was pushed through the column, a methylene chloride solution containing 2,3-DNT, 2,4-DNT, 2,6-DNT, and 3,4-DNT was loaded. To wash or remove any unbound analyte from the sorbent bed of the column, 1 mL of methanol was added. Finally, 1 mL of methylene chloride was added to elute any analyte that was still bound to the column. The methanol wash was evaporated using nitrogen gas and reconstituted using methylene chloride, so it could be analyzed using GC-MS. The final methylene chloride elution was also analyzed.

The extraction process was repeated using pentane as the solvent. 1 mL of the mixed DNT isomers solution was evaporated using nitrogen gas and reconstituted with pentane. The columns were conditioned with 2 mL of pentane, loaded with the 1 mL mixed isomers solution, and then eluted with 1 mL of methanol. The methanol solution was evaporated and reconstituted with pentane. The final elution was analyzed on GC-MS.

## **CHAPTER 4: RESULTS AND DISCUSSION**

### Calibration and Quantitation

In Chapter 3, a calibration curve was created based on the peak area responses from caffeine standards. Linear regression was applied to calculate the caffeine concentrations of samples where the total concentration was unknown. The caffeine standards/solutions used to build the calibration curve were sampled in triplicate using one caffeine templated fiber. Figure 19 is the calibration curve used to calculate the concentrations of a series of tea and coffee samples.

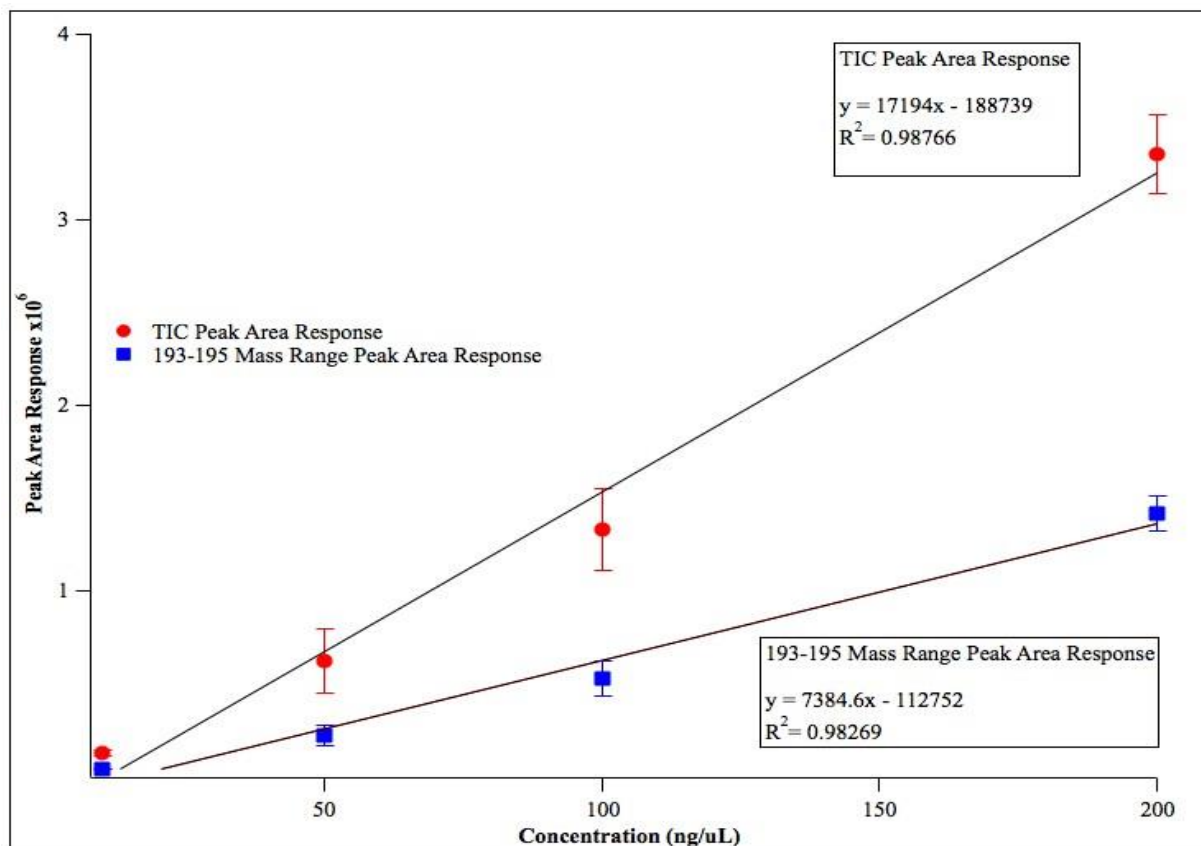


Figure 19: *Caffeine standard calibration curve; solutions prepared at 10, 50, 100, and 200 ng/ $\mu$ L concentrations from a caffeine stock solution (50:50 (v/v) methanol/Epure water)*

Table 8 displays the theoretical and observed concentrations for each sample. The observed concentrations were calculated using the trendline equation from the 193 – 195 mass range peak area response (Figure 19).

**Table 8: Concentrations of Real World Samples**

<b>Sample</b>	<b>Theoretical Concentration (ng/<math>\mu</math>L)</b>	<b>Observed Concentration (ng/<math>\mu</math>L) <sup>±</sup></b>	<b>Averaged Observed Concentration (ng/<math>\mu</math>L)</b>
Chamomile Tea	0	A- 16 <sup>†</sup> B- negligible C- negligible	Negligible
Earl Grey White Tea	140	A- 34 B- 22 C- 23	26
Earl Grey Crème (black) Tea	174	A- 44 B- 15 C- 19	26
Folgers <sup>®</sup> Instant Coffee packet	313	A- 223 B- 431 C- 216	290

<sup>±</sup>Each measurement was repeated three times, denoted by A, B, and C.

\*Concentrations rounded to the nearest whole number.

<sup>†</sup>Residual caffeine present before sampling.

The observed concentrations for each sampling were lower than those of the theoretical values obtained from manufacturers' websites (Appendix A). The only exceptions were the coffee samplings with an observed average concentration close to the theoretical value. The coffee concentration was out of the caffeine calibration range, which could be due to oversaturation of the polymer or detector saturation.

Temperature, steep time, and type of tea can influence overall caffeine extraction into solution.

Using loose leaf tea versus using processed teas (tea bags) is an example of differing types of tea



affecting caffeine concentration.<sup>49</sup> Type of solvent and sample volume can also affect the caffeine concentration during the extraction process. The effect of solvent choice on binding sites will be addressed in the selectivity section below. Other factors to consider are failure of the caffeine molecules to effectively bind to the functional monomer in the active sites or improper templating of target analyte during the polymerization process.

#### Selectivity: Caffeine

Mass spectrometry was used to differentiate between each analyte. The ions of interest for caffeine, theobromine, and theophylline are referenced in Table 7. The mass spectra in Figures 20 through 22 are for each analyte respectively using standard solutions. From these figures the molecular ions were observed to correspond with the base peaks for each analyte.

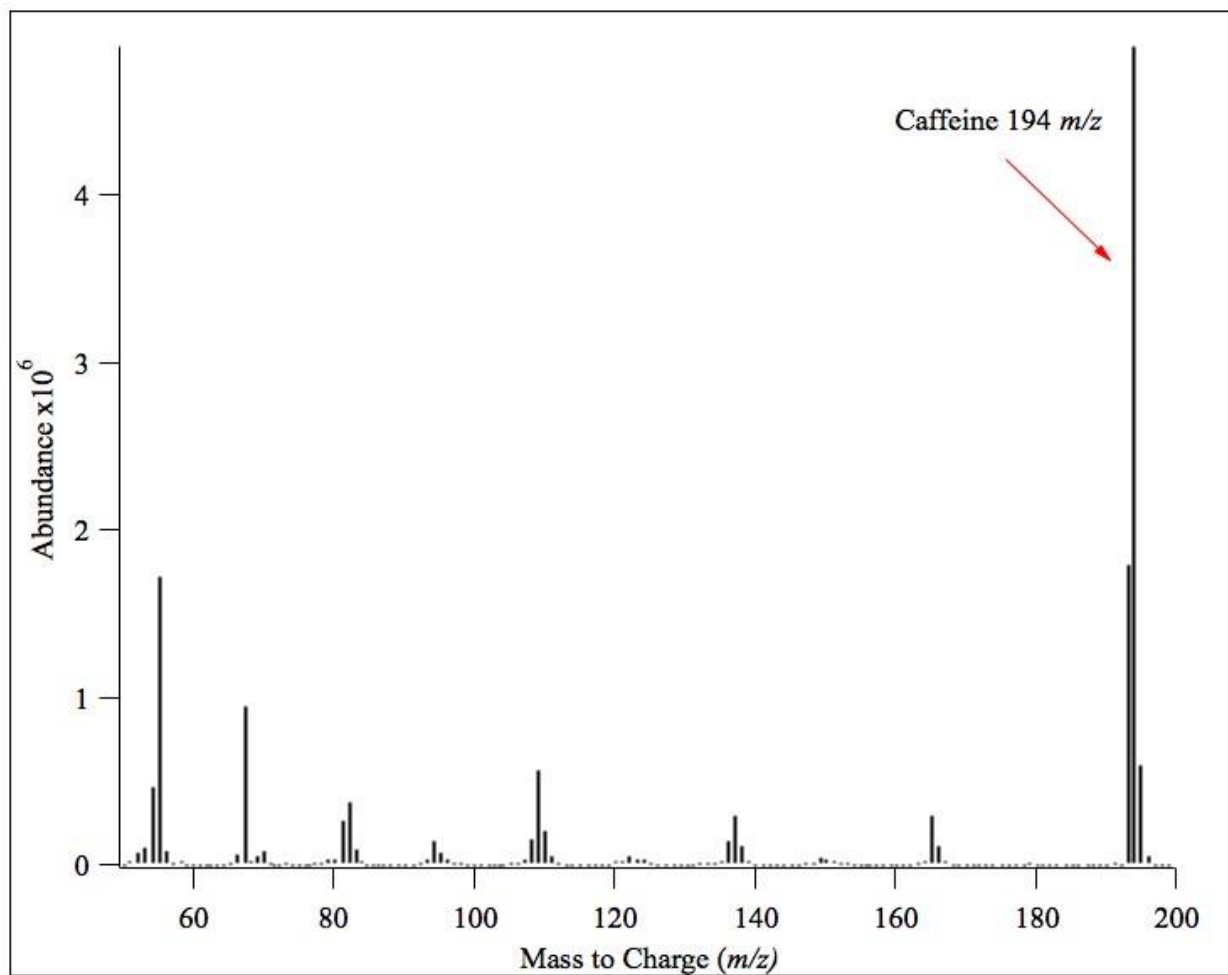


Figure 20: *Caffeine standard mass spectrum with molecular ion and base peak at 194 m/z.*

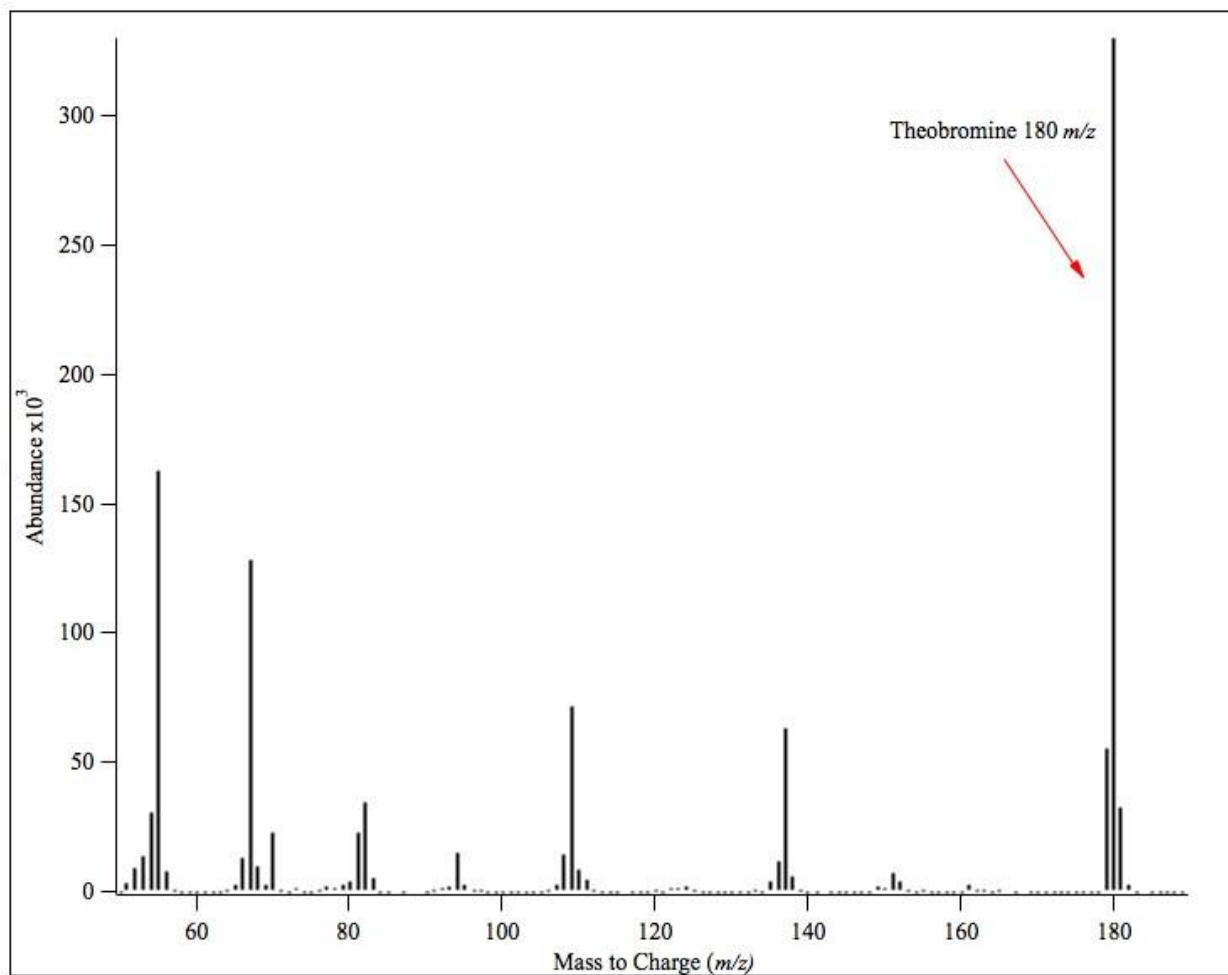


Figure 21: *Theobromine standard mass spectrum with molecular ion and base peak at 180 m/z.*

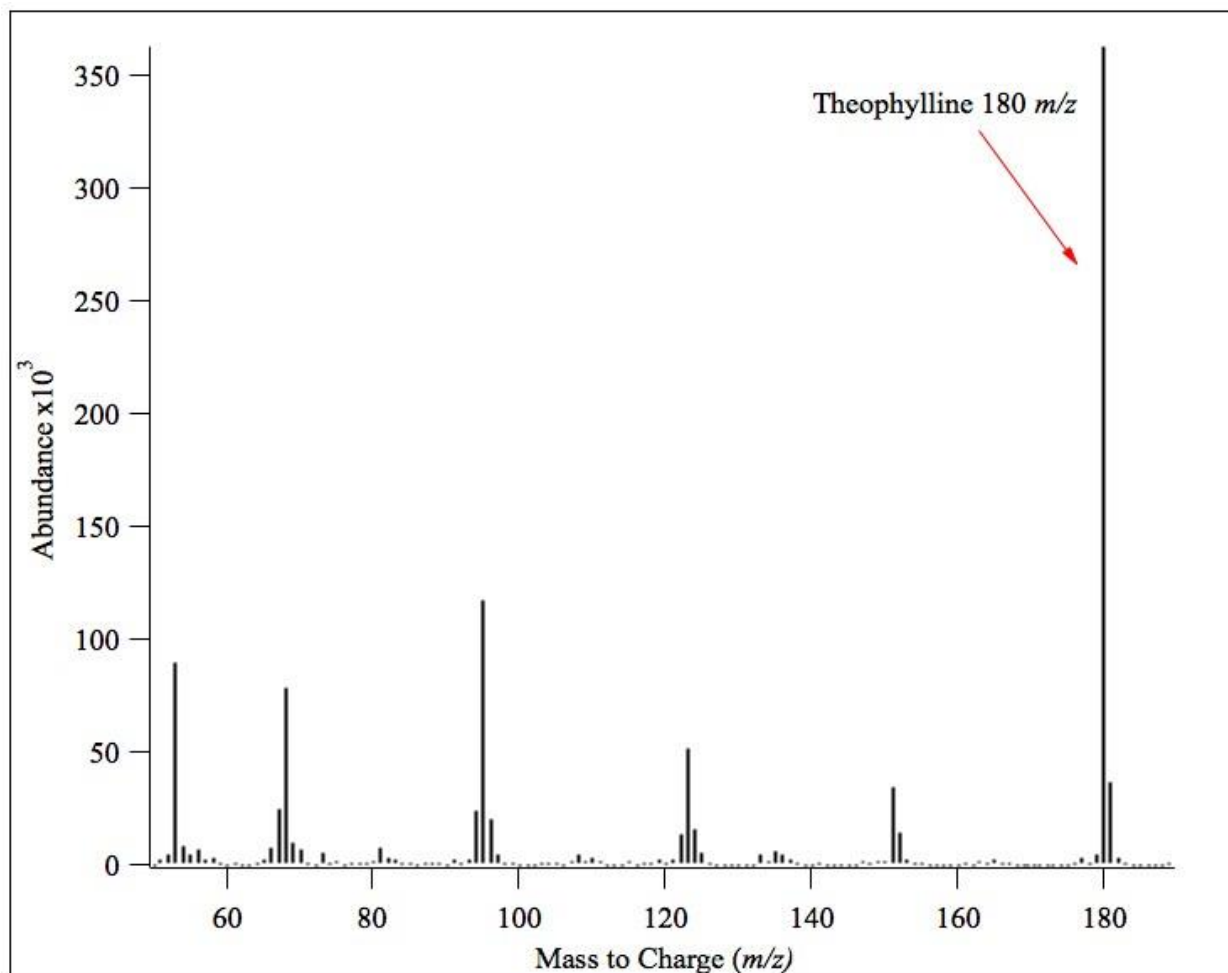


Figure 22: *Theophylline standard mass spectrum with molecular ion and base peak at 180 m/z*

The base peak for caffeine was observed at 194 m/z, while the base peaks for theobromine and theophylline were observed at 180 m/z. When the caffeine template fibers were used to sample solutions containing caffeine, theobromine, and theophylline, only caffeine was visible in the total ion chromatogram (TIC) (Figures 23, 24, and 25).

The caffeine templated fiber was used to sample three solutions of caffeine, theobromine, and theophylline all at 100 ng/ $\mu$ L in the following solvents:

- consisting of purely methanol
- consisting of 50:50 (v/v) methanol to Epure water
- consisting of purely methylene chloride.

These solutions were prepared to determine the extraction efficiency of the polymer.

Theophylline and theobromine were slightly soluble in organic solvents (methylene chloride and methanol) whereas caffeine was soluble in all the solvents used in analysis. In Figure 23, there weren't any peaks that coincided with the retention times of any of the analytes caffeine, theobromine, and theophylline sampled using methanol. Although the chromatogram in Figure 23 shows a peak in the 11 minute area, the retention time does not coincide with caffeine. When examining the mass spectrum of the 11 minute peak, the fragmentation pattern also did not match caffeine.

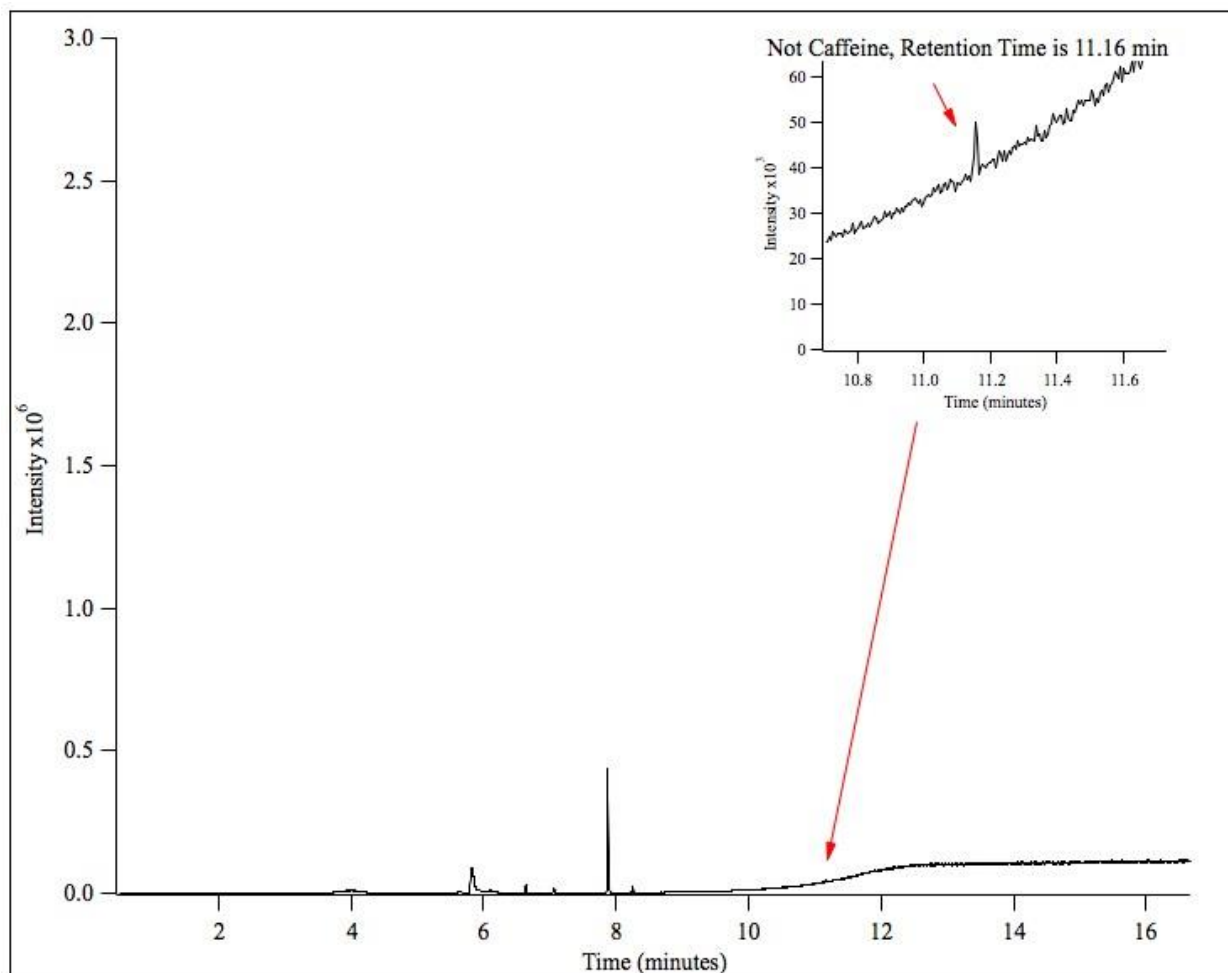


Figure 23: *TIC of caffeine derivatives mix in methanol using caffeine templated fiber*

The 50:50 methanol and water solution resulted in only caffeine being observed on the TIC

shown in Figure 24. The caffeine peak in Figure 24 at 11.09 minutes had an intensity of approximately 13000 counts, weak in comparison to the methacrylic acid peak at 7.72 minutes.

However, the methacrylic acid peak could be large due to degradation of the polymer. Despite the low peak response, the signal to noise ratio for caffeine (11.09 min) was approximately 3:1

satisfying the requirements for qualitative analysis based on limits of detection. Based on these

results, it is likely that caffeine binds more efficiently to the polymer surface as opposed to the other two analytes.

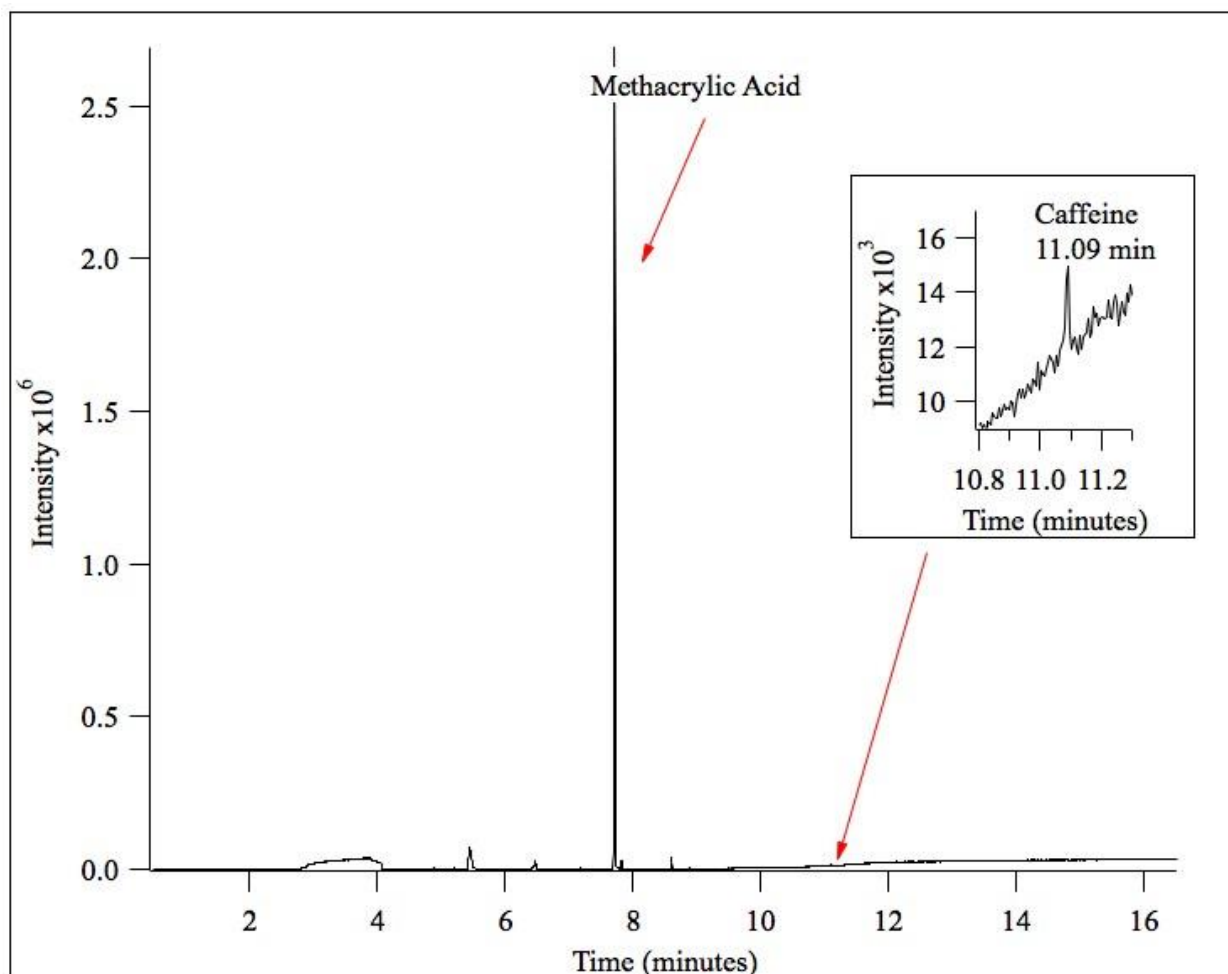


Figure 24: *TIC of caffeine derivatives mix in 50:50 water/methanol solution using caffeine templated fiber*

There are several factors that can affect extraction efficiency. One factor could be the minute structural differences between caffeine (the template), theobromine, and theophylline such as the positioning of the methyl groups on the main xanthine structure. Another factor could be the choice of solvent and its interaction with the polymer and analytes. The solution of 50:50 (v/v)

water to methanol could interfere with hydrogen bonding between analytes and polymer considering that the functional monomer and template analyte bind via hydrogen bonds. The water and methanol solution could bind to the templated polymer instead, preventing the target analytes from populating the active sites. These factors also hold true for the methanol solution motivating consideration of a solvent system that would not interfere with this process. In Figure 25, methylene chloride was used as the solvent where caffeine was observed at 11.09 minutes with other analytes not visible in the chromatogram. The caffeine peak was stronger than was observed in the previous solutions, which may be attributed to methylene chloride being used as the porogen for the polymer to increase extraction efficiency discussed in Chapter 2.

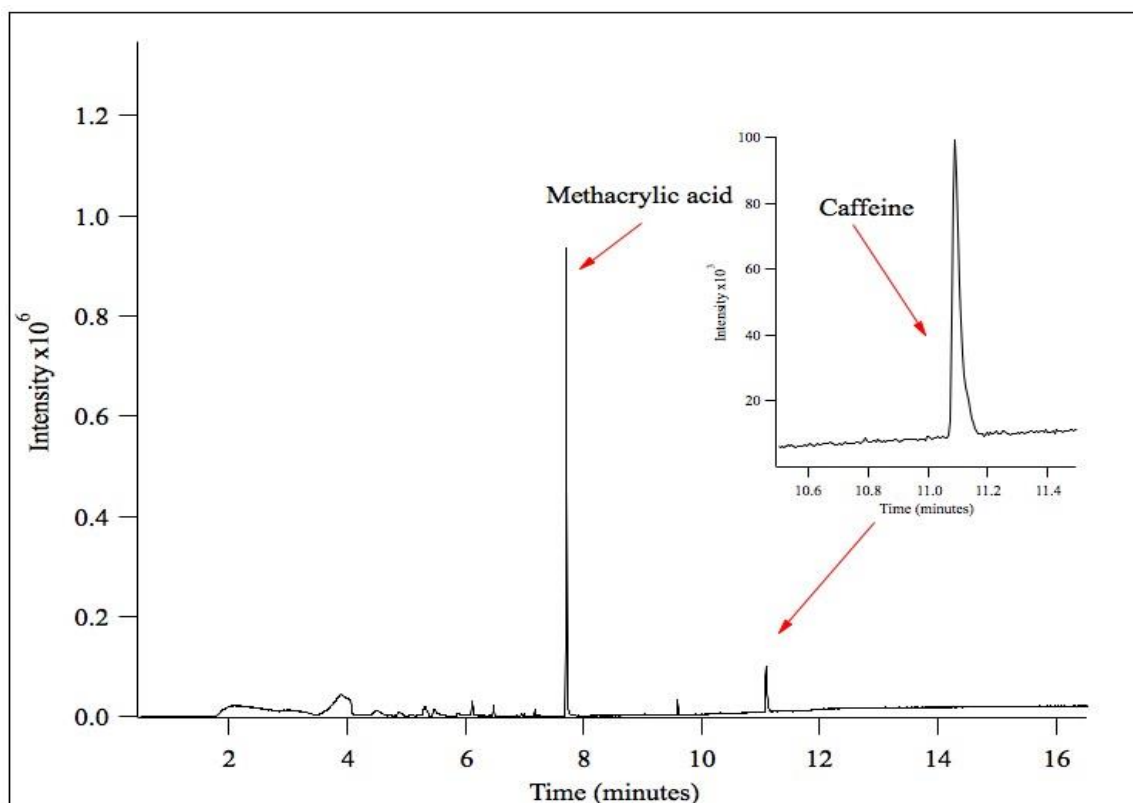


Figure 25: *TIC of caffeine derivatives mix in methylene chloride using caffeine templated fiber*



A comparison between the response of a blank polymer and templated polymer was carried out in order to investigate selectivity of both polymers in the derivatives mix. The preliminary blank polymer sampling, Figure 26, did not demonstrate extraction of any of the analytes. The peaks in the chromatogram before the expected 11 minute range were from possible polymer thermal breakdown products. Small peaks in the 11 minute range were observed, but they were not at the expected retention times and did not meet signal to noise requirements. The analysis was performed using a 30 second inlet desorption after sampling a caffeine solution.

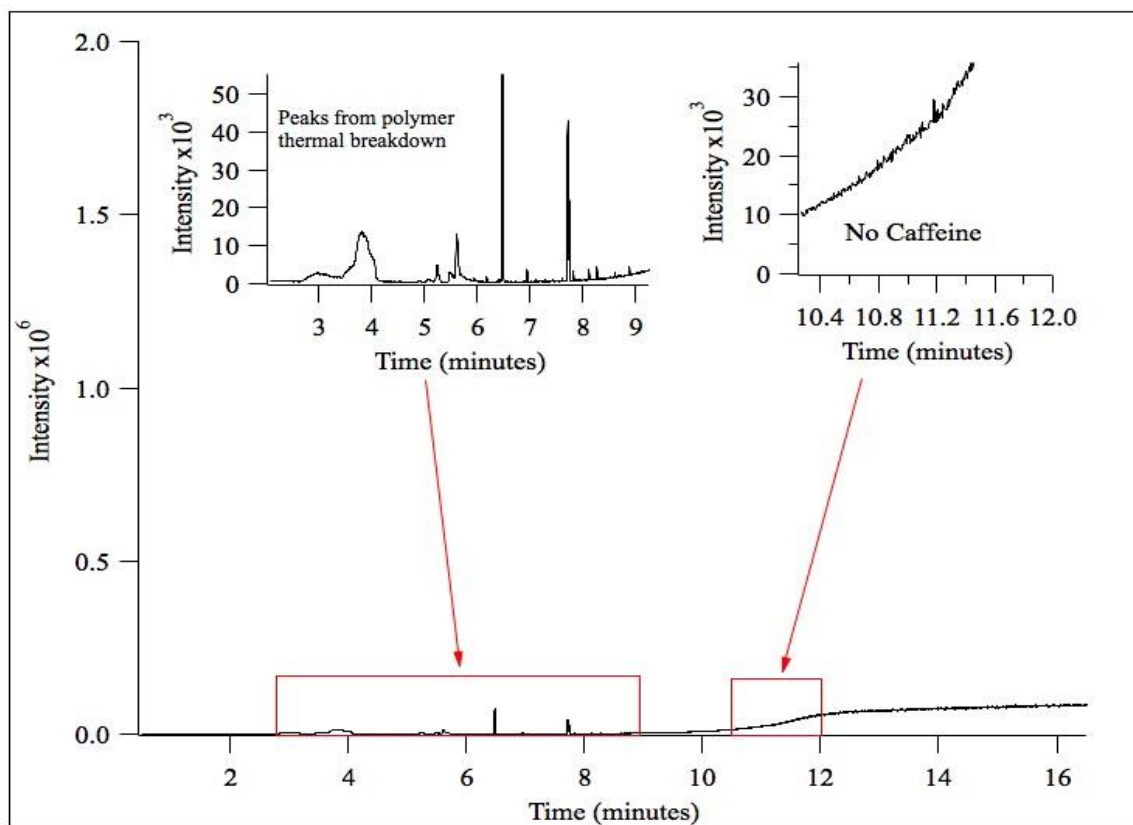


Figure 26: Preliminary blank polymer sampling in caffeine derivatives mix; peaks from degradation of polymer

Another blank polymer was tested, using the one minute inlet desorption, to determine if the polymer would extract any analytes from the Earl Grey Crème Tea solution. The chromatogram shown in Figure 27 exhibited a characteristic peak attributed to caffeine extracted by the blank polymer. The peak intensity of caffeine extracted by the blank polymer was similar to that of the templated polymer in Figure 28. This is contrary to the results shown in Djozan where the blank polymer did not have a similar response of target analyte extracted as compared to the templated polymer.<sup>29</sup>

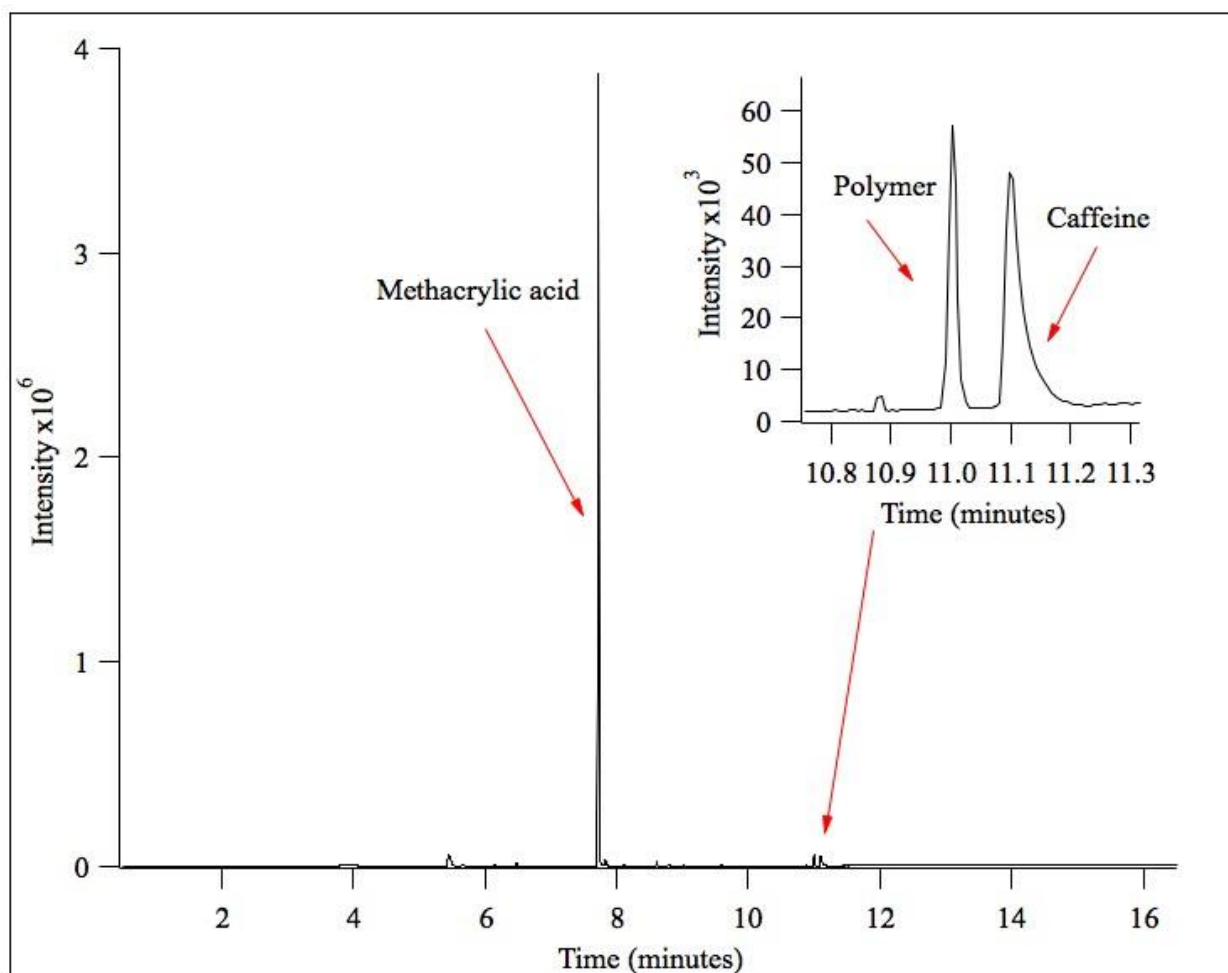


Figure 27: *Blank polymer sampled in Earl Grey Crème tea*

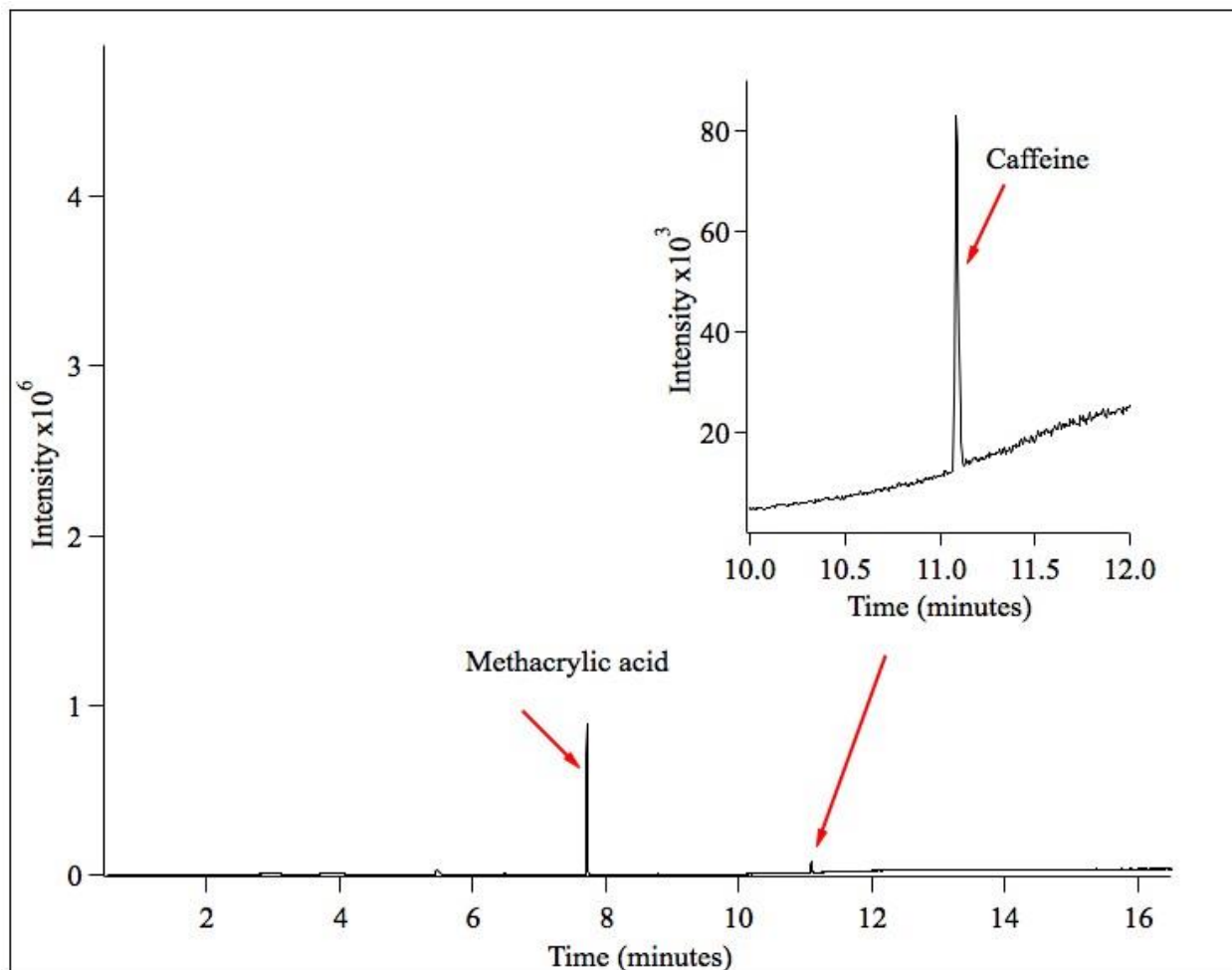


Figure 28: *Caffeine Template Polymer in Earl Grey Crème tea*

#### Selectivity: DNT

The selectivity for 2,4-DNT and 2,6-DNT-  $\alpha,\alpha,\alpha$ - $d_3$  templated fibers were tested following the caffeine trials. The DNT isomers each had multiple ions resulting from their fragmentation patterns, as seen in Table 7. These compounds had similar fragmentation patterns, but due to the position of their substituents, certain fragments were more prevalent than others. 2,4-DNT and 2,3-DNT were the only isomers that coeluted at a retention time of approximately 9.30 min. To differentiate between the two compounds, ions unique to each analyte were used. 2,4-DNT has a

unique ion at 119  $m/z$  and 2,3-DNT has a unique ion at 135  $m/z$ . Figures 29 through 32 are the mass spectra of the DNT standards.

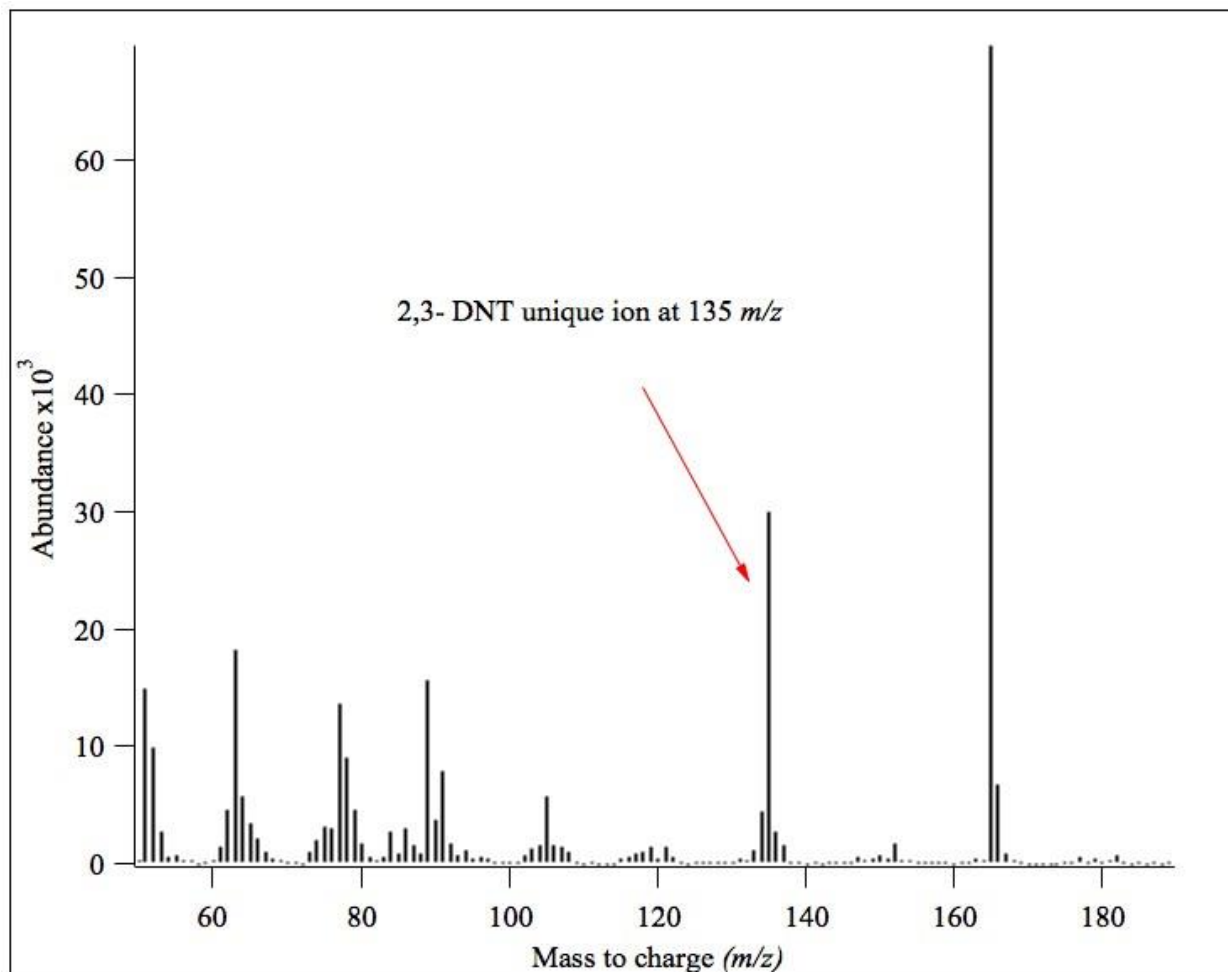


Figure 29: 2,3-DNT standard mass spectrum; unique ion at 135  $m/z$  used to differentiate between 2,4-DNT isomer

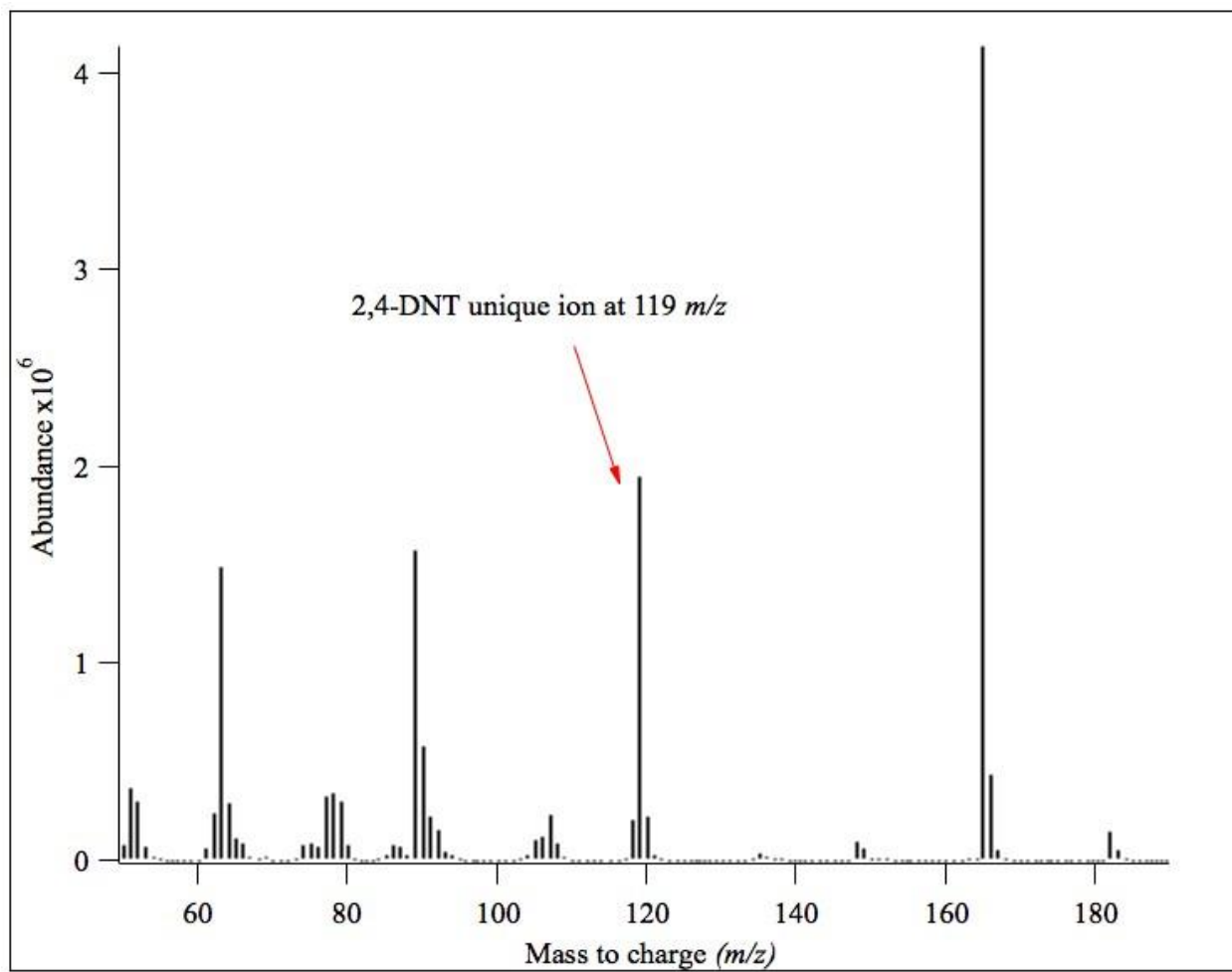


Figure 30: 2,4-DNT standard mass spectrum; unique ion at 119  $m/z$  used to differentiate between 2,3-DNT isomer

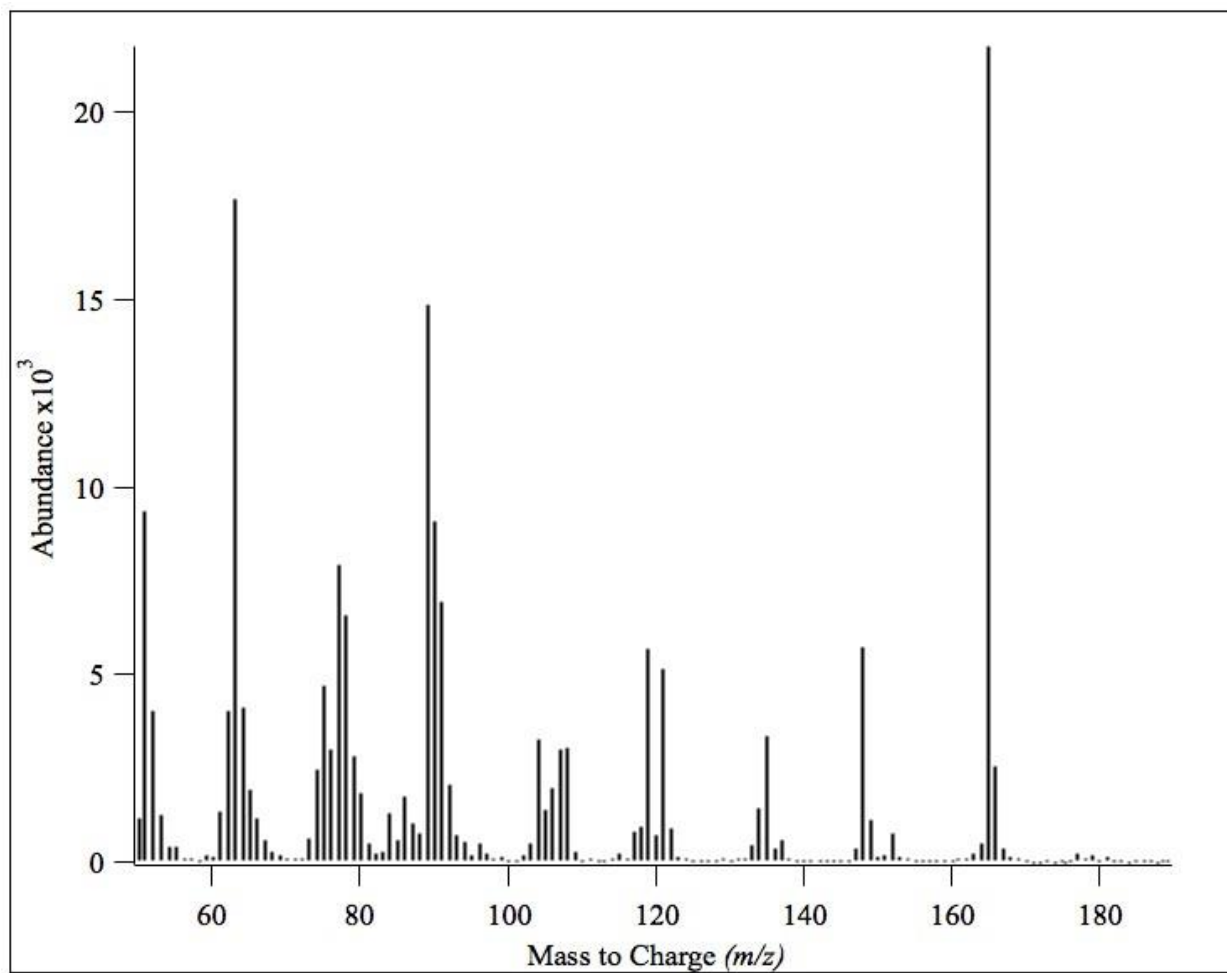


Figure 31: 2,6-DNT standard mass spectrum

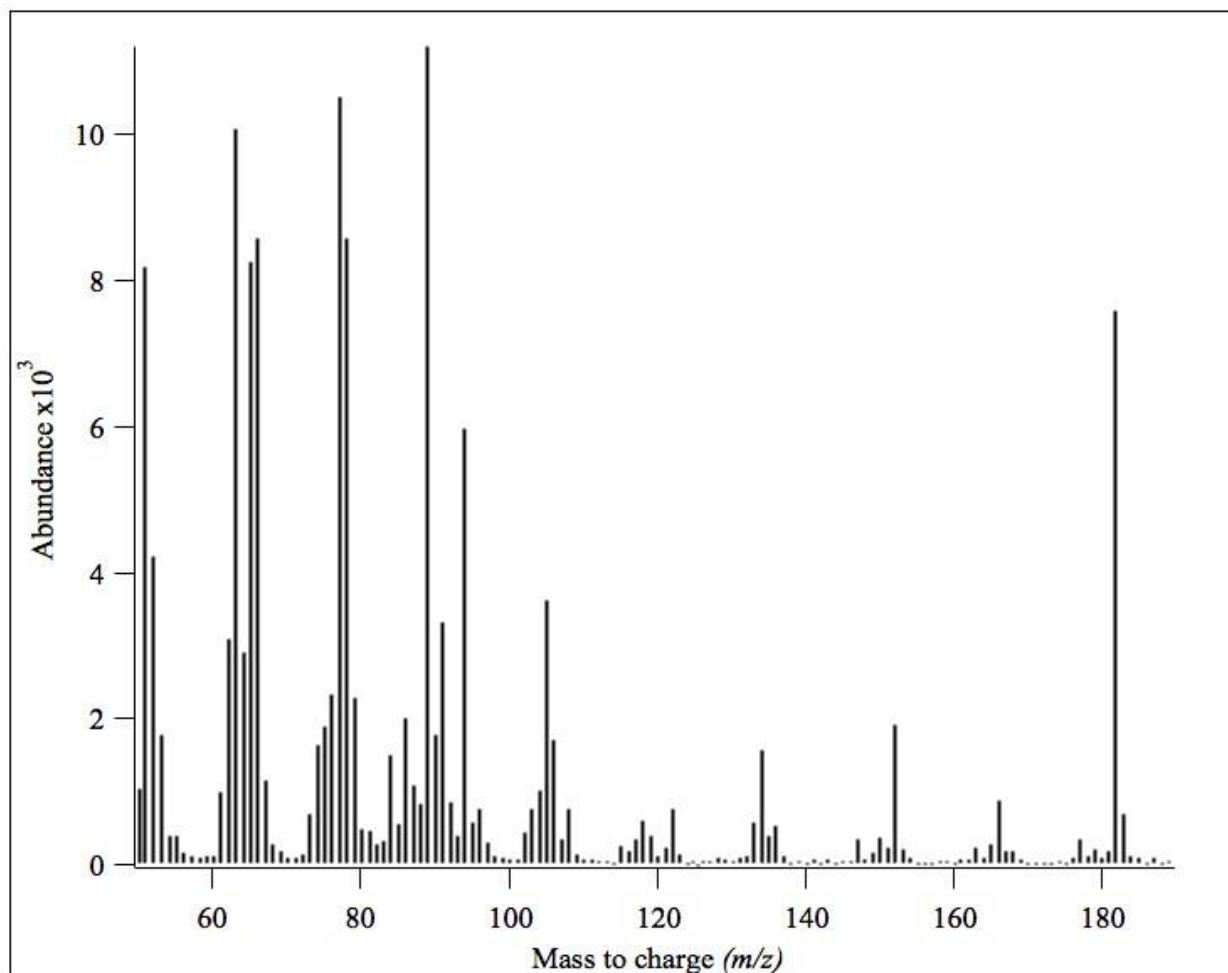


Figure 32: 3,4-DNT standard mass spectrum

In comparison to the chromatograms in the caffeine selectivity section, it appeared that the selectivity for the caffeine templated fibers (Figures 24, 25) was more efficient than the 2,4-DNT and 2,6-DNT- $\alpha,\alpha,\alpha$ - $d_3$  templated fibers (Figures 33, 34). This result may be explained based on interactions between the molecules and the polymer. Caffeine is more readily extracted than theophylline and theobromine despite their minute structural differences. The DNT isomers also have minute differences in structure, but their hydrogen bonding capability and polarity are similar which may attribute to their similar extraction properties on all the polymers.

DNT selectivity was more difficult to determine compared to the caffeine trials. When the 2,4-DNT template fiber was sampled in a solution containing 2,4-DNT, 2,3-DNT, 2,6-DNT and 3,4-DNT, the TIC demonstrated peaks for all analytes (Figure 33).

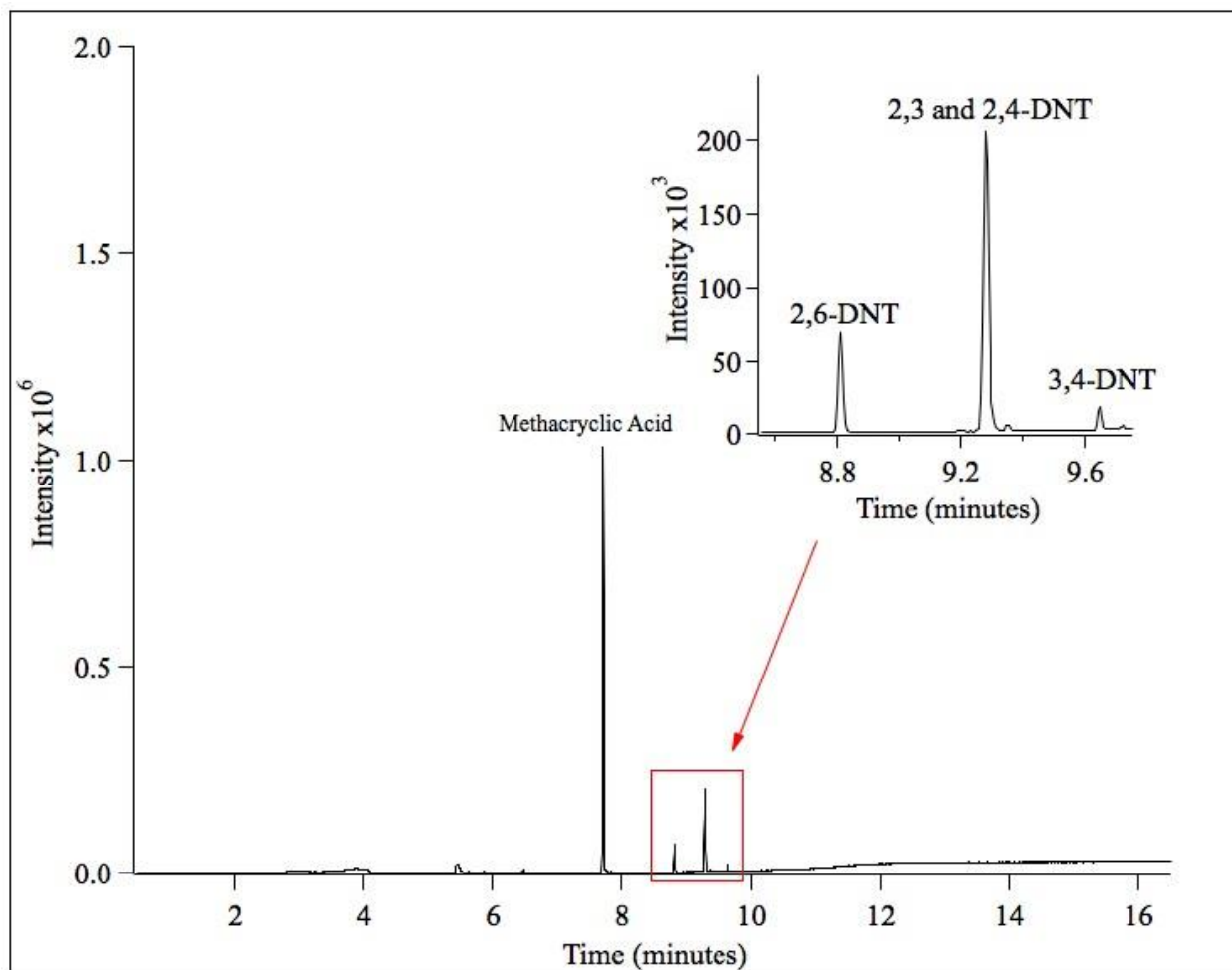


Figure 33: 2,4-DNT templated fiber sampled in DNT isomers mix; 2,3-DNT and 2,4-DNT coelute at approximately 9.3 minutes, while 2,6-DNT elutes at approximately 8.8 minutes and 3,4-DNT elutes at approximately 9.7 minutes

A 2,6-DNT- $\alpha,\alpha,\alpha$ -d<sub>3</sub> template fiber was also used to sample the isomers solution. The TIC for this sampling also demonstrated peaks for all analytes, as demonstrated in Figure 34.



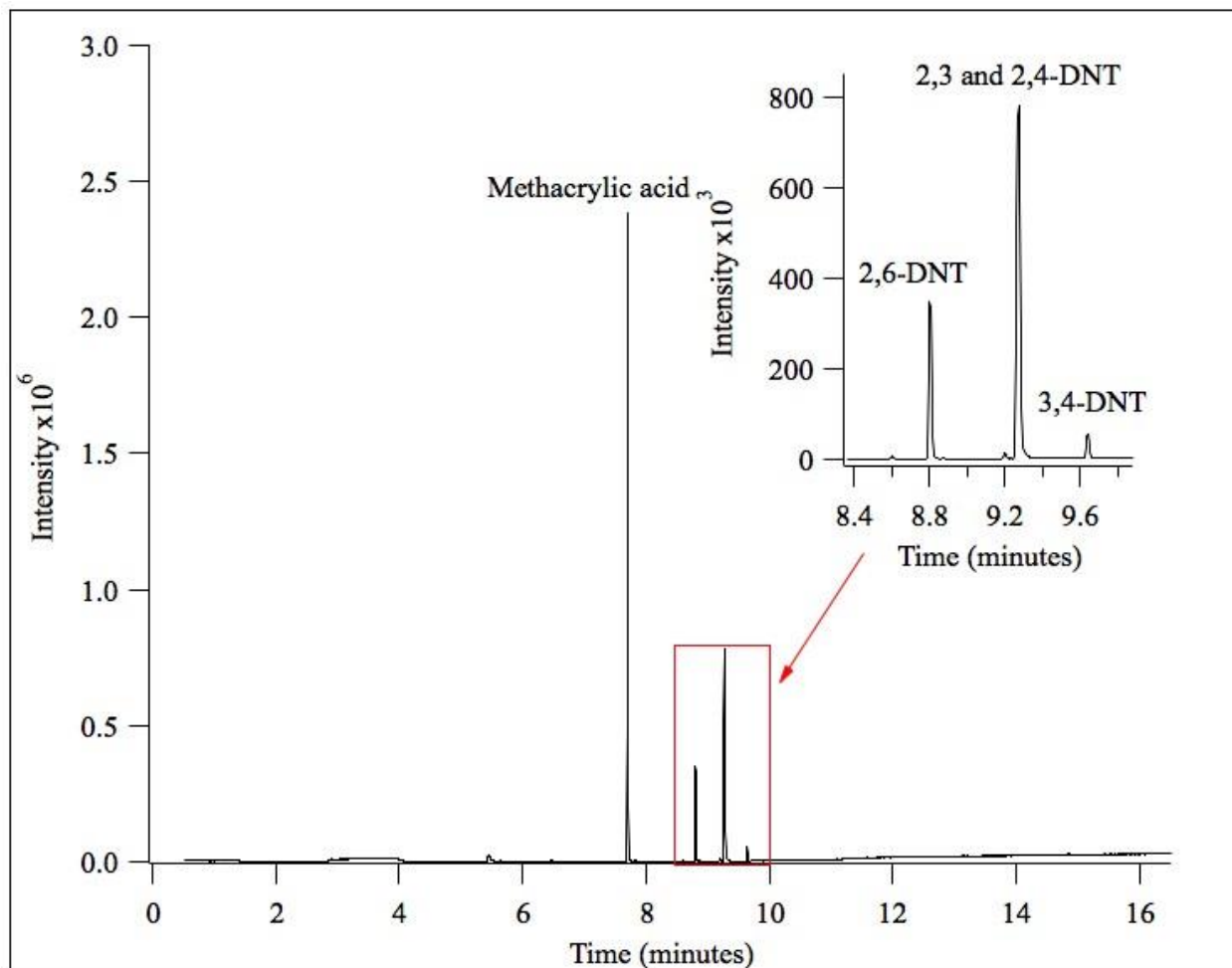


Figure 34: 2,6-DNT-  $\alpha,\alpha,\alpha$ -d<sub>3</sub> templated fiber sampled in DNT isomers mix

The goal was to extract only the analyte for which the fiber was templated, similar to the outcome of the caffeine trials. There was minimal observed difference in selectivity between template molecule and other isomers.

When a blank polymer was used to sample the DNT isomers solution, Figure 34, there was no discernible difference in response between fibers that were templated versus ones which were

not. All four analytes were present on the fiber, much like when a blank polymer was tested in the caffeine solution.

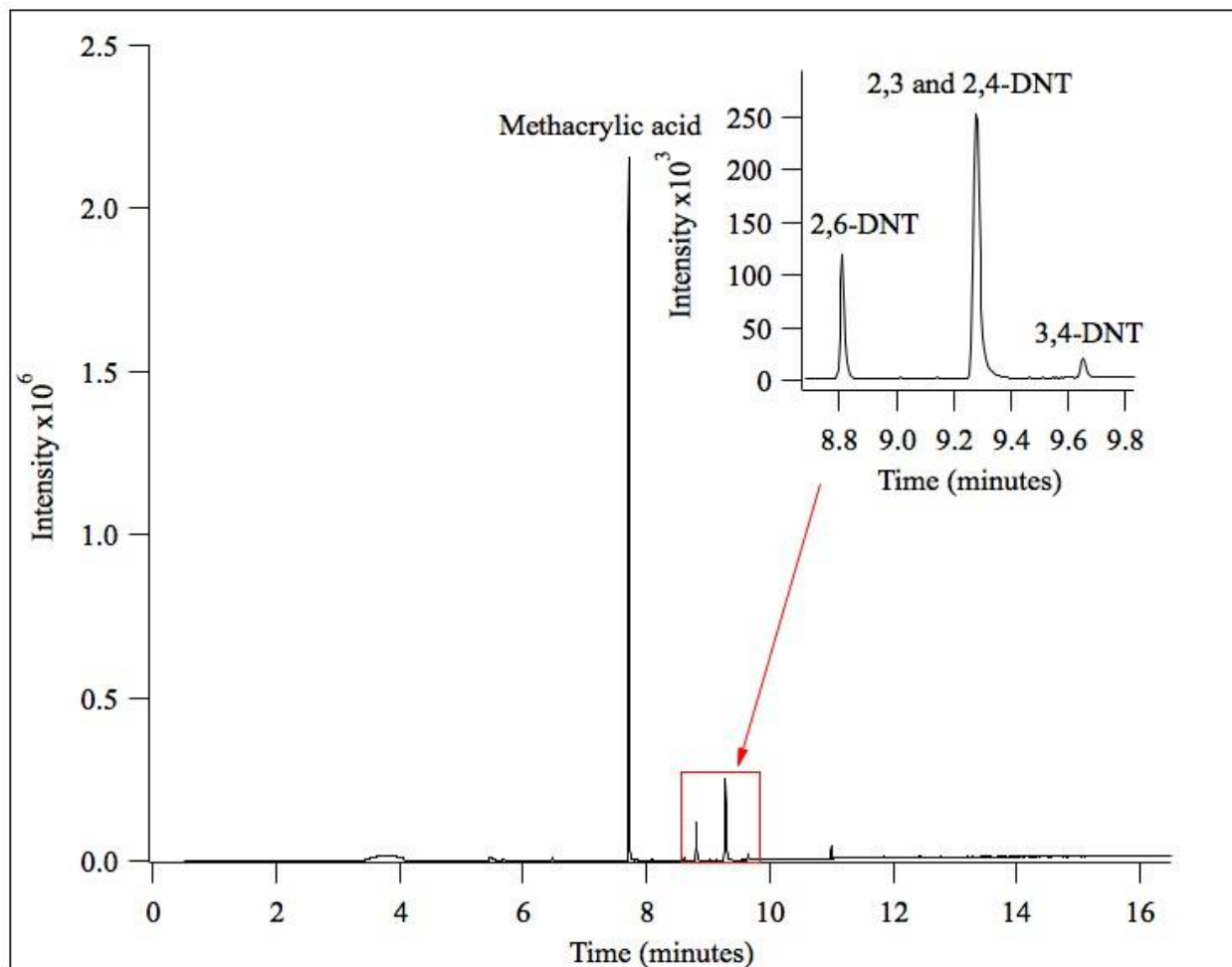


Figure 35: *Blank polymer sampled in DNT isomers mix*

To determine selectivity for the 2,4-DNT and 2,6-DNT- $\alpha,\alpha,\alpha$ -d<sub>3</sub> templated fibers, ratios were calculated between the concentrations of isomers in the solution versus what molecule was templated. Calibration curves were created using the ions of interest from mixed isomer solutions of varying concentrations: 0.5 ng/ $\mu$ L, 1 ng/ $\mu$ L, 5 ng/ $\mu$ L, and 10 ng/ $\mu$ L (Figure 36 and Appendix B). These solutions were sampled in triplicate to produce an average peak area

response. Linear regression was applied to calculate an estimated concentration for each DNT isomer based on the peak area response at their respective unique ions. The unique ion used for 2,4-DNT was 119  $m/z$ , for 2,3-DNT it was 135  $m/z$ , while the 3,4 and 2,6-DNT calibration used 89  $m/z$ . These concentration estimates were then used to obtain ratios to compare between the blank and DNT templated polymers. Figure 36 demonstrates how the selectivity ratios were calculated.

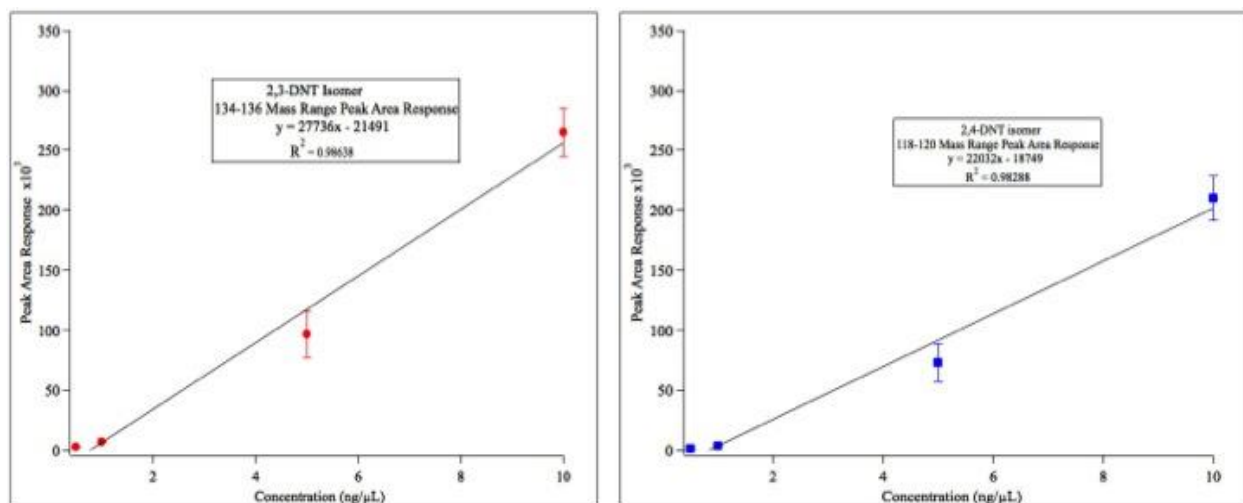


Figure 36: Calibration curves for DNT isomers. (Process for calculating values in selectivity tables. 2,3-DNT/2,4 DNT = 0.4607 (first sampling). Process repeated for each sampling. Final value seen in table: ( $1^{st}$  sampling +  $2^{nd}$  sampling +  $3^{rd}$  sampling)/3; (DNT isomer row/DNT isomer column) = 0.49)

**Table 9: Data used to determine significance of selectivity across the templated and blank fibers**

2,4-DNT Template Fiber			
Sampling	2,3-DNT isomer concentration (ng/uL)	2,4-DNT isomer concentration (ng/uL)	Selectivity Ratios of 2,4-DNT versus 2,3-DNT isomers
1	2.4	5.21	2.17
2	4.14	7.41	1.80
3	2.98	6.64	2.23
average	3.17	6.42	2.06
Standard Deviation	0.89	1.12	0.24

Statistical analysis performed on the concentration values was used to create the selectivity tables (Tables 10-12) in order to determine each fiber's extraction efficiency (Appendix C). The concentrations calculated from the calibration curves for the 2,3-DNT, 2,4-DNT, and 2,6-DNT isomers were averaged and their standard deviations determined. The standard deviations for each isomer were compared to the ratios given in the tables to determine their relationship to one another.

The standard deviations for 2,4-DNT templated and blank fibers were relatively similar, with the 2,4-DNT fiber standard deviation being 0.24 and the blank being 0.26. This results in significant overlap (Figure 37) if considering the  $3\sigma$  standard deviation values with respect to the 2,4/2,3 selectivity ratios at 2.06 for the 2,4-DNT templated fiber and 1.78 for the blank fiber. The

statistical noise in measurement implies little to no selectivity between fibers. The 2,6-DNT- $\alpha,\alpha,\alpha$ -d<sub>3</sub> templated fiber standard deviation value calculated as 0.04 and possessed a selectivity ratio of 1.57, which puts it far below the 2,4-DNT templated and blank fibers. Continuing this type of analysis for the other isomers no significant selectivity is observed for either the 2,4-DNT or 2,6- $\alpha,\alpha,\alpha$ -d<sub>3</sub> templated fibers.

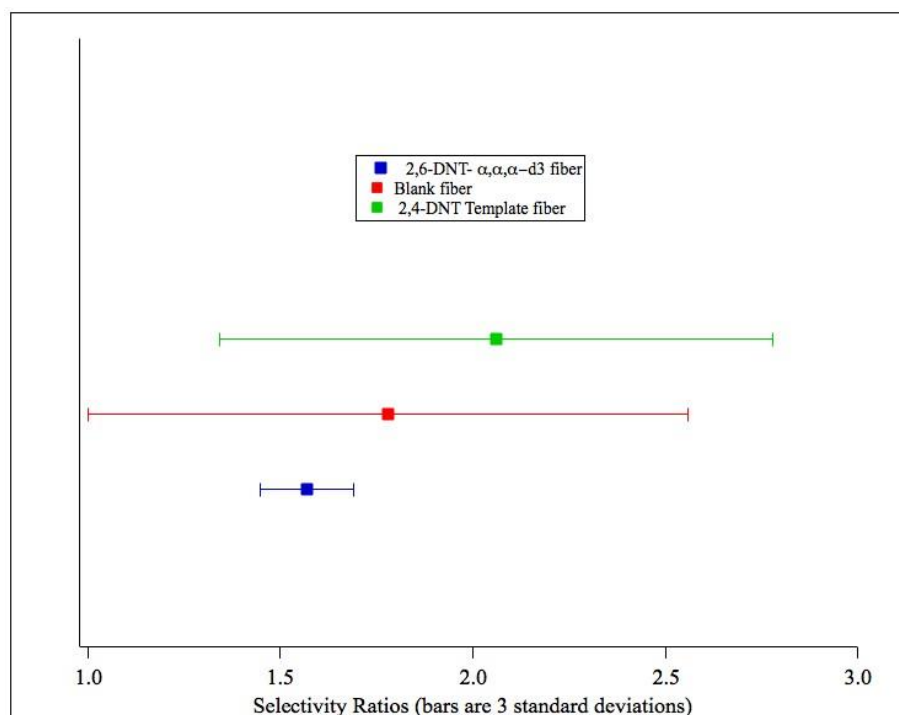


Figure 37: Comparison of the average selectivity ratios of isomers 2,4-DNT versus 2,3-DNT for each fiber

The concentrations for the 2,4-DNT and 2,6-DNT isomers using the 2,6-DNT- $\alpha,\alpha,\alpha$ -d<sub>3</sub> templated fiber were out of the calibration range. This could be due to the sampling method or the fiber.

The concentrations in the second sampling for the blank fiber were also out of range. The Dixon's Q test was performed to determine if these values were outliers. The test for three replicates determined that the values were not outliers and were kept in the calculations. If a

fourth sampling were conducted the second sampling would have likely been an outlier and removed from the calculations.

The selectivity ratios for all the DNT isomers were compared to each other regardless of what was actually templated on the fibers. This comparison was performed in order to determine if one isomer was extracting more efficiently than the others despite whether the isomer was used as the template molecule. The ratios for a 2,4-DNT templated fiber are demonstrated in Table 10, while Table 11 contains the ratios for a 2,6-DNT  $\alpha,\alpha,\alpha\text{-d}_3$  templated fiber. The black cells denote a 1/1 ratio.

**Table 10: Selectivity Ratios using a 2,4-DNT Templated Fiber**

<b>Selectivity = Row / Column isomer designation (2,4-DNT Template)</b>				
<b>row/column</b>	<b>2,3-DNT isomer</b>	<b>2,4-DNT isomer</b>	<b>2,6-DNT isomer</b>	<b>3,4-DNT isomer</b>
<b>2,3-DNT isomer</b>		0.49	0.54	0.81
<b>2,4-DNT isomer</b>	2.06		1.10	1.65
<b>2,6-DNT isomer</b>	1.89	0.91		1.51
<b>3,4-DNT isomer</b>	1.25	0.61	0.66	

**Table 11: Selectivity Ratios using a 2,6-DNT-  $\alpha,\alpha,\alpha$ -d<sub>3</sub> Templated Fiber**

Selectivity = Row / Column isomer designation (2,6-DNT- $\alpha,\alpha,\alpha$ -d <sub>3</sub> Template)				
row/column	2,3-DNT isomer	2,4-DNT isomer	2,6-DNT isomer	3,4-DNT isomer
2,3-DNT isomer		0.64	0.57	1.02
2,4-DNT isomer	1.57		0.89	1.60
2,6-DNT isomer	1.77	1.12		1.80
3,4-DNT isomer	0.98	0.63	0.56	

The values obtained from these calculations did not show increased selectivity for the templated molecules (2,4-DNT or 2,6-DNT -  $\alpha,\alpha,\alpha$ -d<sub>3</sub>). No discernible selectivity differences were observed between the templated fibers and blank fibers. The blank fibers extracted all the isomers with the same selectivity as the templated fibers. Both fibers exhibited the same response levels. The ratios of extracted analytes on a blank fiber are shown in Table 12.

**Table 12: Selectivity Ratios using a Blank Fiber**

Selectivity = Row / Column isomer designation (BLANK)				
row/column	2,3-DNT isomer	2,4-DNT isomer	2,6-DNT isomer	3,4-DNT isomer
2,3-DNT isomer		0.57	0.65	1.00
2,4-DNT isomer	1.78		1.15	1.78
2,6-DNT isomer	1.54	0.88		1.55
3,4-DNT isomer	1.00	0.57	0.65	

Binding affinity was considered as a possible explanation for the lack of selectivity due to solvent effects since the solutions were prepared in methanol. Much like in the caffeine trials, the methanol could be competing for hydrogen bonding between the molecules and the binding active sites. Extraction selectivity did not appear to increase when methylene chloride was used as a solvent. Since methylene chloride was the porogen used in the pre-polymer solution, the binding active sites were expected to accept the molecules more readily. The ratios calculated with methylene chloride as the solvent are shown in Table 12 using the same method as in Tables 10 and 11.

**Table 13: Selectivity Ratios using a 2,4-DNT Templated Fiber in Methylene Chloride**

Selectivity = Row / Column isomer designation (2/4 Template)				
row/column	2,3-DNT isomer	2,4-DNT isomer	2,6-DNT isomer	3,4-DNT isomer
2,3-DNT isomer		0.81	0.88	1.21
2,4-DNT isomer	1.29		1.12	1.53
2,6-DNT isomer	1.14	0.92		1.38
3,4-DNT isomer	0.83	0.66	0.73	

#### Conventional SPME versus MIP-SPME

The carbowax/divinylbenzene (CW/DVB) polymer fiber was sampled in a mixed solution of caffeine, theophylline, and theobromine, and a mixed solution of caffeine and theophylline. Solutions were prepared in a 1:1 (v/v) ratio of methanol to water to avoid damage to the fiber coating from organic solvents (i.e. methylene chloride) as specified by manufacturer instructions.<sup>35</sup> Only caffeine and theobromine peaks were observed in the TIC in Figure 38. The



area where the response for theophylline was expected, a rise in the baseline was observed, but was too weak to confirm despite eluting at the correct retention time. The mass spectrum search also displayed the major ion of interest, however the software gave too low of a match for the baseline rise to be confirmed as theophylline. Theobromine had the best response out of the analytes.

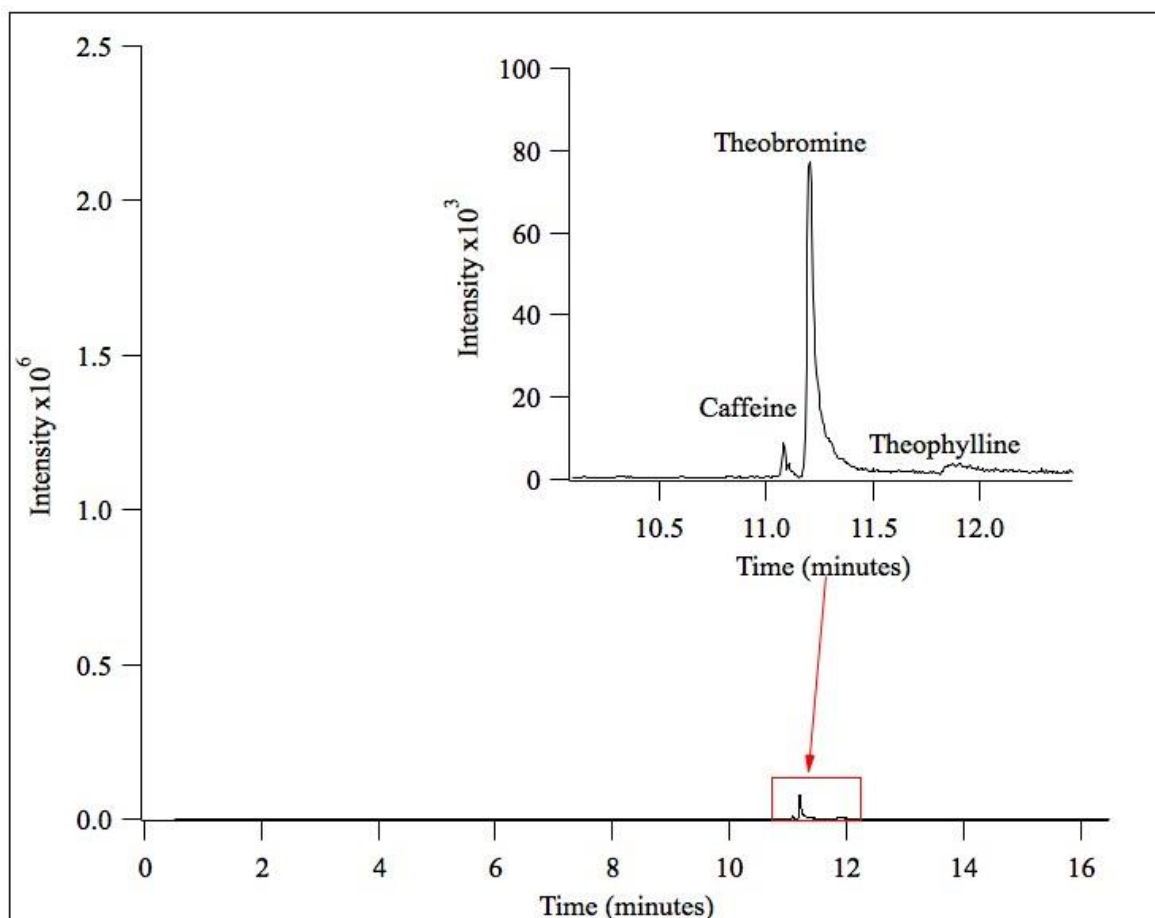


Figure 38: CW/DVB fiber sampled in solution of caffeine, theobromine, and theophylline

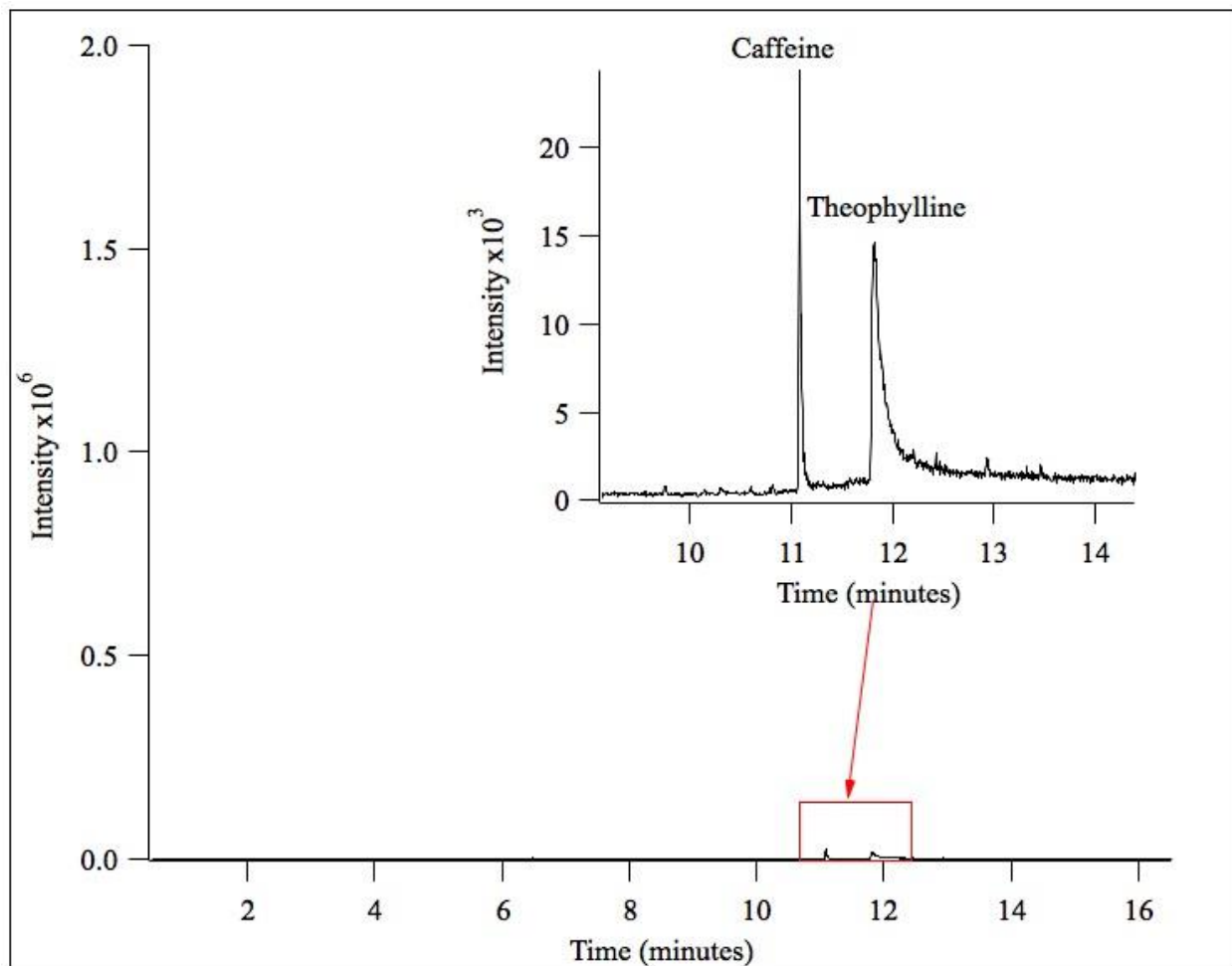


Figure 39: *CW/DVB fiber sampled in solution of caffeine and theophylline*

In the second solution of caffeine and theophylline, both analytes were present, as seen in Figure 39. The manufactured SPME was not as selective as compared to a caffeine templated fiber using the same solvent system (Figure 24). The lack of selectivity could be attributed to factors such as binding affinity, type of solvents used, concentrations of analyte in solution, etc. Both types of fibers, the CW/DVB and the fabricated polymer monolith, use adsorption as their sampling mechanism.<sup>35</sup> The CW/DVB fiber is manufactured for use in a variety of research

areas with the purpose of general selectivity for a wide range of compounds. This could account for the analytes adsorbing to the polymer.

#### MIP-SPE versus MIP-SPME

The extraction of an analyte from a solution using MIP-SPE is based on the same binding principles as that of MIP-SPME. The target analyte templated on the polymer should extract, or be retained. The purpose of this experiment was to see if there were any major differences between MIP-SPME and MIP-SPE to determine which method was better for extraction. There are some advantages MIP-SPE has over MIP-SPME. One advantage is, during Soxhlet extraction, the solvent can have more interaction with the crushed polymer particles versus the polymer monoliths. Due to an increase in surface area obtained through crushing the polymer, improved removal of template molecules from the binding sites through the washing process occurs. This leads to a reduction in overall template bleeding, and more efficient retention of the target analyte during extraction of samples.

In the methylene chloride trial, the DNT isomers were retained on the sorbent bed. The chromatogram of the methylene chloride elution had all the DNT isomers present. Peak intensity for all isomers did not exceed a S/N of 3 as seen in the TIC in Figure 40. The rebinding of 2,6-DNT into the templated active sites should result in a chromatogram reflecting a decreased abundance of the template; however, this did not occur during the methylene chloride trial. The isomers eluted in the methanol wash step instead of the methylene chloride elution step, though the responses were weak.

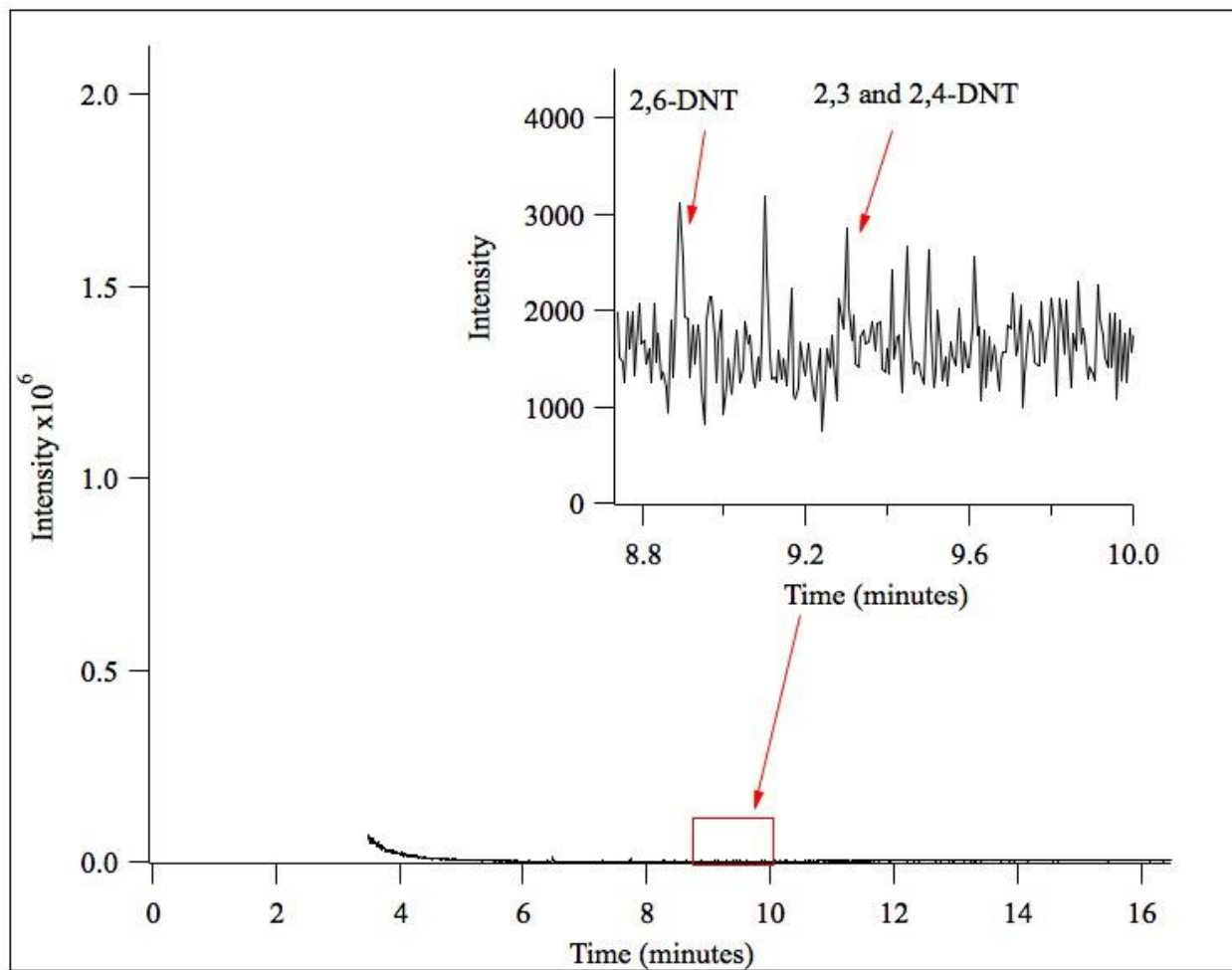


Figure 40: *Methylene chloride elution with templated 2,6-DNT-  $\alpha,\alpha,\alpha$ -d<sub>3</sub> SPE*

The methanol wash, after evaporating with nitrogen gas and reconstituting with methylene chloride, also showed all of the DNT isomers, with the exception of 3,4-DNT, in Figure 41. 3,4-DNT could be present in the baseline and difficult to detect due to response of the isomers being weak overall.

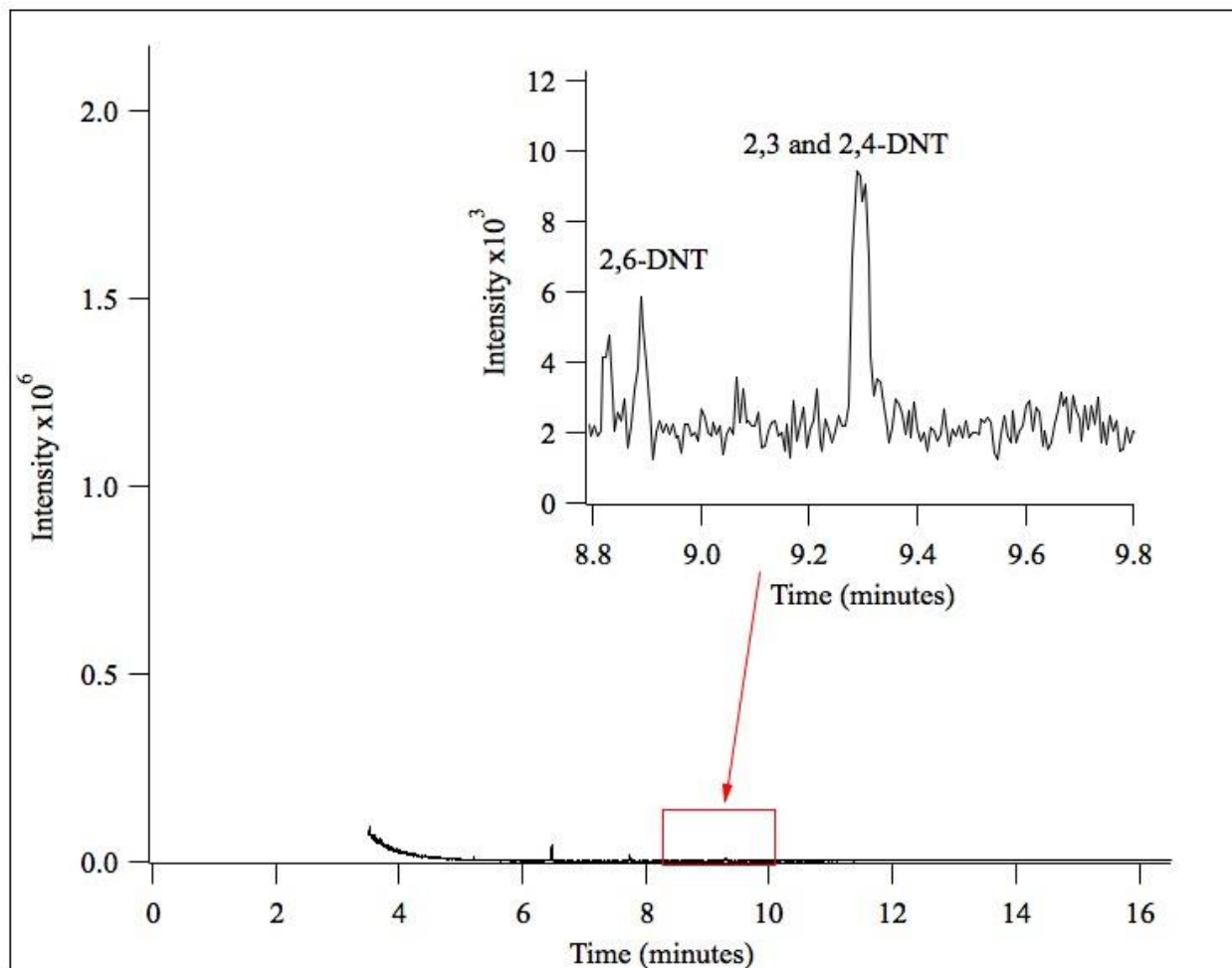


Figure 41: *Methanol wash with templated 2,6-DNT- $\alpha,\alpha,\alpha$ -d<sub>3</sub> SPE*

The extraction process was repeated using pentane as the solvent to condition and load the SPE polymer. Methanol demonstrated better elution efficiency than methylene chloride, so it was used as the elution solvent instead of as a wash solvent. Nitrogen gas was used to evaporate the methanol solution (containing the DNT isomers) and pentane used to reconstitute. Analysis was performed on both the pentane loaded onto the column (before and after) and the methanol wash reconstituted solution as shown in Figures 42, 43, and 44.

To determine the response of the isomers prior to loading onto the column, the pentane solution was analyzed (Figure 42). The combined intensity of 2,3 and 2,4-DNT is large in comparison to the 2,6-DNT and 3,4-DNT isomer intensities.

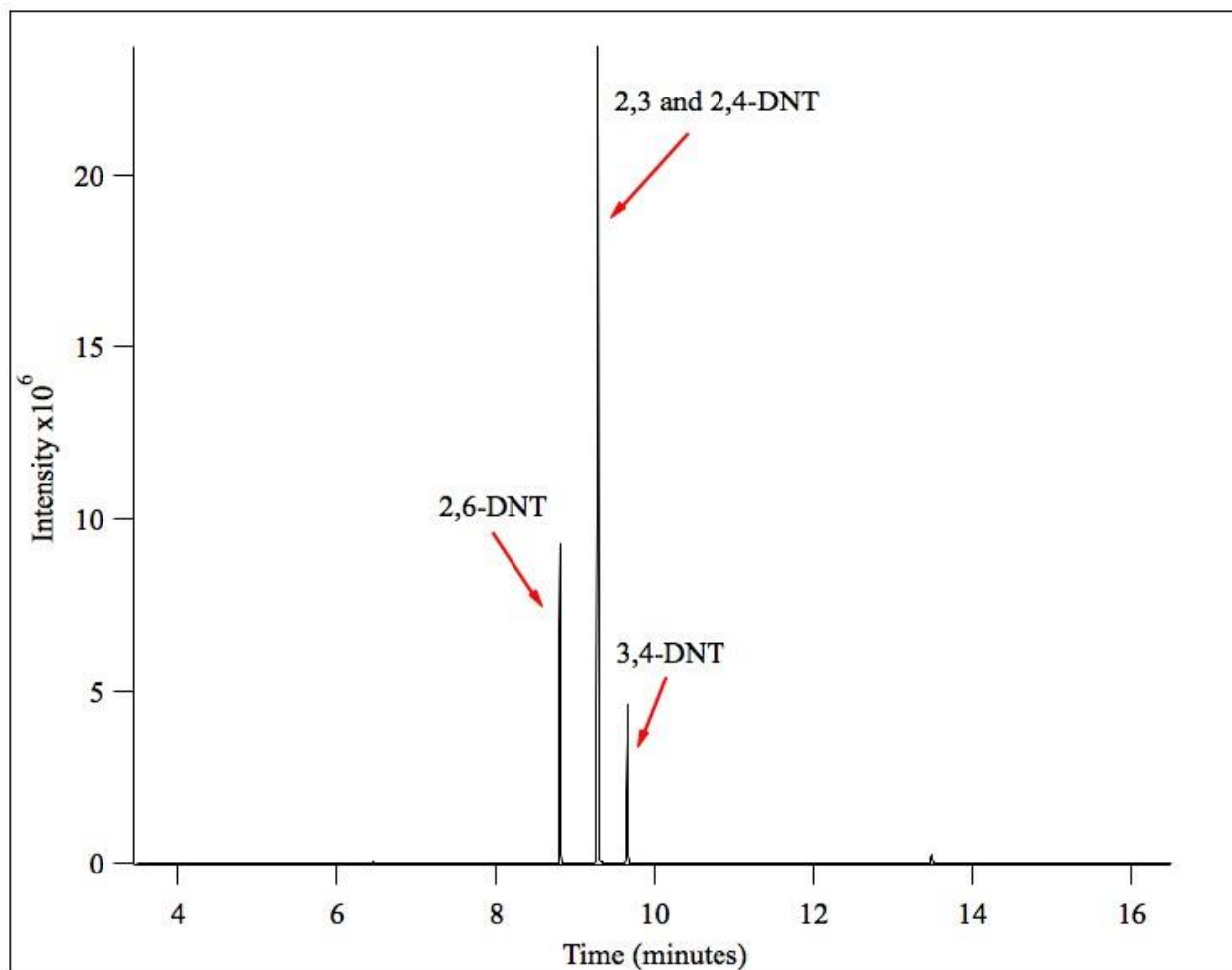


Figure 42: *DNT isomers solution in pentane before loading onto MIP-SPE*

The pentane solution, after loading onto the column, had all of the isomers present in the chromatogram (Figure 43). The 3,4-DNT isomer had a lower abundance than the other three isomers demonstrating that some of 3,4-DNT was retained on the sorbent bed. The other analytes were also retained on the sorbent bed, but to a lesser degree.

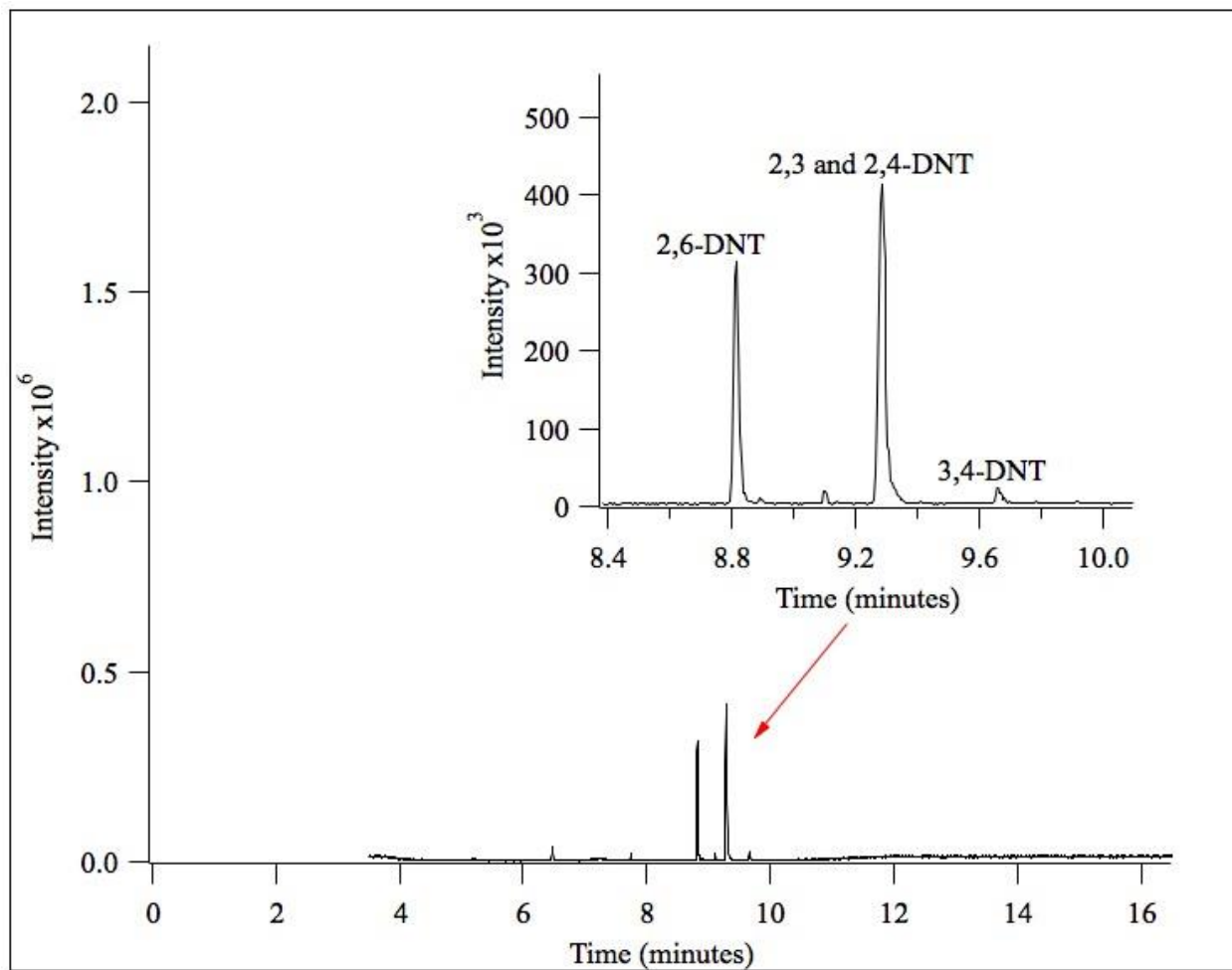


Figure 43: *Pentane sample solution after loading onto MIP-SPE*

All three isomers were observed, as seen in Figure 44. The analytes that were retained on the sorbent bed were eluted using the methanol, similar to what occurred in the methylene chloride trial.

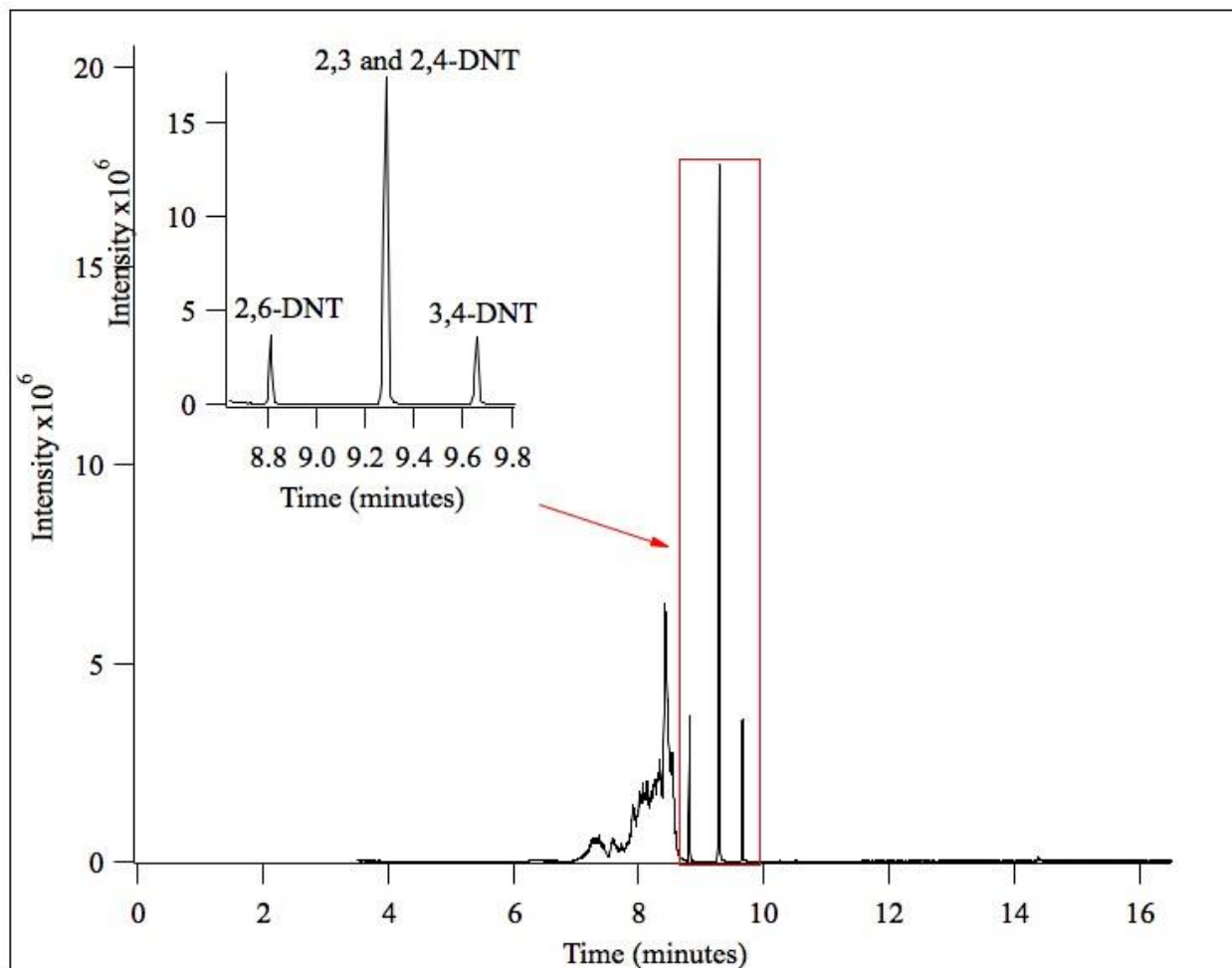


Figure 44: *Methanol elution step dried with nitrogen gas and reconstituted using pentane*

Looking at Figures 43 and 44, the majority of 2,6-DNT stayed with the pentane as the solution was loaded onto the SPE. Isomers retained on the SPE eluted after washing with methanol. The pentane appeared to work better at separating the analytes than the methylene chloride. This could be because pentane is less polar than methylene chloride, or methylene chloride is causing some interference between the target analyte and binding sites. The latter seems to conflict with the increased extraction ability that porogens used in the preparation of polymers are demonstrated to possess. The polarity of the isomers also has an affect on how they interact with



the polymer and the solvent. Changing the polarity of the solvent would provide a better idea on how the polarity of the analyte affects its retention on the sorbent bed. More extractions using different solvents would be required to optimize the extraction process and obtain a better separation and targeted retention of analytes.

## CHAPTER 5: CONCLUSIONS

The objective of developing MIP-SPME fibers is to determine if they could be used to selectively and quantitatively extract a target analyte from solution to leverage their intrinsic properties of efficient analysis of small sample volumes and less destructive extraction procedure. This project was a way of determining an analytical method that could be used in forensic laboratories.

### Conclusion

Caffeine concentrations were quantitatively determined using MIP-SPME fibers in solutions of consumer grade tea and coffee as displayed in Table 14. The caffeine concentrations calculated in Table 8 did not match the theoretical concentrations given by manufacturers with the exception of the coffee sample. Factors, which may have affected concentration, were discussed in Chapter 4 such as steep time and type of tea. The templated polymers extracted caffeine, though competition between the solvents and the analytes was observed. Using more non-polar solvents could change the competitive binding between solvent and analyte.

**Table 14: Summary of Caffeine Extractions**

Solution	Analytes	Fiber	Conditions	Selectivity
Caffeine Analogues Mixture	Caffeine, theobromine, and theophylline	Caffeine (templated)	Methanol	Selective for caffeine
Caffeine Analogues Mixture	Caffeine, theobromine, and theophylline	Caffeine (templated)	50:50 (v/v) Methanol: Epure Water	Selective for caffeine
Caffeine Analogues Mixture	Caffeine, theobromine, and theophylline	Caffeine (templated)	Methylene chloride	Selective for caffeine
Earl Grey Crème Tea	Caffeine	Caffeine (templated)	50:50 (v/v) Methanol: Epure Water	Selective for caffeine
Caffeine Analogues Mixture	Caffeine, theobromine, and theophylline	Blank (non-templated)	50:50 (v/v) Methanol: Epure Water	No caffeine extracted
Earl Grey Crème Tea	Caffeine	Blank (non-templated)	50:50 (v/v) Methanol: Epure Water	Not selective, caffeine extracted

The caffeine MIP-SPMEs demonstrated some selectivity when extracting caffeine from a mixture of caffeine and its analogues, theophylline and theobromine. Neither analogue was visible in the chromatograms using caffeine templated fibers. Their absence could be attributed to caffeine's better binding efficiency. Though this was true for the templated fiber, selectivity between the blank polymer versus the templated polymer was not observed. Despite the blank fiber not being templated, it extracted caffeine as observed in Figure 27, and its response was similar to that of the templated fiber. This outcome does not fit with results obtained in Djozan where the blank fiber extracted significantly less analyte than the templated fiber.<sup>29</sup> This trend was also demonstrated in the DNT templated fibers.

Selectivity was not observed with the 2,4-DNT and 2,6-DNT- $\alpha,\alpha,\alpha$ -d<sub>3</sub> templated fibers. When the polymers were sampled in a solution containing four DNT isomers, the isomers were all extracted. The lack of selectivity may be related to hydrogen bonding on the DNT molecules having a different affinity to the monomer molecule than caffeine and its analogues. More research would be required to fully determine why binding efficiency was the same for all the DNT isomers. Factors that could have affected the binding efficiency are polarity of the analytes and solvent. The MIP-SPE trial outcome resulted in extraction efficiency similar to that of the MIP-SPME trials. The SPE trials demonstrated pentane was a solvent that could be used to condition and load the sorbent bed, and affect retention of the isomers. Methanol was shown as a superior solvent for elution, it performed far better on the DNT isomers in contrast to methylene chloride in the first trial. If the MIP-SPME and MIP-SPE were optimized, a more selective and sensitive method of extraction for forensic samples could be developed.

This project demonstrated that both methods of extraction using molecularly imprinted polymers could extract analytes, even if selectivity was not observed. Other research areas such as the use of MIP-SPME and MIP-SPE in drug analysis have yielded positive results for Djozan *et al.* and Anderson *et al.*, respectively.<sup>30, 48, 50</sup> The MIP-SPME and MIP-SPE fabricated in this experiment could be used as screening tools for groups of compounds that are similarly structured or applied to in-the-field sampling.

### Future Work

In the future, more complex sample solutions such as blood, urine, or explosive residues in soil should be used for analysis. The polymers could extract caffeine from brewed teas without pre-extraction, so the use of more complex samples would test the robustness of these polymers.

The method would need to be optimized to obtain better extraction and selectivity. Changing the solvent, ratio of template molecule to functional monomer, and ratio of functional monomer to cross-linker are some ways to strengthen extraction and selectivity. Experimenting with other functional monomers, such as methyl methacrylate (MMA) or 2-hydroxyethylmethacrylate (HEMA), could also be beneficial; additionally another cross-linker, like DVB and a free radical initiator similar to AIBN, possibly sodium persulfate could prove to be advantageous, as well. Additional cross-linker could make the polymer more rigid, though this could have some drawbacks by making the polymer too rigid to remove the template molecule.

Branching out into other forms of MIP would be worth looking into, as well. Membranes, microbeads, etc. could be manufactured, since these methods have already seen some use in research and have yielded positive results.<sup>13</sup>

Though this project demonstrated that extraction was possible for small volume sizes and quantitation of caffeine concentrations, it had difficulties in selectivity between templated and blank polymers. Overall, the monolith worked, and needs improvement before use as a forensic tool.

## **APPENDIX A: CAFFEINE CONCENTRATION CALCULATION**

An example calculation in order to obtain commercial grade tea and coffee theoretical concentrations (ng/μL) seen in Table 8.

<p><b>DESCRIPTION</b></p> <p><b>Smooth infusion with bergamot overtones and a creamy vanilla finish</b></p> <p>High tea is further elevated with tart bergamot orange tastefully tempered by creamy vanilla then blended with the classic pomp of an Earl Grey black tea. A majestically smooth confection that is now available any time the senses desire. Sprinkled with sunny yellow marigold petals, this full leaf wonder is divinely delicious with a silky sweet finish.</p>
<p><b>TASTING NOTES</b></p> <p>Smooth infusion with bergamot overtones and a creamy vanilla finish</p>
<p><b>CAFFEINE LEVEL</b> ● ● ● ○</p> <p>○ ○ ○ ○</p> <p><b>CAFFEINE-FREE</b></p> <p>● ○ ○ ○</p> <p><b>1-15 MG</b></p> <p>● ● ○ ○</p> <p><b>16-25 MG</b></p> <p>● ● ● ○</p> <p><b>26-39 MG</b></p> <p>● ● ● ●</p> <p><b>40+MG</b></p>
<p><b>INGREDIENTS</b></p> <p>Black tea, natural and artificial flavoring, marigold petals</p>

Figure 45: Caffeine Level estimation chart taken from manufacturer's (Teavana®) website

1 cup = 237 mL

Earl Grey Crème Tea = 34 mg per cup

$34 \text{ mg} / 237 \text{ mL} = 0.14346 \text{ mg/mL}$

$0.14346 \text{ mg/mL} \times (1 \times 10^6 \text{ ng} / 1 \text{ mg}) = 143459.92 \text{ ng/mL}$

$143459.92 \text{ ng/mL} \times (1 \text{ mL} / 1000 \text{ } \mu\text{L}) = 143.46 \text{ ng/ } \mu\text{L}$

Process was repeated for each manufacturer's estimation of caffeine concentration.

## **APPENDIX B: DNT ISOMERS CALIBRATION CURVES**



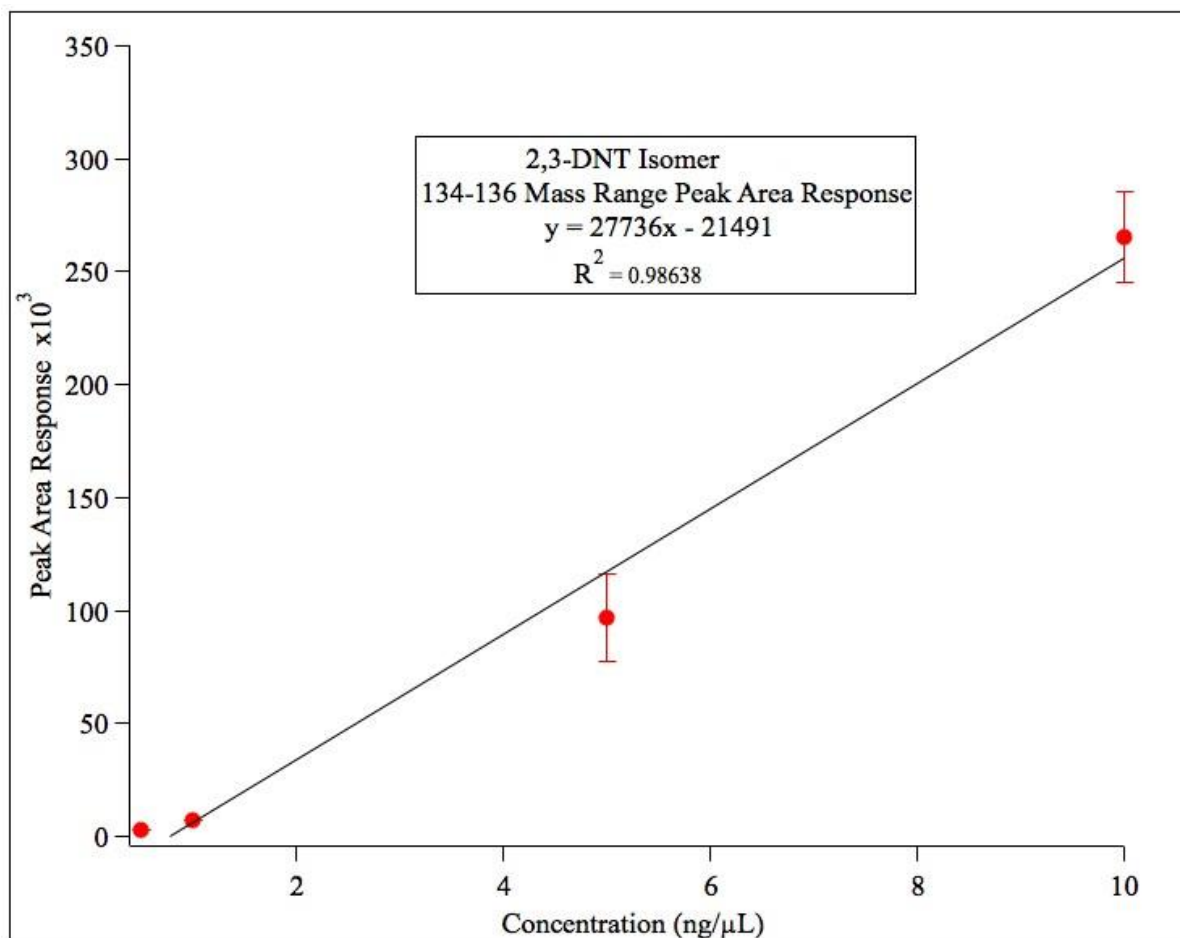


Figure 46: 2,3- DNT Isomer calibration curve used to calculate ratios for determining selectivity in Tables 10-13

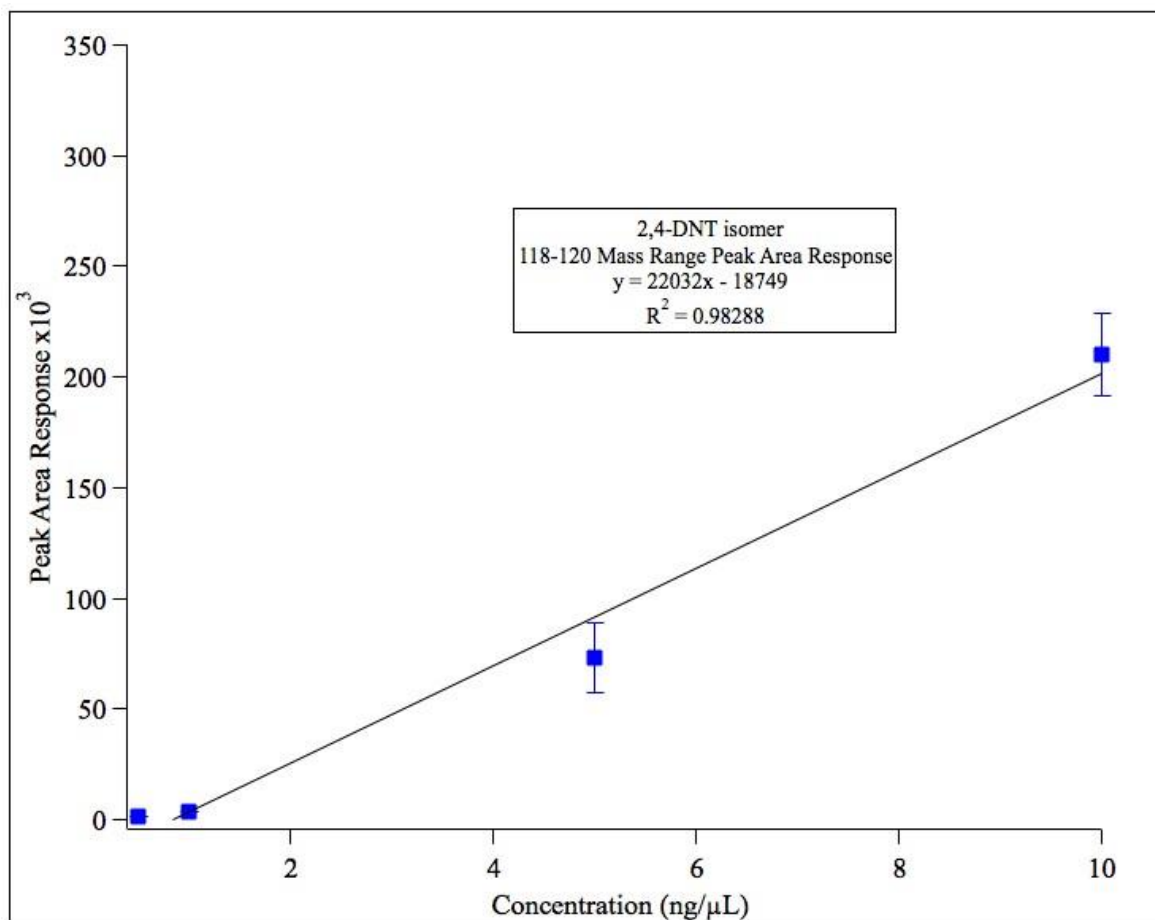


Figure 47: 2,4-DNT Isomer calibration curve used to calculate ratios for determining selectivity in Tables 10-13

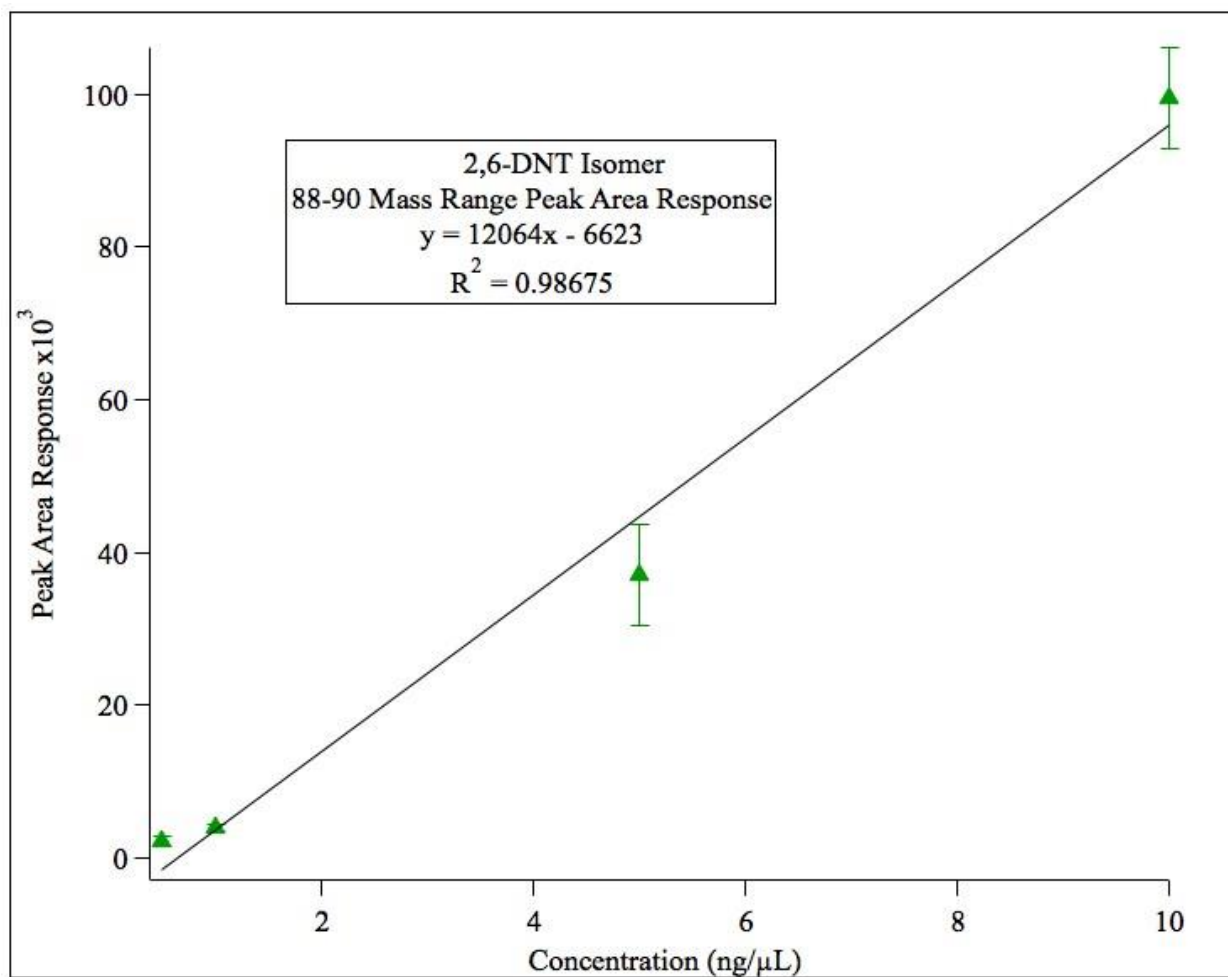


Figure 48: 2,6-DNT Isomer calibration curve used to calculate ratios for determining selectivity in Tables 10-13

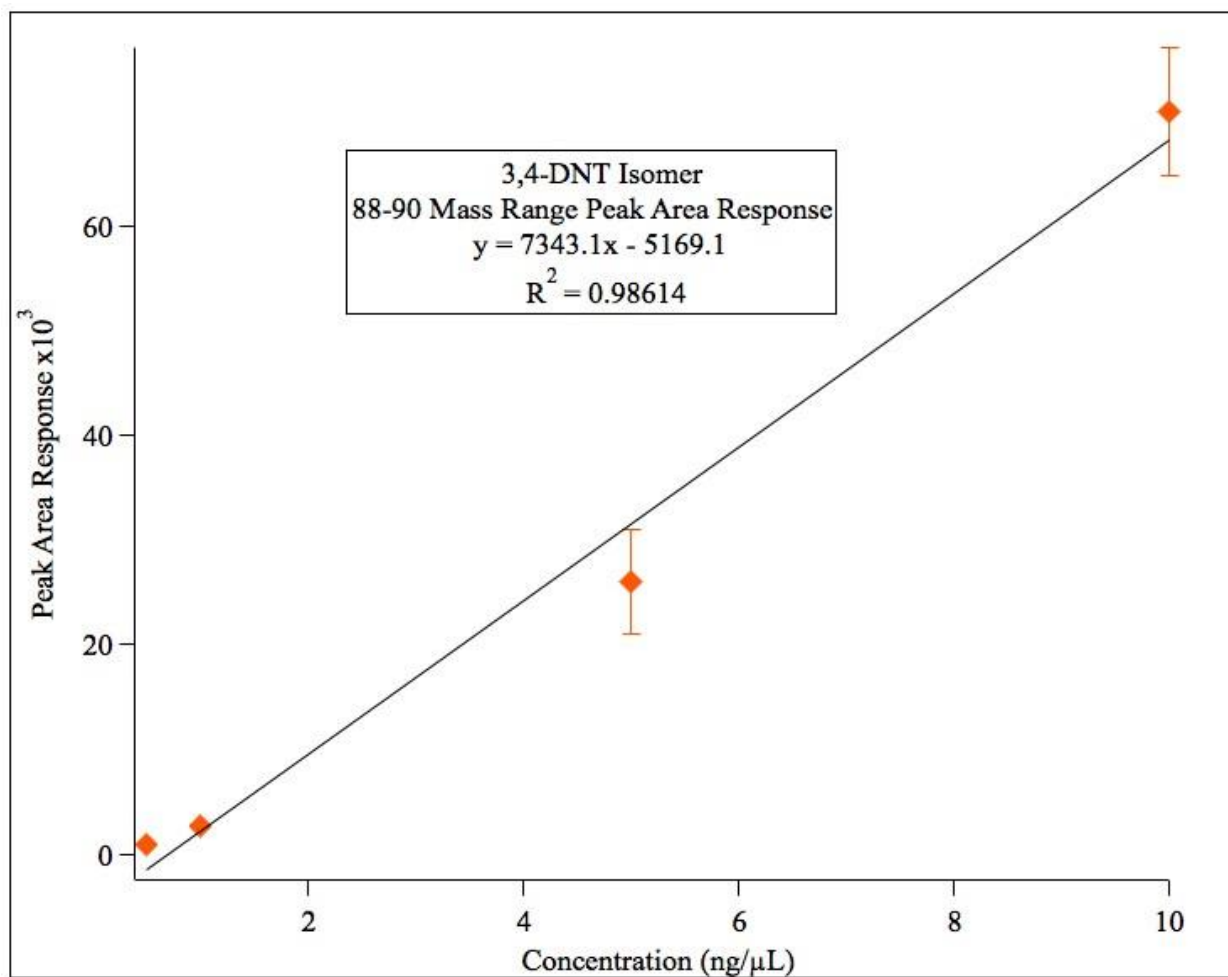


Figure 49: 3,4-DNT Isomer calibration curve used to calculate ratios for determining selectivity in Tables 10-13

## **APPENDIX C: STATISTICAL ANALYSIS FOR SELECTIVITY TABLES**

**Table 15: Data used to determine significance of selectivity across the templated and blank fibers**

2,4-DNT Template Fiber

Sampling	2,3-DNT isomer concentration (ng/uL)	2,4-DNT isomer concentration (ng/uL)	Selectivity Ratios of 2,4- DNT versus 2,3-DNT isomers
1	2.4	5.21	2.17
2	4.14	7.41	1.80
3	2.98	6.64	2.23
average	3.17	6.42	2.06
Standard Deviation	0.89	1.12	0.24

**Table 16: Data used to determine significance of selectivity across the templated and blank fibers**

2,6-DNT- $\alpha,\alpha,\alpha$ -d<sub>3</sub> Template Fiber

Sampling	2,3-DNT isomer concentration (ng/uL)	2,4-DNT isomer concentration (ng/uL)	Selectivity Ratios of 2,4-DNT versus 2,3- DNT isomers
1	8.23	13.34	1.62
2	8.54	13.37	1.57
3	7.60	11.66	1.53
average	8.12	12.79	1.57
Standard Deviation	0.48	0.98	0.04

**Table 17: Data used to determine significance of selectivity across the templated and blank fibers**

Blank Fiber			
Sampling	2,3-DNT isomer concentration (ng/uL)	2,4-DNT isomer concentration (ng/uL)	Selectivity Ratios of 2,4-DNT versus 2,3- DNT isomers
1	4.42	8.26	1.87
2	11.89	17.64	1.48
3	3.70	7.36	1.99
average	6.67	11.09	1.78
Standard Deviation	4.53	5.69	0.26

**Table 18: Dixon's Q test performed on second sampling of blank fiber to determine if its values are considered outliers**

Dixon's Q Test (Determining whether blank fiber second sampling is an outlier)

Isomer	135 m/z	119 m/z	89 m/z
2,3-DNT	0.912087912		
2,4-DNT		0.912451362	
2,6-DNT			0.886307054
3,4-DNT			0.899728997

Q critical = 0.94 for 90% CL (for 3 replicates)



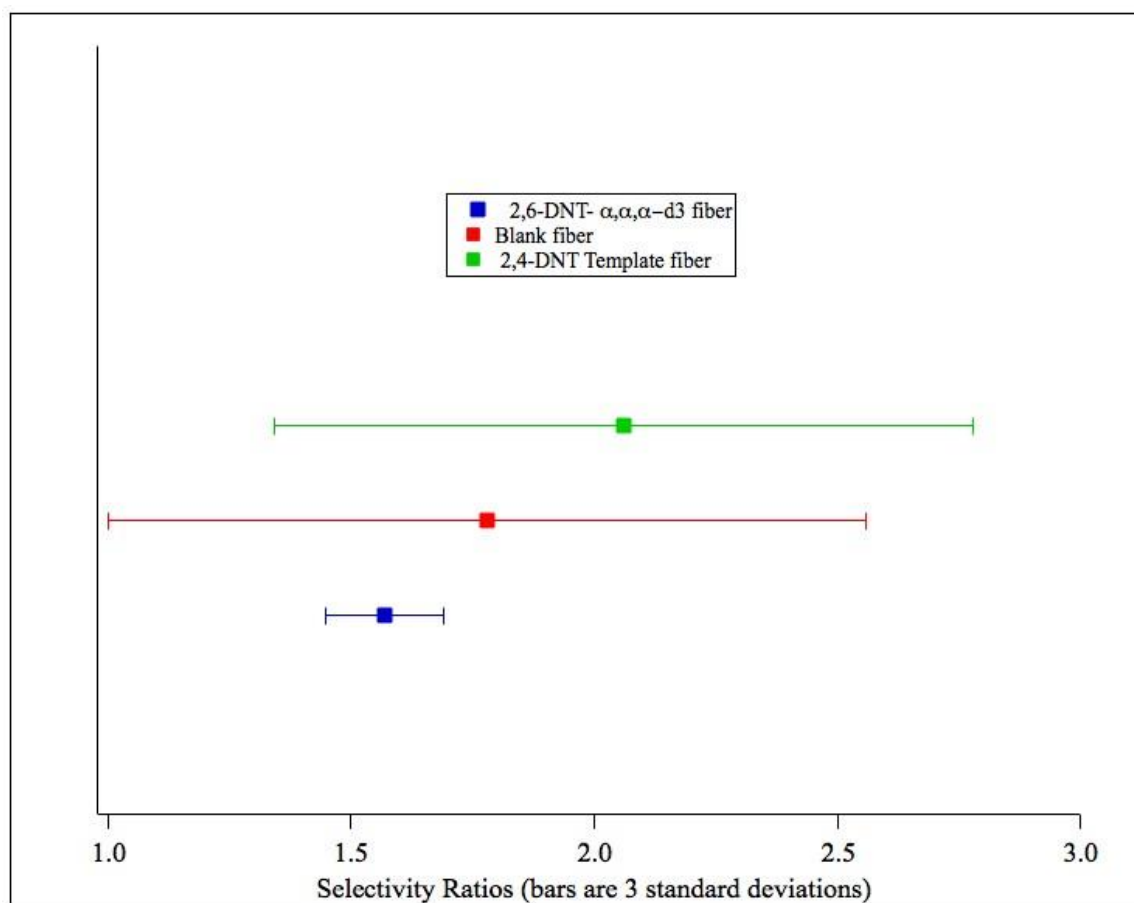


Figure 50: Comparison of the average selectivity of isomers 2,4-DNT versus 2,3-DNT for each fiber

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