A REVIEW ON THE ADVANCEMENTS IN THE FIELD OF MERCAPTURIC ACIDS AND THE DEVELOPMENT OF AN ULTRA-HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY HIGH-RESOLUTION MASS SPECTROMETRY APPROACH FOR PROFILING URINARY MERCAPTURIC ACIDS

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ABSTRACT

The exposure to mercapturic acid precursors contributes to several reported human health effects. These electrophilic highly reactive precursors undergo several metabolic steps for detoxification and excretion as a mercapturic acid. Liquid chromatography coupled with high-resolution mass spectrometry is a technique recently used for the screening of these metabolites in biofluids since it allows detection with high confidence and mass accuracy. In this work, a novel UHPLC-HRMS nanoflow method was developed aiming to screen for mercapturic acids using a data-dependent untargeted approach, to combine the detection of known MAs with the potential identification of previously unknown ones. The method was optimized using a standard mixture of MAs typically found to be elevated in cigarette smokers and it was tested on urine samples from smokers and nonsmokers. The analysis allowed the detection of 316 putative MA signatures and differences in the profiles of MAs were observed when comparing the two groups.

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Abbreviations		Definition
i.	AAMA	N-acetyl-S-(2-carbamoylethyl)-L-cysteine
ii.	Acetyl-CoA	Acetyl coenzyme A
iii.	ACN	Acetonitrile
iv.	CEMA	N-acetyl-S-(2-cyanoethyl)-L-cysteine
v.	CNL	Constant Neutral Loss
vi.	CYP450	Cytochrome P450
vii.	DHBMA	N-acetyl-S-(3,4-dihydroxybutyl)-L-cysteine
viii.	ESI	Electrospray Ionization
ix.	FA	Formic Acid
x.	GC	Gas Chromatography
xi.	GGT	γ-glutamyl transpeptidase
xii.	GSH	Glutathione
xiii.	GST	Glutathione-S-transferase
xiv.	HMPMA	N-acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine
xv.	HPMA	N-acetyl-S-(hydroxypropyl)-L-cysteine
xvi.	HRMS	High Resolution Mass Spectrometry
xvii.	IS	Internal Standard
xviii.	LC	Liquid Chromatography
xix.	MA	Mercapturic Acid
xx.	MAX	Mixed-Mode Anion Exchange
xxi.	MeOH	Methanol
xxii.	MHBMA	N-acetyl-S-(2-hydroxy-3-buten-1-yl)-L-cysteine
xxiii.	MS	Mass Spectrometry
xxiv.	NH_4OAc	Ammonium Acetate
xxv.	PAH	Polycyclic Aromatic Hydrocarbon
xxvi.	SBMA	N-acetyl-S-benzyl-L-cysteine
xxvii.	SPE	Solid-Phase Extraction
xxviii.	SPMA	N-acetyl-S-(phenyl)-L-cysteine
xxix.	TIC	Total Ion Chromatogram
xxx.	UDP	Uracil-diphosphate
xxxi.	UHPLC	Ultra-High-Performance Liquid Chromatography
xxxii.	VOC	Volatile Organic Compounds
xxxiii.	XIC	Extracted Ion Chromatogram

1. INTRODUCTION

Mercapturic acid excretion in urine was initially observed in 1879 when researchers Baumann and Presse administered bromo and chloro-benzene to dogs. The discovery was confirmed when both S-(bromophenyl) and S-(chlorophenyl)-mercapturic acid were identified and isolated from the urine of the dogs exposed to the halogenated benzenes [6]. Follow-up research by Jaffé the same year showed that iodobenzene could also be metabolized and excreted as a mercapturic acid [37]. At this time there was much about mercapturic acids that was still unknown, however, a similar moiety was being observed in the structure of mercapturic acids that have been discovered thus far. It was not until about 5 years later in 1884 when Baumann coined the term acetylcysteine conjugates for mercapturic acids [5]. It was observed that all mercapturic acids share this N-acetylcysteine portion, and it was hypothesized that the last step before excretion is acetylation. Since their discovery in 1879, mercapturic acids have been known to be correlated with exposure to benzene and its toxic derivatives. After these discoveries there was not much breakthrough in the world of mercapturic acids until 1934, when researchers Bourne and Young successfully isolated 1-α-napthylmercapturic acid from the urine of rabbits exposed to naphthalene [9]. This was very important in the field of mercapturic acids because up to this point excretion of mercapturic acids was only attributed to benzenes, but now 1-α-napthylmercapturic acid was observed in the urine of rabbits exposed to the aromatic hydrocarbon, naphthalene. More specifically naphthalene is part of the polycyclic aromatic hydrocarbon (PAH) group of compounds which are compounds that naturally occur in fossil fuels. PAHs are very prevalent in today's research with the more recent discoveries of their harmful effects on the environment and the human body [63]. Until the mercapturic acid pathway was discovered only a few benzene derivatives and naphthalene showed evidence of mercapturic acid excretion. However, as researchers unraveled the workings of the mercapturic acid pathway, more mercapturic acid precursors were discovered originating from occupational, environmental, or diet-based exposure sources.

It was not until 1939, about 60 years after the initial discovery of mercapturic acids, when researchers proposed a mechanism for the acetylation that occurs before excretion [76]. And a year later, in 1940, Stekol presented some of the first indirect evidence on glutathione (GSH) being a key step in the metabolic pathway by feeding rats S-benzylglutathione and observing N-acetyl-S-benzyl-1-cysteine (S-benzylmercapturic acid) in the urine of the exposed rats [77]. Researchers at the University of Birmingham in 1959 presented the first direct evidence of the role of glutathione by showing the decrease in glutathione levels in the liver when administered the mercapturic acid precursors [3]. Two years later in 1961 Booth and other researchers described glutathione conjugation and the enzymatic role of glutathione-S-transferases (GST) in the formation of glutathione-S-conjugates in isolated rat liver preparations [8]. γ-glutamyltransferase (γ-glutamyl transpeptidase/GGT) was isolated and its mechanistic activity was shown in 1964 by researchers Orlowski and Meister [58]. They observed that when a glutathione-S-derivative conjugate substrate is administered to hogs' it can act as a donor of the γ-glutamyl when it is cleaved by GGT producing a cysteinyl-S-glycine conjugate [58]. There was very little known about the metabolic fate of the cysteinyl-S-glycine conjugates, until 1981 when Kozak and Tate discovered that dipeptidases could hydrolyze the cysteinyl-S-glycine conjugates producing cysteine-S-conjugates [44]. The last step of the mercapturic acid pathway was already theorized very early due to the presence of the N-acetyl group on all mercapturic acids, but it wasn't until 1969 when Barnsley and others detected acetyltransferase activity in the liver of rats exposed to known cysteine-S-conjugates [4].

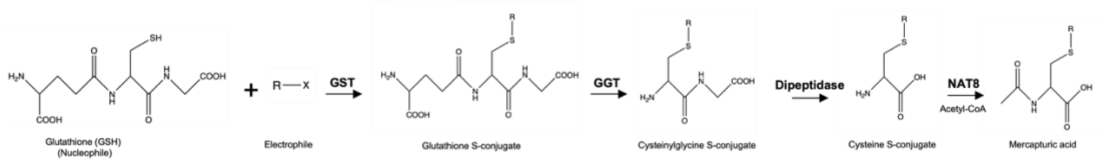


Figure 1. The four main steps in the mercapturic acid pathway. **(STEP 1)** Glutathione conjugation with electrophilic substrate catalyzed by glutathione S-transferase (GSH) **(STEP 2)** γ-glutamyltransferase (GGT) cleavage of glutathione S-conjugate **(STEP 3)** Removal of glycine from cysteinylglycine S-conjugate by dipeptidase hydrolysis **(STEP 4)** Acetylation of cysteine S-conjugate with Acetyl-CoA catlayzed by N-acetyltransferase 8 (NAT8) to form a mercapturic acid.

The mercapturic acid pathway involves four steps that electrophilic compounds follow to be detoxified into a mercapturic acid (**Figure 1**). Although some precursor compounds follow some unique additional steps either before or after the mercapturic acid pathway steps, the mercapturic acid pathway heavily revolves around glutathione conjugation [32]. For glutathione conjugation to take place the precursor compound must possess an electrophilic center to allow the strong nucleophile, glutathione, to conjugate with the toxic precursor compound, the electrophile [20]. A lot of the compounds that follow this pathway are already electrophilic enough to undergo GSH conjugation directly but in some cases, there needs to be an additional step prior to the GSH conjugation [32,20]. For example, benzene lacks an electrophilic center and must undergo oxidation by phase I CYP enzymes, in the case of benzene it is CYP450 which oxidizes benzene to form benzene oxidase which is electrophilic enough for attack by GSH [20]. Some precursor compounds even have the chance to biotransform into different mercapturic acids, for example, acrylamide and acrylonitrile are both electrophilic enough to undergo GSH conjugation directly to start the mercapturic acid pathway producing N-acetyl-S-(2-carbamoyl ethyl)-L-cysteine (AAMA) and N-acetyl-S-(2-cyanoethyl)-L-cysteine (CEMA) respectively. Although the direct GSH conjugation is the primary route, acrylamide and acrylonitrile can also under oxidation by phase I

CYP enzymes to generate their epoxide forms which then follow the mercapturic acid pathway to generate mercapturic acids structurally different than AAMA and CEMA [20]. Acrolein is another xenobiotic that can generate different mercapturic acids. Acrolein is already electron-deficient and can be directly conjugated to GSH to start the mercapturic acid pathway. However, acrolein produces S-(3-oxopropyl)-N-acetylcysteine (OPMA) but this mercapturic acid is not excreted but rather is introduced to one of two enzymes: aldehyde dehydrogenase to form CEMA or aldo-keto reductase to form 3-hydroxypropylmercapturic acid (3-HPMA) [20]. There are times when mercapturic acids are formed by different reactions, but the importance is they all follow a similar pathway that generates the mercapturic acid structure when excreted. As mentioned, these highly reactive electrophilic compounds follow four steps that are involved in the metabolic processing of all mercapturic acids (**Figure 1**). These four steps produce the N-acetylcysteine portion that is present in all mercapturic acids and is a moiety that has been crucial in discovery mercapturic acid profiling studies.

The four steps are as follows (**Figure 1**): the first step is phase II enzymatic GSH conjugation by the enzyme glutathione-S-transferase (GST) which forms a thioether bond between GSH and the electrophile (toxicant) producing a glutathione-S-conjugate [32]. Glutathione conjugation is the most important step of the pathway as the glutathione is the source of the cysteine (sulfur atom) portion of mercapturic acids and occurs in the liver while other steps occur in the small intestine and kidney [2,20,32,35]. The second step involves the enzyme γ-glutamyltransferase (GGT) cleaving glutamate from the glutathione-S-conjugate to convert it to a cysteinylglycine-S-conjugate [32]. The third step involves enzymes known as peptidases which hydrolyze the cysteinylglycine-S-conjugate removing the glycine to form a cysteine-S-conjugate [32]. The peptidase involved in the third step is either aminopeptidase N (APN), cysteinyl glycine peptidase, or leucyl aminopeptidase [20]. The fourth and last step in the mercapturic acid pathway involves the enzyme N-acetyltransferase 8 (NAT8) that catalyzes the reaction between cysteine-S-conjugate and acetyl-CoA to form N-acetyl-L-cysteine-S-conjugate (mercapturic acid) [20,32]. It is important to note that after the cysteine-S-conjugate is formed, there is potential for the intermediate to not be acetylated but rather undergo separate pathways. Those separate pathways are the thiomethyl shunt and glucuronidation. For these pathways to be more favorable the cysteine-S-conjugate needs a good leaving group to be converted by cysteine-S-conjugate β-lysases to pyruvate, ammonium, and a mercaptan derivative of the mercapturic acid precursor [32]. The mercaptan can either be converted into a thiomethylated compound by the enzyme known as thiomethyltransferase which can be excreted intact, or following oxidation can be S-glucuronidated by the enzyme known as UDP-glucuronosyltransferases to form glucuronyl-S-conjugate that can be readily excreted [32]. Studies have shown that acetylation of the cysteine-

S-conjugates is the preferred route of excretion, but it is noted that the thiomethyl shunt and glucuronidation also play a cooperative role with the mercapturic acid pathway for the elimination of mercapturic acids [32].

This research focuses on a specific class of biomarkers that are excreted in urine when an individual is exposed to highly reactive electrophiles from volatile organic compounds (VOCs) as “exposure to VOCs is pervasive and has been a topic of concern, due to the mutagenic, neurotoxic, genotoxic, and carcinogenic potentials of these chemicals” [48]. Research has been done validating these specific mercapturic acids for biomarkers of tobacco exposure as studies have showed increased levels of MAs produced when exposed to VOCs such as ethylene oxide, acrolein, crotonaldehyde, butadiene, benzene, acrylonitrile, and acrylamide in the urine of smokers and researchers report a significant decrease in urinary MA levels after an individual has ceased smoking [14,17]. Mercapturic acids have been heavily studied in the context of profiling exposure from smoking cigarettes and according to the CDC and the International Agency for Research on Cancer (IARC) 9 out of 10 lung cancer cases are linked to cigarette usage but not all smokers end up developing lung cancer [36,57,63]. This leads to questions such as what factors promote lung cancer development in an individual and is there any way to predict an individual's chances of developing lung cancer from smoking. There are current risk assessment indicators such as family history of cancer, how much and how long you have smoked, and age which provide decent understandings about your risk of lung cancer; however, these types of measures will not provide enough information to determine a person’s susceptibility. Nowadays the discovery and use of tobacco exposure biomarkers have a multitude of uses from evaluating type of exposure, length of exposure, and “assessing new and modified risk tobacco products and identifying and evaluating potential product standards” [17]. Although one of the most studied exposure sources is from tobacco smoke, individuals can be readily exposed to the VOCs that produce mercapturic acids as they originate from several sources in your occupation, environment, and your diet. The standard mix that is used in our experiment contains six heavy labeled versions of mercapturic acids that are known to derive from the toxicants in tobacco smoke; they are D_3-CEMA, D_3-2-HPMA, D_3-3-HPMA, D_3-AAMA, D_3-HMPMA, & D_5-SPMA (**Figure 2**). This is the same standard mix that Hecht’s group uses [13,15], as these mercapturic acids have been observed in their studies and others being excreted more in the urine of people who smoke. The toxicant precursors they originate from are classified as carcinogens or possible carcinogens by the International Agency for Research on Cancer ranging from group 1 to group 2B; benzene is classified as carcinogenic group 1 and propylene oxide as possibly carcinogenic group 2B [38]. There are many exposure sources in environment, occupation, and diet for each of these toxicants besides just tobacco smoke (**Table 1**), for example, acrylonitrile is widely used in industrial manufacturing of acrylic, fibers, adhesives, and nitrile rubber products [19,34,53,57]. It is important to note that exposure to occupational toxicants can occur by just living near the site of production through air pollutants and drinking water. While acrolein, crotonaldehyde, and benzene are all found in our environment as they originate from the breakdown of fossil fuels [53,57]. Acrylamide is one that you will find in food sources at higher levels due to its release upon heating carbohydrate rich foods, the FDA reports that acrylamide is found in 40% of the calories in an average American diet [85].

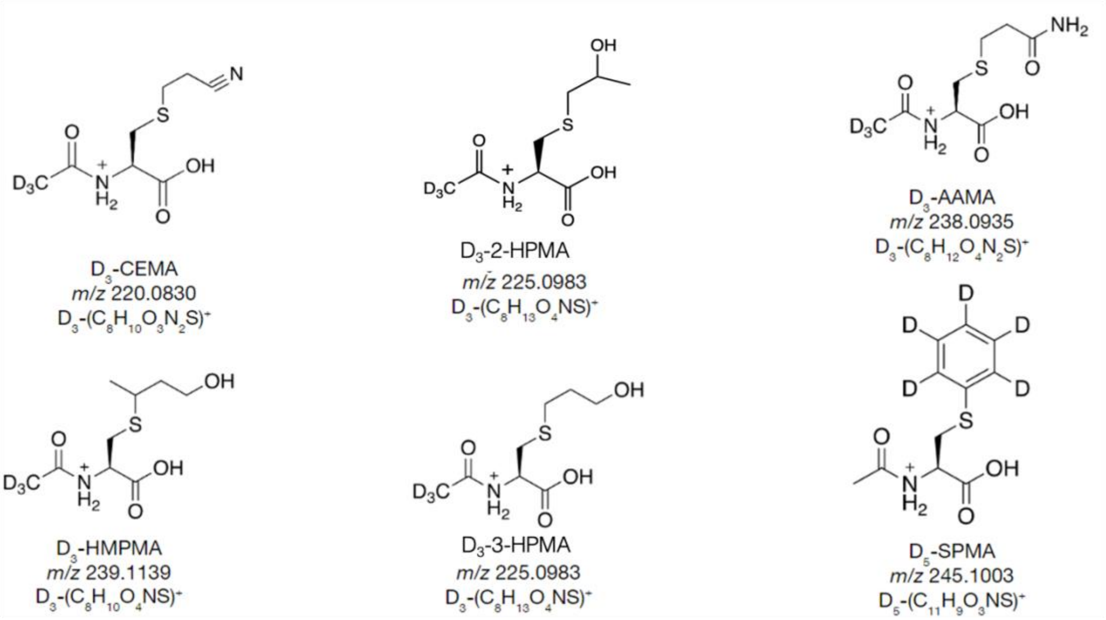
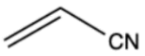
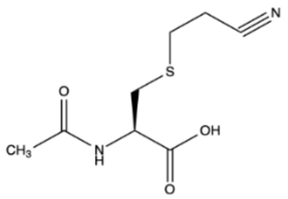
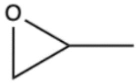
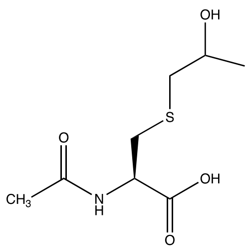
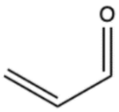
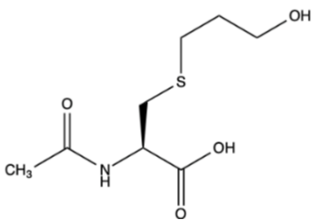
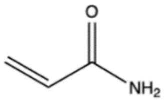
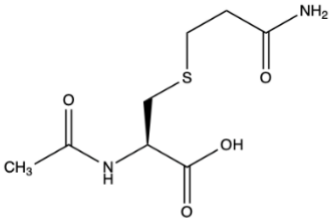
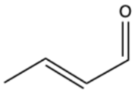
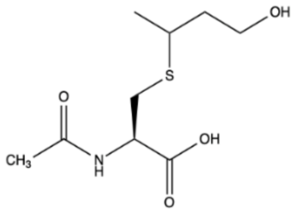
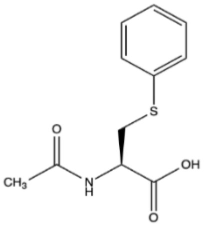


Figure 2. Structures of the internal standards used in our positive ion mode method: D_3-CEMA, D_3-2-HPMA, D_3-3-HPMA, D_3-AAMA, D_3-HMPMA, & D_5-SPMA.

With numerous exposure sources for the many toxicants that produce mercapturic acids it is important that there is an accurate way to measure an individual's level of exposure to really get an understanding of a person's mercapturome and a deeper understanding on the biotransformation of these mercapturic acids. Since their discovery many highly reactive electrophilic compounds have been shown to be mercapturic acid precursors and are usually found to be toxicants and/or cancer-causing agents. With so many mercapturic acid precursors it is important to refine the detection methods for mercapturic acids in effort to develop a method that can effectively scan for all mercapturic acids in a sample, essentially to one day map out the complete mercapturome. Mercapturome profiling is important to gain a better understanding of the detoxification and enzymatic processes that play a role in mercapturic acid production, ultimately giving insight into an individual's response to toxic substances and utilize the information to understand the strength and toxicity of the exposure to aid in safety and risk assessment studies.

Table 1. The structures of the precursor compounds and their respective mercapturic acid metabolites. These six mercapturic acids are the light versions of the heavy labeled mercapturic acids used in our internal standard mix. Including the common exposure sources for each precursor compound [34,53,57].

Precursor Compound	Mercapturic Acid	Exposure Source(s)
Acrylonitrile 	 CEMA = N-Acetyl-S-(2-cyanoethyl)-L-cysteine	Industrial component for acrylic fibers, plastics, nitrile rubber, latex products, coatings, and adhesives. Tobacco smoke.
Propylene Oxide 	 2-HPMA = N-Acetyl-S-(2-hydroxypropyl)-L-cysteine	Component for polyurethane foams. Used to produce propylene glycol, that is used in e-cigarette liquid. Used as an to produce polyether polyols, propylene glycols and propylene glycol ethers that are used in several industrial settings. Tobacco smoke.
Acrolein 	 3-HPMA = N-Acetyl-S-(3-hydroxypropyl)-L-cysteine	Used in agriculture products like biocide. Produced during the breakdown/burning of pollutants & organic matter. Found in some foods such as oils, coffee, and fried foods.

		Used in military gas production and gas masks. Tobacco smoke.
Acrylamide 	 AAMA = N-acetyl-S-(2-carbamoylethyl)-L-cysteine	Susceptible to enter drinking water during treatment process and dam construction. Industrial component used in plastics and dyes. Tobacco smoke. Produced during the cooking of foods that are high in carbohydrates.
Crotonaldehyde 	 HMPMA = N-Acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine	Industrial component used in the production of some cotton, plastic, and paper products. Used to produce the food preservative known as sorbic acid. Found in some cooking oils. Tobacco smoke and automobile exhaust.
Benzene 	 SPMA = N-Acetyl-S-(phenyl)-L-cysteine	Produced from burning coal, oil, gas car exhausts, industrial solvents, and during the evaporation of gasoline. Tobacco smoke is a huge source for benzene exposure accounting for nearly half of national exposure to benzene. Susceptible to entering the drinking water.

2. Review on Mass Spectrometry Methods for Mercapturic Acid Detection

Mass spectrometry has been used for studies involving mercapturic acids for years but many of the analyses target one or a few MAs which fail to provide a full picture of the mercapturome. However, as technology advances it is becoming easier to detect multiple mercapturic acids simultaneously. Traditionally, methods have used urine but sometimes blood or plasma and have focused on only a few MAs at a time, but that improvement of technology has pushed now to develop untargeted screening techniques. The development of a MS screening method for the detection of mercapturic acids over the years has been built on the use of standards and a unique characteristic neutral loss to aid in the detection of and evaluate the levels of mercapturic acids in an individual. Researchers have been able to use mercapturic acids as biomarkers due to their unique structures and similar fragmentation. Historically, mercapturic acid discovery studies employ the use of the "N-acetylcysteine" constant neutral loss (CNL) fraction as a critical part of discovery and quantitative analyses (**Figure 3**). Some more recent studies strongly suggest using additional fragmentation patterns to help when discovering mercapturic acids. The terminology is misleading when referring to the N-acetylcysteine CNL. The fragment does not contain sulfur, rather it is a feature of the N-acetyl-L-cysteine structure, and the literature often calls it the "N-acetylcysteine" fragment due to lack of a better term.

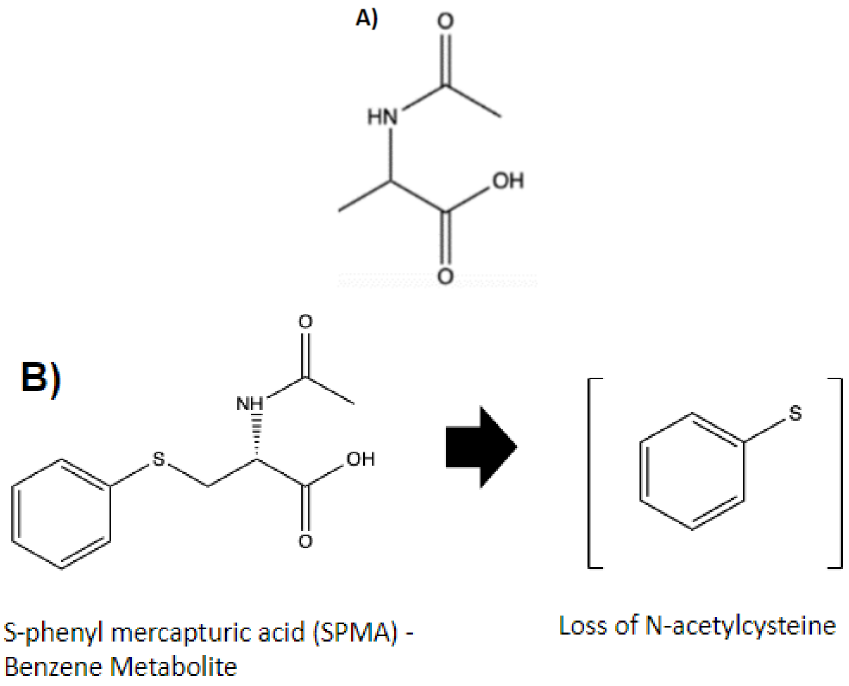


Figure 3. (**A)** The N-acetylcysteine constant neutral loss fragment reported corresponds with this structure. **(B)** The fragmentation of SPMA when losing the N-acetylcysteine moiety.

The first publication presenting the use of this N-acetylcysteine CNL for detection of glutathione conjugates was in 1988 using positive fast atom bombardment ionization and tandem mass spectrometry to combine constant neutral loss scans and daughter ion scanning, which researchers concluded was useful for the detection and identification of mercapturic acids in rat bile [33]. In 1993 Jones and others utilized gas chromatography/electron capture mass spectrometry with positive FAB ionization and a separate method using negative FAB ionization for the analysis of mercapturic acid conjugates of products of lipid peroxidation in rat urine samples [41]. After testing both negative and positive ionization modes, researchers concluded that one of the reasons why negative ionization was performing better is that positive mode seemed to be diminishing sensitivity and [M-H] ions were all found yielding daughter ions via N-acetylcysteine CNL in negative mode but not in positive mode [41]. These two papers were the earliest studies employing unique fragmentation patterns of mercapturic acids to identify them. Fragmentation plays a key role in the discovery of mercapturic acids in tandem mass spectrometry (MS/MS) procedures. Aside from the normal losses researchers scan for e.g., loss of H_2O, COOH, CO_2, or other patterns that provide information about the structure of organic compounds. However, most of the studies involving the use of MAs as biomarkers look for daughter ion fragments and losses of fragments that are features of the N-acetyl-L-cysteine moiety.

Due to urine having the complex matrix the use of tandem mass spectrometry is of utmost importance to allow the accurate identification and quantification of different mercapturic acids. In a review on LC-MS methods for detection of mercapturic acids they report that “mercapturates are often minor metabolites, tandem (MS/MS) offers the greatest level of analytical sensitivity for analysis, and its application has expanded the utility of urinary mercapturates from biomarkers of exposure to indicators of toxicant metabolism, biotransformation and elimination” [54]. To expand our understanding on the best way to extract, collect, and scan for mercapturic acids, this thesis will describe the use of established methods and a novel untargeted screening approach to contribute to the research of comprehensively profiling urinary mercapturic acids.

This thesis includes a table listing several previous methods for the determination of mercapturic acids as biomarkers of occupational, environmental, and dietary exposures to harmful toxicants (**Appendix Table 2)**. With the numerous studies on mercapturic acids as biomarkers for exposure there has yet to be one specific method that outperforms the rest. Past reviews have detailed summaries of detection methods for single or a few mercapturic acids, however, expanding the mercapturome is becoming more possible. It is very important for assessing human exposure to electrophilic agents because "In theory, an unlimited number of mercapturic acids can be synthesized by the human body. Many products, both endogenous and exogenous, can be partly conjugated with glutathione during their biotransformation processes, producing mercapturic acids" [22]. Successful sample preparation techniques are critical for the determination of mercapturic acids by mass spectrometry as the normal sample type used in this area of research is urine. As mentioned, the urine matrix is complex as it contains many interfering compounds from electrolytes, metabolites from diet & medications, proteins, and salts that negatively impact the detection of our compounds of interest. There are a few commonly used sample preparation techniques reported on such as dilute and shoot, protein precipitation, and solid phase extraction. Solid phase extraction more recently seems to be the most efficacious technique for metabolite extraction from urine samples but even less rigorous clean up techniques are still being utilized, especially in targeted work [54]. Methods for the analysis of mercapturic acids seem to vary slightly from paper to paper but there is a clear trend of using reversed-phase LC-MS equipped with electrospray ionization in negative mode and very few published methods involving positive ion mode.

An earlier mentioned paper that tested both positive and negative ionization modes concluded that negative mode performed better since the N-acetylcysteine CNL wasn't observed for all compounds in positive mode [41]. As mentioned, most of the literature contains analyses using negative ion mode due to its observed superiority in MA research and when researchers Scholz and others assessed both ionization modes their results were similar using rat urine samples [71]. Scholz and researchers employed constant neutral loss combined with enhanced product-ion scans on a linear ion trap mass spectrometer to screen for MAs using both positive and negative ionization modes [71]. They too found that the N-acetylcysteine CNL for some of their MA standards was not observed in positive ion mode. There is supporting evidence, presented even in very early papers, which suggests negative ion mode is superior for MA identification by diagnostic MS^2 loss of N-acetylcysteine, meaning a possible limitation to our study is running in positive ion mode.

It has been observed that untargeted approaches focus on screening for the N-acetylcysteine moiety CNL to detect and identify MAs. Unfortunately, it has been previously described and is shown in our experiments that not all MAs undergo a loss of the N-acetylcysteine fragment and screening for only one CNL may also lead to false positives. As the research has continued untargeted approaches combine characteristic fragment screening with database searches and comparison between test and control groups (such as smokers & non-smokers) to increase the detection accuracy and identification of new MAs. The field of untargeted MA analysis is still in its early stages as there is a lack of a database containing all identified MAs and there is a limited number of untargeted approaches in the literature. Most of the reported studies on MAs involves targeted and lower resolution methods to either assess the efficacy of internal standards, or to evaluate known MAs in a group of people who are at high risk of exposure to highly reactive chemicals in populations such as smokers or workers at specific occupations like factories or gas stations.

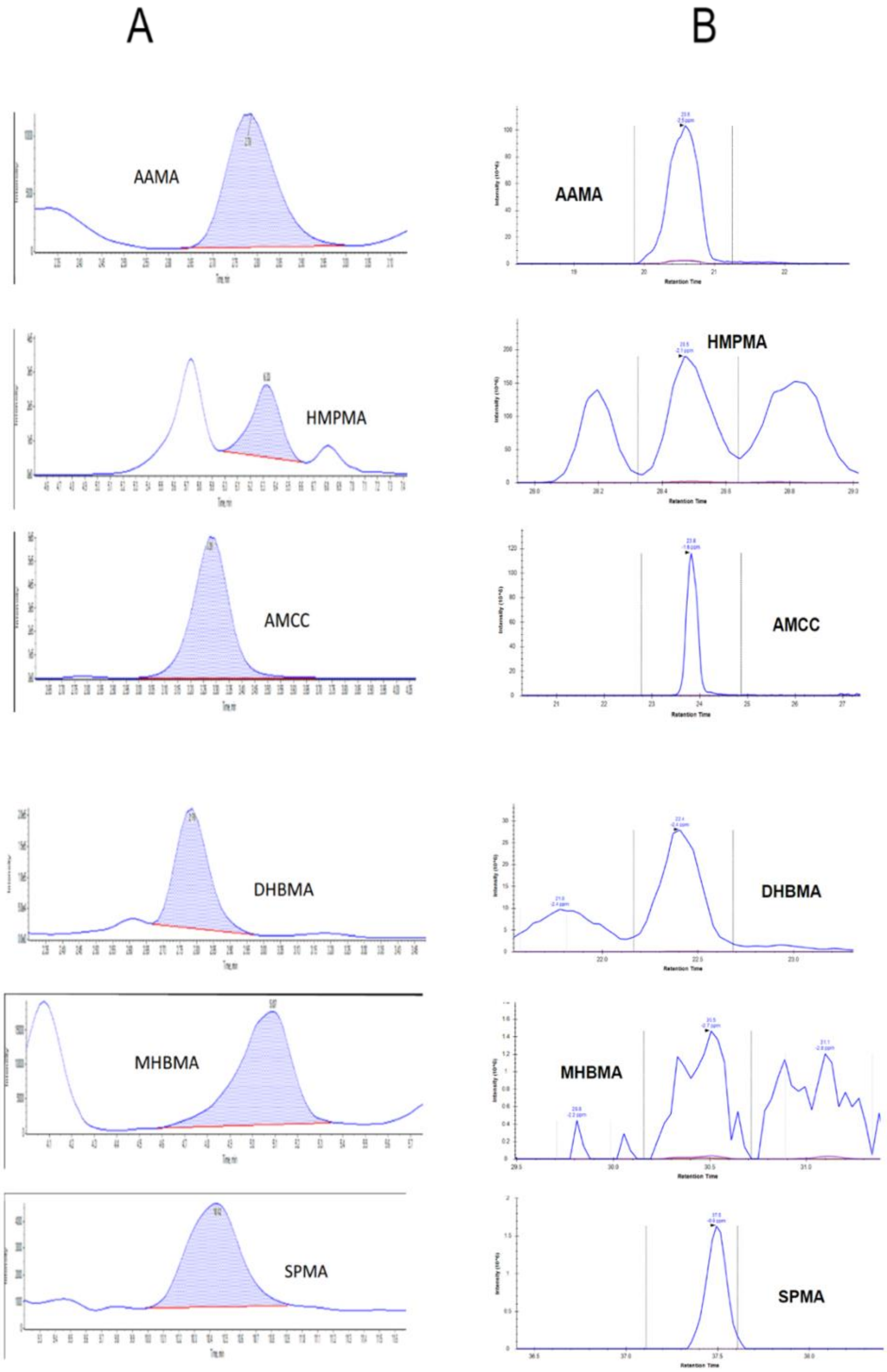
Liquid chromatography has been the most prevalent separation technique used in mercapturic acid analysis however gas chromatography is also utilized but is reported on much less. Researchers published a method in 1995 for the sensitive determination of the two mercapturic acid metabolites of benzene and toluene (SPMA & SBMA) using GC [25]. Researchers also specified they used human urine instead of human blood “due to short half-lives of VOCs in blood and volatility of the compounds, causing possible contamination of the sample from ambient air or loss of analytes during storage. Determination of metabolites of VOCs excreted with urine avoids these disadvantages” [25]. The importance of this research was that they compared their newly developed GC method with a past HPLC method to evaluate the advantages and disadvantages of each method. According to their results the concentrations of S-phenylmercapturic acid and S-benzylmercapturic acid (SBMA) were fairly similar in both methods, however, the detection limit was about 6 times higher for the HPLC method [25]. Overall, during the time of the experiment the GC method was observed to have higher reproducibility and higher sensitivity in comparison to their HPLC method [25]. These results were published over 20 years ago and only targeted two mercapturic acid metabolites but effectively provided information on different chromatographic techniques. One of the reasons why GC appears less in mercapturic acid studies is that it requires derivatization to improve chromatographic separation and peak shape, which adds more time to the sample preparation, which is already the rate-limiting step in mercapturic acid analysis. However, to say one chromatographic technique is better than the other would be deceiving as GC has observed performing better in studies targeting one or a few metabolites, such as this one, where the detection of benzene and toluene MA metabolites was superior using GC versus HPLC. Nowadays, LC is one of the most used techniques for metabolite screening due to its ability to discover and quantify a broader range of compounds in a sample when compared to GC. With this in mind LC is the technique that would be best suited for untargeted work aiming at discovery and global profiling studies as the physiochemical properties of the numerous mercapturic acids vary and LC can separate compounds from a wide range of polarities [62].

There is an assortment of sample preparation techniques used for the extraction of mercapturic acids, but it depends on the sensitivity and type of analytical method. However, the acidification of the urine prior to sample clean-up is used frequently regardless of the type of sample preparation technique. A study analyzing 18 urinary mercapturic acids by two high-throughput multiplex-LC-MS methods concluded that acidification of the urine samples is critical for the detection of SPMA, and the acidic conditions allow pre-SPMA to convert to SPMA [62]. For our assay and many published methods, an acid such as HCl is used for acidification of the urine prior to analysis as it has been shown to increase the recovery of specific mercapturic acids, such as SPMA. Also, due to the large range of polarities of mercapturic acids they found success in splitting the assay into two parts and reported that peak separation and sensitivity was better when the assay was split in two [62]. This is like the initial method used for the workup of our urine samples where there are two sample sets containing different mercapturic acids [13,15]. For our initial fractionated sample work up two fractions were collected, one fraction collected with 30% MeOH, 2% FA containing CEMA, 2-HPMA, 3-HPMA, AAMA, and HMPMA and the second fraction was collected with 90% MeOH, 2% FA containing SPMA [13,15]. The work they published addressed some of the challenges when screening for metabolites with different physiochemical properties and their discussion highlights the importance of developing a sample prep method that caters to mercapturic acid extraction from urine and an LC-MS method that will allow for good peak separation and recovery for the analysis of a large set of MAs.

Mathias and B’hymer published a review in 2016 describing previously used LC-MS methods for mercapturic acid analysis [54]. Their review highlights the need to progress this research as the simultaneous detection of multiple mercapturic acids is still in its infancy as most of the methods described included only a small number of target mercapturic acids. Their review also highlights that there has yet to be a consensus for what method works best for mercapturic acid screening. Numerous papers use a multitude of techniques that have shown success in detecting mercapturic acids. However, the future of this field is moving towards employing high resolution mass spectrometry as there is a shared theory in the field that it would improve identification and quantitation of minor metabolites making analyses less difficult.

A study involving the urine of rats’ fed diets to induce oxidative stress was analyzed by UPLC coupled with HRMS with a negative ESI source and is important as it is one of the few papers published recently using a high-resolution mass spectrometry technique [38]. Researchers observed that HRMS yielded better results compared to traditional untargeted metabolomics approaches they have used in the past [38]. The neutral loss of 129 Da (N-acetylcysteine) fragmentation pattern was observed in seven mercapturic acids in their study originating from lipid peroxidation products. However, they also point out that if studies only use one characteristic fragment for mercapturic acid analysis and subsequent processing of data could potentially lead to either false-positives or missing mercapturic acids that for an unknown reason are not yielding the N-acetylcysteine NL ion. They mention the use of MS^3 to gather more structural information on mercapturic acids but explain that weak signals from the N-acetylcysteine fragment ion were preventing MS^3 detection [38]. Researchers concluded that urinary mercapturic acids are successful for monitoring an individual's exposure to certain reactive electrophilic precursors and labeled the research as a proof of concept for mercapturic acid monitoring using untargeted HRMS ESI in negative mode to monitor the N-acetylcysteine characteristic neutral loss [38]. Researchers also concluded that using ESI in negative mode produced the most intense signals of the loss of N-acetylcysteine moiety and explained it was due to the carboxylic acid of the mercapturic acid ability to easily undergo deprotonation [38]. This paper further proves how mercapturic acid identification solely based on the N-acetylcysteine fragment loss can be a limitation for untargeted mercapturic acid profiling as the N-acetylcysteine NL was only observed in 7 mercapturic acids when they list many more mercapturic acids that are known oxidative stress biomarkers [38]. Nonetheless, this research only enriches the foundation for mercapturic acid screening methods as it highlights limitations and possible future directions involving HRMS in the field of metabolites and biomarkers.

A study using human urine samples from unexposed workers and gasoline station attendants to profile mercapturic acids of known occupational and environmental toxicants utilized hybrid triple HPLC-MS with negative ESI to separate the metabolites of interest [29]. You will notice that the peak shapes of the MAs detected in our work are similar, excluding MHBMA. Figure 10 shows that our method is successful in detecting the commonly reported mercapturic acids for occupational and environmental exposures and any decrease in intensity could be due to a deficiency in our untargeted DDA approach. There additionally seems to be a difficulty of separating the two HPMA isomers which is a common challenge in these analyses, our method appears to be more successful in HPMA isomer separation which is most likely due to our much longer runtime of 68 minutes compared to their runtime of 12 minutes (**Figure 10B**). Researchers mentioned one of the primary challenges was chromatographic separation of multiple mercapturic acids when using single chromatographic run and it especially hindered separation of the more polar mercapturic acids such as AAMA [29]. Our method, using urine from tobacco smokers, showed good chromatographic separation and peak shapes for 10 mercapturic acids that are also presented in their paper (**Figure 4**). Researchers reported the detection of 17 unique mercapturic acids using their method and report the detection of a mercapturic acid that has been rarely reported , S-(4-nitrophenyl)mercapturic acid (NANPC), “NANPC, a urinary metabolite of 4-chloronitrobenzene, which is a chlorinated nitroaromatic intermediate used in the production of drugs, pesticides, dyes, and antioxidants” [29].


A
B
AAMA
AAMA
HMPMA
HMPMA
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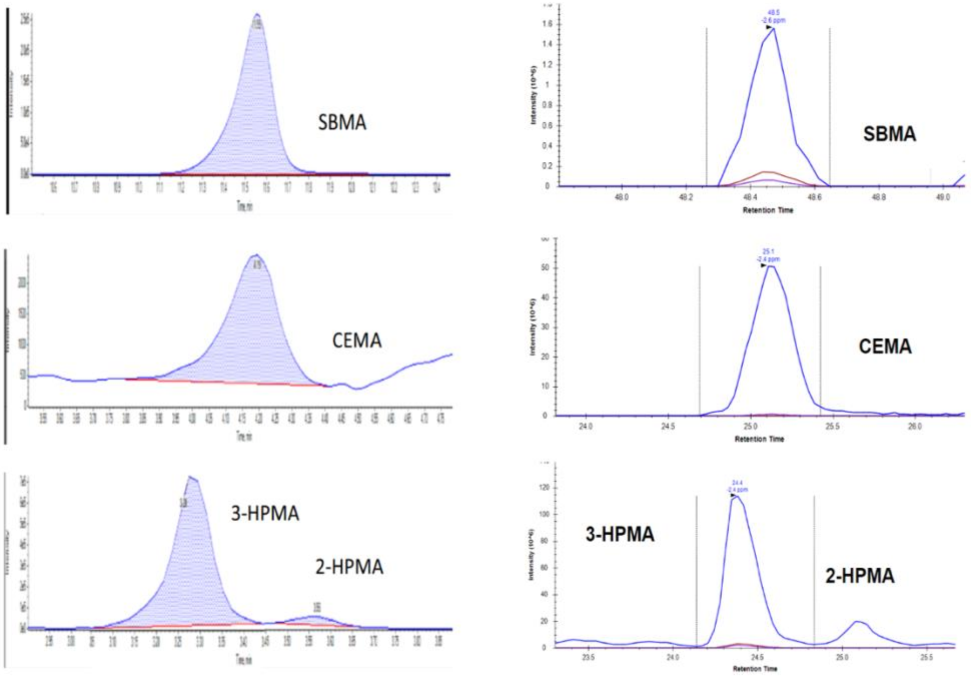


Figure 4. Extracted ion chromatogram traces from a reported method and our method which are presented as a proof of principle that our method can detect commonly reported mercapturic acids associated with environmental and occupational exposures. **(A)** Extracted ion chromatograms of 10 unique MAs from a 2019 paper aiming at profiling urinary mercapturic acids from urine samples of individuals working at a gas station using a LC-MS negative electrospray ionization method (SBMA, CEMA, 2-HPMA, 3-HPMA AAMA, HMPMA, AMCC, DHBMA, MHBMA, and SPMA) [29]. **(B)** Extracted ion chromatograms of the same 10 unique MAs detected using our LC-MS positive nanospray ionization method (SBMA, CEMA, 2-HPMA, 3-HPMA AAMA, HMPMA, AMCC, DHBMA, MHBMA, and SPMA.

Mercapturic acids are still being discovered from precursor compounds that are harmful to your health. Novel mercapturic acids have been discovered as biomarkers for exposure in the past decade, for example, in 2010 researchers identified S-(acetamidomethyl)mercapturic acid (AMMA) in human urine samples as a novel biomarker for the exposure to N,N-dimethylacetamide [65]. Another more recent example, in 2014 researchers identified 1,4-bis-(N-acetyl-L-cystein-S-yl)butane-2,3-diol (bis-BDMA) in rat urine samples as a novel biomarker for the metabolic activations of 1,3-butdiene to 1,2,3,4-diepoxybutane [46]. A 2022 study using urine samples collected from healthy children ages 0-7 children living in south and central China to assess their exposure to 21 metabolite VOCs that induce oxidative stress, 14 of them being mercapturic acids precursors [73]. The VOCs used in this study that produce mercapturic acids included acrolein, acrylamide, acrylonitrile, vinyl chloride, ethylene oxide, benzene, 1-

bromopropane, 1,3-butadiene, crotonaldehyde, N,N-dimethylformamide, methyl isocyanate, propylene oxide, toluene, and benzyl alcohol. The samples were run on an UPLC coupled triple quadrupole MS using ESI in negative mode [73]. Interestingly, the most abundant metabolite in their study was the crotonaldehyde metabolite, HMPMA, and the heavy-labeled version of HMPMA used in our studies was the best performing standard by far. Crotonaldehyde's magnitude of exposure sources is described coming from tobacco smoke, fossil fuels, some foodstuffs, endogenous lipid peroxidation, and "crotonaldehyde can also form in vivo as a metabolite of N-nitrosopyrrolidine and 1,3-butadiene" [73]. With many exposure sources researchers suggest that more research be done on crotonaldehyde as it "can cause oxidative damage, genetic mutations, lung injury, and hepatocellular carcinoma" [73]. Researchers also observed that DHMBA, the metabolite of 1,3-butadiene was the number 1 contributor to the increase of the oxidative stress biomarkers 8-hydroxy-2′-deoxyguanosine (8-OHdG) and 8-hydroxyguanosine (8-OHG) and concluded that research should consider "the importance of oxidative stress in the pathogenesis of various chronic diseases such as cancer, diabetes, and Alzheimer's disease, more studies are required to illustrate the potential role of oxidative stress in chronic diseases during long-term exposure to VOCs" [73]. They also added that since DHBMA was linked to an increase in levels of 8-OHdG and 8-OHG which are biomarkers for oxidative stress that causes DNA and RNA damage it should be listed as a priority VOC for the risk management of young children [73]. This paper which is only a year-old highlights that more studies are needed even for already known mercapturic acid precursor compounds like crotonaldehyde but also underlining that more research is needed on how potential important biomarkers, like DHBMA, should be monitored for health management.

Over the past two decades mercapturic acids analysis has seen major accomplishments of discovering novel mercapturic acids and developing methods for the increased detection of mercapturic acids. Currently there are numerous unique mercapturic acids that are known to derive from various compounds. The compounds that produce mercapturic acids when a person is exposed are still being studied and further studies on them are warranted to identify their direct effects on the human body and how the body detoxifies the compounds. Profiling studies are important to pursue in exposed and non-exposed groups to assess levels of excreted mercapturic acids and aid in developing a better understanding of what influences specific mercapturic acid excretion.

3. SIGNIFICANCE AND APPROACH

Screening for biomarkers is important because it can provide early detection of health problems by characterizing human exposure to occupational and environmental toxicants, which can lead to risk management and earlier treatment by identifying the toxins and other hazards that are harmful to human health. The measurement of mercapturic acids in a biological sample is also important for assessing toxicological effect and mechanism of action which can be used to assess multiple groups and identify metabolic imbalances gaining insight on specific detoxification processes. Exposure to MA precursors and the biological response of an individual has been investigated throughout the years and LC-MS has been the most popular analytical technique for that. With the development of HRMS in the recent years' researchers have agreed that employing the use of HRMS in untargeted work, like ours, could help with detection and discovery of MAs with high mass accuracy and has potential for screening for both known and unknown MAs. In this work, a highly sensitive LC-MS nanoflow method with a positive nanospray source was developed employing the use of UHPLC and HRMS with data-dependent acquisition aiming to screen for mercapturic acids and show differences in specific MA excretion in smoker and nonsmoker urine samples. The hypothesis is that this novel HRMS nanoflow method will provide more accurate mass measurements of a large set of mercapturic acids in a complex matrix with increased selectivity for their detection. This work presented here is fundamental in efforts to discover the most efficient way to screen for these mercapturic acids and to essentially one day provide a global picture of the mercapturome.

Why are mercapturic acid biomarkers important and why is it important to expand the mercapturome? The answer is that the mercapturic acid pathway is an important biotransformation route that protects against exposure to highly reactive electrophilic compounds. With mercapturic acids being the product to a semi-known detoxification process, the use of metabolites like mercapturic acids as biomarkers can provide insight into the specific exposures, mechanisms behind detoxification, and the development of proper analytical methods. Why are electrophilic compounds bad? The answer is explained in Lopachin's Mechanisms of Soft and Hard Electrophile Toxicities, when a person is exposed to electrophiles, they "can cause cytotoxicity since the formation of covalently bonded adducts with specific nucleophilic sites can irreversibly disable the functions of enzymes, DNA, cytoskeletal proteins and other biological macromolecules" [51]. It is important to emphasize that the problem is not limited to the effects of exogenous chemical exposures. "Endogenous electrophiles associated with oxidative stress and metabolic imbalances result in protein modifications that contribute to diverse diseases and understanding these mechanisms of injury represents an important opportunity for toxicology to impact human health" [49].

To consolidate the importance of screening for mercapturic acids it has been shown that exposure to mercapturic acid precursors is directly related to cancer, respiratory effects, nephrotoxicity, oxidative stress, and cellular damage. Aside from tobacco smoke other exposures investigating mercapturic acids reported range from red meat consumption, natural gas from nearby exploitation, fossil fuels of urban traffic, alkylating agents, omega-6 and saturated fatty acids, lipid peroxidation, cerebrovascular accidents, and many more [16,24,36,43,55,62,69,73]. Like all exposure cases the health problems vary depending on route & duration of exposure and the individual's biology. It is important to note that the presence of these mercapturic acids in your urine does not indicate immediate health problems but serve as biomarkers for exposure to toxic substances and provide evidence of extended and/or repeated exposure indicating the possible risk of developing a future health problem.

The next question is why use urine? It is simple, the analysis of urine is a prominent technique used for metabolic profiling in many clinical settings as it is known to provide information that directly reflects the condition of a biological system and could possibly lead to the discovery of other potential biomarkers. Some other reasons would include that it is non-invasive, collecting samples is more accessible, can be stored long-term in freezers, and is a very metabolite rich biofluid. Unfortunately, urine has a complex and complicated matrix which can cause several issues in mass spectrometry analysis. The stability of mercapturic acids is higher in urine and the lack of studies using other biofluids is because mercapturic acids degrade rapidly in human blood and plasma [25, 40]. The urine matrix is an evident problem in the whole world of metabolomics so developments on untargeted methods, like ours, is not only important for MA research but for other exposure metabolomic and small molecule studies.

The biggest difficulty in this research is owing to the different compounds in urine that can co-elute at the same time as some MAs and compete for ionization making quantification and quantitation difficult. Research involving biofluids and LC-MS is still working to prevent matrix effects. Unfortunately, even LC-MS methods of high sensitivity like the one presented in this work still suffer from matrix induced ion suppression. In efforts to reduce the limiting effects of the urine matrix and increase extraction of mercapturic acids from urine we began working on the development of a rigorous cleanup procedure and HRMS analysis of mercapturic acids employing internal standards. To find the best way to analyze these samples using this method, the way the samples are prepared was changed as well as different parameters of the LC-MS method, gradient, reconstitution buffers, mobile phases, column, and injection amount. These tests are to see if any of the parameters in the method can be adjusted to maximize our recovery while minimizing any suppression. The unbiased screening of mercapturic acids by an UltiMate 3000 RLSCnano UHPLC utilizing a linear gradient coupled with a Thermo Orbitrap Fusion Tribrid HRMS in positive nanospray ionization with data-dependent acquisition is a novel technique so all results, positive or negative, were helpful during the process of refining this method.

4. MATERIALS AND METHODS

4.1 - Chemicals and Reagents

Six deuterium labeled MA standards were used including D_3-N-acetyl-S-(2-cyanoethyl)-L-cysteine (D_3-CEMA), D_3-N-acetyl-S-(2-carbamoylethyl)-L-cysteine (D_3-AAMA), D_3-N-acetyl-S-(2-hydroxypropyl)-L-cysteine (D_3-2-HPMA), D_3-N-acetyl-S-(3-hydroxypropyl)-L-cysteine (D_3-3-HPMA), D_3-N-acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine (D_3-HMPMA), and D_5-N-acetyl-S-(phenyl)-L-cysteine (D_5-SPMA). The six standards were purchased from Toronto Research Chemicals. Solutions and solvents used were purchased from U-Stores, University of Minnesota including HPLC grade methanol, LC-MS grade water, UHPLC-grade acetonitrile, 30% HCl, 98% formic acid, and 30% NH_4OH. All solutions used for sample preparation were prepared fresh on the same day as the assay.

4.2 - Study Population

The smoker and non-smoker urine samples were from an ongoing tobacco biomarker research study that sampled healthy volunteers in Minnesota and stored at -80°C. All urine samples were retrieved from the Biorepository of the University of Minnesota Tobacco Research Programs. All urine collection procedures were approved by the University of Minnesota Institutional Review Board. The individuals did not change any smoking habits or routines for the purpose of the study and smoked an average (±SD) of 16.04 ± 7.48 cigarettes per day. Detailed information on the participants and their demographics is included (**Appendix Table 1)**.

4.3 - Sample Preparation

The protein precipitation we tested but abandoned went as follows: pipette 100 µL of urine and 100 µL of IS mix to a 2 mL microcentrifuge tube. Next add 400 µL of chilled (-20°C) 90:10 MeOH:Acetone to each sample and vortex at high speed for 1 minute. Once vortexed, incubate the samples at -20 °C for 15 min then centrifuge at 13,000 x g for 15 min at 4 °C. °C. Lastly, transfer the supernatant to a new 1.2 mL vial, speedvac to dryness without heat (drying usually around 4 hours), and now the samples are ready to be reconstituted in 20 µL of 98:2 (H_2O:ACN), 0.1% FA and 1 µL is injected for analysis. The different preconditioning wash methods tested are shown in Table 2 including the normal wash of only one wash of MeOH and one wash of 2% NH_4OH [13,15]. Table 3 lists the test on normalizing urine samples to total volume prior to preparation but was not used in final procedure due to lack of 24-hour urine samples and lack of better results from normalization.

Table 2. The three different preconditioning wash steps tested.

Normal Preconditioning Wash	+H_2O Preconditioning Wash	+FA Preconditioning Wash
1: 1x 0.7 mL MeOH	**1:** 2x 0.7 mL MeOH	**1:** 2x 0.7 mL MeOH
2: 1x 0.7 mL 2% NH_4OH	**2:** 2x 0.7 mL H_2O	**2:** 1x 0.7 mL H_2O
	3: 2x 0.7 mL 2% NH_4OH	**3:** 1x 0.7 mL 2% FA
		4: 1x 0.7 mL H_2O
		5: 2x 0.7 mL 2% NH_4OH

Table 3. Dilutions of 5 smoker 24-hour urine samples which were normalized to total volume prior to loading the samples onto the Oasis MAX SPE 96-well plate. The volume of urine and H_2O added was added to the wells after preconditioning them.

24hr Urine Volume (mL)	Dilution Factor	Urine Added (uL)	H_2O Added (uL)
2350	1	200	0
1300	1.807	200	161.54
1080	2.175	200	235.19
960	2.447	200	289.58
620	3.790	200	558.06

All solvents, samples, and LC-MS grade water samples were applied using an 8 channel multi-pipette (Eppendorf). The sample preparation technique originates from a previously described 96-well Oasis MAX SPE assay for the detection mercapturic acids in urine [13,15]. Our final method includes changes to the preconditioning wash methods which are shown in Table 2. The fractionation technique was also changed by instead of collecting two fractions, a 30% MeOH collection and a 90% MeOH collection our method included only a 90% MeOH fraction collection (**Figure 5**). The steps are as follows: Add 0.04 mL of a 1:5 dilution of 30% HCl and 0.2 mL of urine to the 2 mL wells of a 96-well collection plate and let sit for 15 min. Then a 0.1 mL mixture of heavy mercapturic acid internal standards consisting of 50ng of D_3-3-HPMA, 10ng D_3-2-HPMA, 10 ng D_5-SPMA, 50 ng D_3-HMPMA, 20ng D_3-AAMA and 20ng D_3-CEMA is added to each well with HCl and urine. The 96-well collection plate is then covered with a silicone cover and vortexed very gently to ensure proper mixing of the internal standards and HCl with the urine. Before the urine mixture is added to the Oasis MAX 96-Well SPE Plate 60µm (60mg, 2 mL reservoir size) (Waters Corp., Milford, MA) the wells are preconditioned with double the number of washes in the FA wash method (**Table 2**). That includes four washes of 0.7 mL of MeOH, make sure the solvents go down or slightly below the surface of the frit/packing using a Cerex N_2 assisted pressure processor (Chromtech, Apple Valley, MN) before adding another solvent. The drip flow should be about one drop per second with assistance of N_2, “adjustable flow” about 4-10 PSI. Next the wells are washed twice with 0.7 mL of H_2O, twice with 0.7 mL 2% FA, and two more washes of 0.7 mL H_2O. Lastly the wells are washed with four washes 0.7 mL of 2% NH_4OH and the pH of the cartridge is checked to ensure it is at basic conditions before adding the urine. The urine mixture from the collection plate is then applied to the Oasis MAX 96-well SPE plate. The SPE plate was then washed with 0.7 mL of 2% NH_4OH with gentle assistance from N_2 and once with 0.7 mL MeOH and dried under “max flow” N_2 at 60 PSI for 20 minutes. The plate was then washed once with 0.7 mL of 2% FA before collecting with two washes of 0.5 mL of 90% MeOH in 2% formic. The samples in the collection plate are then transferred to 1.2 mL silanized vials and the solvents were removed in a speedvac without heat and were dried overnight. Once dried the samples are stored at −20 °C until ready for analysis.

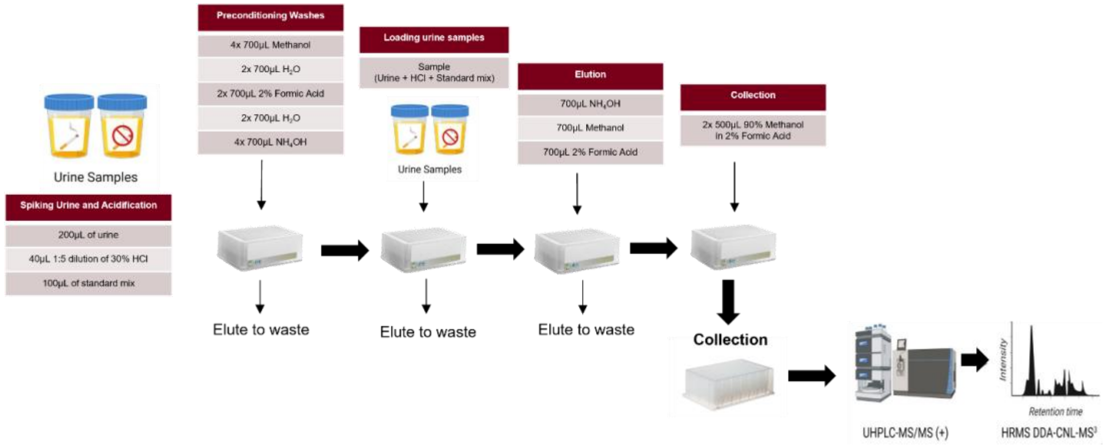
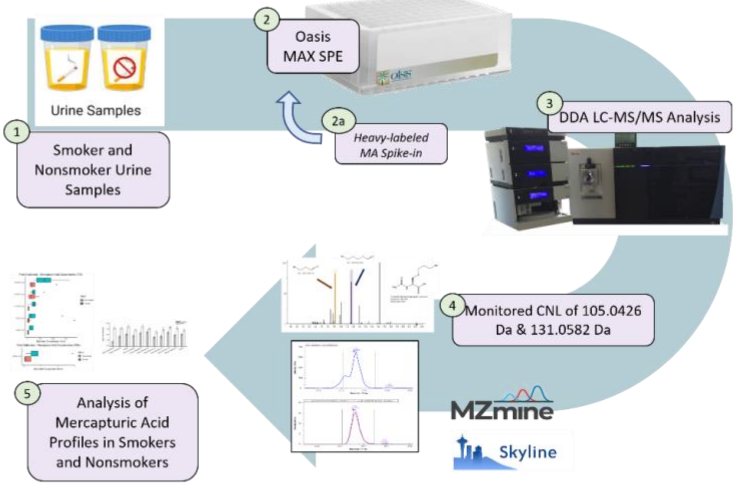


Figure 5. Sample preparation schematic showing the final developments of our workflow. Samples are prepared using Oasis MAX SPE and analyzed using a UHPLC-MS/MS platform generated in positive ion mode.

4.4 - Instrumental Analysis

Throughout the development of our LC-MS method multiple different parameters were tested but not used in final method including: a Luna C18 column (20 cm length x 75 µm ID, 5 µm particle size), a 42 minute gradient (5% B held for 2 min, 10% B held for 26 min, 50% B held for 2 min, 90% B for 5 min, and 5% B held for the remainder of the run), a 98:2 H_2O:ACN buffer system with mobile phase A of 0.1% FA in aqueous solution and mobile phase B of 0.1% FA in ACN solution, a NH_4OAc buffer system with mobile phase A of 2mM NH_4OAc and mobile phase B of ACN, and reconstitution in 98:2 (H_2O:ACN), 0.1% FA. The series of diluted 30% MeOH fraction samples of 1 µL injections used for assessment were reconstituted in 20 µL of H_2O, 0.1% FA (1:20 dilution), 80 µL of H_2O, 0.1% FA (1:80 dilution), 200 µL of H_2O, 0.1% FA (1:200 dilution), 1000 µL of H_2O, 0.1% FA (1:1000 dilution).

The final developments of our LC-MS method led to the following method shown in Figure 6: dried down samples are reconstituted in 100 µL of H_2O (0.1% FA), centrifuged quickly and spiked with 100 fmol/µL of Pierce^{TM} Peptide Retention Time Calibration Mixture (Rockford, IL) to assess quantitative reproducibility. Injection was 1 µL (1%) of sample for analysis on a 10 µL injection loop on a Quadrupole-Orbitrap Fusion Instrument (Thermo Scientific, San Jose, CA) coupled with a Dionex UltiMate 3000 RLSCnano UHPLC system (Thermo Fisher Scientific, Waltham, MA) equipped with a self-packed C18 column (Dr. Maisch GmbH ReproSil-PUR 1.9 µm 120 Å C18aq, 100 µm ID x 45 cm length) kept at a constant 55°C. The flow rate was 325 nL/min, and a Nanospray Flex ion source was used with a source voltage of 2.1 kV and an ion transfer tube temperature of 250 °C. The column was eluted with mobile phase A (H_2O, 0.1% FA) and B (ACN, 0.1% FA). LC-MS was equipped with a 68 min linear gradient operated in nanoflow mode. The gradient was as follows: 0% B held for 40 min, then 50% B held for 20 min, then 90% B held for 8 min, then 0% B held for the remainder of the run with the total run duration 77 minutes total with wash. All analyses were collected by data dependent acquisition over a full scan range of 170-600 m/z with an Orbitrap resolution of 120K, AGC targeted setting of 4×10^5 , a maximum ion injection time of 50 ms, and an S-Lens RF setting of 40%. MS^2 spectra were collected using a DDA design with a 2 sec cycle time in centroid mode with a 15 sec dynamic exclusion list and acquired with quadrupole isolation of 0.8 m/z, 30k Orbitrap resolution utilizing HCD fragmentation, an AGC setting of 1×10^5 , and a 100 ms maximum injection time with a full-scan MS isolation of 1.5 m/z and MS^2 isolation of 2.0 m/z. MS^3 spectra were collected using a conditional neutral loss design that acquired an additional fragmentation scan on ions exhibiting a neutral loss of the N-acetylcysteine, 131.0582 Da ($C_5H_9NO_3$), and the loss of the N-acetyl and carboxyl group, 105.0426 Da ($C_3H_7NO_3$) from the selected precursor. MS^3 spectra were acquired with a full-scan MS isolation of 1.5 *m/z* and MS^2 isolation of 2.0 m/z, Orbitrap detection at a resolution of 30,000, AGC setting of 2×10^5, and a 200 ms maximum injection time. The MS^3 analysis utilized HCD fragmentation at a fixed collision energy of 50%. Mercapturic acid data processing was done using fragmentation filtering in MZmine3 using diagnostic Putative mercapturic acids required the observation of 131.0582 Da or 105.0426 Da neutral loss in MS/MS spectra to be classified as a mercapturic acid.



Final method parameters

Ultimate 3000 RLSCnano UHPLC

- Reversed-phase separation
- Reconstitution in 100µL H_2O, 0.1% FA
- (A) H_2O, 0.1% FA; (B) ACN, 0.1% FA
- Self-packed C18 column (Dr. Maisch GmbH ReproSil-PUR 1.9 µm 120 Å C18aq, 100 µm ID x 45 cm length)
- Linear 68 min gradient
- 325 nL / min flow rate
- 1 uL (1%) injection volume

Thermo Orbitrap Fusion Tribrid HRMS

- Positive ion mode acquisition
- 120K resolution full scan (170-600 *m/z*)
- HCD activation strategy (30% fixed CE)
- 30K resolution DDA MS^2 collection
- NL-triggered MS^3 acquisition

Figure 6. Final UHPLC-HRMS workflow instrumental parameters included.

5. RESULTS AND DISCUSSION

5.1 – Initial conditions

Contaminant discovered. The previously described sample preparation technique using the Oasis MAX 96-well SPE plate [13,15], was used to prepare the first round of smoker samples and analyzed with our LC-MS method. A large contaminant of ~102 m/z was observed eluting at ~3 minutes in both the spike water and urine samples that went through SPE. However, the contaminant was not observed when urine samples are prepared the same way but analyzed using Hecht's groups LC-MS method indicating a sample prep limitation for our LC-MS method [13,15].

5.2 – Method optimization

Elimination of an interfering contaminant. Since the contaminant was only detected in high abundance in our LC-MS analysis, the contaminant is possibly originating from the sorbent of the Oasis MAX SPE cartridges. To better clean the sorbent, additional preconditioning washes were implemented to elute any contaminants from the cartridge before introducing our samples, three different preconditioning wash methods were compared (**Table 2**). All our internal standards were observed in the water and urine samples of each wash method. The FA wash showed the best recovery and signal intensity for our standard peaks (**Figure 7C**). The peak for D_3-HMPMA in SPE prepared water samples had an area of 1.817×10^7 when the plate was washed more rigorously using the FA preconditioning wash method compared to a peak area of 2.245×10^6 when doing the normal preconditioning wash method (**Figure 7A & C**). The contaminant (102 m/z) was still observed and suppressing the signal of compounds of interest, especially ones that elute early such as D_3-AAMA.

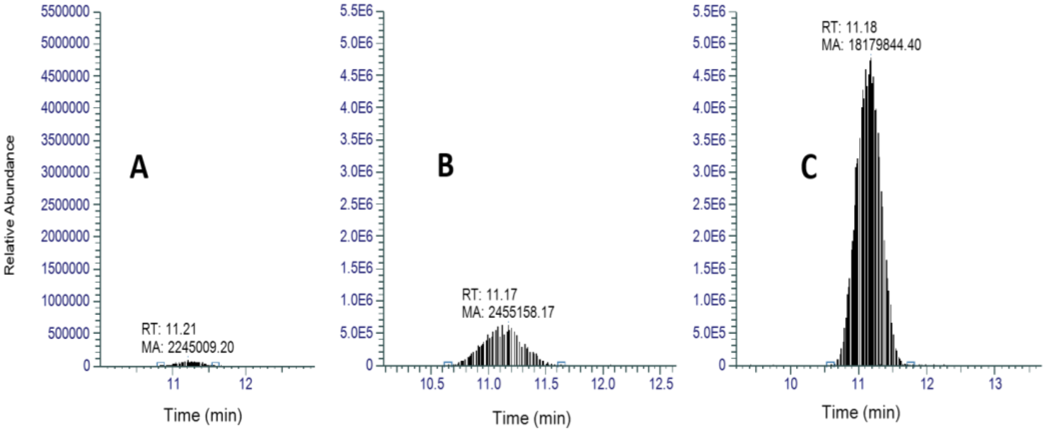


Figure 7. Extracted ion chromatograms of D_3-HMPMA (239.1138 m/z) from each spiked water sample and its respective preconditioning wash technique shown in Table 2. **(A)** Normal wash **(B)** + H_2O wash **(C)** + H_2O & FA wash.

Testing of a sample preparation method typically used in metabolomics analysis. Using the protein precipitation sample clean up procedure described in Sample Preparation in Materials and Methods, is often used when screening for mercapturic acids and other metabolites, this allows us to test if the SPE sorbent is producing the contamination. Although SPE is known to be one of the most effective sample preparation techniques for the extraction of metabolites, this is a novel method of using Oasis MAX SPE paired with UHPLC-HRMS running in positive nanoflow mode, so testing a less intensive sample clean-up is important to validate the use of SPE for this assay. The results for the protein precipitation prep performed much worse than the SPE prepared samples. The protein precipitation prep was not enough to control the urine matrix since chromatographic peaks for the standards in the water samples were observed but the peaks of the standards in the urine samples were not observed. The metabolomics prep was informative in the fact that the contaminant was not observed in the TICs of the protein precipitation prepped water sample and urine sample indicating that the contaminant is in fact coming from the Oasis MAX sorbent (**Figure 8**).

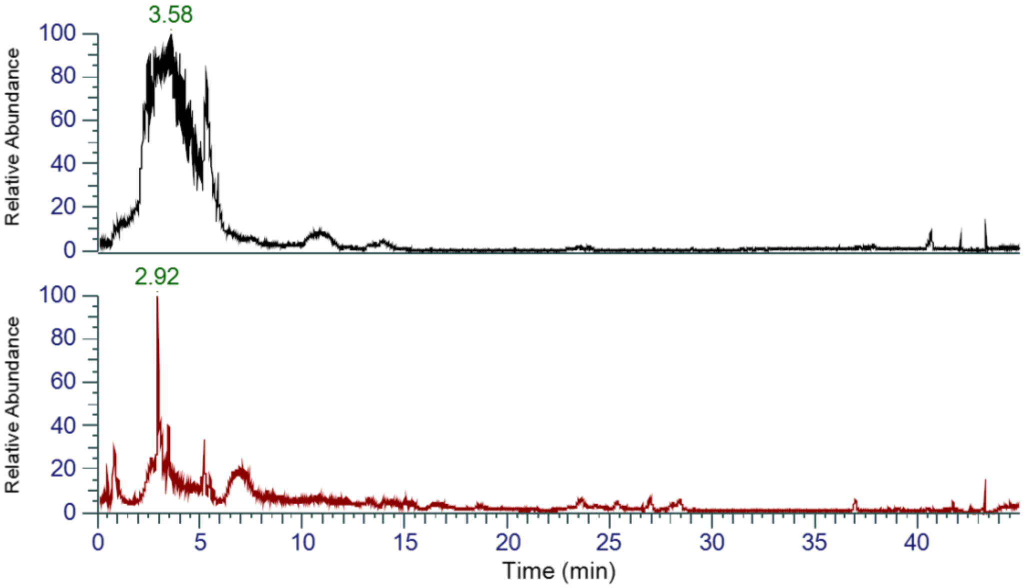


Figure 8. Total ion chromatogram **(in black)** of a SPE prepped smoker urine sample. Total ion chromatogram **(in red)** of a metabolomics prepped smoker urine sample showing that the large contaminant is not present in the metabolomics prepped samples.

Optimization of the Purification steps. Since the contaminant was confirmed to be eluting into our samples from the SPE Oasis MAX sorbent the number of preconditioning washes was increased even more. This time the FA preconditioning wash method shown in Table 2 was doubled. Although traces of the contaminant were still detected in lower abundance, the increase of the preconditioning washes was successful in increasing the intensity of our standard peaks, the D_3-HMPMA peak area was larger when more preconditioning washes were used with an area of 4.464×10^8 compared to a peak area of 7.294×10^7 (**Figure 9A & B**). Nonetheless the results were still not the best chromatographically, lacking signal response in urine samples hypothesizing that most of our suppression is because of the urine matrix. To assess the suppression caused by the urine matrix a prepped urine sample was injected and then it was injected a second time only after spiking in an additional 1 µL of our internal standard mixture. The spiked sample was lower in intensity with a peak height of 9.22×10^6 compared to a peak height of 1.04×10^7 for the unspiked sample signifying suppression induced by the urine matrix. Urine matrix suppression was also observed in our results as some standards, such as D_3-CEMA, were missing in the urine sample but were present in the water sample that went through the Oasis MAX SPE.

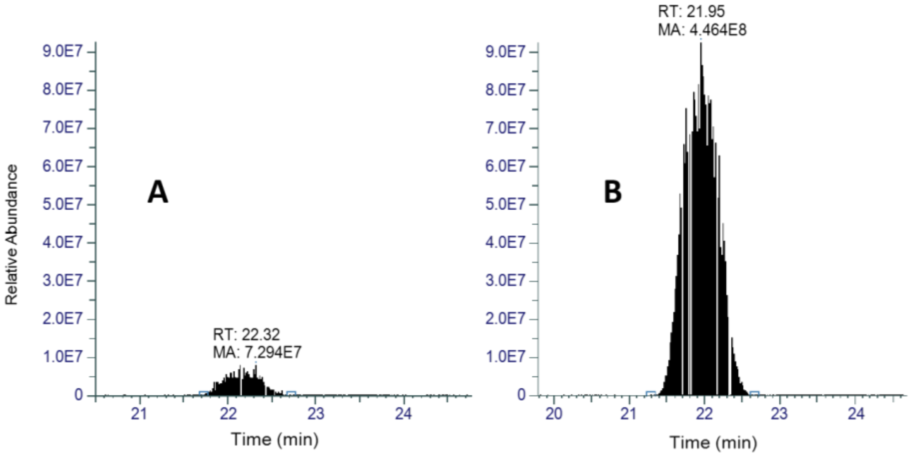


Figure 9. Extracted ion chromatograms for D_3-HMPMA (239.1138 m/z) from spiked water samples, of **(A)** FA preconditioning wash method and **(B)** doubled FA preconditioning wash method.

Chromatographic conditions optimization. In efforts to improve the chromatography the columns were switched and only the IS mixture was used to do the following assessments on the new system. The longer Dr. Maisch column length allowed for longer runtimes that increases the chromatographic separation, and a smaller particle size improving separation as well but not affecting the runtime. The buffer system used for the prior runs was a 98:2 (H_2O:ACN), 0.1% FA, however, in several metabolomics and mercapturic acid studies an ammonium acetate (NH_4OAc) buffer system is used. The IS mixture contained the following concentrations of standards: 9119.93 fmol of D_3-CEMA, 4460.30 fmol of D_3-2-HPMA, 22302.52 fmol of D_3-3-HPMA, 8428.51 fmol of D_3-AAMA, 20990.76 fmol of D_3-HMPMA, & 4092.99 fmol of D_5-SPMA. Dilutions were done on the IS mix to achieve the different concentrations of standards and the fmol of D_5-SPMA is listed as a reference. The initial IS assessment was performed using an estimated injection of 25 fmol D_5-SPMA. A very low response for multiple of the standards was observed and immediately increased the load to 100 fmol D_5-SPMA and tried as high as 500 fmol D_5-SPMA injections (**Figure 10**). The standards were not focusing on the columns and the peaks were broad, fronted, and barely discernible from noise for the 5 µL 500 fmol D_5-SPMA injections in both buffer systems (**Figure 10B & D**). The observed peaks for the standards using the 98:2 buffer system was much better in peak intensity and shape as it is shown that the 1 µL 100 fmol D_5-SPMA injection was much better on the 98:2 system compared to the NH_4OAC system (**Figure 10A & C**). The 98:2 buffer system was the best performing system with the 1 µL 100 fmol D_5-SPMA injection being much better in shape and much more discernible from noise in comparison

(**Figure 10A**). This raises the question, are the standards too concentrated increasing noise and decreasing resolution or was the injection volume too much resulting in wider peaks?

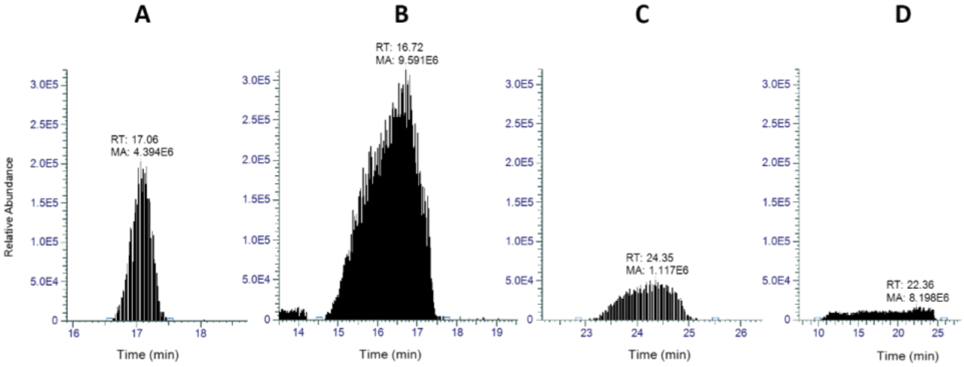


Figure 10. Extracted ion chromatograms of D_3-CEMA (220.083 m/z) from IS mix injection using high and low concentrations of our standards with both the 98:2 buffer system and the 2mM NH_4OAc buffer system. **(A)** 98:2 buffer system 1uL 100 fmol injection **(B)** 98:2 buffer system 5uL 500 fmol injection **(C)** NH_4OAc buffer system 1uL 100 fmol injection **(D)** NH_4OAc buffer system 5uL 500 fmol injection.

Different injection amounts and different concentrations of internal standards were used to assess if the column is being overloaded with too much fmol or volume of our standard mix. When you inject more than 1 µL of sample the chromatography suffers as the 1 µL injections of both the 100 and 500 fmol D_5-SPMA concentrations produce good peaks with the 1 µL 100 fmol D_5-SPMA injection being much more superior in intensity with a peak area more than 3 times the size (**Figure 11A & C**). When looking at the 1 µL of 100 fmol D_5-SPMA and comparing it to the 4 µL 100 fmol D_5-SPMA injection there is possibly a matrix effect observed that happens when more than 1 µL is injected resulting in massive degradation in peak shape for the standards (**Figure 11B & C**). This phenomenon is possible in capillary-LC when screening for polar compounds and the peak broadening and decreased intensity when over 1 µL injected is due to volume overload on the column (**Figure 11B**).

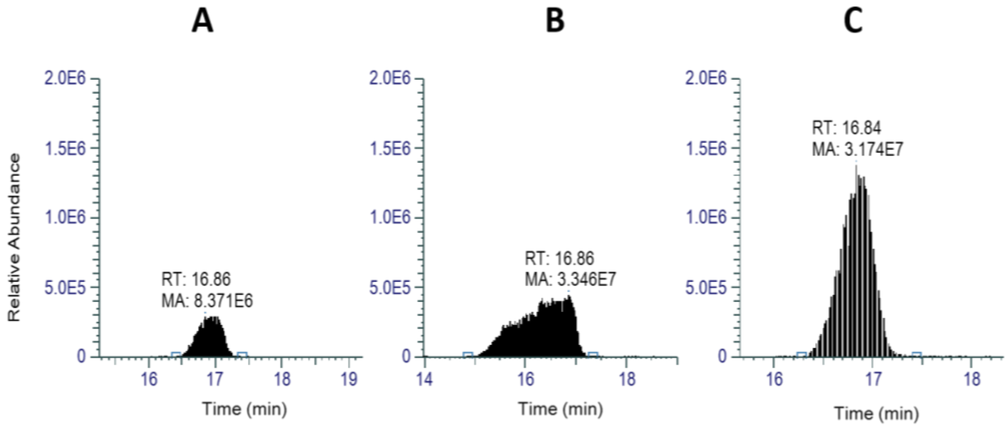


Figure 11. Extracted ion chromatograms for D_3-CEMA (220.083 m/z) showing different concentration and injection amounts of IS mix. **(A)** 1uL 400 fmol SPMA injection **(B)** 4uL 100 fmol D_5-SPMA injection **(C)** 1uL 100 fmol D_5-SPMA injection.

Before urine samples were injected on the new system a final IS assessment was performed by reconstituting the internal standard mix in H_2O, 0.1% FA. In our prior runs the IS mixture on this system and urine samples on the previous system were always reconstituted in 98:2 (H_2O:ACN), 0.1% FA, however, it was hypothesized that since there is a limited number of binding sites within the column, the ACN and the mercapturic acids might be competing for binding of the column. Also aiming for better separation, the LC method runtime was increased from a 42-minute gradient to a 68-minute gradient. The aqueous reconstitution in 0.1% FA and longer LC method provided more Gaussian shaped peaks and better separation of our standards, specifically better resolution of the two HPMA isomers (**Figure 12B**). The peak area for the standard peaks of D_3-2-HPMA & of D_3-3-HPMA in the 98:2 (H_2O:ACN), 0.1% FA reconstitution was 3.954×10^7 compared to a peak area of 4.620×10^7 when reconstituted in H_2O, 0.1% FA (**Figure 12A & B**).

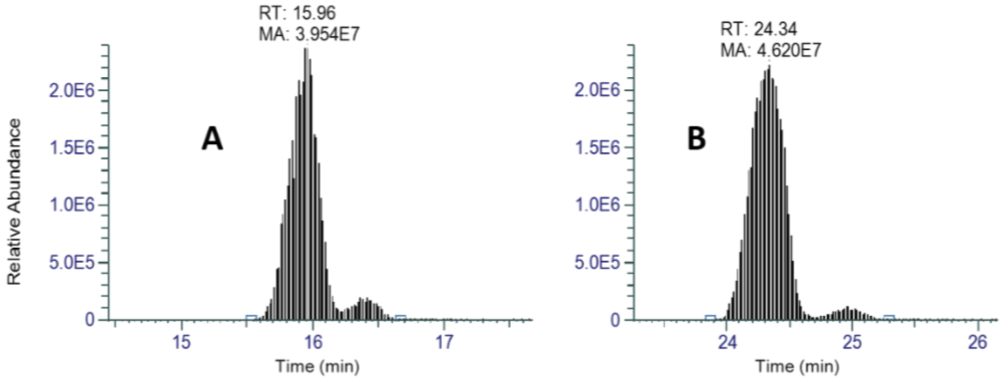


Figure 12. Extracted ion chromatograms of D_3-2-&3-HPMA (225.0983 m/z) from IS mix injections. **(A)** The 98:2 (H_2O:ACN), 0.1% FA reconstitution with shorter LC method. **(B)** The H_2O,

0.1% FA buffer system using a longer LC method producing a more Gaussian shape of the 3-HPMA peak and the 2-HPMA is more separated in comparison.

Validation of the method to analyze urine. The combined optimization of changing the IS concentrations, injection amounts, mobile phases, reconstitution buffers, column, and runtime was successful as the chromatographic results now provided optimal signal response and peak shape for each of our standards. By observing good signal response for each standard, it was time to begin running prepped urine samples using this newly optimized method on the new system. The assessment of our samples was done by using the series of diluted 30% MeOH, 2% FA fraction samples (1:20, 1:80, 1:200, and 1:1000). The contaminant is not seen impacting the resolution of our peaks of interest as the increased washing has decreased its presence and the increased separation using a new column with a much longer runtime allowed for our peaks of interest to favor the latter half of the chromatogram, away from the highest source of contamination. Looking at the XIC's for the standards from each of the dilutions as the sample was increasingly diluted the standard peaks were becoming more visible and more symmetric. The best signal response of each standard was consistently the 1:80 and/or the 1:200 dilution.

Preliminary Test on urine from 5 Smokers and 5 Nonsmokers. To begin our preliminary tests, 5 smoker and 5 non-smoker samples were prepared and reconstituted in 100 µL of H_2O, 0.1% FA (1:100 dilution). The detection of mercapturic acids varied between samples much more than anticipated. There is a lot of variability especially in between smoker samples, some variability is expected given the matrix but not expecting so many differences between samples. Due to the chromatographic variability observed the sample preparation was repeated but this time normalizing 5 smoker 24hour urine samples to total volume prior to preparation as it could head off some of the variability (**Table 3**). However, the tests for normalizing to total volume did not show much difference in comparison and it could just be that urine is such a dynamic and heterogeneous matrix that individual profiles are a greater source of variability than we thought. Following experiments in the future could include normalizing the urine samples to creatinine, however, creatinine measurements were not collected for the samples used in this work. It was also hypothesized that some analytes may be lost to the 50% MeOH, 2% FA wash as some standards that were supposed to be eluted and collected with the 30% MeOH, 2% FA wash were showing up in the 90% MeOH, 2% FA fraction. It was hypothesized that collecting only one fraction would enable all our analytes of interest to be in one fraction to increase MA recovery or potentially run into the issue a previous study encountered when screening for a large set of MAs before splitting the assay in two [62]. The analysis of the single 90% MeOH, 0.1% FA collection using our longer and stepper gradient method may however produce better chromatographic results compared to the study that suggested that splitting the assay in two allows for increased detection of MAs due to their wide range of physicochemical properties [62]. The results from the unfractionated samples revealed promising quantitative reproducibility benefits. The coefficient of variation using the peak areas of our internal standard peaks in the unfractionated samples with an outlier removed showed better quantitative reproducibility as more standards had coefficient of variation percentages below 30% in the unfractionated prep compared to the normal prep. The COE's for the fractionated sample prep were as follows: 40% for D_3-3-HPMA, 41% for D_3-2-HPMA, 26% for D_5-SPMA, 21% for D_3-HMPMA, 66% for D_3-AAMA and 34% for D_3-CEMA. The coefficient of variation percentages for the unfractionated sample prep were as follows: 26% for D_3-3-HPMA, 33% for D_3-2-HPMA, 24% for D_5-SPMA, 38% for D_3-HMPMA, 33% for D_3-AAMA and 19% for D_3-CEMA. More analyses will need to be done to determine the influence of our longer runtime for separating a large set of MAs and whether collecting only one fraction hinders the response of a large set of MAs as reported previously or will that not be as much of an issue when using our much longer method.

Despite the variability between the smoker samples, all our standards and their light equivalents were observed in the urine samples and our unfractionated method was successful in showing that smokers have higher concentrations of mercapturic acids in their urine compared to non-smokers urine with only one collection using 90% MeOH, 0.1% FA (**Figure 13**). The difference in concentrations for a set of mercapturic acids was estimated using the concentrations of standards on column and peak areas of the standard and their light equivalent MA peaks from the urine samples of smokers and nonsmokers. The smoker samples were observed to have higher concentrations of mercapturic acids besides 3-HPMA as equally high concentrations of 3-HPMA were detected in both smoker and non-smoker samples. It has been reported that the urinary levels of 3-HPMA are higher in smokers compared to non-smokers and the reason why our method shows similar levels isn't quite known but most likely is due to the variability involved in our method. It is believed that this may be a deficiency with our method as reproducibility is a problem in nanoflow methods. It may also be the presence of a coeluting isomer that is observed eluting prior to 3-HPMA possibly shadowing the signal of 3-HPMA. Further analysis of a different and larger set of smoker and non-smoker samples set to understand if this is a common problem with our method.

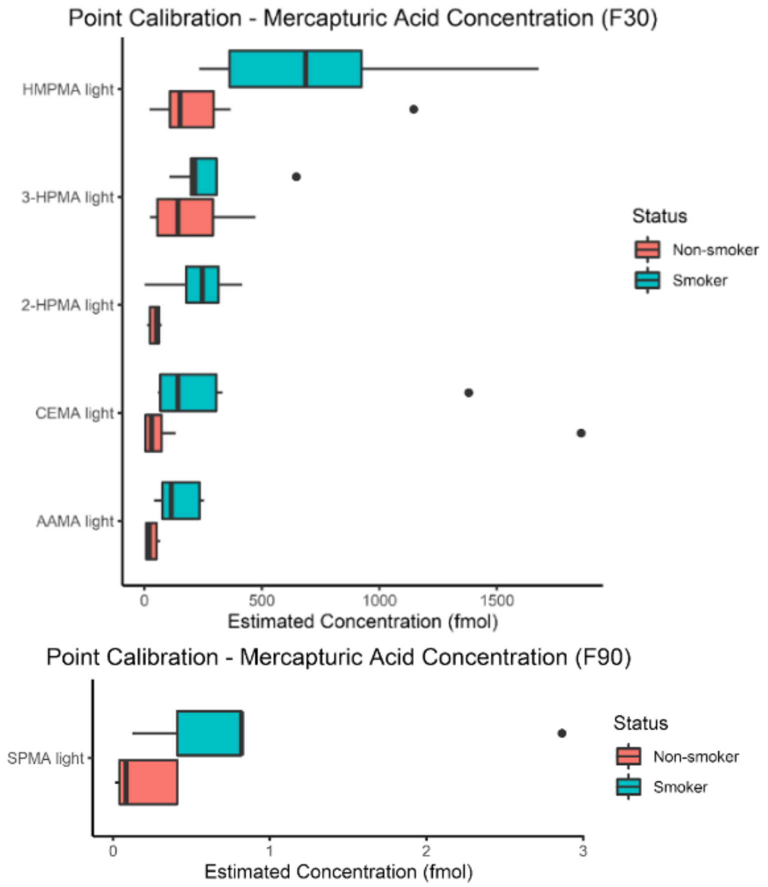


Figure 13. Box and whisker diagram showing the concentrations of mercapturic acids in smoker and non-smoker samples derived from point calibration using the concentration on column and peak areas of our heavy labeled mercapturic acids to estimate the concentration of their light equivalents in our unfractionated samples. Showing how the concentrations of HMPMA, 2-HPMA, CEMA, AAMA, and SPMA are higher in smoker samples compared to non-smoker samples indicating they are suitable biomarkers for tobacco smoke exposure using our method. F30 is the standards that were originally in the 30% fraction of HMPMA, 2-HPMA, CEMA, AAMA. F90 is standard SPMA which was originally collected in the 90% fraction. 3-HPMA had similar urinary levels observed in both smokers and nonsmokers which could be due to a deficiency in our nanoflow untargeted DDA method as 3-HPMA has been reported in higher levels in smokers compared to nonsmokers in multiple studies.

5.3 Analysis of urine from 20 smokers and 20 nonsmokers.

Moving on from our preliminary tests 20 smoker and 20 nonsmoker first void only urine samples were prepared using our unfractionated SPE method with only a 90% MeOH fraction collection. There were over 300 putative mercapturic acid signatures detected using fragmentation filtering, and data analysis done by Kevin Murray at CMSP on identifying the signatures that differ in smoker and nonsmokers led to the discovery of a potential mercapturic acid biomarker for tobacco exposure. A potential novel mercapturic acid of 269.0952 m/z was observed and MS^2 detection shows a loss of N-acetylcysteine indicating its possibility of being a mercapturic acid (**Figure 14**).

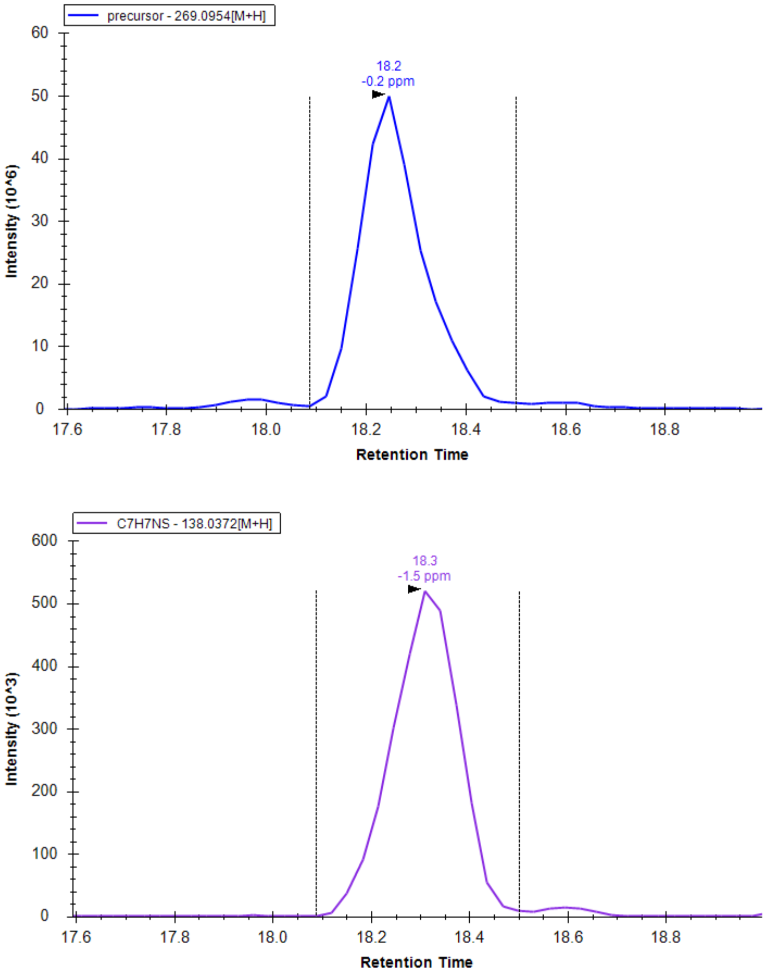


Figure 14. The extracted ion chromatogram from smokers' urine sample of the potential novel MA biomarker on top and on the bottom is the MS$_2$ spectra fragmentation showing the transition of m/z 269.0954→138.0372 from the loss of N-acetylcysteine moiety (131.0582 Da).

This potential MA biomarker is strongly suggested to be linked to tobacco exposure as increased levels of it are observed in smokers in comparison to nonsmokers (**Figure 15**). The predicted formula for this potential novel MA is $C_{12}H_{16}N_2O_3S$ and the predicted structure based on annotation of the spectra corresponds with the PubChem ID of 79267016, however, it is not described in any studies, leading to the hypothesis that the physicochemical properties of this specific MA may make it easier to detect in positive ion mode on a nanoflow system. Future tests are planned to look at the same sample or set of samples when injected on a LC-MS system employing both positive and negative modes to compare data sets for this specific MA.

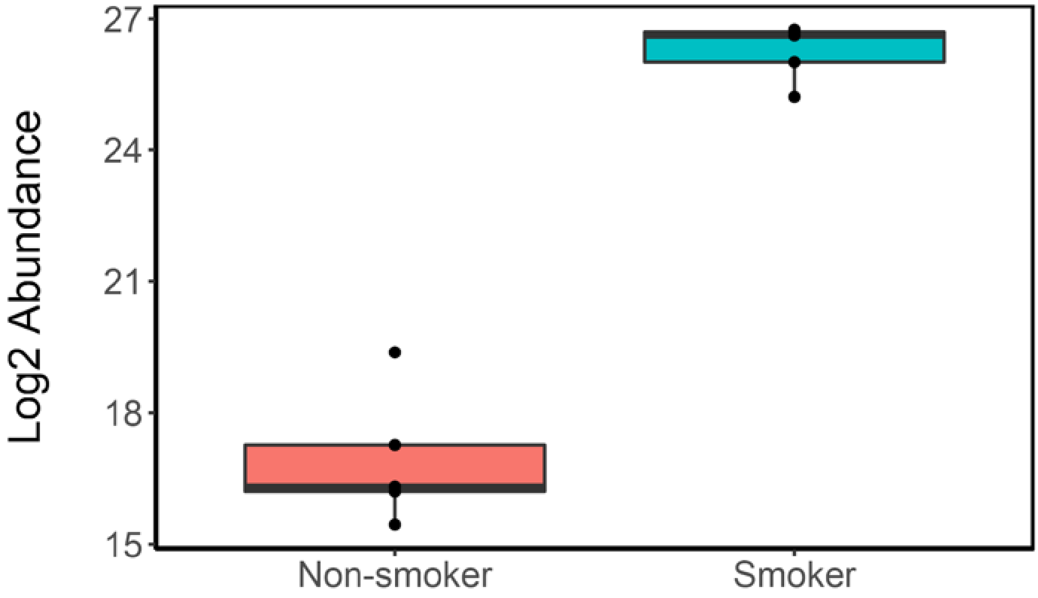


Figure 15. Box and whisker diagram showing the increased abundance derived from peak area of the possible MA tobacco biomarker at 269.0952 m/z in the urine of 20 smokers and 20 nonsmokers.

The use of the N-acetylcysteine fragment for mercapturic analysis has shown success but researchers in the field suggest that one CNL nowadays is not enough to classify a compound as a mercapturic acid. Our method consisted of scanning for a diagnostic fragmentation pattern for mercapturic acids, the loss of the N-acetylcysteine moiety (131.0582 Da), and the loss of the N-acetyl + carboxyl functional groups (105.0426 Da) (**Figure 16**). The 131.0582 mass shift reflects an additional H_2 loss in the fragment ion following the disassociation of the N-acetylcysteine moiety. This structural rearrangement has yet to be reported as a common observation for mercapturic acid conjugates in negative mode. Research on important diagnostic fragments for mercapturic acids is important since it has been mentioned that some mercapturic acids may not fragment the same as others and employing the use of additional characteristic fragmentation patterns for mercapturic acids is crucial for discovering any novel mercapturic acid that doesn’t lose the N-acetylcysteine fragment as well as evaluating exposures for already known MAs. A paper that was published this year that reported the detection of about 500 MA candidates monitored for not only the N-acetylcysteine CNL but also common ion fragments of the N-acetylcysteine moiety including 74.020 m/z (C_3H_6S), 84.045 m/z (C_4H_6NO), 128.035 m/z ($C_5H_6NO_3$), and 162.023 m/z ($C_5H_8NO_3S$) in MS^2 spectra and this implementation of more criteria to classify a detected compound as a mercapturic could prove useful when confirming if a peak of the detected compound is a mercapturic acid [83]. From our analysis all the mercapturic acids in our standard mix display the N-acetylcysteine NL in high abundance as observed in the MS^2 spectra of 3-HPMA (**Figure 17**), with the exception of CEMA where the most abundant NL was the loss of carboxyl + N-acetyl functional groups (-105 Da) and the N-acetylcysteine (-131 Da) NL was missing in the MS^2 spectra (**Figure 18**). This diagnostic fragment could help for mercapturic acid discovery and identification; however, more research is needed to understand the behavior of this neutral loss in positive mode as it hasn't been described previously. The application of positive ion acquisition may prove useful for discovery techniques and deserves re-evaluation for mercapturic acid discovery as there is an increase in fragmentation compared to negative mode which might help to increase confidence in mercapturic acid structural predictions.

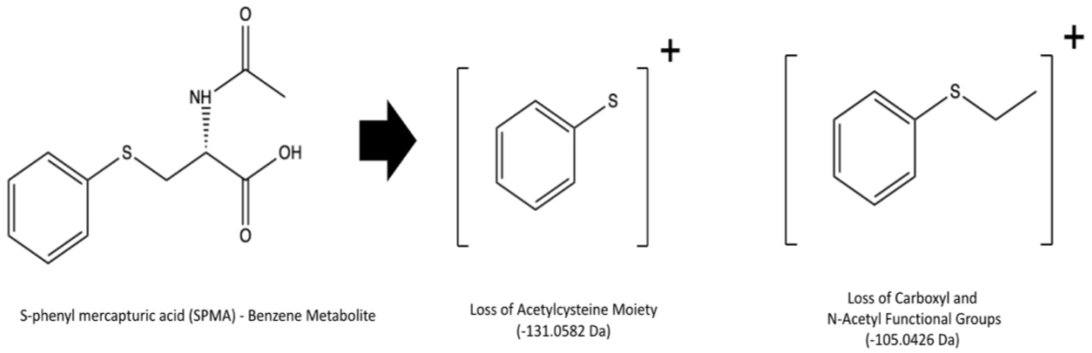


Figure 16. The fragmentation patterns common to mercapturic acids (using SPMA as an example) that are used in our analysis.

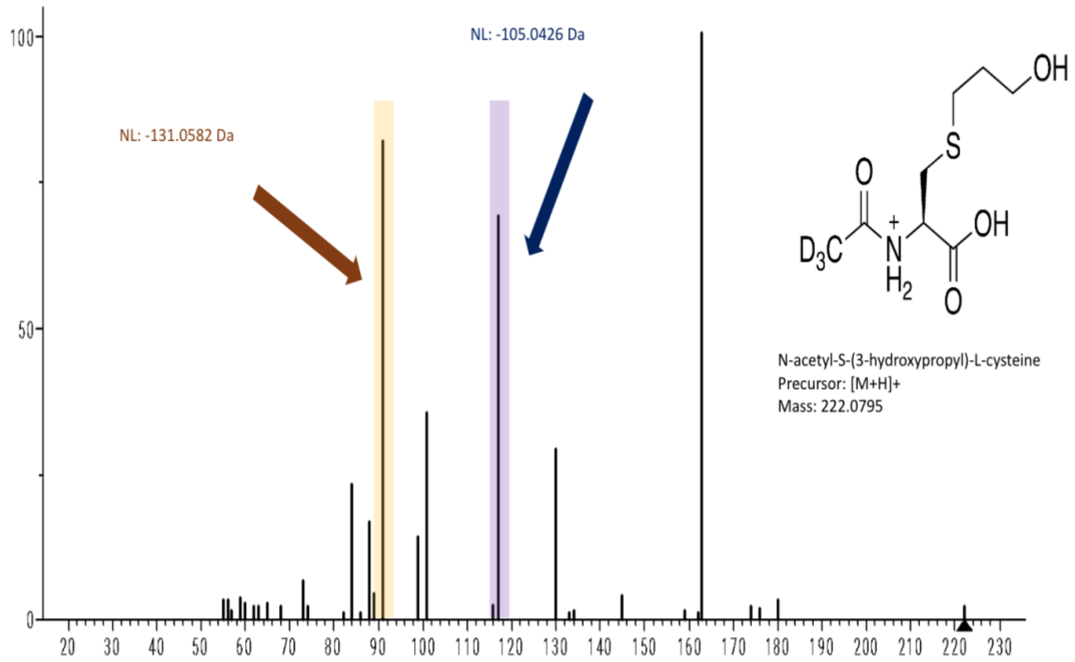


Figure 17. MS^2 spectra of 3-HPMA (222.0795 m/z) showing the neutral loss (NL) of both the N-acetylcysteine (-131.0582 Da) and the carboxyl + N-acetyl (-105.0426 Da) fragment

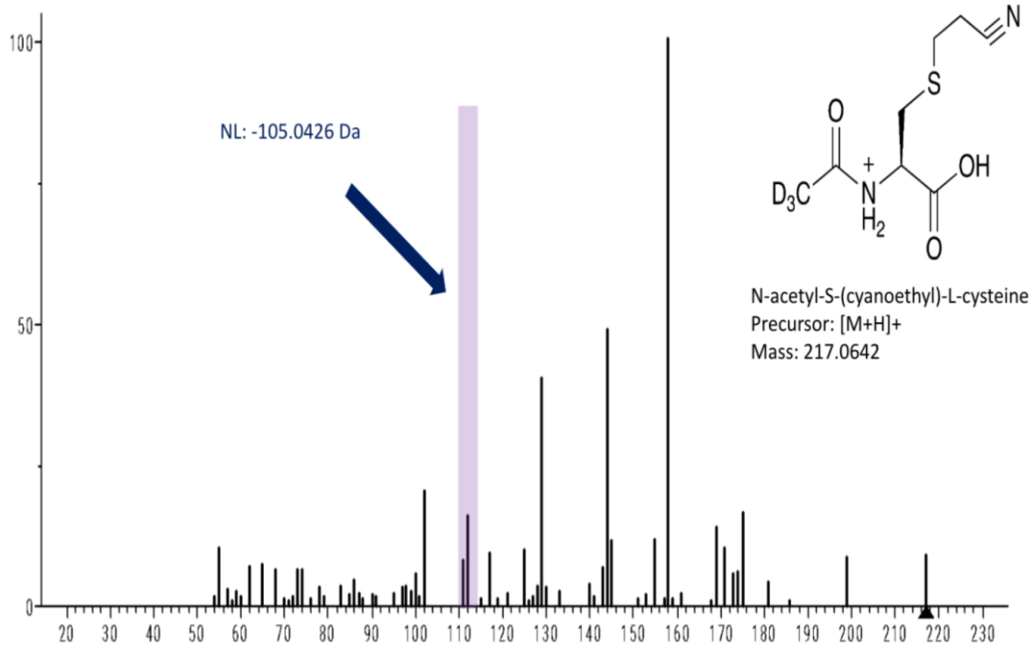


Figure 18. MS^2 spectra of CEMA (217.0642 m/z) showing the neutral loss (NL) of the carboxyl + N-acetyl (-105.0426 Da) fragment.

6. CONCLUSIONS AND FUTURE WORK

Our method successfully reported detection of all our standards, their light equivalents, and other putative MA signatures using our novel method. From total and extracted ion chromatograms inspections shows that our assay produced consistent chromatographic profiles free of impeding contamination with suitable signal stability for untargeted detection with minimal retention time shift between replicates. Success was found in improving the chromatography by adding extra H_2O and FA preconditioning washes to the Oasis MAX 96-well plate assay [13,15]. Experiments testing a protein precipitation technique demonstrated that the Oasis MAX sorbent contains a significant contaminant that co-elutes in our samples and negatively impacts results by taking up column and impeding signal reponse of early eluting MAs, specifically in high resolution nanoflow methods. To improve our chromatography on a new column, the method was first optimized using only our IS mix. The column was changed from a Luna C18 column (20 cm length x 75 µm ID, 5 µm particle size) to a self-packed C18 column (Dr. Maisch GmbH ReproSil-PUR 1.9 µm 120 Å C18aq, 45 cm length x 100 µm ID) and the new column was tested with two different buffer systems. The NH_4OAc buffer system produced very poor results and it was clear that the 98:2 2 (H_2O:ACN), 0.1% FA was superior. With the tests performed using different injection amounts and different concentrations of our internal standard mixture the conclusion was when injecting over 1 µL of sample the peak shape and intensity suffer and when too high of a concentration is injected the intensity suffers. It was confirmed that the best injection was 1 uL. The final optimization done on our internal standard mixture was testing a new reconstitution due to the theory that ACN was competing for binding in the column. The new aqueous reconstitution of 100% H_2O, 0.1% FA provided the optimal signal response and chromatographic peaks of each of our standards and the issue of overloading was solved with less concentrated injections derived from doing a 1:100 dilution of the dried down sample with H_2O, 0.1% FA prior to injection. Our alterations to the gradient, buffers, injection volume, and standard concentrations produced promising results by improving chromatographic separation and peak shape indicating compatibility with the nanoflow UHPLC system. Monitoring of Pierce^{TM} iRT peptide standards showed quantitative reproducibility of peak heights and areas between independent injections. Including the light equivalent of our standard MAs, 316 MA candidates were detected many with MS/MS spectra. However, a possible limitation to our method is the use of positive ionization as it is reported in studies that employed both techniques suggesting that negative ionization is better for mercapturic acid analysis when using tandem mass spectrometry. However, it is observed that positive mode results in increased fragmentation of MAs while negative mode was mainly superior for fragmentation from loss of the N-acetylcysteine moiety. This work and other studies that screen for mercapturic acids in urine and other biofluid samples could help advance research on and improve early diagnosis from at risk exposures, risk stratification, and monitoring response treatments. Future experiments using our method are warranted to investigate the potential 269.0952 m/z MA biomarker for tobacco exposure and the applications of other common ion fragments. This thesis has highlighted how MA analysis has improved over the years which allowed for the placement of our research within the context of existing literature making a case for why further mercapturic acid analysis studies are still needed.

There is an extended number of well-established studies on mass spectrometry analysis of mercapturic acids but there is still potential for development to improve this area of research. One effort to improve analysis methods to increase separation and detection is testing different chromatographic techniques and employing the use of HRMS. Research will continue to focus on developing targeted methods to quantify known mercapturic acids of interest and more work will be needed to improve untargeted approaches that can identify novel MAs and quantify a broader range of MA metabolites. Future untargeted work may also involve method development work on high resolution mass spectrometry as it is seldom seen in studies screening for mercapturic acids. In principle, to get accurate mass measurements of mercapturic acids HRMS and multiple internal standards and fragmentation patterns are needed as increasing mass resolution will consequently increase false detections of mercapturic acids. It has been observed that not all mercapturic acids exhibit the N-acetylcysteine daughter ion, and it isn’t even the most abundant fragmentation pattern for some mercapturic acids. Employing the use of the N-acetylcysteine CNL has been successful over the years for discovering new mercapturic acids but for mercapturome research to advance more characteristic fragmentation patterns are needed and with more time and development additional novel mercapturic acids can be discovered that are generated from exogenous or endogenous compounds. Future applications of positive ion mode outperforming negative mode in fragmentation will require further evaluation in the context of discovery. Research on the metabolism of mercapturic acids has been studied in enough detail that the general enzymatic steps are known, however, specific enzyme kinetics, variations in enzyme activity, transporters, or interactions with other compounds that might play a role in MA metabolism are still not completely figured out. In addition, it is not fully understood how MA metabolism plays out in different tissues of the body, as it is understood the liver is the major site, but the role other organs play is not fully understood. Developments on methods to expand our knowledge and understanding of the mercapturome in specific populations will not only provide information on new mercapturic acids and new exposure sources but importantly will provide insight on the detoxification processes behind their production.

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Appendices

Appendix Table 1. Description of urine samples from smoker and nonsmoker volunteers.

Subject #	Smoking status	Type of urine collection	Age	Gender	Race	Ethnicity	Cigarettes per day?	How long have you smoked at this rate?	Have you ever smoked more than your current rate of smoking?	When you were smoking the heaviest, how many cigarettes did you smoke per day?	How long did you smoke at that rate?	Cigarette Brand?	What age did you smoke your first cigarette?	What age did you become a regular smoker?
079	NS	1st void	22	F	Asian/ white	NH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
082	NS	1st void	30	F	White (Caucasian)	NH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
087	NS	1st void	29	M	African American	NH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
088	NS	1st void	27	M	White (Caucasian)	NH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
094	NS	1st void	21	F	African American	NH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

096	NS	1st void	22	M	Asian/Pacific Islander	NH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
128	NS	1st void	35	F	Asian/Pacific Islander/Indian	NH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
133	NS	1st void	44	F	White (Caucasian)	NH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
134	NS	1st void	26	F	Indian, Spaniard	H	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
140	NS	1st void	49	M	White (Caucasian)	NH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
141	NS	1st void	38	F	White (Caucasian)	NH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
142	NS	1st void	42	M	White (Caucasian)	NH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
149	NS	1st void	47	F	White (Caucasian)	NH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

161	NS	1st void	26	F	White (Caucasian)	NH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
162	NS	1st void	41	F	White (Caucasian)	NH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
167	NS	1st void	47	F	White (Caucasian)	NH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
189	NS	1st void	60	M	White (Caucasian)	NH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
194	NS	1st void	61	F	White (Caucasian)	NH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
207	NS	1st void	53	F	White (Caucasian)	NH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
215	NS	1st void	22	F	White (Caucasian)	NH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
538	NS	spot	29	F	Asian/Pacific Islander	NH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

101	S	1st void	33	M	N/A	NH	20	5	no	40	3 years	Newports	18	20
110	S	1st void	39	F	African American	NH	17	5	yes	34	1 year	Marlboro 72's	18	18
117	S	1st void	48	F	White (Caucasian)	NH	10	10	yes	20	20 years	Salem Slim Lights (100s)	13	18
131	S	1st void	52	F	African American	NH	15	7	yes	20	7 years	Newports	11	11
132	S	1st void	53	F	White (Caucasian)	NH	10	11	no	N/A	N/A	Camel Lights	22	24
150	S	1st void	23	F	White (Caucasian)	NH	10	2	no	N/A	N/A	Camel lights	15	16
151	S	1st void	24	F	Hispanic (Mexican) / Caucasian	H	10	7	no	N/A	N/A	Marlboro Light Menthols	17	17
152	S	1st void	45	M	African American	NH	20	15	no	20	15 years	Mavericks	24	25

156	S	1st void	33	F	Black/ White	NH	20	15	yes	50	5	Newport 100's	14	14
157	S	1st void	53	F	White (Caucasian)	NH	30	10	yes	40	3 years	Marlboro - basic	14	14
135	S	1st void	29	M	Black/ American Indian	NH	12	1	no	N/A	N/A	Newport 100's	17	20
136	S	1st void	42	M	White (Caucasian)	NH	10	4	yes	20	15 years	Camel Lights	6	17
138	S	1st void	23	M	African American	NH	15	6	no	N/A	N/A	Newport	17	18
144	S	1st void	62	F	White (Caucasian)	NH	10	7	yes	20	12 years	American Spirit	17	22
145	S	1st void	51	M	White (Caucasian)	NH	40	42	no	N/A	N/A	Grand Prix Full - Flavor	8	9
146	S	1st void	53	F	White (Caucasian)	NH	10	1	yes	20	5 years	Camel Lights	10	14

147	S	1st void	41	F	African American	NH	18	5	no	N/A	N/A	Marlboro	16	16
163	S	1st void	56	M	African American	NH	10	42	yes	20	5 years	Mavericks	14	15
171	S	1st void	36	F	Chicano/ Mexican, Puerto Rican, Cuban Caucasian, east Indian	NH	20	20	yes	40	3 months	Newport 100's	13	13
190	S	1st void	51	F	White (Caucasian)	NH	20	30	no	N/A	N/A	Marlboro Light	15	17
451(v3)	S	Spot	66	F	White (Caucasian)	NH	20	47	2	N/A	N/A	Virgina Slim - Filtered - Non Menthol - Regular	18	19
246	S	spot	20	M	American Indian, Alaskan Native	NH	10	6	yes	20	3 months	Newport Menthol 100's	10	14

293	S	spot	26	M	African American	NH	12	5	no	N/A	16 years	Newport	15	15
332	S	24 hour	35	F	White (Caucasian)	NH	20	15	No	N/A	N/A	Camel Blue	12	17

*NS=Nonsmoker, S=Smoker, M=Male, F=Female, H=Hispanic, NH=Non-Hispanic.

Table 5. Description of previous mass spectrometry methods for the determination of mercapturic acids as biomarkers for exposure to occupational, environmental, and dietary toxicants.

Reference	**Exposure**	**Mercapturic Acids Detected & Standards**	**Sample preparation**	**Chromatography & Mobile Phases**	**Column (Length x ID, Particle Size)**	**MS instrument**	**Injection Vol. / Flow Rate.** **Ionization / LOD**
Paci et al., 2007 [59]	Urine from smokers and nonsmokers that also worked at oil plant.	SPMA	SPE on Sep-pack C18 cartridges and filtered on a 0.2-mm filter device	Series 200 HPLC quaternary pump A: MeOH B: 70% 0.1 M acetic acid	Discovery C18 (150 x 4.6 mm)	PE Sciex API 4000.	20uL / 1mL/min Negative ESI / 0.05 ug/L
Sabatini et al., 2008 [66]	Urine from occupational exposed individuals	SPMA SBMA o-MBMA	Supernatant of urine extracted via Evolute^{TM} ABN SPE, eluted methanol.	Agilent 1100 - HPLC system	Synergi 4u Max-RP capillary	Sciex API 2000	0.5uL / 10uL/min

				A: H_2O B: MeOH	column (50 × 0.5 mm, 4 µm, 80 ˚A)		Negative ESI / 0.30ug/L
Schettgen et al., 2008 [68]	Smoker and non-smoker human urine samples	SPMA SBMA	Protein precipitation with 20 L of FA (100%) samples.	Agilent 1100 Series HPLC A: H_2O, 0.05% FA, pH 2.5 B: ACN.	Reversed-phase HPLC column (Luna C8 (2) 150 × 4.6 mm, 3 µm)	Sciex API 3000	100uL / 0.3mL/min Negative ESI / 0.02ug/L
Yoshida et al., 2012 [84]	Urine of stroke patients	3-HPMA	Protein precipitation with 2 mL NH_4HCO_2 buffer, 0.04 ml FA. Next SPE column (ISOLUTE ENV+, 100 mg, 3 mL).	HILIC LC A: 5 mM NH_4OAc B: MeOH	XBridge HILIC (150 × 2.1 mm, 3.5 µm)	Sciex API 2000	- / 0.3mL/min Negative ESI / -
Scheuch et al., 2015 [70]	Human urine samples after single dose of flupirtine	Flupirtine metabolites M-424 & M-466	STRATA SPE Giga tubes, Phenomenex, C18E 20g	HPLC Agilent 1100 A: H_2O, 0.1% FA B: ACN, 0.1% FA	Synergi® Fusion-RP (50 × 2.0 mm, 2.5 µm)	Sciex API3000	10uL / 0.2mL/min Negative ESI / 5ng/mL
Caron-Beaudoin et al., 2018 [16]	Human urine samples with high	SPMA SBMA	SPE using a Perkin Elmer Janus Automated Workstation	HPLC	x	LC-MS/MS	-/-

	levels of natural gas exploitation			A: H_2O, 0.5% acetic acid B: MeOH			Negative ESI / 0.8ug/L
Wu et al., 2012 [82]	Smokers and Non-smokers	CEMA CHEMA HEMA	Protein precipitation with FA and NH_4HCO_2 buffer (50 mM), filtered through a 0.22-μm poly(vinylidene difluoride) membrane filter	UPLC A: ACN, 0.1% FA B: H_2O, 0.1% FA	Waters Acquity HSS T3 (100 × 2.1 mm, 1.8 μm)	TSQ Quantum	- / 200uL/min Negative ESI / 1ug/L
Linhart et al., 2012 [50]	Workers exposed to styrene	2-VPMA 3-VPMA 4-VPMA	OasisTM HLB 60 mg SPE columns (Waters)	Normal Phase HPLC A: H_2O, 0.1% FA B: ACN, 0.1% FA	Hypersil Gold C18 column (150 × 2.1 mm, 5 μm.	Linear trap mass spectrometer	10uL/ 150uL/min Negative ESI / 0.3ng/mL
Kotapati et al., 2011 [45]	Smoker and nonsmoker urine samples	MHBMA THBMA DHBMA	Protein precipitation with 50 mM NH_4HCO_2 buffer (pH 2.5) and 98% 10 FA and centrifuged and the supernatant was loaded onto Isolute ENV+ SPE cartridges eluted	Agilent 1100 HPLC A: H2O, 0.1% FA B: ACN, 0.1% FA	A SIELC Primesep B2 column (50 × 2.1 mm, 5 μm)	TSQ Quantum Discovery	- / 150uL/min Negative ESI / 0.1ng/mL

			with 1.2 mL of 2% FA in methanol.				
Kotapati et al., 2014 [46]	Smokers and nonsmokers and 1,3-butadiene exposed workers	DHBMA THBMA *bis*-BDMA	Protein precipitation with 50mM NH_4HCO_2 buffer (pH 2.5) and FA and loaded on Isolute ENV+ SPE cartridges (1ml/50mg)	Agilent 1100 HPLC A: H_2O B: 50% ACN, 50% H_2O, 1% FA	a SIELC Primesep D column (100 × 2.1 mm, 5 µm)	TSQ Vantage	3uL / 200uL/min Negative ESI / 1ng/mL
Zhang et al., 2015 [85]	Human urine samples from healthy college students and urine from acrylamide dosed rats	AAMA AAMA-Sul GAMA iso-GAMA	For rat urine, filtrated through the 0.22-mm millipore filter For human urine, protein precipitation with FA & NH_4HCO_2 and SPE using Isolute® ENV+ SPE and passed through the 0.22-mm millipore filter.	1290 Infinity quaternary UHPLC A: H2O, 0.1% (v/v) FA B: ACN	Acquity UHPLC HSS T3 column (150 x 2.1 mm, 1.8 um)	Agilent 6460	2uL / 0.2mL/min Negative ESI / 0.1 - 0.3ng/mL
Wagner et al., 2007 [79]	No specific exposure looked at. Pool of healthy human urine samples.	Acrylamide-MA (AAMA) S-(4-tert-Butylbenzyl) MA S -phenyl MA (SPMA)	Dilute and shoot with 5uL of 25% HCl and 10uL standard mix	HPLC Agilent Series 1100 A: 95% H_2O 0.1% FA	ReproSil-Pur C18-AQ (150 × 2 mm, 3 µm)	Sciex QTRAP 2000	400uL / 0.2mL/min Negative ESI / -

				B: 5% ACN 0.1% FA			
Eckert & Göen, 2014 [23]	Smokers and nonsmoker human urine samples	Methyl MA (MeMA) Ethyl MA (EtMA) n-Propyl MA (PrMA) iso-Propyl MA (iPrMA)	Protein Precipitation with 1 mL NH_4HCO_2 buffer (50 mmol/L, pH 2.5) and 40 µL of FA, 25 µL of the of the internal standard	Reversed Phase HPLC Agilent series 1100 A: 0.2% acetic acid in H_2O/ACN, 9/1, v/v B: 0.2% acetic acid in H_2O/ACN, 2/8, v/v	Aqua C18 (150 × 4.6 mm, 3 µm)	API 2000	100uL / 0.4mL/min Negative ESI / 2ug/L
Eckert et al., 2011 [24] Eckert et al., 2010 [22]	Smoker and nonsmoker human urine samples	2 HEMA 2-HPMA 3-HPMA DHPMA DHBMA MHBMA	Protein precipitation with 2 mL NH_4HCO_2 buffer and FA. Then Isolute ENV+ SPE (100 mg, 3 mL).	HPLC Agilent series 1100 A: 5 mM NH_4OAc pH 4.5 in ACN/H_2O (88/12, v/v) B: 5 mM NH_4OAc in ACN/H_2O (5/95, v/v).	Strategy® HILIC Silica (100 × 2.1 mm, 2.2 µm)	Sciex API 2000	10uL / - Negative ESI / 2ug/L

Ding et al., 2009 [21]	Smoker and non-smoker human urine samples	CEMA HPMA MHBMA DHBMA HEMA SPMA	Dilute and shoot with 0.5% acetic acid water solution.	Agilent 1100 LC A: H_2O, 0.5% acetic acid B: ACN	Waters Xterra C18 MS column (50 x 4.6 mm, 5 μm)	API 4000	10uL / - Negative ESI / 0.2ug/L
Schettgen et al., 2008 [69]	Smoker and non-smoker human urine samples	HEMA 2-HPMA 3-HPMA AAMA AMCC	SPE	Hewlett-Packard HP 1100 series HPLC A: H_2O, 0.1% FA B: 40% ACN, 0.1% FA	Luna C8 (50 x 4.6 mm, 3 μm)	Sciex API 3000	100uL / 0.3mL/min Negative ESI / 0.5mg/L
Moriwaki et al., 2000 [56]	Healthy volunteers	SBMA o-MBM 2,3-DMB 2,6-DMB 3,4-DMB	Protein precipitation and SPE using a Sep-Pak Plus tC18 cartridge.	Hewlett-Packard HP 1100 Series HPLC A: 20 mM NH_4OAc, B: ACN	Develosil C30-OG column (150 x 4.6 mm, 5 μm)	x	20uL / 0.5mL/min Negative ESI / 3.2ng/mL
Kuiper & Stevens, 2010 [47]	Oxidatively stress rat urine and smokers	ONE-MA HNE-MA ONO-MA DHN-MA	Dilute and shoot with ethyl acetate.	Reversed Phase HPLC	Synergi Max RP C12 column (250 × 2 mm)	Sciex QTrap 4000.	10 uL / 0.2 mL/min

	and non-smoker human urine.	ONA-MA HNA-MA HNAL-MA		A: H_2O, 0.1% FA B: ACN, 0.1% FA			Negative ESI / -
Song et al., 2022 [73]	Urine samples from fasting healthy patients after exposed to polluted air.	CEMA HMPMA SPMA HPMA HEMA	Online SPE Cartridge C18 MGII (S3) (4.0 × 10 mm)	UPLC UltiMate 3000 A: H_2O, 0.1% FA B: ACN, 0.1% FA	Shiseido ADME column (150 x 2.1 mm, 3 µm)	MassAR RAY Compact 96	- / 0.3 mL/min Negative ESI / -
Bloch et al., 2019 [7]	Urine samples from mothers during pregnancy.	Carbamoylethyl-MA Carboxy-MA Chlorophenyl-MA Dinitrophenyl-MA Ethoxy-MA Ethyl-MA Farnesyl-MA Fluoro-MA Furyl-MA Hydroxyethyl-MA Hydroxynonenal-MA Methyl-MA Metolachlor-MA Phenyl-MA Simazine-MA Thienyl-MA	Urine samples were filtered using 0.22 µm polyether sulfone filters and stored until use.	Agilent 1290 system A: H_2O, 0.1% FA B: MeOH. 0.1% FA	Kinetex XB-C18 (3 × 50 mm, 1.7 µm).	SCIEX QTrap 6500	- / 450uL/min Negative ESI / 5ng/mL

Frigerio et al., 2020 [26] Frigerio et al., 2019 [29]	Urine from humans exposed to VOCs of coke oven workers	2-HPMA 3-HPMA AAMA AMCC CEMA CMEMA DHBMA EMA GAMA HEMA HMPMA MHBMA MMA NANPC PHEMA SBMA SPMA	Diluted in FA then filtered with a 0.45-µm regenerated cellulose membrane filter	Reversed Phase HPLC Agilent 1260 A: 5mM NH_4HCO_2, 0.1% FA B: ACN	Betasil C18 column (150 ×, 2.1 mm, 5 µm)	Sciex QTRAP 5500	20 uL / 200 uL/min Negative ESI / -
Pluym et al., 2015 [62]	Urine from smokers and nonsmokers	SPMA SBMA EMA MMA HEMA CEMA PHEMA HMPMA, CMEMA 2-HPMA 3-HPMA AAMA AMCC	Sample prep was split into two different methods. Method 1 protein precipitation with NH_4HCO_2 and 50% NaOH Method 2 protein precipitation with methanol.	Reversed Phase UPLC Method 1: A and C: H_2O, 0.1 % FA B: ACN, 0.1 % FA D: A and B (40/60, v/v).	Method 1 used a Luna C8 column (150 × 4.6 mm, 3 µm) Method 2 BEH C18 column (150 × 3 mm, 1.7 µm).	API 5000.	2uL / 500µL/min for pump 1 and to 300µL/min for pump 2 Negative ESI / 5 ug/mL

		GAMA MHBMA DHBMA		Method 2: A: 5 mM NH_4OAc B: ACN, 5 % 100 mM NH_4OAc			
Scherer et al., 2022 [67]	Combustible cigarettes, electronic cigarettes, heated tobacco products, oral tobacco, nicotine replacement therapy products, and nonusers' urine.	HEMA 2-MHBMA DHBMA SPMA MMA EMA AMCC 3-HPMA AAMA GAMA DHPMA PHEMA-1 PHEMA-2 CEMA 2-HPMA HMPMA CMEMA IPMA3 SBMA	Urine evaporated to dryness and protein precipitation with methanol.	Reversed Phase UPLC Method 1: A and C: H_2O, 0.1 % FA B: ACN, 0.1 % FA D: A and B (40/60, v/v). Method 2: A: 5 mM NH_4OAc B: ACN, 5 % 100 mM NH_4OAc	C18 column (150 × 3 mm, 1.7 µm)	API 5000.	2uL / 300 uL/min Negative ESI / 5 ng/mL
Song et al., 2022 [74]	Urine from healthy children ages 0-7	AAMA AMCC BMA BPMA	Urine incubated with β-glucuronidase/sulfatase, diluted with	ExionLC A: H_2O, 0.05% FA	ACQUITY UPLC® HSS T3 (2.1 × 150 mm, 1.8 µm).	SCIEX QTrap 6500+	10uL / 0.25 mL/min

	living in China.	CEMA CYMA DHBMA GAMA HEMA 2-HPMA 3-HPMA HPMMA MHBMA3 PMA HNEMA	FA water solution (0.05%).	B: MeOH			Negative ESI / 0.02ug/L
Carmella et al., 2009 [14]	Urine from smokers and nonsmokers	MHBMA DHBMA HPMA HBMA SPMA HEMA	SPE using Oasis MAX (Waters)	HPLC A: 15 mM NH_4OAc, pH 6.8 B: MeOH	Synergi C12, , Max-RP, 80 Å (250 × 4.6 mm, 4µm)	a TSQ Quantum Discovery Max	- / 0.8mL/min Negative APCI / 0.013-3.2 pmol/mL
Xie et al., 2023 [83]	Urine samples from smokers and nonsmokers	MPhMA 3HPMA 23HPMA 2CaEMA MCaMA BzMA 1PMA) 2CoEMA 2CyEMA 34HBMA 2CaHEMA 3HMPMA 2HPhEMA)	Dilute and shoot with 0.1% FA.	Acquity I-Class UPLC A: H_2O 0.1% FA B: ACN, 0.1% FA	Acquity Premier HSS T3 (150 x 2.1 mm, 8µm)	QTOF-MS Synapt XS HDMS	7.5µL / 0.45mL/min Negative ESI / -

Janobi et al., 2006 [40]	Human plasma and urine following a strict broccoli diet	Mercapturic acid metabolites of Sulforaphane and iberin	Filtered (0.2 µm pore size) and dilute and shoot with NH_4OAc	Agilent 1100 A: 13mM NH_4OAc, 0.1% acetic acid B: ACN, 0.1% acetic acid.	Zorbax SB-Aq (100 × 2.1 mm, 3.5 µm)	A Quattro Ultima tandem mass	10µL / 0.25mL/min Postive ESI / -
Jamin et al., 2019 [38]	Rats on diets with heme iron (0.094% w/w) to catalyze lipid peroxidation .	8 pentanol-MA hexanol-MA ethylbenzene-MA ethyllactone-MA 4-hydroxyhexanal-MA (HHE-MA) heptanol-MA 4-hydroxyhexanol-MA (DHH-MA) hydroxyhexanol-MA octanal-MA hydroxyheptanal-MA octanol-MA hydroxypropyllactone-MA hydroxyheptanoic ac-MA decenal-MA hydroxynonenol-MA dihydroxyoctanal-MA 4-hydroxynonanol-MA (DHN-MA) dihydroxyoctanoic ac-MA ω-COOH-hydroxydecanal-MA	Dilute and shoot in 95% H_2O, 5% CH_3OH, 0.1% CH_3CO_2H	Reversed Phase UPLC A: 95% H_2O, 5% MeOH, 0.1% acetic acid B: MeOH, 0.1% acetic acid	Hypersil Gold C18 (100 × 2.1 mm, 1.9 µm)	Q-ToF Synapt G2-Si	10 uL / 0.3 mL/min Negative ESI / 10ng/mL

Alwis et al., 2012 [1]	Urine samples from human smokers and non-smokers	AAMA AMCC BMA BPMA CEMA CYMA 1,2-DCVMA 2,2-DCVMA DHBMA DPMA GAMA HEMA 2-HPMA 3-HPMA HPMMA MHBMA1 MHBMA2 MHBMA3 PHEMA SPMA TCVMA	Dilute and shoot with 1:10 with buffer (50 L urine + 25 L working mixed internal standard + 425 L 15 mM ammonium acetate pH 6.8).	UPLC A: 5 mM NH_4OAc, pH 6.8 B: ACN	Acquity UPLC® HSS T3 (150 × 2.1 mm, 1.8 µm)	Sciex API 5500	- / 300uL/min Negative ESI / 2 ng/mL
Scholz et al., 2005 [71]	Rats exposed to VOCs benzene, toluene, xylene, styrene, butadiene, naphthalene	N-acetyl-(2,2-dichlorovinyl)-L-cysteine N-acetyl-(4-chlorobenzyl)-L-cysteine N-acetyl-(2-fluorobenzyl)-L-cysteine N-acetyl-(4-bromobenzyl)-L-cysteine	Protein precipitation with ACN	Reversed Phase HPLC A: 5 mM NH_4OAc, pH 6.8 B: ACN	Synergi polar RP18 column (150 × 2 mm, 4 µm).	QTRAP API 2000	5 uL / 0.25 mL/min Negative & Positive ESI / 20 ng/mL

	, & biphenol A.	N-acetyl-(4-tert-butylbenzyl)-L-cysteine 3-(N-acetylcystein-S-yl)-acetaminophen N-acetyl-(1,1-Dichloro-2,2-difluoroethyl)-L-cysteine N-acetyl-phenyl-L-cysteine N-acetyl-(2,4-dinitrophenyl)-L-cysteine N-acetyl-(1,2,2-trichlorovinyl)-L-cysteine N-acetyl-(3-hydroxypropyl)-L-cysteine N-acetyl-(1,2-dichlorovinyl)-L-cysteine					