11-23-2015

High Performance Liquid Chromatography Tandem Mass Spectrometry for Trace Analysis in Environmental Samples

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY FOR TRACE ANALYSIS IN ENVIRONMENTAL SAMPLES

by

Heather L. Fleming

A Dissertation

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

Major: Chemistry

The University of Memphis

December 2015

Dedicated to:

My Mom and Dad

&

Lionel

Acknowledgments

I would like to thank my advisor, Dr. Paul Simone, Jr., for his guidance, support, and encouragement. His strong belief in my talents and abilities shaped my research career and was essential in my personal development. I would also like to express my appreciation to Dr. Gary Emmert, if not for his advocacy I might have never pursued this path in life.

I would like to sincerely thank Dr. Patricia Ranaivo for her mentorship and her contributions to portions of this dissertation. I would like to express gratitude to my committee members, Dr. Randy Buddington, Dr. Xiaohua Huang, and Dr. William Alexander for their counsel and their time. I would also like to thank the members of the MAMML group, past and present, for their advice, support, and friendship.

Finally, I gratefully acknowledge the support of the University of Memphis Department of Chemistry, USPlabs for funding the work discussed in Chapters 2 and 3, and the FedEx Institute of Technology for funding portions of the work in Chapter 4.

Abstract

Fleming, Heather L. Ph.D. The University of Memphis. December 2015. High Performance Liquid Chromatography Tandem Mass Spectrometry for Trace Analysis in Environmental Samples. Major Professor: Paul Simone, Jr., Ph.D.

1,3-dimethylamylamine (1,3-DMAA) is a stimulant commercially sold in a variety of dietary supplements as a chemical species derived from geranium plants (Pelargonium graveolens). Whether 1,3-DMAA naturally occurs in geranium plants or other dietary ingredients, it has important regulatory and commercial ramifications. An extraction method combined with HPLC-MS/MS is used to determine 1,3-DMAA and 1,4-dimethylamylamine (1,4-DMAA) concentrations in geranium plants with both external calibration and standard addition methods. Samples from the Changzhou, Kunming and Guiyang regions of China during both winter and summer were analyzed. Following the detection of DMAA in the Changzhou sample, an extraction and pre-column, chiral derivatization chemistry method was developed for the separation and analysis of the four stereoisomers of 1,3-DMAA and two enantiomers 1,4-DMAA. Two chiral derivatizing agents (CDAs) were investigated: (-)-1-(9-fluorenyl)ethyl chloroformate [(-)-FLEC] and (R)-(-)α-methoxy-α-(trifluoromethyl) phenylacetyl [(-)-MTPA]. Optimization studies and detailed method detection limit (MDL), accuracy, precision and linearity studies are presented for analysis of the DMAA-FLEC species in geranium plants. The DMAA-FLEC product was found to be unstable and a second, more stable CDA [(-)-MTPA] was employed. A preparatory scale HPLC separation was added prior to derivatization with (-)-MTPA to aid in the separation of all DMAA stereoisomers. The propagation of error prohibited a confident analysis of individual DMAA stereoisomers, but the DMAA-

FLEC and DMAA-MTPA methods both confirmed the presence of DMAA in the Changzhou plant samples.

Haloacetic acids (HAAs) are formed during the chlorination of drinking water. Previous work detected HAAs in bulk sodium hypochlorite solutions with post column reaction ion chromatography (PCR-IC). A HPLC-MS/MS method was designed to provide confirmation of the presence of HAAs in the sodium hypochlorite solutions. Detailed MDL, accuracy, and precision studies are presented for the analysis of nine haloacetic acids in sodium hypochlorite solutions. Due to the complex nature of the sample matrix a solid phase extraction step was added to the HPLC-MS/MS procedure. The HAAs monochloroacetic acid, dichloroacetic acid, and trichloroacetic acid were detected in the bulk sodium hypochlorite solutions by both the HPLC-MS/MS and the PCR-IC although the two methods disagree on reported concentrations.

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List of Symbols and Abbreviations

~	approximately
[ ]	molar concentration
ºC	degrees Celcius
ºC min^{-1}	degrees Celcius per minute
=	equals
<	less than
µg	microgram
µg L^{-1}	microgram per liter
µL	microliter
µM	micromolar
µm	micrometer
/	per (division)
%	percent
+	plus
±	plus or minus
ACS	American Chemical Society
AR	Arkansas
b	y-intercept
BCAA	bromochloroacetic acid
BDCAA	bromodichloroacetic acid
CH_3CN	acetonitrile
CH_3COOH	acetic acid
C.L.	confidence level
Cl^-	chloride ion
Cl_2	chlorine gas
cm	centimeter
Conc.	concentration
DBA	dibutylamine
DBAA	dibromoacetic acid
DBCAA	dibromochloroacetic acid
DBPs	disinfection by-products
DC	direct current
DCAA	dichloroacetic acid
DIA	diastereomer
DMAA	dimethylamylamine
ECD	electron capture detector
ESI	electrospray ionization
eV	electron volt
FAC	free available chlorine

FLEC	(-)-1-(9-fluorenyl)ethyl chloroformate
g	gram; gravity
GC	gas chromatography
GC-ECD	gas chromatography-electron capture detector
GC-FID	gas chromatography-flame ionization detector
GC-MS	gas chromatography-mass spectrometry
H^+	hydrogen ion
H_2O	water
H_2SO_4	sulfuric acid
HAAs	haloacetic acids
HAA5	the five regulated HAAs
HAA9	all nine HAAs found in drinking water
HCl	hydrochloric acid
HILIC	hydrophilic interaction chromatography
HOCl	hypochlorous acid
HPLC-MS/MS	high performance liquid chromatography-tandem mass spectrometry
I^-	iodide ion
I_3^-	triiodide ion
KI	potassium iodide
KIO_3	potassium iodate
KOH	potassium hydroxide
KOT	knitted open tubular
kV	kilovolt
LC	liquid chromatography
L hr^{-1}	liter per hour
LIT	linear ion trap
LOD	limit of detection
LOQ	limit of quantification
M	moles per liter
m	slope
m/z	mass to charge ratio
MΩ·cm	megaohms per centimeter
MA	Massachusetts
MBAA	monobromoacetic acid
MCAA	monochloroacetic acid
MCL	maximum contaminant level
MDL	method detection limit
mg	milligram
mg L^{-1}	milligram per liter

MHA	methylhexaneamine
min	minute(s)
mL	milliliter
mL min^{-1}	milliliters per minute
mm	millimeter
mM	millimoles per liter
MO	Missouri
MRM	multiple reaction monitoring
MS	mass spectrometry; Mississippi
MS1	first mass analyzer
MS2	second mass analyzer
MTPA	(R)-(-)α-methoxy-α-(trifluoromethyl) phenylacetyl
MTBE	methyl tert-butyl ether
N	normality
$Na_2S_2O_3$	sodium thiosulfate
Na_2SO_4	sodium sulfate
$NaHCO_3$	sodium bicarbonate
NaOCl	sodium hypochlorite
NaOH	sodium hydroxide
NJ	New Jersey
nm	nanometer
NMR	nuclear magnetic resonance
NY	New York
OCl^-	hypochlorite ion
OH^-	hydroxide ion
PAH	polycyclic aromatic hydrocarbon
PCR-IC	post-column reaction-ion chromatography
ppb	parts per billion
ppm	parts per million
psi	pound-force per square inch
QQQ	triple quadrupole
QTOF	quadrupole time of flight
R.S.D.	relative standard deviation
r^2	correlation value
Rec.	recovery
rf	radio frequency
$S_2O_3^{2-}$	thiosulfate ion
$S_4O_6^{2-}$	tetrathionate ion
SPE	solid phase extraction

TBAA	tribromoacetic acid
TCAA	trichloroacetic acid
THMs	trihalomethanes
TN	Tennessee
Trad.	traditional
Unc.	uncertainty
UPLC	ultra performance liquid chromatography
USA	United States of America
USEPA	United States Environmental Protection Agency
UV-Vis	ultraviolet-visible
V	voltage
vs.	versus

Chapter 1

Introduction

Determining the concentrations of multiple analytes in complex environmental samples relies upon the use of analytical separations. The analytical separations can take the form of a chromatographic separation, extraction or a combination of both. These separations take advantage of the partition coefficient of an analyte between two phases of a heterogeneous mixture. The partition coefficient (K) is an equilibrium constant based on the concentration ratio of the analyte in Phase 2 ($[S]_2$) versus Phase 1 ($[S]_1$) (Equation 1).

$$K = \frac{[S]_2}{[S]_1} \qquad \text{(Equation 1)}$$

The distribution of the analyte in either phase is directly associated with the pH of the solution when the solute is an acid or base. In this scenario the distribution of analyte is better described by the distribution coefficient (D), defined as

$$D = \frac{[S]_2}{[S]_1 + [S_i]_1} \qquad \text{(Equation 2)}$$

where $[S_i]_1$ is the concentration of the ionized species (Equation 2). The equilibrium of ionized and uncharged molecules for a particular solute is defined by the dissociation constant K_a (pK_a = -log K_a). The ionized species are more soluble in aqueous phases and neutral species are more soluble in organic phases. In other words, a sufficient change in pH resulting in the conversion of the uncharged solute species to the ionized species facilitates it's extraction into an aqueous medium (Harris, 2007). For example, lowering the pH for a basic analyte and increasing the pH for an acidic analyte will increase their solubility into water. In the same manner, the pH of the mobile phase will play a role in

the chromatographic separation of ionic analytes. As molecules become more ionized they become more hydrophilic and are less retained by a reversed phase column (Synder, Kirkland, & Dolan, 2010). For this reason, knowledge of the chemical properties (K_a, pH, etc.) of both the target analyte and the sample matrix is crucial for determining distribution in an extraction and retention time and resolution in a chromatographic separation.

Chromatographic separations used in environmental analytical chemistry are typically gas chromatography (GC), high performance liquid chromatography (HPLC), and most recently ultra-performance liquid chromatography (UPLC) due to their robustness and ruggedness when presented with multiple sample types. The trend in detection over the last 30 years has been to replace chemically specific detectors (e.g. flame ionization detection for GC, or ultraviolet-visible absorption and fluorescence detectors for HPLC) with mass spectrometry (MS) techniques (Dass, 2007). Chromatographic separations combined with mass spectrometry, such as the HPLC-MS/MS discussed and presented in this dissertation, are powerful analytical techniques for the qualitative and quantitative determination of trace level compounds in environmental samples. Trace analyses are defined by analyte concentrations ranging from approximately 1 $\mu g\ L^{-1}$ to 100 $mg\ L^{-1}$. Typically, GC-MS methods are limited to volatile and thermally stable compounds. Occasionally, non-volatiles can be analyzed by GC-MS, but only if they are derivatized to a volatile species first (Skoog, Holler, & Crouch, 2007). An excellent example of this is esterification of carboxylic acids to the corresponding methyl esters for GC analysis (USEPA, 2003). The advantage of HPLC-MS is that it is not limited to volatile analytes and therefore does not require complex

derivatization steps to volatilize the analytes. The HPLC-MS (Figure 1) technique analyzes and identifies compounds based on two independent chemistries. The first chemistry is separation via chromatography using retention times as an identifying characteristic (Skoog et al., 2007). The second chemistry is mass spectrometric analysis where the molecular ion and/or one or more product ions (Vogeser & Seger, 2008) provide one (MS) or more (MS/MS) identifying characteristics. As compared to a HPLC-MS with a single quadrupole, the HPLC-MS/MS is capable of providing a second level of selectivity due to instrument design. A HPLC-MS/MS is equipped with two additional quadrupoles that perform as a collision cell and a second mass analyzer. The additional quadrupoles allow for the detection of the target analyte while avoiding compounds and interferences that might co-elute or have isobaric interferences in the sample matrix (Kim & Carlson, 2005; Skoog et al., 2007). These characteristics make the HPLC-MS/MS an excellent choice for the analysis of trace level compounds found in the complex matrices of environmental samples.

This introductory chapter is dedicated to explaining the fundamental steps essential for the development of an HPLC-MS/MS method for the analysis of an environmental sample. These steps include sample preparation, liquid chromatographic separation and mass selective detection. Sample preparation techniques are imperative in the analysis of environmental sample matrices which can contain superfluous compounds that interfere with detection. The work presented here describes the separation and detection of analyte compounds by reverse-phase chromatography and tandem mass spectrometry (triple quadrupole) detection.

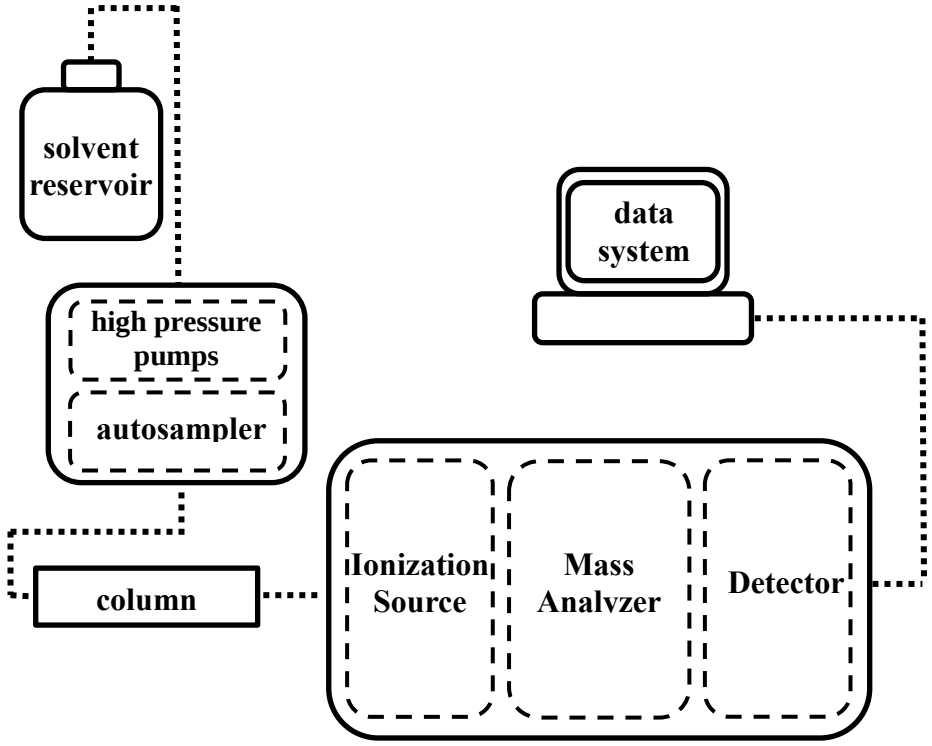

Figure 1. HPLC-MS instrument diagram.

High Performance Liquid Chromatography

Since its introduction in the 1960s high performance liquid chromatography (HPLC) has become an invaluable analytical technique that is practiced all around the world (Snyder et al., 2010). HPLC is a liquid chromatography technique characterized by the use of high-pressure, reciprocating piston pumps, columns with < 10 μm particles, and a variety of universal and chemically specific detectors. These characteristics make HPLCs applicable to a large variety of assays while providing fast separations and reproducible results. HPLC separations can be accomplished in multiple modes: adsorption, partition, ion-exchange, size exclusion, and affinity chromatography (Harris, 2007). Adsorption chromatography is characterized by a solid stationary phase and a liquid or gas mobile phase. The speed at which an analyte travels through the column is

dependent upon its equilibrium between adsorption onto the stationary phase and solubility in the mobile phase solvent. Strongly adsorbed analytes will travel slower through the column and therefore increasing the elution time (Faust, 1997). Two types of adsorption chromatography exist: normal phase and reverse phase. Normal phase chromatography is characterized by a polar stationary phase and less polar mobile phase. In comparison, reverse phase chromatography uses a non-polar stationary phase and a polar mobile phase. Much like adsorption chromatography, ion-exchange chromatography is also characterized by a solid stationary phase and a liquid mobile phase (Harris, 2007). The difference is found in the modified stationary phase (also called a resin): anions and cations are covalently bonded to this phase and govern the adsorption of the solute ions. Partition chromatography is characterized by a non-volatile liquid stationary phase that is adhered to an inert solid surface. Size exclusion chromatography (also called gel permeation or gel filtration chromatography) separations are governed by the pore size of the stationary phase and molecules are sorted based on their size (Snyder et al., 2010). The smaller pore size allows larger solute particles to pass while retaining smaller solute particles. Affinity chromatography is similar to ion-exchange chromatography in that the stationary phase has been modified to include covalently attached molecules that interact specifically with the target compound. For example, the covalently attached molecule could be a substrate for a particular enzyme (Harris, 2007).

In the work presented here, adsorption chromatography was performed using a versatile, reverse-phase C_{18} column to separate two different classes of small, polar analyte molecules that are ionic and/or neutral. As mentioned previously, in reverse phase chromatography the column stationary phase is nonpolar and the mobile phase is a

homogeneous mixture of polar solvents. In a traditional separation, a pH buffered, aqueous solution is mixed with methanol or acetonitrile. The buffer maintains a narrow pH range, and the methanol and acetonitrile serve to solvate the neutral compounds.

Reverse-phase chromatography can also be combined with derivatization and ion pairing methods to achieve even more complex separations of stereoisomers and ionic species. Separation of enantiomers on an achiral column is impossible because the enantiomers have the same chemical and physical properties. However, if a pair of diastereomers exist – then separation is possible because the diastereomers have differing chemical and physical properties. Thus, separation of enantiomers (and stereoisomers) on an achiral column can be accomplished via a specialized chiral stationary phase (direct method) or via derivatization to diastereomers using enantiomerically pure reagents (indirect method). In the direct method the enantiomers will form diastereomeric complexes with the stationary-phase selectors. Chiral stationary phases include Pirkle-type, cellulose triesters or carbamates on silica, cylclodextrins, polyacrylates, pollyacrylamides, crown ethers, and protein phases (Ahuja, 1997; Snyder et al., 2010). In the indirect method, the enantiomer analyte reacts with an enantiomerically pure reagent to form multiple diastereomers. The diastereomer products can then be separated with an achiral reversed-phase chromatography column (Snyder et al., 2010). Finally, reverse-phase chromatography can be used for separation of ionic analytes by mobile phase modification. This is achieved by adding an ion-pairing reagent to the mobile phase. The ion-pairing reagent interacts via electrostatic forces with the ionized analyte compound to form a neutral ion pair. With the correct ion-pairing agent, the ion pair species will have

hydrophobic properties resulting in the retention of analytes on the column. The separation type can be altered based on mobile phase components.

The three works presented in this dissertation (Chapters 2, 3, and 4) were conducted on the same type of C_{18} column but all employed a unique chemistry for the final separation of analytes. Chapter 2 describes the reverse-phase separation of two dimethylamylamine compounds (1,3-DMAA and 1,4-DMAA). In these studies, formic acid was added to the mobile phase to aid in the ionization of the analytes at the electrospray interface. Chapter 3 describes the separation of enantiomers with the indirect method: the two dimethylamylamine compounds (1,3-DMAA and 1,4-DMAA) are reacted with two chiral derivatizing agents (CDAs) to produce diastereomer products that are separated with the C_{18} reverse-phase column. Chapter 4 focuses on the separation of nine haloacetic acid compounds with the aid of an ion-pairing reagent dibutylamine (DBA). Following their separation, the analytes are detected with a triple quadrupole mass spectrometer.

Tandem Mass Spectrometry

Mass spectrometry is widely used for the analysis of environmental samples due to its enhanced selectivity and ability to provide sensitivity without complete resolution of individual components. In the work presented here a triple quadrupole instrument was used with electrospray ionization (ESI) and multiple reaction monitoring (MRM) mode (Figure 2).

The ESI is a specialized interface that converts the HPLC eluate into gaseous ions before entering the mass spectrometer (Dass, 2007). The ESI process first begins when the HPLC eluate enters a heated capillary positioned near a counter electrode. Here a

potential difference is formed and produces an electrostatic field. This electrostatic field disperses the HPLC eluate into a fine mist of charged droplets. A flow of nitrogen, termed desolvation gas, then assists in evaporation of the solvent from the charged droplets. This process results in the release of dissolved ions into the atmosphere that can then enter the mass analyzer. The electrospray process also has the ability to perform in positive and negative mode depending on the polarity of the target analyte ions.

The MRM mode uses the full functionality of tandem mass spectrometry (MS/MS) to detect a multiple target compounds in a single analysis. In MRM mode a compound is detected based on a precursor ion *m/z* (usually the pseudo molecular ion) and a product ion *m/z* (a fragment ion produced in the collision cell). The first quadrupole (MS1) uses an electric field, created by applying direct-current (dc) and radio-frequency (rf) potentials to the electrodes, to select the precursor ion *m/z* ("A^+" in Figure 2) (Dass, 2007). Only this ion will exit MS1 and enter the second quadrupole. In the second quadrupole, known as the collision cell, an rf only field transmits all ions while the ions collide with argon gas to impart collisional energy and induce fragmentation. All of the fragment ions ("A_1^+, A_2^+, A_3^+" in Figure 2) enter the third quadrupole (MS2) where specific product ion(s) ("A_1^+" in Figure 2) are allowed to exit and enter the detector (Figure 2). For example, monochloroacetic acid has a molecular weight of 94.49 amu. The compound is ionized at the electrospray interface (operated in negative mode for this scenario) to produce a precursor ion (93 *m/z*), a $[M-H]^-$ pseudomolecular ion. This ion is selected to pass through the first quadrupole (MS1) and then enters the collision cell for fragmentation. Monochloroacetic acid will then fragment at the chlorine-carbon bond to produce a chloride ion (35 *m/z*). This ion, along with any other fragment ions produced in

the collision cell, will enter the third quadrupole, or the second mass analyzer (MS2). In this case the chloride ion acts as the product ion and is the only ion allowed to reach the detector. This type of instrument design allows the operator one of the highest levels of specificity and greatly increases the signal to noise ratio of the analysis, thus improving the detection limits.

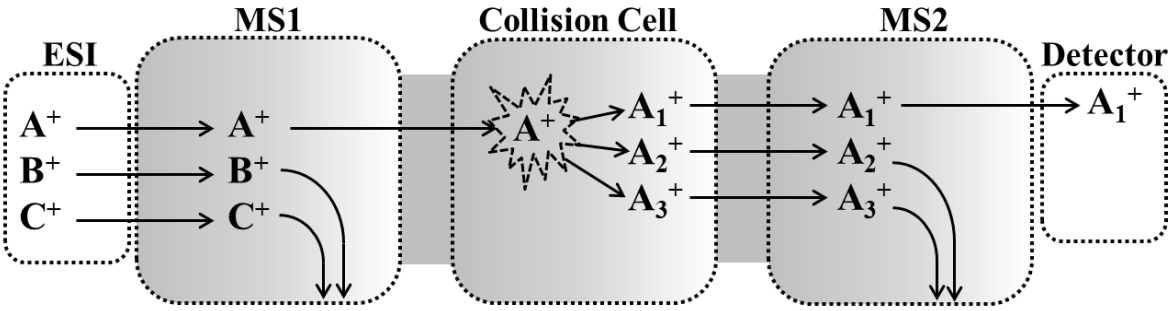

Figure 2. Tandem mass spectrometer diagram illustrating the multiple reaction monitoring process. "A^+" represents the precursor ion and "A_1^+, A_2^+, A_3^+" represent the fragment ions. "A_1^+" represents the target product ion.

Sample Preparation

Analytical separations are often combined with sample preparation techniques to increase the concentration of the analyte prior to the analysis, derivatize the analyte to change chemical properties, or minimize interfering species. During method development, sample preparation techniques are considered based on the chemical properties and concentrations of the analyte as well as interfering species in the sample matrix. Typically, environmental matrices vary in composition and, in the research presented here, contain trace concentrations of the target compound. The inconsistency in matrices leads to a large variety of interferents that can result in "false positives or false

negatives" (Pozo, Sancho, Ibanez, Hernandez, & Niessen, 2006). These interferents can also influence the quantification of the target analyte (ion suppression or enhancement); this phenomenon is commonly termed as "matrix effects" (Bylda, Thiele, Kobold, & Volmer, 2014). Special considerations to matrix effects are required when performing analysis via HPLC-MS/MS with electrospray ionization (ESI). Studies have shown that the ESI mechanism can result in ion suppression caused by higher relative concentrations of matrix compounds co-eluting with the target analyte (King, Bonfiglio, Fernandez-Metzler, Miller-Stein, & Olah, 2000).

One approach to minimize matrix effects is to employ sample preparation techniques such as liquid-liquid extraction and solid phase extraction. Liquid-liquid extraction (LLE) can be characterized by three definitions: Primarily, LLE is described as "the process of transferring a dissolved substance from one liquid phase to another (immiscible or partially miscible) liquid phase in contact with it" (Rice, Irving, & Leonard, 1993). LLE can also be defined as a process used to concentrate target analytes or as a "clean-up" step to remove impurities (Clement & Hao, 2012). Solid-phase extraction (SPE) is the process of passing the sample through a cartridge containing the desired stationary phase or molecularly imprinted polymer to isolate the target compound from the matrix compounds (Harris, 2007). SPE is typically performed via the following steps: the cartridge phase is conditioned (Figure 3a), the sample is applied (Figure 3b), the stationary phase is rinsed (Figure 3c), and the analyte is eluted (Figure 3d).

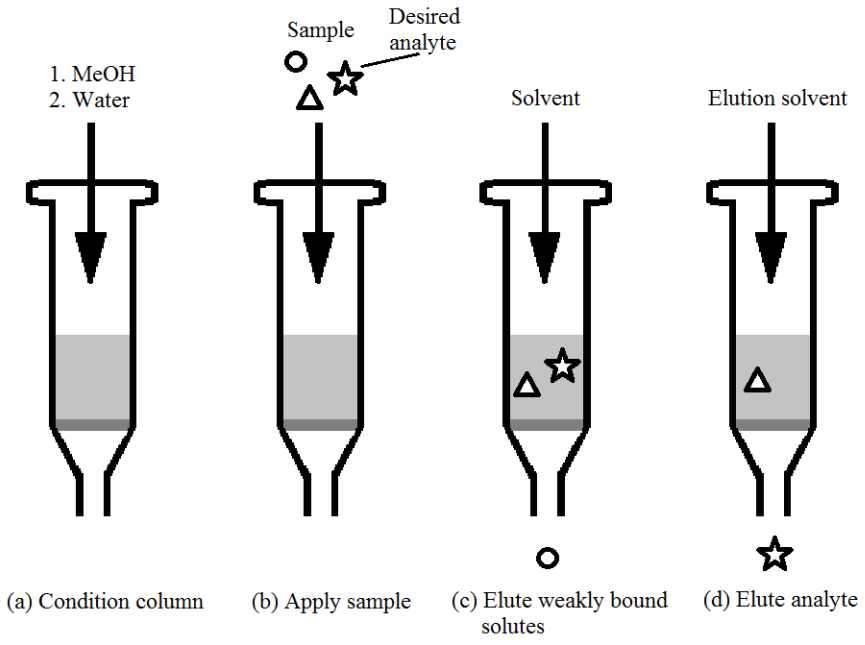

Figure 3. Solid-phase extraction steps diagram (adapted from Harris, 2007).

The studies reported in chapter two and three describe a LLE step (used for sample clean up) that is performed prior to HPLC-MS/MS analysis of the sample. In these studies the target analytes are present in a plant matrix that has been extracted into an acid solution. The acid solution is subjected to a sample clean-up step with hexane in order to reduce matrix effects. Also, in chapter 3 LLE is used to concentrate samples having analyte concentrations below the method detection limit of the analysis. In chapter four the target analytes are haloacetic acids found in sodium hypochlorite solutions. A solid-phase extraction (SPE) step is incorporated into the method to separate the target analytes from the high ionic strength matrix prior to HPLC-MS/MS analysis.

Overall, the goal of this work is to develop and implement methods for the qualitative and quantitative detection of target analytes present in complex environmental matrices. This objective is achieved for the analysis of two dimethylamylamine

compounds in geranium plant matrices and for the analysis of nine haloacetic acids in sodium hypochlorite solutions. Instrument design and versatility make HPLC-MS/MS the most suitable technique for the trace analysis of compounds in environmental samples.

Chapter 2

Analysis and Confirmation of 1,3-DMAA and 1,4-DMAA in Geranium Plants Using High Performance Liquid Chromatography With Tandem Mass Spectrometry

Introduction

There has been significant discussion of 1,3-dimethylamylamine (1,3-DMAA) in the literature concerning the presence of 1,3-DMAA in geranium plants, specifically of the genus and species *Pelargonium graveolens* (ElSohly et al., 2012; Li, Chen, & Li, 2012; Lisi, Hasick, Kazlauskas, & Goebel, 2011; Perrenoud, Saugy, & Saudan, 2009; Vorce, Holler, Cawrse, & Magluilo, 2011; Zhang, Woods, Breitbach, & Armstrong, 2012). 1,3-DMAA, also known as 4-methyl-2-hexaneamine (MHA), 1,3-dimethylpentylamine or 2-amino-4-methylhexane can be labeled as geranium extract in dietary supplements. Confirming the presence or absence of 1,3-DMAA as a natural product in geranium plants has important regulatory and commercial consequences for many dietary supplement companies (FDA, 2012). For this reason, geranium plant samples from China are analyzed for the presence of 1,3-DMAA (and 1,4-DMAA) in the work presented here.

The chemical properties and concentrations of 1,3-DMAA and the associated matrix do not allow for simpler LC detection methods (UV-Visible absorption or refractive index). Typically, GC-MS analysis requires derivatization to a higher molecular weight to increase boiling point and retention time. The geranium oil and plant matrix are sufficiently complex that the most universal detectors, such as refractive index and flame ionization detectors are likely to encounter significant matrix interferences. Thus, research and analytical effort for 1,3-DMAA analysis has focused on GC-MS

(ElSohly et al., 2012; Lisi et al., 2011; Perrenoud et al., 2009; Zhang et al., 2012) and HPLC-MS/MS (ElSohly et al., 2012; Li et al., 2012; Perrenoud et al., 2009; Vorce et al., 2011; Zhang et al., 2012) analysis protocols for matrices, such as urine, geranium oil extracts and geranium plants.

The World Anti-Doping Agency requires that compounds with chemical structure and biological activity similar to banned substances must be analyzed by anti-doping laboratories. 1,3-DMAA and 2-aminoheptane (a banned stimulant) have similar chemical structures and physiological stimulant effects (Figure 4). The laboratory of Saudan (Perrenoud et al., 2009) developed a high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method for detection of 1,3-DMAA in urine samples. The method was calibrated over the range of 50 to 700 ng mL^{-1} with excellent intraday precision and accuracy of less than 6%. The results from the Saudan laboratory found that 1,3-DMAA could be detected in urine samples up to 105 hours after administration of a 40 mg dose.

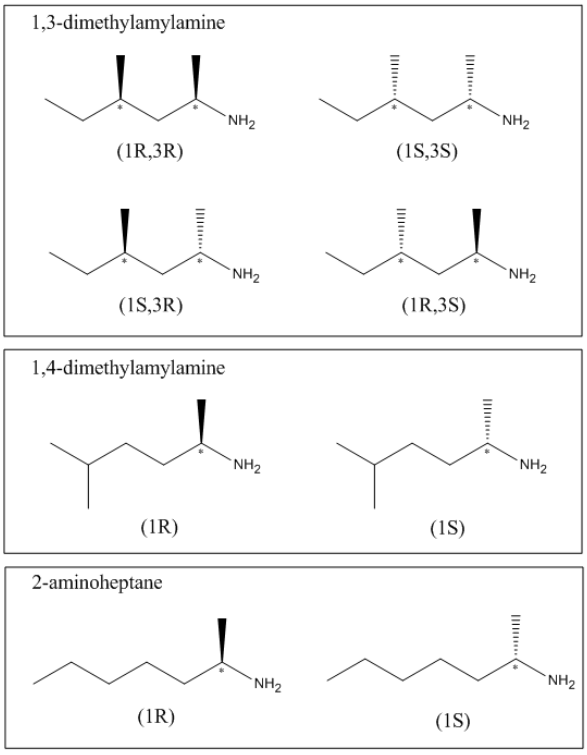

Figure 4. Chemical structures of the stereoisomers of 1,3-DMAA, 1,4-DMAA, and 2-aminoheptane with stereogenic carbons labeled (*) and their respective *(R,S)* configurations.

Subsequent research by Vorce, Holler, Cawrse, and Magluilo (2011) used HPLC-MS/MS to confirm 1,3-DMAA as the cause of false positives in amphetamine screening kits used by the Department of Defense drug-screening laboratories. 1,3-DMAA was suspected due to its inclusion in body-building energy supplements available over-the-counter. Vorce et al. (2011) reported that 1,3-DMAA would cause false positives at urine concentrations above 6.0 mg L^{-1}, and confirmed the presence of 1,3-DMAA concentrations over the 6.0 mg L^{-1} limit in 92.3 % of the false positive results for amphetamines.

The laboratory of Lisi et al. (2011) conducted an analysis of five geranium oils which had origins in France, Egypt and New Zealand. The geranium oils were analyzed using a derivatization and extraction procedure for 1,3-DMAA. None of these samples were reported to have 1,3-DMAA, but no limit of detection (LOD) was reported for the method. Supplements containing 1,3-DMAA were then administered and tested in a urine excretion study using a GC with a nitrogen-phosphorous detector. The results showed that 1,3-DMAA is excreted for at least 29 hours in agreement with a previous report (Perrenoud et al., 2009).

The research team of ElSohly et al. (2012) used GC-MS, LC-MS/MS and high resolution ultra-performance LC with quadrupole-time of flight- MS (UPLC-QTOF-MS) to analyze geranium oils and leaves from India as well as geranium leaves, stems and freshly extracted oil from plants grown in Oxford, MS, United States. The GC-MS and LC-MS/MS based methods used similar extraction procedures with a reported extraction efficiency of 35% (relatively low). However, the extraction was shown to have excellent accuracy (75%) and precision (less than 5%) on the control sample using GC-MS analysis. The limits of detection for the GC-MS, LC-MS/MS, and UPLC-QTOF-MS were 0.1 ppm, 2.5 ppb and 10 ppb, respectively. The GC-MS analysis of the 0.1 ppm spikes of 1,3-DMAA in the geranium oil clearly shows the characteristic double peaks of the 1,3-DMAA diastereomer pairs. The authenticated geranium plant material shows a similar pattern to the spiked geranium oil; whereas the negative geranium oil and authenticated geranium oil do not. The GC-MS chromatograms of the authenticated geranium plant material suggest the presence of the 1,3-DMAA. However, the two more sensitive LC-MS/MS methods did not detect 1,3-DMAA in any of the samples analyzed.

The LC-based methods do not exhibit the characteristic diastereomer double peak–possibly due to the chromatographic separation conditions (Li et al., 2012; Vorce et al., 2011; Zhang et al., 2012).

The laboratory of Armstrong (Zhang et al., 2012) has recently reported the analysis of eight different geranium oils, four from China and four from Egypt, and analysis of thirteen dietary supplements containing 1,3-DMAA. The goal of the paper was to determine whether the 1,3-DMAA in dietary supplements had synthetic or natural origins. The supplements were analyzed using GC-FID analysis with a chiral column. The 1,3-DMAA in the standards and supplements were derivatized by pentafluoropropionic anhydride (PFPA). The derivatized stereoisomer separation of 1,3-DMAA by GC-FID was excellent–showing all four stereoisomers present. The GC-FID analysis protocol did not have an LOD reported; however, the calibration curve range was 0.2 to 0.8 mg mL^{-1} of 1,3-DMAA. The dietary supplements were reported to contain the same stereoisomer ratios as the synthetic standards.

The laboratory of Armstrong then used two LC-MS based methods to analyze the geranium oils for 1,3-DMAA (Zhang et al., 2012). The LOD of the linear ion trap method (HPLC-ESI-LIT) was 50 ppb and the LOD of the triple quadrupole instrument (HPLC-ESI-QQQ) was 10 ppb for derivatized 1,3-DMAA. The HPLC-ESI-LIT used a chiral-phase HPLC separation column and the HPLC-ESI-QQQ used a standard C18 separation phase. In both methods, 1,3-DMAA was not detected above the LOD and both lack the characteristic diastereomer double peak as expected (both possibly due to chromatographic separation choices).

Finally, the research team of Li et al. (2012) developed an extraction and LC-MS/MS based method for the analysis of 1,3-DMAA and 1,4-DMAA in geranium plants and oils (three distinct samples of each). The method validation was detailed and conducted according to United States Pharmacopeia guidelines. The traditional instrument LOD (Skoog et al., 2007) reported was 1 – 2 pg g^{-1} with a reported method quantification limit (LOQ) of 1 – 2 ng g^{-1} in the geranium sample. Li et al. (2012) reported concentrations of 1,3-DMAA and 1,4-DMAA as present in three samples of geranium plants ranging from 13 to 365 ng g^{-1} and 3 to 35.3 ng g^{-1}, respectively. In the geranium oil, Li et al. (2012) reported all three samples contained 1,3-DMAA ranging from 167 to 13,271 ng g^{-1}. In the sample containing 13,271 ng g^{-1} of 1,3-DMAA, 1,4-DMAA was detected at 220 ng g^{-1}. The other two geranium oil samples did not contain 1,4-DMAA above the LOD.

The research and sample analysis presented here use an adapted extraction and LC-MS/MS analysis (Li, 2011; Li et al., 2012) to analyze both 1,3-DMAA and 1,4-DMAA in geranium plants. Linearity, method detection limit (MDL), accuracy and precision studies were carried out followed by analysis of geranium plants from 3 distinct regions in China (Changzhou, Guiyang, and Kunming) during winter and summer months. An improved analysis protocol was developed that used standard addition analysis to re-analyze samples and confirm the reported concentrations of 1,3-DMAA and 1,4-DMAA. One of the Changzhou, China samples was analyzed by another laboratory (Li et al., 2012) and to the best of the author's knowledge represents the first inter-laboratory analysis and confirmation of 1,3-DMAA in an identical geranium sample. Additionally, the diastereomer ratio of 1,3-DMAA in geranium plants was

measured and compared to synthetic standards and previously reported research (Zhang et al., 2012).

Experimental

Chemicals and reagents. All chemicals and reagents have a purity of 97% or greater. All standards and eluent were prepared in reagent-grade water with a resistivity of 18.2 MΩ·cm produced by a Barnstead e-pure four cartridge system. Glassware was cleaned with concentrated detergent and rinsed with reagent-grade water three times. 1,3-DMAA was purchased from 2A Pharmachem USA (purity confirmed by NMR) and 1,4-DMAA was purchased from Sigma-Aldrich. LC-MS grade acetonitrile and formic acid; HPLC grade ethanol and hexane; and ACS Certified Plus concentrated hydrochloric acid where purchased from Fisher Scientific.

Standard preparation. A combined stock solution was first prepared containing both standards (1,3-DMAA and 1,4-DMAA) with a concentration of 1000 mg L^{-1} each in ethanol. An intermediate standard solution is then diluted from the stock to prepare a standard with a concentration of 1000 µg L^{-1} in 0.5 N HCl for both 1,3-DMAA and 1,4-DMAA. Two external calibration curves were prepared for each analysis, due to the unknown concentrations of 1,3-DMAA. The low range calibration was 1 to 20 µg L^{-1}, and the high range calibration was 3 to 100 µg L^{-1}. The standard addition curves were prepared by analyzing sample spikes of 1,3-DMAA and 1,4-DMAA at 15.0 µg L^{-1} and 25.0 µg L^{-1} for each sample.

Sample preparation: preliminary homogenization and extraction protocol. The preliminary extraction method was adapted from a standard analysis method (Li, 2011). The method was scaled from 200 g to 50 g of geranium plant for analysis and each

subsequent step was appropriately scaled by a factor of four. The geranium plants were first cut into pieces having a mass ranging from 40 to 50 g and subsequently placed into a blender (Black & Decker; Towson, Maryland, USA). 15 mL of 0.5 N HCl solution was added to extract the 1,3-DMAA and 1,4-DMAA analytes present in the plants. The mixture was homogenized at high speed for two minutes, filtered and re-extracted with 7.5 mL of 0.5 N HCl. Both extracts were combined and diluted to a final volume of 25.00 mL. The solution was then sonicated, filtered and analyzed by HPLC-MS/MS. A blank (no geranium plant) and spiked samples containing an additional 10.0 μg L^{-1} of the standard solution were also prepared by following the same procedure as those of the plant preparation. The spiked sample provides a percent (%) recovery estimate for each sample matrix.

Sample preparation: optimized homogenization and extraction protocol. The preliminary analysis method was further modified (Li et al., 2012) to reduce matrix effects by adding a hexane partitioning step (hexane clean-up step). The geranium samples remained frozen at −20 ºC prior to analysis, and thawed for sample preparation. The wet geranium leaves and stems were cut into 1-2-cm pieces and subsequently ground with a high speed grinder (Cuisinart; Stamford, CT, USA) into finely chopped pieces. Then, 10 g of the chopped sample were weighed and placed into a standard food blender with 80 mL of 0.5 N HCl, and homogenized at the highest blend setting for two minutes. The blended mixture was transferred into a 100-mL volumetric flask and the blade and blender cup were rinsed with 15 mL of 0.5 N HCl and poured into the 100 mL volumetric flask. The blended geranium mixture was extracted by sonication for one hour at 50 °C. This solution was centrifuged at 3700 × g for 10 min after cooling and filling to volume

with 0.5 N HCl. Four mL of the supernatant and 2 mL of hexane were added to a 15-mL glass centrifuge tube with screw cap. This mixture was shaken by a vortex mixer for thirty seconds. The mixture was then centrifuged at 2000 × g for 5 min. The aqueous layer was filtered (0.45 µm syringe filter) and analyzed by LC-MS/MS. For all sample analysis, a blank was analyzed with each sample to verify no carryover occurred from the previous analysis. For standard addition analysis, spiked samples were prepared by spiking standard prior to the blending process, such that the final added concentration was 15.0 and 25.0 µg L^{-1} in the volumetric flask.

This optimized method added and modified existing steps (grinding, sonication, and centrifuging) to the original extraction protocol to maximize the extraction efficiency of 1,3-DMAA and 1,4-DMAA from the plant matrix. The reduction of plant material extracted and increased volume of extractant resulted in a more practical extraction procedure and minimized sample handling errors. The sonication temperature was increased to 50 °C to increase the breakup and dissolution of the plant material in the acid extract and increase solvation of the analytes. The additional hexane extraction step minimized concentrations of the non-polar plant material in the 0.5 N HCl extraction solution. The non-polar plant material likely caused matrix effects during analysis by causing ion suppression in the ESI source. The combination of these steps provides an extract which contains a more representative concentration of 1,3-DMAA and 1,4-DMAA and a reduction of matrix effects. This means that the performance of the extraction method improves and this is demonstrated by the large improvement in percent recovery.

Instrumentation

The LC-MS/MS system consists of an Agilent 1100 HPLC system equipped with an autosampler, coupled to a triple quadrupole mass spectrophotometer (Waters Quattro Ultima) operated in ESI+ mode. The injection volume was 100 µL with separation performed on a Phenomenex Kinetex C18 phase column (4.6 × 150 mm, 2.6 µm) with a column temperature set at 25 °C and flow rate at 0.4 mL min^{-1}. The HPLC eluent ratio was 82:18 of mobile phase A (1% of formic acid in reagent water) to mobile phase B (acetonitrile). The column effluent was split at a ratio of 1:1 prior to introduction to the mass spectrometer.

The mass spectrometer operating conditions were as follows: the capillary voltage was 3.0 kV; the cone voltage was 20 V; the source temperature was set at 120 ºC with a flow of 108 L hr^{-1}; the desolvation temperature was 350 ºC with a flow of 635 L hr^{-1}. The dwell time was 0.5 s, the inter-scan delay was 0.1 s. The collision voltage was set to 8 eV with a collision gas (argon) pressure at 7 psi. The detection of the analytes was done using the MRM function with a pair of mass transitions of 116/99.7 *m/z* and 116/57 *m/z* to produce a single chromatogram for both 1,3-DMAA and 1,4-DMAA.

All chromatogram integrations were performed with Waters MassLynx MS software. Each chromatogram was pre-filtered with a peak-to-peak noise amplitude of 2000. Also, chromatograms were submitted to a Savitzky Golay (Savitzky & Golay, 1964) smoothing method within the MassLynx software. The Savitzky Golay method takes an average of the intensities of the data points weighted by a quadratic curve.

The HPLC-MS/MS total analysis time was 10 minutes. Figure 5 presents a typical standard chromatogram of a 20 µg L^{-1} standard of 1,3-DMAA and 1,4-DMAA. It is

important to mention that the compound 1,3-DMAA has two chiral centers that result in four stereoisomers (Figure 4). These stereoisomers include two diastereomers that have different physical properties and can be separated. Therefore 1,3-DMAA is detected as two peaks in the chromatogram. All values referenced to '1,3-DMAA_total' or '1,3-DMAA' are calculations based on the summation of both peak areas. The compound 1,4-DMAA exists as two enantiomers which cannot be separated. Therefore only one peak was detected for 1,4-DMAA.

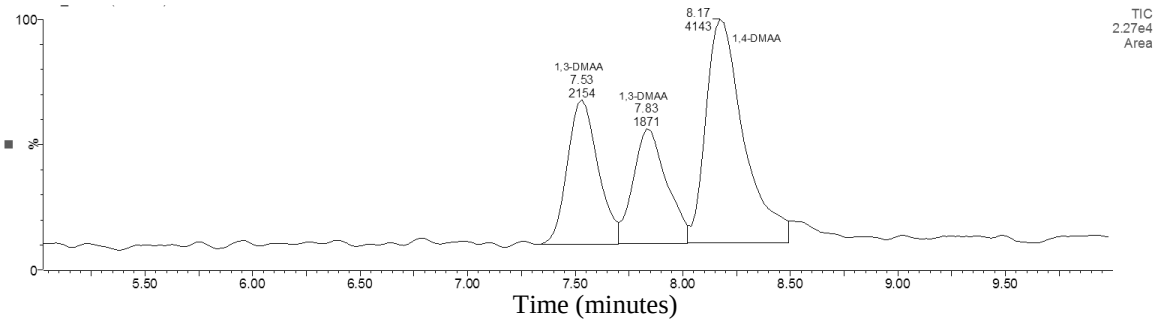

Figure 5. Typical MRM chromatogram at 20 µg L^{-1} each for 1,3- and 1,4-DMAA analytes. The retention times for the 1,3-DMAA diastereomers are 7.53 and 7.83 min, and 1,4-DMAA retention time is 8.17 min.

Results and Discussion

Detection limits, accuracy, precision, and linearity studies. Before sample analysis is conducted, detection limit, accuracy, precision, and linearity studies were completed to evaluate and ensure acceptable instrument performance (Glaser, Forest, McKee, Quave & Budde, 1981; Harris, 2007; Skoog et al., 2007; USEPA, 1984; USEPA, 1996). The typical practice for USEPA MDL studies in the laboratory is to construct a 5-point calibration curve and analyze a check standard halfway between the two lowest calibration points. The USEPA MDL reported here represents the lowest concentration distinguishable from noise and determined on the variation of the analytical signal of a check standard expected to be within a factor of 2 to 5 of the detection limit. At these analytical conditions, the MDL study provides a worst-case estimate of the analyzer performance. The accuracy of the analysis is estimated using the mean % recovery of the check standard analysis (USEPA, 1996). The precision is estimated as the % relative standard deviation (USEPA, 1996).

Another estimate for the detection limit is the propagation of uncertainty MDL (Unc. MDL) (Harris, 2007). The Unc. MDL is determined using the standard deviations of the slope (m), y-intercept (b), and signal (y) as determined by the LINEST function in Microsoft Excel (2010). These standard deviations are then used to propagate and determine the error on "x" in the linear regression line (Harris, 2007). The propagated error represents the lowest concentration of analytical significance.

Detailed MDL, accuracy and precision studies of 1,3-DMAA and 1,4-DMAA are presented in Table 1 and Table 2, respectively, for all sample analysis conducted (Analysis Set 1-3). The reported values for Analysis Set 1 were based on the preliminary

extraction protocol. Analysis Set 2 and 3 were conducted using a hexane clean-up step as well as standard addition analysis. Typically, an MDL, accuracy and precision study was conducted with two different check standard concentrations prior to each set of sample analysis. For Analysis Set 1 and 2, the MDLs at 3.0 µg L^{-1} were based on the calibration curves from 1 to 20 µg L^{-1} (low range calibration). The MDLs at 8.0 µg L^{-1} were based on the 3 to 100 µg L^{-1} calibration curves (high range calibration). In Analysis Set 3, the calibration curve for the 2.0 µg L^{-1} check standard was 1 to 100 µg L^{-1}, and the calibration curve for the 3.0 µg L^{-1} check standard was 2 to 100 µg L^{-1}. The r^2 values for all studies with both DMAA species were greater than 0.99.

The MDL values for 1,3-DMAA range from 0.6 to 3.2 µg L^{-1} and for 1,4-DMAA range from 0.8 to 2.7 µg L^{-1}. Accuracy (USEPA, 1996) for 1,3-DMAA ranges between 60 and 126% and 1,4-DMAA ranges between 48 and 127%. The precision, estimated as %RSD, for 1,3-DMAA is in the range of 9 to 35 % for 1,4-DMAA ranges between 10 to 30%. With the exception of one mean % recovery analysis in Analysis Set 2, the reported mean % recoveries and %RSD are within the guidelines set by the USEPA for check standard analysis. The USEPA reports that mean % recovery can range from 50 to 150 % and the %RSD can be up to 30 % when samples are analyzed within a factor of 2 to 5 of the MDL. As the MDL factor decreases, the % RSD of the check standard analysis increases and below an MDL factor of 2, the % RSD can dramatically increase beyond 30 % (Ranaivo, Henson, Simone, & Emmert, 2011).

Ideally, MDL, accuracy and precision studies should provide estimates that are similar to each other (Brown & Emmert, 2007; Ranaivo et al., 2011; Simone, Ranaivo, Geme, Brown, & Emmert, 2009). Further confidence of these MDL values is gained

when the USEPA MDLs are compared to the Unc. MDL. Both sets of detection limit values are within 2 µg L^{-1} of each other in absolute terms and within a factor of 5 in all cases. This similarity indicates the MDL values for the calibration and analysis protocols are realistic estimates for both 1,3-DMAA and 1,4-DMAA.

A linearity study was conducted to estimate the upper limit of linearity for the LC-MS/MS analysis. A calibration curve was prepared and analyzed over the range of 1 to 250 µg L^{-1} for 1,3-DMAA and 1,4-DMAA, with both species being linear over the entire range as evidenced by the excellent r^2 values (> 0.99). The linearity study resulted in an linear regression equation for 1,3-DMAA of "y = 149.08x + 380.91" and for 1,4-DMAA of "y = 148.05x + 473.94".

Table 1
Detection limits, accuracy, and precision studies for 1,3-DMAA for all sample analysis.

	Check Standard (µg L^{-1})	USEPA MDL (µg L^{-1})	Unc. MDL (µg L^{-1})	Mean % Recovery	% RSD	MDL Factor	r^2
Analysis Set 1	3.0	1.1	0.4	126	9	2.8	0.999
	8.0	1.8	3.4	73	10	4.5	0.999
Analysis Set 2	3.0	2.3	0.5	71	35	1.3	0.998
	3.0	1.8	0.8	95	20	1.7	0.994
	8.0	2.5	1.5	62	16	3.2	0.999
	8.0	3.2	1.4	60	21	2.5	0.999
Analysis Set 3	2.0	1.4	2.6	103	21	1.5	0.996
	3.0	0.6	1.4	63	10	4.9	0.999

Table 2
Detection limits, accuracy, and precision studies for 1,4-DMAA for all sample analysis.

	Check Standard (µg L^{-1})	USEPA MDL (µg L^{-1})	Unc. MDL (µg L^{-1})	Mean % Recovery	% RSD	MDL Factor	r^2
Analysis Set 1	3.0	1.4	0.7	127	12	2.1	0.999
	8.0	2.7	4.6	60	18	2.9	0.999
Analysis Set 2	3.0	2	0.6	73	30	1.5	0.998
	3.0	0.9	0.6	93	10	3.4	0.994
	8.0	2.4	2.9	48	20	3.3	0.999
	8.0	2.1	0.9	81	10	3.9	0.999
Analysis Set 3	2.0	0.8	2.6	98	13	2.4	0.996
	3.0	0.8	1.6	76	11	3.7	0.999

DMAA concentrations in the plant material. The reported concentration of the DMAA species in the geranium herb was determined using the calculated concentration from the calibration curve, final extraction volume and mass of geranium (Equation 3). The MDL, accuracy and precision studies (Table 1 and 2) were conducted with prepared standards in solution (no extraction). However, the MDLs in the analyzed plant would vary with the amount of plant mass used and the final extracted volume. For Analysis Set 1, the amount of plant material used was 50 g and extracted into 25.00 mL. This resulted in MDLs that ranged from 0.6 to 1.4 ng DMAA g^{-1} $geranium^{-1}$. In Analysis Set 2 and 3, 10 g of plant material was extracted into 100.00 mL which resulted in MDLs ranging from 6 to 32 ng DMAA g^{-1} $geranium^{-1}$. While the MDLs increased for the second extraction method the % recovery of DMAA analysis also increased for all samples. The increase in % recovery is likely due to the hexane clean-up step as well as a more practical increase in the extraction solvent volume. If the mass of plant material were doubled, the MDLs of the optimized extraction protocol would likely increase by a factor of two.

$$DMAA_{Geranium}\left(\frac{ng}{g}\right) = \frac{DMAA_{CalCurve}\left(\frac{\mu g}{L}\right) * Extraction\ Volume\ (L)}{Geranium\ Mass\ (g)} * 1000 \qquad \text{(Equation 3)}$$

The *Pelargonium graveolens* (geranium) samples were collected and authenticated as all belonging to the genus and species *Pelargonium graveolens* by Xu YouKai of the Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences. Samples were collected from three regions in China: Changzhou, Guiyang, and Kunming and during three different harvest seasons. The Chinese Academy received the geranium herbs as potted plants originally grown in the field. Multiple plants (ranging from two to

ten) were collected from each location. The plants from each location were combined prior to shipment to The University of Memphis. Therefore concentrations of 1,3-DMAA and 1,4-DMAA in individual plants and variations thereof are not reported here. The samples were sent by express airmail from Dr. Yi Jin of Yunnan University directly to the University of Memphis where the samples were immediately stored at −20 ºC. Analysis Set 1 and 2 consisted of a Changzhou sample collected on June 9, 2011 (Changzhou S11-1 and Changzhou S11-2), a Kunming, China sample collected March 20, 2012 (Kunming 1 and 2); a Guiyang, China sample collected March 16, 2012 (Guiyang 1 and 2); and an additional Changzhou, China sample collected on March 10, 2012 (Changzhou 1). Analysis Set 3 consisted of a Changzhou sample collected on May 18, 2012 (Changzhou 3), a Guiyang sample collected May 20, 2012 (Guiyang 3), and a Kunming sample collected May 23, 2012 (Kunming 3). The Changzhou S11 sample was received from Intertek Labs (Detroit, MI, USA) and frozen upon arrival. The Changzhou S11 sample is an identical sample previously analyzed and reported by Li (Li et al., 2012) providing an inter-laboratory analysis of a sample. The numbers for each region identifier signify the various analysis sets.

Analysis set 1: preliminary extraction protocol. The concentrations of 1,3-DMAA and 1,4-DMAA in the three winter geranium samples and Changzhou S11 sample are presented in Table 3. The Changzhou S11-1 analysis was conducted in duplicate and the winter samples were analyzed in singlet. A spike sample was analyzed to determine the % recovery for that particular plant sample. There is no reported spike analysis for Changzhou 1 due to lost sample during analysis – and no additional sample

was available. The percent recovery of the spike was calculated using equation 4 (Harris, 2007).

$$\%\ Recovery = \frac{Spike\ Conc\ (\mu g\ L^{-1}) - Unspiked\ Conc(\mu g\ L^{-1})}{10\ (\mu g\ L^{-1})} \times 100\% \qquad \text{(Equation 4)}$$

Of the four samples in Analysis Set 1, only the Changzhou S11-1 and Changzhou 1 sample contained 1,3-DMAA and 1,4-DMAA above the MDLs of the method (Table 3). Figures 6 and 7 present an MRM chromatogram of the Changzhou S11-1 and Changzhou 1 samples, respectively. The average concentration of 1,3-DMAA in the Changzhou S11-1 sample was 94.7 ± 15.1 ng g^{-1} geranium, with a % recovery of 19 % on the 10 µg L^{-1} spike. The average concentration of 1,4-DMAA in Changzhou S11-1 was 13.5 ± 1.8 µg L^{-1} with a 65 % recovery on a 10 µg L^{-1} spike. The concentration of 1,3-DMAA and 1,4-DMAA in the Changzhou 1 samples were 213 and 52 ng g^{-1} respectively. The reported 1,3-DMAA concentrations for the Changzhou S11-1 and Changzhou 1 samples were outside the calibration range, but within the linearity of the analyzer. A 1:1 dilution of both samples was analyzed and resulted in calculated concentrations within 9% of the original concentration reported in Table 3.

While the % recovery of the DMAA species is not ideal, the relative concentrations should be considered for the spike. For the Changzhou S11-1 sample, the concentrations of 1,3-DMAA in volumetric flask after extraction averaged 190 µg L^{-1} of 1,3-DMAA. The % RSD error of analysis from the MDL study was of 9 – 10 % for Analysis Set 1, and translates to ~18 µg L^{-1} error. This is more than twice the 10 µg L^{-1} spike and thus a likely contributor to the low % recovery (high error). When 1,4-DMAA is examined, the 10 µg L^{-1} spike addition is outside the error of analysis (2.7 µg L^{-1}) and

gives a more reasonable 65% recovery. Additionally, the low % recoveries across all samples indicate the presence of a matrix effect. Previous reports (Li et al., 2012) have suggested that extraction protocols are likely to be extracting lipids from the cell membranes and contributing to ion suppression in the ESI source.

Table 3
*Analysis Set 1: preliminary extraction protocol results of geranium samples from Changzhou, Kunming, and Guiyang. *The results are less than the MDL values.*

	1,3-DMAA			1,4-DMAA		
	Sample (ng g^{-1})	Spike Level (µg L^{-1})	Percent Recovery (%)	Sample (ng g^{-1})	Spike Level (µg L^{-1})	Percent Recovery (%)
Changzhou S11-1	94.7 ± 15.1	10	19	13.5 ± 1.8	10	65
Kunming 1	< 0.5*	10	44	< 0.7*	10	32
GuiYang 1	< 0.5*	10	36	< 0.7*	10	23
Changzhou 1	213	N/A	N/A	52	N/A	N/A

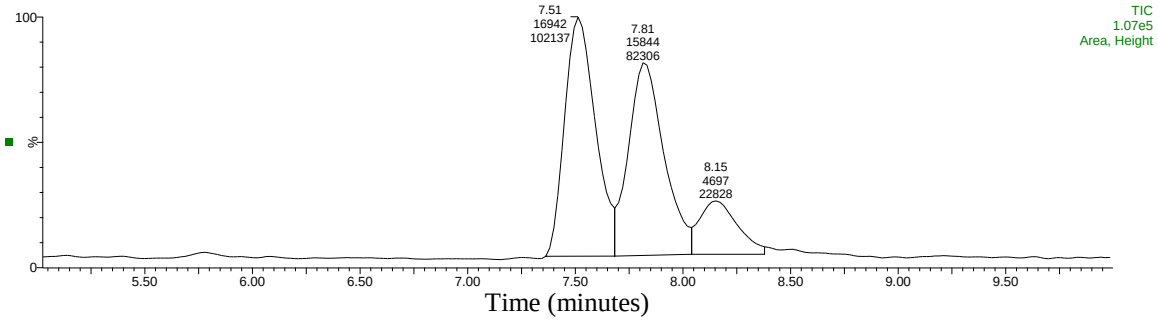

Figure 6. A MRM chromatogram of the Changzhou S11-1 sample.

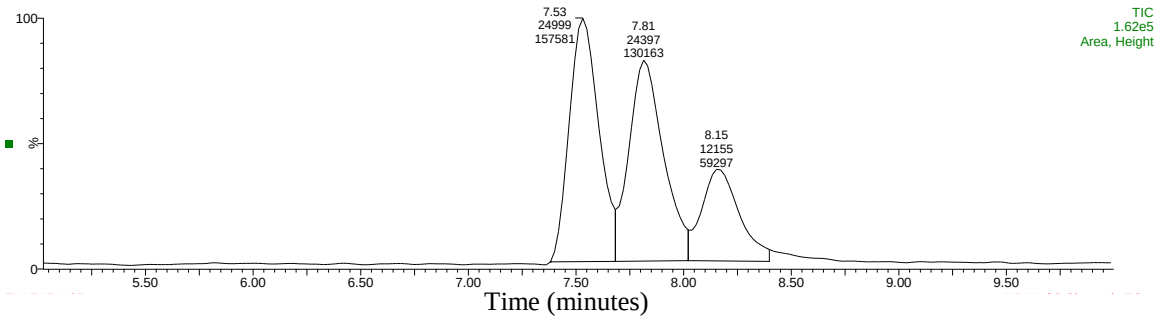

Figure 7. A MRM chromatogram of the Changzhou 1 sample.

Analysis set 2: optimized extraction protocol analysis of the Changzhou S11 and winter geranium samples. The matrix effect identified in Analysis Set 1 was minimized by the addition of a hexane clean-up step. Additionally, the optimized method was more efficient as it used less plant sample mass per extraction. This efficiency provided an opportunity to re-analyze the Changzhou S11, Kunming, and Guiyang winter samples. Each sample was extracted and analyzed with two different spike concentrations (15.0 µg L^{-1} and 25.0 µg L^{-1}) for both 1,3-DMAA and 1,4-DMAA in duplicate. The spiked samples were analyzed concurrently with the unspiked ones, and the % recovery was subsequently calculated (Harris, 2007). Detailed analysis results are presented in Table 4.

The Changzhou S11 concentrations were expected to be high, thus analyzed on the high range calibration of 3 to 100 µg L^{-1} of both DMAA species. The concentrations of 1,3-DMAA and 1,4-DMAA were 254 ng g^{-1} and 39.8 ng g^{-1} respectively and an optimized extraction chromatogram of Changzhou S11-2 is presented in Figure 8. The % recovery for 1,3-DMAA was approximately 55% for both spike levels. Both Kunming and the Guiyang (Figure 9) samples were analyzed using the low range calibration curves (1 to 20 µg L^{-1} of each DMAA species). The concentrations of 1,3-DMAA and 1,4-

DMAA are reported in Table 2.4 and all are less than the MDL of the analysis. The % recovery for all remaining samples ranged from 63 to 107%, indicating the matrix effect previously identified was substantially mitigated by the optimized extraction protocol.

A comparison of the two extraction protocols using the Changzhou S11 geranium sample demonstrates that the preliminary extraction protocol underestimates the concentrations of both DMAA species as indicated by the % recovery results. However, it is clear that the Changzhou S11 geranium samples contain 1,3-DMAA species and the concentrations are well above the MDL of both analysis. In contrast, the Kunming and Guiyang samples did not contain 1,3-DMAA or 1,4-DMAA species at significant concentrations above the MDL of analysis (20 ng g^{-1}).

Table 4

*Analysis Set 2 – Optimized Extraction Protocol Results of Geranium Samples from Changzhou S11, Kunming and Guiyang. *The results are less than the MDL values; **one duplicate was less than the MDL for the sample (23.9 ng g^{-1}).*

	1,3-DMAA			1,4-DMAA		
	Sample (ng g^{-1})	Spike Level (µg L^{-1})	Percent Recovery (%)	Sample (ng g^{-1})	Spike Level (µg L^{-1})	Percent Recovery (%)
Changzhou S11-2	254 ± 17	15.0	54 ± 5	39.8 **	15.0	76 ± 2
		25.0	55 ± 8		25.0	65 ± 1
Kunming 2	< 20 ± 4*	15.0	83 ± 11	< 14 ± 8	15.0	78 ± 10
		25.0	67 ± 1		25.0	63 ± 5
Guiyang 2	< 20 ± 4*	15.0	107 ± 23	< 14 ± 8	15.0	82 ± 16
		25.0	81 ± 2		25.0	78 ± 6

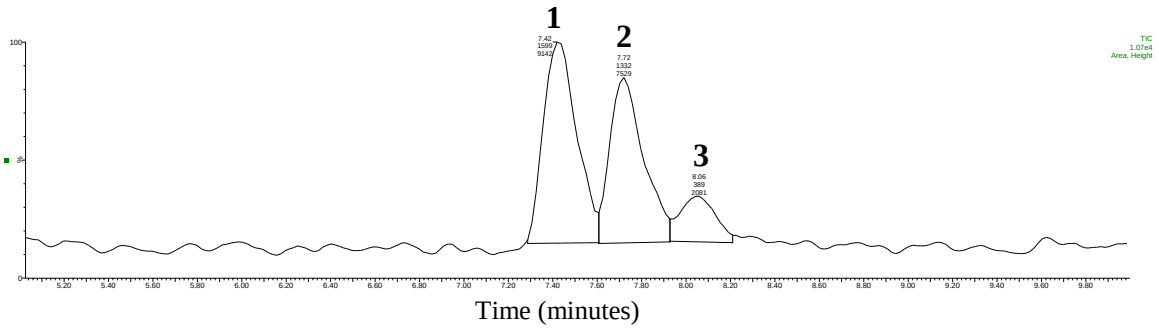

Figure 8. A MRM chromatogram of the optimized extraction protocol for Changzhou S11-2 showing the presence of 1,3-DMAA diastereomers (peaks 1 and 2) and 1,4-DMAA (peak 3).

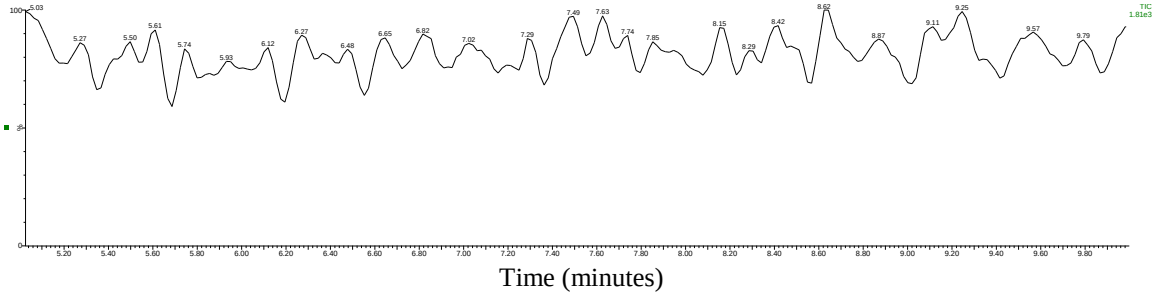

Figure 9. A typical MRM chromatogram of the Guiyang 2 sample demonstrating the absence of 1,3-DMAA and 1,4-DMAA in the geranium plant.

Analysis set 3: optimized extraction protocol of summer geranium samples. An additional round of samples was collected from a "Summer" harvest of geranium plants and analyzed using the same protocols from Analysis Set 2 (with two spike levels, in duplicate). The Changzhou 3 sample (Figure 10) contained 1,3-DMAA and 1,4-DMAA concentrations of 68.8 ± 36.5 ng g^{-1} and 118 ± 45 ng g^{-1}, respectively (Table 5). Both the Kunming 3 and Guiyang 3 had concentrations of 1,3-DMAA and 1,4-DMAA below the MDL (less than 10 ng g^{-1}). These results are consistent with the previous winter sample analysis. Both DMAA species were detected and quantified in the Changzhou samples, but no DMAA species were detected above the MDL in the Kunming and Guiyang samples. The % recovery for all samples was acceptable and ranged then between 64 and 86%.

Table 5
*Analysis Set 3 – Optimized Extraction Protocol Results of Geranium Summer Samples from Kunming, Guiyang and Changzhou. *The results are less than the MDL values.*

	1,3-DMAA			1,4-DMAA		
	Sample (ng g^{-1})	Spike Level (µg L^{-1})	Percent Recovery (%)	Sample (ng g^{-1})	Spike Level (µg L^{-1})	Percent Recovery (%)
Kunming 3	< 10 ± 6*	15.0	68 ± 3	< 8.2 ± 0.3*	15.0	64 ± 2
		25.0	74 ± 6		25.0	75 ± 9
Guiyang 3	< 10 ± 6*	15.0	75 ± 4	< 8.1 ± 0.2*	15.0	78 ± 1
		25.0	81 ± 8		25.0	84 ± 6
Changzhou 3	68.8 ± 36.5	15.0	76 ± 13	118 ± 45	15.0	86 ± 4
		25.0	79 ± 13		25.0	77 ± 7

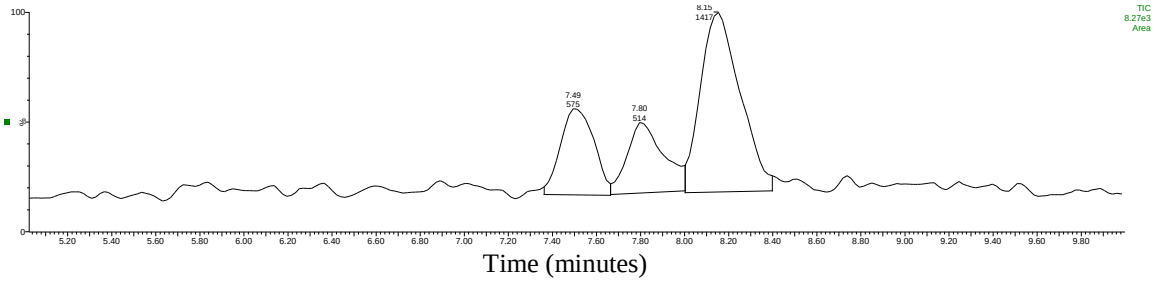

Figure 10. A MRM chromatogram of Changzhou 3 sample showing the presence of 1,3-DMAA at a lower concentration than 1,4-DMAA.

***Winter vs. summer sample analysis*.** Previous research has shown that concentrations of chemical species in natural products can be highly variable (Khan, 2006; Burns et al., 2012). A seasonal comparison is possible between the "winter" harvest (March 2012) and the "summer" harvest (May 2012) for the Kunming, Guiyang, and Changzhou samples. Neither the winter nor summer harvest samples of Kunming and Guiyang samples contained 1,3-DMAA or 1,4-DMAA species above the MDLs of the analysis. However, the Changzhou sample resulted in similar concentrations of 1,3-DMAA and 1,4-DMAA in the June 2011 and March 2012 samples. From March 2012 to May 2012, 1,3-DMAA results in a factor of 3 decrease in concentration while 1,4-DMAA about doubles in concentration. These results indicate a potential seasonal effect of 1,3-DMAA and 1,4-DMAA concentrations in agreement with previously reported research discussing environmental effects on chemical composition (Burns et al., 2012; Khan, 2006). It is also possible the concentrations of 1,3-DMAA in the Changzhou winter samples are higher due to an apparent underestimation of 1,3-DMAA concentrations by the preliminary extraction protocol as evidenced by the Changzhou S11 analysis.

Standard addition analysis of 1,3-DMAA and 1,4-DMAA. A standard addition analysis protocol was developed for sample analysis. Standard addition analysis compensates for matrix effects found in geranium plant caused by chemical species other than DMAA affecting analytical signal (either positive or negative) (Harris, 2007). In the standard addition method, known quantities of the 1,3-DMAA and 1,4-DMAA standards are added to the sample extract. This is termed "spiking" the sample. The added standard is affected by matrix effects just as the analyte in the sample. The unknown concentration can then be derived from a plot of signal versus spike concentration as long as the analyte has been previously established to have a linear signal response. Thus, the standard addition method resolves matrix interferences present in the complex geranium sample composition (Harris, 2007).

The standard addition protocol was applied to both Analysis Set 2 and 3 (Changzhou S11, Kunming, and Guiyang winter samples and the Changzhou, Kunming and Guiyang summer samples). For this study a three point standard addition plot was constructed using the unspiked sample, a 15.0 μg L^{-1} spike each of 1,3-DMAA and 1,4-DMAA, and a 25.0 μg L^{-1} spike each of 1,3-DMAA and 1,4-DMAA. The signal was plotted against the spike concentration (0, 15, and 25 μg L^{-1}) and a linear regression analysis was performed. The slope (m) and y-intercept (b) of the calibration curve were used to calculate the concentration of analyte (x) in the sample. The equation for determining the x-intercept is "$x = -b/m$" and in standard addition, the negative of the x-intercept is the concentration present in the unspiked sample.

The standard addition analysis results showed some matrix effects are still present in the optimized procedure and the external calibration analysis likely underestimates

DMAA concentrations. However, the standard addition analysis agreed overall with the external calibration results. Samples reported to contain 1,3-DMAA by external calibration also contained 1,3-DMAA by standard addition. Concentrations of 1,3-DMAA species were quantified in both the Changzhou S11-2 and Changzhou 3 samples at 496 ± 46 ng g^{-1} and 97 ± 20 ng g^{-1}, respectively. The concentrations of 1,4-DMAA in the Changzhou S11-2 and Changzhou 3 samples were 68 ± 7 ng g^{-1} and 162 ± 48 ng g^{-1}, respectively. All concentrations are well above the MDL of the analysis, and clearly demonstrate 1,3-DMAA and 1,4-DMAA are present in geranium plants from the Changzhou region.

The standard addition results for winter and summer samples of the Kunming and Guiyang agree with the external calibration results. Concentrations of 1,3-DMAA and 1,4-DMAA are all less than the MDL previously reported, or so close to the MDL that the confidence of analysis is extremely low. One of the Kunming 3 duplicates resulted in a 1,3-DMAA concentration of 21 ng g^{-1}, while the other duplicate was below the MDL of 14 ng g^{-1}. Similarly, one of the Kunming 2 duplicates resulted in a 1,4-DMAA concentration of 10 ng g^{-1}; whereas the other duplicate had concentrations less than the 20 ng g^{-1} MDL of that particular analysis.

Measurement of the diastereomer ratios of 1,3-DMAA in the Changzhou geranium samples. The laboratory of Armstrong measured the diastereomer ratios (reported as first peak/second peak) of synthetic standards and dietary supplements containing 1,3-DMAA using GC-FID analysis (Zhang et al., 2012). The reported results showed the diastereomer ratio of a Sigma-Aldrich standard of 1,3-DMAA was 1.22 ± 0.06 and the ChromaDex standard ratio was 1.42 ± 0.09. The dietary supplement ratios

were in the same range as those of the standards suggesting that both standards and supplements were of synthetic origin.

In this report, both pairs of diastereomers were detected in the Changzhou region samples as well as the synthetic calibration standards. By inspection of the chromatograms (Figures 6, 7, 8, and 10), both the standards and geranium samples present similar diastereomer ratios. Quantitatively, the average ratio of 1,3-DMAA diastereomers (first peak/second peak) in typical 20, 50 and 100 μg L^{-1} calibration standards is 1.14 ± 0.08. The diastereomer ratio of the Changzhou S11-1 sample was 1.10 ± 0.01, Changzhou 1 was 1.02, Changzhou S11-2 was 1.25 ± 0.03, and Changzhou 3 was 1.16 ± 0.10. The results of the geranium plant diastereomer ratios are similar to the ratios of the synthetic standards presented here, as well as the standards and supplements analyzed by the laboratory of Armstrong. This indicates that supplements containing both 1,3-DMAA diastereomer pairs could be naturally produced and extracted from geranium plants.

Conclusions

Geranium plants (*Pelargonium graveolens*) from three different regions of China (Kunming, Guiyang, and Changzhou) and three different harvests (June 2011, March 2012 and May 2012) were analyzed for 1,3-DMAA and 1,4-DMAA. An extraction and HPLC-MS/MS analysis method was used to determine concentrations of 1,3-DMAA and 1,4-DMAA with both external calibration and standard addition analysis. The extraction and external calibration analysis likely suffered from matrix effects and thus underestimated concentrations of 1,3-DMAA and 1,4-DMAA in geranium plants. The matrix effects were largely solved by the standard addition analysis, as expected. This

demonstrates that future analysis should use standard addition to minimize matrix effects and increase confidence of analysis with little additional labor. All extraction and calibration protocols reported 1,3-DMAA and 1,4-DMAA concentrations in geranium plants from the Changzhou region of China above the reported MDLs. The reported concentrations of 1,3-DMAA ranged from 68 to 496 ng g^{-1} and 1,4-DMAA ranged from 13 to 162 ng g^{-1} . Similarly, 1,3-DMAA and 1,4-DMAA were not detected above the MDL in samples from the Guiyang and Kunming regions. To the best of the author's knowledge, this is the first reported inter-laboratory analysis confirming the presence of 1,3-DMAA in a geranium plant (specifically the Changzhou S11 sample). Finally, the diastereomer ratios of the 1,3-DMAA in geranium plants from Changzhou are similar to those of the synthetic standards. This indicates that 1,3-DMAA could be a natural product extract, fulfilling a requirement of the Dietary Supplement Health and Education Act (National Institutes of Health, 1994).

The results reported here provide evidence that 1,3-DMAA naturally occurs in geranium plants in agreement with Li (Li et al., 2012) but clearly in disagreement with other previously reported articles by well-respected chemists and organizations (Elsohly et al., 2012; Zhang et al., 2012). However, this may not be a case of right or wrong. As an analytical chemist, the critical review of data is important for explaining differences in reported results. These differences can also provide insight into why analysis of seemingly identical plant species can result in very different outcomes. Khan (2006) has published an extensive review showing that it is not uncommon for plants of different locations to exhibit variations in their chemical compositions. For example, studies show that fluctuating geographical dynamics such as water stress and nutrient availability in the

soil are associated with the variations in cyanide concentration in the Cassava plant (Burns et al., 2012).

The published research to date includes a substantial amount of Geranium plant and oil analysis (ElSohly et al., 2012; Li et al., 2012; Lisi et al., 2011; Perrenoud et al., 2009; Vorce et al., 2011; Zhang et al., 2012). However, until now, none of the samples analyzed have been identical, or reported as from the same region. Thus, regional environmental variations (Burns et al., 2012; Khan, 2006) could explain the presence of 1,3-DMAA in the Changzhou S11, Changzhou March 2012, and Changzhou May 2012 samples and the absence of 1,3-DMAA concentrations in the Kunming and Guiyang geranium samples reported here; the Indian and Mississippi samples reported by ElSohly et al. (2012); the France, Egypt, and New Zealand samples reported by Lisi et al. (2011); and the China and Egypt samples reported by Armstrong (Zhang et al., 2012). A possible solution to this discrepancy would be a multiple laboratory, blind analysis of identical samples expected to have 1,3-DMAA (such as the Changzhou region samples) as well as samples which are not expected to contain 1,3-DMAA. Using this approach, a satisfactory answer for the national regulatory agencies as well as the commercial interests could be provided.

Chapter 3

Analysis of 1,3-Dimethylamylamine (DMAA) and 1,4-DMAA Stereoisomers in Geranium Plants With (-)-1-(9-Fluorenyl) Ethyl Chloroformate (FLEC) and (R)-(-)-α-(Trifluoromethyl) Phenylacetyl (MTPA) Derivatization Using HPLC With Tandem Mass Spectrometry

Introduction

As discussed in Chapter 2, the occurrence of 1,3-dimethylamylamine (1,3-DMAA) concentrations in geranium plants as a dietary supplement is under scrutiny from regulatory agencies (FDA, 2013; Venhuis & de Kaste, 2012; World Anti-Doping Agency, 2010) and researchers (Austin, Travis, Pace, & Lieberman, 2013; Di Lorenzo, Moro, Dos Santos, Ubert, & Restani, 2013; ElSohly et al., 2012; Fleming, Ranaivo, & Simone, 2012; Li et al., 2012; Lisi et al., 2011; Perrenoud et al., 2009; Vorce et al., 2011; Zhang et al., 2012). The World Anti-Doping Agency has banned 1,3-DMAA from use by athletes since 2010 (World Anti-Doping Agency, 2010), and in April of 2013, the Food and Drug Administration (FDA) required that products containing 1,3-DMAA be taken off the market (FDA, 2013). One of the primary questions driving this scrutiny is whether 1,3-DMAA is a naturally-occurring chemical component of geranium plants, specifically *Pelargonium graveolens (P. graveolens*). Many research groups have reported the absence (less than method detection limits) of 1,3-DMAA in geranium plants and oils from many regions of the world including France (Lisi et al., 2011), Egypt (Lisi et al., 2011; Zhang et al., 2012), New Zealand (Lisi et al., 2011), India (ElSohly et al., 2012), China (Zhang et al., 2012), Oxford, MS (ElSohly et al., 2012), Barre, MA (Austin et al., 2013), and Dover, DE (Austin et al., 2013). However, two analytical laboratories have

reported concentrations of 1,3-DMAA and 1,4-DMAA in authenticated *P. graveolens* from the Guiyang, Changzhou and Kunming regions of China (Fleming et al., 2012; Li et al., 2012) at concentrations ranging from 3 to 365 ng g^{-1}. The Li et al. (2012) and Fleming (Chapter 2) studies were the first reported concentrations of 1,3-DMAA and 1,4-DMAA in an authenticated *P. graveolens* split sample using the same analytical method. Multi-laboratory studies like these are important for validating analytical methods (Prior et al., 2012; Travis et al., 2011; USEPA, 2012). A detailed review of the literature has been recently published (Gauthier, 2013) that covers evidence for the presence of 1,3-DMAA in geranium plant materials.

The 1,3-DMAA species determined by previous reports (Austin et al., 2013; ElSohly et al., 2012; Fleming et al., 2012; Li et al., 2012; Lisi et al., 2011; Perrenoud et al., 2009; Vorce et al., 2011; Zhang et al., 2012) appears as a pair of peaks, whereas 1,4-DMAA is determined as a single peak (Figure 5). The 1,3-DMAA species has two stereogenic centers, and thus four possible stereoisomers that exist as a pair of diastereomers. The 1,4-DMAA species has one stereogenic center and exists as a pair of enantiomers (ElSohly et al., 2012; Fleming et al., 2012). The pair of peaks for 1,3-DMAA species indicates the presence of diastereomers, i.e., separable by traditional chromatography, while the single peak of 1,4-DMAA does not indicate whether one or both enantiomers are present.

The chromatographic analysis of stereoisomer ratios requires a chiral resolving agent which is able to preferentially interact with one stereoisomer over the others (Skoog et al., 2007). Chiral resolving agents can be introduced via the chromatographic stationary phase, via the mobile phase as a pairing agent, or as a covalently bonded

derivatizing agent. Derivatizing agents for DMAA analysis are routinely used (ElSohly et al., 2012; Lisi et al., 2011; Perrenoud et al., 2009; Zhang et al., 2012). 1,3-DMAA and 1,4-DMAA are small, six-carbon chain molecules with low molecular weights. The derivatizing agents are typically used to change separation chemistry as well as improve the limits of detection by increasing the mass of fragment ions for mass spectrometry. For example, stereoisomer analysis of 1,3-DMAA has been previously reported by Zhang et al. (2012) in dietary supplements containing 1,3-DMAA. The 1,3-DMAA stereoisomers were derivatized with pentafluoropropionic acid and separated using a gas chromatograph with flame ionization detection on an Astec ChiralDex column. The concentrations of 1,3-DMAA in the dietary supplements were high rather than the trace levels in the plants and no method detection limit (MDL) was discussed for the GC-FID analysis.

The goals of the research here are to develop and use an enantiomerically-pure, chiral derivatizing reagent to produce and then separate diastereomer derivatives of 1,3-DMAA and 1,4-DMAA for stereoisomer ratio analysis in geranium plants. Two chiral derivatization reagents were investigated, (-)-1-(9-fluorenyl)ethyl chloroformate [(-)-FLEC] and (R)-(-)α-methoxy-α-(trifluoromethyl) phenylacetyl [(-)-MTPA] chloride, also referred to as Mosher's acid chloride. Both (-)-FLEC and (-)-MTPA are enantiomerically-pure, chiral derivatizing reagents that produce four diastereomers of 1,3-DMAA and two diastereomers of 1,4-DMAA. The derivatized 1,3-DMAA and 1,4-DMAA diastereomer products are separated using a traditional, C_{18} reverse-phase high performance liquid chromatography (HPLC) column and detected using tandem mass spectrometry (MS/MS). Optimization and stability studies for the derivatization

procedures are reported, along with method detection limit, accuracy and precision studies for the DMAA-FLEC analysis. Authenticated geranium plant extracts from Changzhou, China and Memphis, TN (USA) are analyzed with the (-)-FLEC procedure. These analyses will confirm the presence or absence of 1,3-DMAA and 1,4-DMAA species, previously reported in authenticated *P. graveolens* (Chapter 2) and a locally grown sample. The stereoisomer analyses can also determine if the 1,3-DMAA and 1,4-DMAA detected in the geranium plants are present as racemic mixtures or enriched in a particular stereoisomer. The DMAA-FLEC derivatives were ultimately unstable, rendering the stereoisomer analysis using (-)-FLEC as labor intensive. The (-)-MTPA chemistry was subsequently investigated for stability and use as a DMAA derivatization agent. DMAA standards and an extract of geranium plant from Changzhou (with a different harvest date than the Changzhou plant of the previous analysis) are analyzed with the (-)-MTPA procedure to provide further confirmation and to determine stereoisomer ratios.

Experimental

Chemicals and reagents. All chemicals and reagents have a purity of 97% or greater, except the (-)-FLEC which is commercially sold as an 18 mM solution from Sigma-Aldrich. Standards and eluent are prepared in reagent-grade water with a resistivity of 18.2 $M\Omega \cdot cm^{-1}$. Glassware is cleaned with concentrated detergent and rinsed with reagent-grade water three times. 1,3-DMAA is from 2A PharmaChem USA; 1,4-DMAA and Mosher's acid chloride are from Sigma-Aldrich; LC-MS grade acetonitrile, ethanol, concentrated hydrochloric acid (HCl), formic acid and sodium

hydroxide (NaOH) are from Fisher Scientific. The boric acid was purchased from Matheson Coleman & Bell.

DMAA standards and derivatization procedures. Stock standards of 1,3-DMAA and 1,4-DMAA were prepared separately. The 1 mg mL^{-1} 1,3-DMAA stock standard was prepared by weighing 25.0 mg of 1,3-DMAA into a 25.00 mL volumetric flask and diluting to volume with ethanol. The 1,4-DMAA stock was prepared similarly with 33 µL of 1,4-DMAA into 25.00 mL of pure ethanol in a volumetric flask. The stocks are diluted accordingly with 0.5 M HCl to prepare a working standard of 500 µg L^{-1} for each DMAA species. For both (-)-FLEC and (-)-MTPA derivatization procedures: 0.5 mL of 0.5 M NaOH, a suitable amount of the DMAA standard solution, 0.5 mL of 0.1 M borate buffer adjusted to pH 9.2 and a specific amount of pure derivatizing reagent (18 mM) solution are successively added to a 15-mL graduated centrifuge tube. The concentrations of DMAA in the standards and expected concentrations in the geranium plants are low, thus a molar ratio of 1:1000 for DMAA: (-)-FLEC or DMAA: (-)-MTPA is kept to ensure a maximum yield of the derivatized product. After each addition, the solution is mixed for at least 30 seconds with a vortex mixer. Finally, acetonitrile is added to obtain a total final volume of 2.0 mL, followed by a final 1 minute vortex mixing. The derivatization reaction time is 30 minutes at ambient laboratory temperature.

Geranium plant samples are also analyzed using a similar procedure described above. In a 15-mL graduated centrifuge tube, 0.5 mL of 0.5 M NaOH, 200 µL of extracted (extraction procedure outlined in Chapter 2) plant sample, 0.5 mL of 0.1 M borate buffer adjusted to pH 9.2 and 50 µL of 18 mM (-)-FLEC or (-)-MTPA solution are successively added. After each addition, the solution is mixed for at least 30 s with a

vortex mixer. Finally, acetonitrile is added to obtain a total final volume of 2.0 mL and the solution is again mixed for 1 minute with a vortex. The derivatization reaction time is 30 minutes and the solution is filtered before analysis. The extracted plant sample was pre-concentrated for samples with low DMAA concentrations described below.

Pre-concentration procedure. A simple pre-concentration step was adapted for samples containing low (< 10 ng g^{-1}) concentrations of 1,3-DMAA and 1,4-DMAA. 100 mL of a standard (or plant extract) solution was neutralized with NaOH, and extracted with 5 mL of chloroform three times. The chloroform phase was collected and evaporated to 3 mL. Then, 2 mL of the evaporated chloroform was placed into a centrifuge tube and back extracted with 2 mL of 0.5 M HCl. The aqueous phase was collected and analyzed (without derivatization) with the HPLC-MS/MS, resulting in a pre-concentration factor of 50. The calibration curve was prepared with 3 points at 1, 5, and 10 μg L^{-1}. The check standard has a concentration of 0.5 μg L^{-1} and was analyzed seven times. The MDL values for 1,3-DMAA are 0.4 ng g^{-1}, with a mean % recovery of 451 % and % relative standard deviation (%RSD) of 0.6 %. The MDL for 1,4-DMAA was 0.2 ng g^{-1} with a mean % recovery of 454 % and the %RSD was 0.3 %. The high recoveries are likely a result of the propagation of error inherent in the multi-step pre-concentration procedure. Despite the high recoveries, the pre-concentration procedure provides an independent analysis for confirmation and comparison to the (-)-FLEC analysis for 1,3-DMAA and 1,4-DMAA in the Memphis geranium plant.

Geranium samples. Two geranium plant samples were analyzed with the (-)-FLEC analysis. One was from Changzhou, China and was previously authenticated, analyzed (Fleming et al., 2012), and stored at 4°C for approximately one year. The

second sample was sold as geranium plant, purchased from Home Depot in Memphis, TN (USA) in April 2013. The Memphis geranium plant was authenticated by Dr. Randall Bayer, professor of Biology at The University of Memphis, as *Pelargonium zonale*. The plant was initially grown in Mills River, NC USA at the Van Wingerden Intl. nursery and then shipped to a Home Depot in Memphis, TN. After purchasing from Home Depot, the Memphis geranium plant was placed under fluorescent lighting and watered routinely to continue growth in a room separate from the analysis laboratories to minimize potential contamination. Immediately before analysis, the Memphis plant is freshly cut and extracted in the laboratory. Also, a new Changzhou sample (harvest date of May 2013) was pre-concentrated and analyzed with the (-)-MTPA procedure.

Instrumentation. The derivatized standard solutions and geranium plant extracts are analyzed using an Agilent 1100 HPLC coupled to a Waters Quattro Ultima triple quadrupole MS. The derivatized products are separated on a Phenomenex Kinetex C18 column (4.6 x 150 mm, 2.6 µm particle size) with two eluents: eluent A is 1 % formic acid in reagent water and eluent B is acetonitrile. The injection volume is 100 µL. The flow from the HPLC was split such that 300 µL min^{-1} was introduced into the ESI source and 500 µL min^{-1} went to waste. The DMAA-FLEC derivatives were separated with an isocratic mode (40:60 eluent A: eluent B) at 0.8 mL min^{-1}. The total analysis time for DMAA-FLEC derivatives is 40 min.

The DMAA-MTPA derivatives were separated with 1 % formic acid in methanol as eluent A and 75%/25% methanol/acetonitrile as eluent B. The DMAA-MTPA separation requires a gradient program with a flow rate of 0.6 mL min^{-1}. The gradient program is as follows: initial conditions (80 % eluent A and 20 % eluent B), hold 0.5

minutes; from 0.5 to 20 min, eluent B increases to 60 %; from 20 to 40 min eluent B is held at 60 %; and from 40 to 45 min, eluent B is decreased back to initial conditions of 20 %. The total analysis time for DMAA-MTPA derivatives is 45 min. The tandem MS analysis is performed with ESI in positive mode. The capillary voltage is set at 3 kV and the cone at 40 V. The source temperature was set at 120 °C with a flow of 108 L hr^{-1} and a desolvation temperature of 350 °C at a flow of 635 L hr^{-1}. The collision voltage was set to 8 eV with an argon collision gas pressure at 7 psi. The exact mass for the FLEC derivative of 1,3-DMAA and 1,4-DMAA is 351.22 g mol^{-1}. The MRM transition is set to 353/194 *m/z* where 353.65 $(M+2H)^+$ is the precursor ion and 194.52 is the product ion, corresponding to $[C_{15}H_{14}]^+$. The exact mass for both DMAA-MTPA derivatives is 329.36 g mol^{-1}. The MRM transition is set to 332/300 *m/z* where 332.4 $(M+2H)^+$ corresponds to the precursor ion and 300.4 is the product ion. All data analyses were conducted in Waters Mass Lynx MS software. The chromatograms were smoothed using a Savitzky-Golay algorithm (Savitzky & Golay, 1964) within the MassLynx software.

Prior to derivatization with Mosher's acid chloride the DMAA standards or plant extracts were separated using a Parallex Flex preparatory scale HPLC (Biotage; Charlottesville, VA, USA) and monitored using a split flow to the tandem MS. The eluent gradient program is generated at a constant flow rate of 7.0 mL min^{-1} and is comprised of two components: 0.5 % formic acid in reagent water (eluent A) and 1:3 reagent water: acetonitrile (eluent B). Initial conditions were 0 % eluent B which was held for 3 min. The injection time was 1.43 minutes with initial conditions. From 4.43 to 9.43 min eluent B was increased to 10 %. From 9.43 to 19.43 min eluent B was increased from 10 to 12 %. From 19.43 to 39.43 min eluent B was held at 12 %. The total

chromatographic run time was 39.43 min. Fractions of the standard (or plant extract) were collected in 10 mL well plates.

Results and Discussion

Overview of the extraction and derivatization of 1,3-DMAA and 1,4-DMAA.

The extraction of the two DMAA species in geranium plants is done in acidic solution (0.5 M HCl) to take advantage of their amine chemistry (Austin et al., 2013; ElSohly et al., 2012; Fleming et al., 2012; Li et al., 2012; Lisi et al., 2011; Zhang et al., 2012). However, the (-)-FLEC and (-)-MTPA derivatization of 1,3-DMAA and 1,4-DMAA occurs in basic solution (Figure 11 and Figure 12, respectively) as a nucleophilic addition and elimination reaction between the acid chloride functional group of the derivatizing agent and the amine functional group of the DMAA species. Thus the geranium plant extracts are first neutralized with 0.5 M NaOH before derivatization. The 1,3-DMAA- and 1,4-DMAA derivatives have three and two chiral centers, respectively (Figure 11 and 12, asterisk "*" labels). The enantiomerically-pure derivatizing reagent creates a fixed chiral center in the derivative molecule to produce a set of diastereomers and allows for separation using a non-chiral, reverse phase HPLC column. This approach creates four diastereomers of 1,3-DMAA [e.g. (*R*,*R*,*S*); (*R*,*S*,*S*); (*S*,*R*,*S*); and (*S*,*S*,*S*)] and two diastereomers for 1,4-DMAA [e.g. (*R*,*S*) and (*S*,*S*)], which can be separated from each other because each diastereomer has different chemical and physical properties (Skoog et al., 2007). In the following sections, the (-)-FLEC and (-)-MTPA chemistry will be discussed individually.

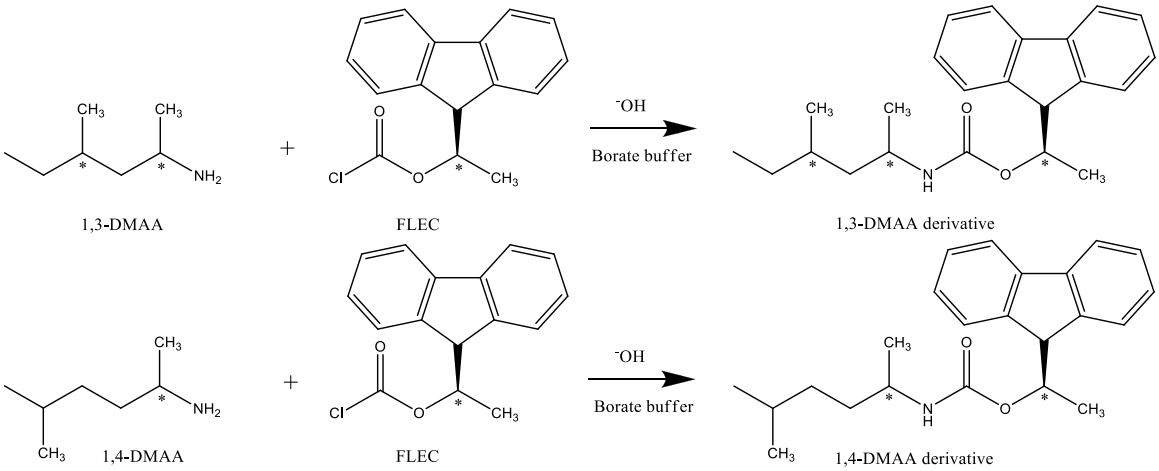

Figure 11. Schematic reaction of 1,3-DMAA and 1,4-DMAA with (-)-FLEC to produce 1,3-DMAA- and 1,4-DMAA-FLEC derivatives. "*" represents a chiral carbon.

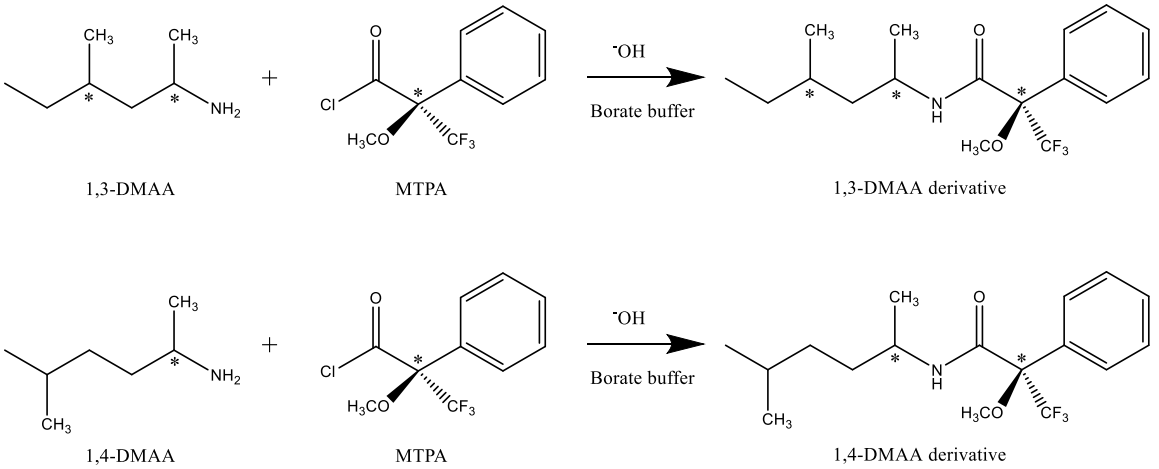

Figure 12. Schematic reaction of 1,3-DMAA and 1,4-DMAA with (-)-MTPA to produce the 1,3-DMAA- and 1,4-DMAA-MTPA derivatives. "*" represents a chiral carbon.

DMAA-FLEC stereoisomer separation overview. Figure 13-A is a chromatogram that demonstrates the separation of the four 1,3-DMAA-FLEC diastereomers at a concentration of 10 µg L^{-1}, labeled as 1,3-DIA 1, 1,3-DIA 2, 1,3-DIA 3 and 1,3-DIA 4 which correspond to the four 1,3-DMAA stereoisomers. Their respective retention times are 32.8, 33.3, 34.5 and 35 minutes. Figure 13-B demonstrates the separation of 10 µg L^{-1} of 1,4-DMAA-FLEC diastereomers that are resolved into two distinct peaks (1,4-DIA 1

and 1,4-DIA 2) and correspond to the two 1,4-DMAA enantiomers. The retention times of 1,4-DMAA-FLEC diastereomers are 33.5 and 35.2 minutes, which overlap with two of the 1,3-DMAA-FLEC diastereomers, 1,3-DIA 2 and 1,3-DIA 4. Figure 13-C demonstrates the co-elution of the 1,3-DMAA-FLEC diastereomers and the 1,4-DMAA-FLEC diastereomers at 10 μg L^{-1}. In these chromatograms, the ratio of the four 1,3-DMAA stereoisomers in the synthetically produced 1,3-DMAA are not equal (Figure 13-A). The ratios are 32:21:31:16 in numerical order for the four diastereomers. While the individual stereoisomers are not equal, the ratio of DIA 1+DIA 2: DIA 3+DIA 4 is 53:47 (1.13) matching the previously reported diastereomer analysis of 1.14 ± 0.08 for this particular 1,3-DMAA standard (Chapter 2, "Measurement of the diastereomer ratios of 1,3-DMAA in the Changzhou geranium samples").

The retention times of the six DMAA-FLEC diastereomers range from 33 to 35 minutes (Figure 13). This is a very small window to accurately measure the relative enantiomeric concentrations of 1,3-DMAA and 1,4-DMAA species in plant materials simultaneously. However, if only one of the DMAA species is present then the analysis is straightforward. In this study, due to the co-elution of the DMAA derivatives, two approaches are taken for the analysis of DMAA species in geranium plants. The first approach uses the (-)-FLEC derivatization chemistry to qualitatively determine the presence or absence of DMAA species and confirm a previous analysis (Chapter 2). The second approach uses the (-)-FLEC chemistry to provide a quantitative measure of the DMAA concentrations and stereoisomer ratios in geranium plants.

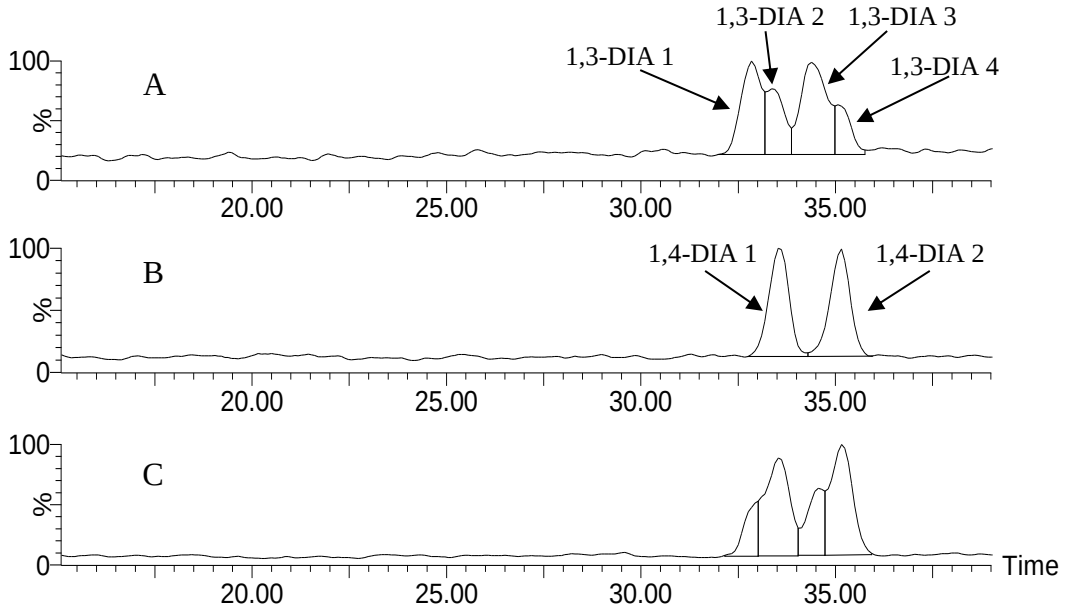

Figure 13. 1,3-DMAA-FLEC and 1,4-DMAA-FLEC derivatives separation. Each DMAA species has a concentration of 10 µg L^{-1}. Chromatogram A: 1,3-DMAA-FLEC derivative. Chromatogram B: 1,4-DMAA-FLEC derivative. Chromatogram C: Combined 1,3-DMAA- and 1,4-DMAA-FLEC derivatives.

Reaction time optimization and stability studies of 1,3-DMAA-FLEC and 1,4-DMAA-FLEC derivatives. The derivatization of 1,3-DMAA and 1,4-DMAA are carried out at room temperature, and a reaction time optimization study was conducted from 5-45 min with the DMAA-FLEC derivative. The 30-minute reaction time provided the highest analytical signal for the derivatized product. Empirical observations during method development for (-)-FLEC derivatization suggested the DMAA-FLEC derivative was decomposing in solution. Thus, stability studies were carried out for 1,3-DMAA and 1,4-DMAA derivatives separately at concentrations of 5, 25, and 50 µg L^{-1} for 1,3-DMAA species and 10, 25, and 50 µg L^{-1} for 1,4-DMAA to systematically explore these observations. The stability study was conducted for 12 hr for the 1,3-DMAA-FLEC derivatives and 7 hr for the 1,4-DMAA derivatives (Figure 14). The DMAA species were

derivatized with (-)-FLEC solution for 30 minutes followed by analysis of each solution at 2 hr time intervals for 1,3-DMAA and 45-minute intervals for 1,4-DMAA. In Figure 14 the results of the 25 µg L^{-1} of 1,3-DMAA and 1,4-DMAA are presented which are representative of the 5, 10, and 50 µg L^{-1} studies. The peak area is plotted as a function of the elapsed time to ascertain the stability of the DMAA-FLEC products. For 1,3-DMAA, the total peak area of the diastereomer pairs is plotted, thus 1,3-DIA1 and 1,3-DIA2 are plotted as 1,3-Peak1, and 1,3-DIA3 and 1,3-DIA4 are plotted as 1,3-Peak2. In all cases, the peak area decreases over 50% of the original value by the 2 hr time mark. This stability study shows the DMAA-FLEC derivative must be produced just prior to HPLC-MS/MS analysis for accurate determination of the DMAA species concentrations.

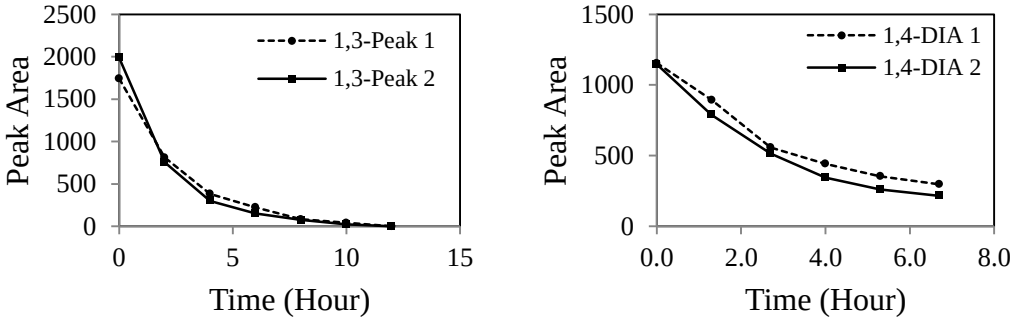

Figure 14. 1,3-DMAA-FLEC and 1,4-DMAA-FLEC derivative stability study. Left plot: 25 µg L^{-1} 1,3-DMAA-FLEC derivatives. Right plot: µg L^{-1} 1,4-DMAA-FLEC derivatives.

Method detection limit, accuracy, precision, and linearity studies for DMAA-FLEC. Detailed method detection limit (Glaser et al., 1981; USEPA, 1984), accuracy and precision (USEPA, 1996) studies were carried out for the DMAA-FLEC derivatives according to the USEPA protocols. The USEPA MDL is determined by multiplying the standard deviation of the check standard concentration with Student's t-value at (n−1) degrees of freedom where "n" is equal to the number of check standards (Glaser et al., 1981; Harris, 2007; USEPA, 1984). The USEPA MDL provides an estimate on the error of the instrumental analysis. Concentrations reported within a factor of 2 – 5 of the MDL can have accuracy values ranging from 50 – 150 % and precision values up to 30 % relative error (USEPA, 1996). Two USEPA MDL values are reported here: the Instrumental MDL and the Extraction MDL (Table 6). The Instrumental MDL is the minimum concentration distinguishable from the noise of the instrument. The Extraction MDL takes into account the extraction volume and mass of plant used to determine the minimum concentration which can be detected in the analysis. The MDL, accuracy and precision studies for 1,3-DMAA and 1,4-DMAA were conducted separately to minimize the effects of co-elution and the detailed results are presented in Table 6. A 5-point calibration curve was constructed separately for both DMAA species from 2 to 50 μg L^{-1} followed by analysis of five, 3 μg L^{-1} 1,3-DMAA check standards and four, 3 μg L^{-1} 1,4-DMAA check standards. Since the DMAA-FLEC derivatives are not stable, each check standard was derivatized just prior to HPLC-MS/MS analysis. As in the "Reaction and Stability Studies" section, the MDL for 1,3-DMAA was calculated based on the combination of 1,3-DIA1 and 1,3-DIA2 as "Peak 1" and 1,3-DIA3 and 1,3-DIA4 as "Peak 2." The Instrumental MDL values ranged from 1.2 to 2.0 μg L^{-1} for the 1,3-

DMAA-FLEC and 1,4-DMAA-FLEC derivatives, respectively. The accuracy, estimated using mean % recovery, ranged from 122 to 131 %, and precision, estimated using % relative standard deviation, ranged from 8 to 12 % for all diastereomers analyzed. The Extraction MDL of the DMAA-FLEC analysis in the geranium plants (Table 6) can be calculated from multiplying the "MDL in μg L^{-1}" by the volume of extraction (0.1 L) and dividing by the mass of plant used in the extraction, typically 10 g. This produces an MDL of analysis in units of (μg DMAA) (g of plant)$^{-1}$ which is converted to (ng DMAA) (g of plant) $^{-1}$ by multiplying by 1000. The extraction MDL ranges from 12 to 20 ng g^{-1} for both 1,3-DMAA and 1,4-DMAA.

A linearity study was conducted with concentrations ranging from 2.5 to 50 μg L^{-1} for 1,3-DMAA-FLEC derivatives and from 1 to 50 μg L^{-1} for 1,4-DMAA-FLEC derivatives. A high linear range is desired, but the amount of (-)-FLEC solution needed to maintain a 1:1000 ratio increases rapidly at high DMAA concentrations. The results of the linearity study demonstrated the analytical responses for all DMAA-FLEC diastereomers were linear ($r^2 > 0.96$) up to and including 50 μg L^{-1} for both DMAA species. The detailed MDL, accuracy, precision and linearity results demonstrate the (-)-FLEC derivatization chemistry and analysis is sufficient for qualitative and quantitative analysis of 1,3-DMAA and 1,4-DMAA concentrations that are typically found in geranium analysis (FDA, 2013; World Anti-Doping Agency, 2010; Li et al., 2012; Fleming et al., 2012). If higher DMAA concentrations are determined, the geranium plant extracts are readily diluted for repeat analysis to be within the practical linear limit of 50 μg L^{-1}.

Table 6

MDL, accuracy and precision studies for 1,3-DMAA-FLEC and 1,4-DMAA-FLEC derivatives. [a] *The Instrumental MDL is calculated by multiplying the standard deviation of the concentration by the associated t-value at the 98% C.L. (t-value for seven check standards is 3.143);* [b] *The Extraction MDL is calculated by multiplying the instrumental MDL by the volume of the extraction (0.1 L0 and dividing by the mass of plant used in the extraction (10 g). The µg* g^{-1} *unit is then converted to ng* g^{-1}.

	Instrumental MDL ($\mu g\ L^{-1}$)[a]	Extraction MDL ($ng\ g^{-1}$)[b]	Mean % Recovery	%RSD
1,3-DMAA				
1,3-Peak 1	1.2	12	131	8
1,3-Peak 2	1.4	14	127	10
1,4-DMAA				
1,4-DIA1	2.0	20	122	12
1,4-DIA2	1.6	16	122	10

1,3-DMAA and 1,4-DMAA concentration determination using DMAA-FLEC analysis. Following the detailed MDL, accuracy and precision studies, two geranium plant samples were analyzed using the described (-)-FLEC method and the extraction method previously described in Chapter 2: one from Changzhou, China and one from Memphis, TN. The Changzhou geranium plant was harvested in May 2012 and previously extracted and analyzed in June 2012 (Chapter 2). The Changzhou sample extract was refrigerated for one year at 4 °C prior to FLEC analysis. In the previous study, the Changzhou sample extract (labeled Changzhou 3) was reported as the average of the DMAA concentration from duplicates. In the results presented here, the Changzhou sample previously analyzed is labeled as "†" (e.g. Changzhou†). One of the extract vials was analyzed and the concentration from that particular vial is included here. Aliquots from the stored extract were analyzed in duplicate by the (-)-FLEC analysis.

For the HPLC-MS/MS analysis, 200 µL of the plant extract was derivatized with 50 µL of 18 mM (-)-FLEC solution to maintain a 1000-fold excess of (-)-FLEC at an

expected maximum concentration of 50 µg L^{-1} of total DMAA in the extract. The concentrations of DMAA species in the Changzhou geranium extract were not expected to exceed 50 µg L^{-1} based on previous results. The Memphis geranium sample was not expected to contain DMAA concentrations, since no geraniums outside of the Chinese regions have been demonstrated to contain 1,3-DMAA or 1,4-DMAA (Austin et al., 2013; ElSohly et al., 2012; Lisi et al., 2011; Zhang et al., 2012). The detailed results from the Changzhou and Memphis analyses are presented in Table 7, with respective chromatograms in Figures 15 and 16.

The results from the sample analysis are complex due to the partial co-elution of the 1,3-DMAA-FLEC and 1,4-DMAA-FLEC derivatives (Figure 15). However, details concerning the qualitative presence or absence of 1,3-DMAA and 1,4-DMAA can be elucidated from the chromatograms. The presence of 1,3-DMAA is *qualitatively confirmed* by the (-)-FLEC chemistry in the Changzhou sample, with the presence of 1,4-DMAA as *highly probable*. A detailed analysis of the chromatogram (Figure 15) shows the presence of 1,3-DMAA as shoulder peaks at 32.9 and 34.6 min, and matching the retention times of the combined 1,3-DMAA- and 1,4-DMAA-FLEC derivatives (Figure 13-C). The calibration curve did not take into account the effects of peak splitting, thus each peak is able to provide a concentration estimate independent of the others. For 1,3-DMAA in the Changzhou[a] sample, an average concentration of 39.5 ± 0. 2 ng g^{-1} was calculated based on the 1,3-DIA1 peak and 40.1 ± 5.8 ng g^{-1} based on the 1,3-DIA3 peak, and agree with the original analysis.

The presence of 1,3-DMAA indicates the 1,4-DMAA-FLEC derivative peaks may also contain concentrations of 1,3-DMAA-FLEC derivatives. Regardless, if all of the

analytical signal is assumed to be 1,4-DMAA, then the reported concentration averages 67.9 ± 7.0 ng g^{-1} based on 1-4-DIA1 and 61.5 ± 4.9 ng g^{-1} based on 1,4-DIA2 for Changzhou[a]. In this case, the (-)-FLEC analysis reports similar concentrations to the previous analysis of the individual vial of 86.0 ng g^{-1}.

Table 7
*Concentration and stereoisomer ratios for 1,3-DMAA and 1,4-DMAA derivatives from authenticated geranium plants. [a] Samples measured using (-)-FLEC analysis; [†]Changzhou geranium plant extract previously extracted and analyzed (Chapter 2); *Memphis geranium plant extracted using pre-concentration method; [‡]DMAA concentrations from one duplicate.*

	1,3-DMAA (ng g^{-1})		1,4-DMAA (ng g^{-1})	
	1,3-DIA 1	1,3-DIA 3	1,4-Peak 1	1,4-Peak 2
Changzhou[a]	39.5 ± 0. 2	40.1 ± 5.8	67.9 ± 7.0	61.5 ± 4.9
Changzhou[†]	43.0[‡]		86.0[‡]	
Memphis[a]	< 12	< 14	35.7 ± 3.8	37.3 ± 4.4
Memphis*	14 ± 15*		18 ± 13[*]	

The Memphis geranium plant extract was prepared and analyzed using three different approaches: (1) the freshly cut and extracted plant was derivatized with (-)-FLEC solution and analyzed in duplicate; (2) the same plant extract was analyzed using the method reported by Fleming *et al*.(Chapter 2); and (3) the plant extract was pre-concentrated and then analyzed with HPLC-MS/MS.

The detailed (-)-FLEC sample analysis results for the Memphis sample reported 1,3-DMAA concentrations as less than the MDLs of 12 and 14 ng g^{-1} for the respective peaks (1,3-DIA1 and 1,3-DIA 3). The concentration of 1,4-DMAA diastereomers was reported as ranging between 35 and 37 ng g^{-1} (Table 7) and is within a factor of 2 of the MDL of the analysis. Chromatograms from the analysis demonstrate the presence of

predominantly 1,4-DMAA species by the (-)-FLEC analysis, though the small shoulders on the 1,4-DMAA peaks indicate small concentrations of 1,3-DMAA (Figures 16-A and 16-B). To confirm the analysis, the pre-concentration method was used and determined the concentrations of 1,3-DMAA as 14 ± 15 ng g^{-1} and 1,4-DMAA as 18 ± 13 ng g^{-1}. The error of the analysis is high, but does confirm the presence of 1,3-DMAA and 1,4-DMAA in the Memphis geranium plant (Figures 16-C and 16-D), a first for a plant outside of China. The 1,3-DMAA concentrations are at or less than the MDL of the (-)-FLEC analysis with Fleming *et al*. (2012) analysis, indicating agreement. The concentrations of 1,4-DMAA by pre-concentration are within a factor of 2 of the (-)-FLEC analysis and agree reasonably well.

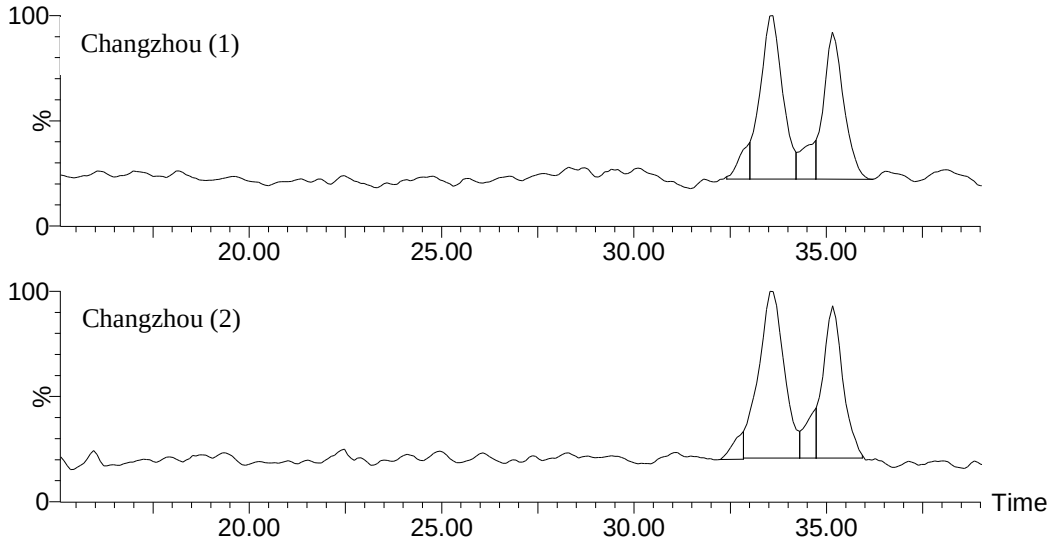

Figure 15. (-)-FLEC derivatized Changzhou plant extract analyzed in duplicate.

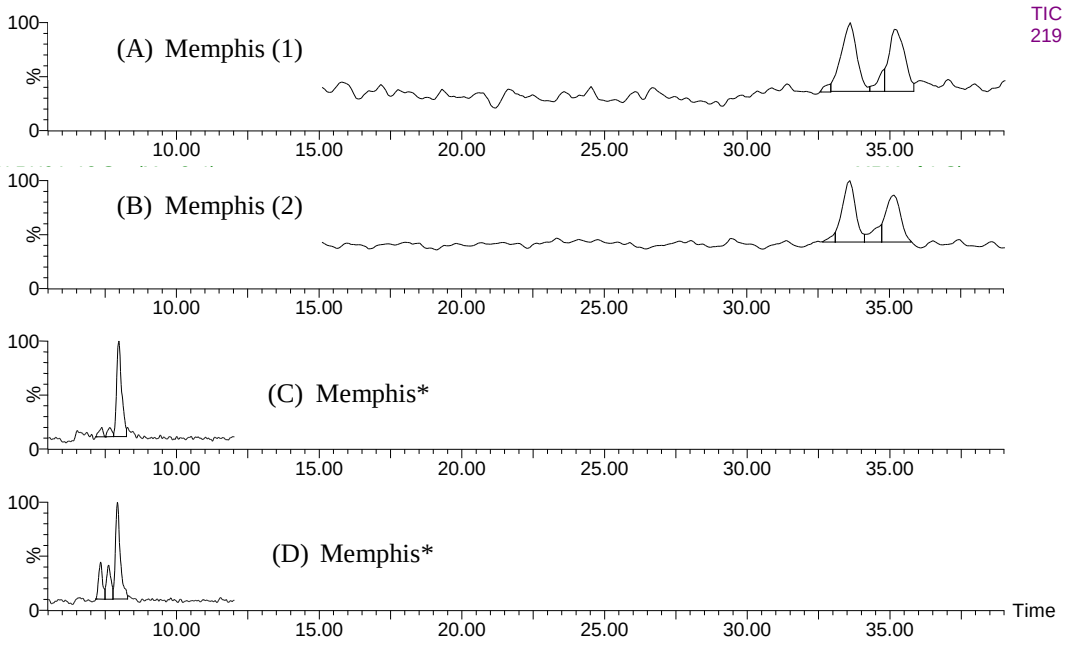

Figure 16. (A & B) Memphis, TN plant extract analyzed in duplicate using (-)-FLEC. (C & D) *Memphis geranium plant analyzed by pre-concentration with HPLC-MS/MS.

Stereoisomer ratio determination for 1,3-DMAA and 1,4-DMAA using DMAA-FLEC chemistry. The (-)-FLEC derivatization chemistry creates a fixed chiral center in the DMAA-FLEC derivative to produce four diastereomers of 1,3-DMAA and two diastereomers of 1,4-DMAA, which are separated on a traditional HPLC column with MS/MS detection. Traditionally, HPLC measurements are made with the peak area of the eluting component. Therefore, stereoisomer ratios for 1,4-DMAA were calculated by dividing the individual peak area of each 1,4-DMAA stereoisomer by the sum of the total peak area. However, due to the co-elution of the 1,3-DMAA-FLEC diastereomers peak height is used to calculate the stereoisomer ratio. The stereoisomer ratios were determined by dividing the individual peak height of each 1,3-DMAA stereoisomer by the sum of total peak height.

The stereoisomer ratios were computed in the linearity study for 1,3-DMAA and 1,4-DMAA standards (Table 8), though the values at 50 µg L^{-1} were not included because the diastereomer peaks co-eluted. Overall, the 1,3-DIA1 and 1,3-DIA3 have a higher ratio than 1,3-DIA2 and 1,3-DIA4. The stereoisomer ratio for 1,3-DIA1 ranges from 23 to 32% with an average of 27 ± 3.4%; 1,3-DIA2 ranges from 19 to 25% with an average of 22 ± 2.6%; 1,3-DIA3 ranges from 24 to 43% with an average of 29 ± 4.7%; and 1,3-DIA4 ranges from 16 to 28% with an average of 22 ± 5.2%.

At low concentrations (2 – 5 µg L^{-1}), the average stereoisomer ratio of 1,3-DMAA is 24 ± 1.4 : 24 ± 1.4 : 24 ± 0.7 : 28 ± 0.7. At higher concentrations, 10 µg L^{-1} and higher, the average stereoisomer ratio is 29 ± 2.6 : 20 ± 1.2 : 32 ± 2.1 : 19 ± 2.5. The differences between the two measurements may be a result of high relative error in the analysis. The error of the analysis can be contributed to the fact that the measurements were conducted within a factor of 2 – 5 of the MDL.

For the 1,4-DMAA standard, the average enantiomeric ratio was 48 ± 8: 52 ± 8 with all concentrations included. The high apparent error on the enantiomeric analysis results from the two measurements at 1 µg L^{-1} and 2 µg L^{-1} which are near the MDL, thus have a high relative error (USEPA, 1996; Ranaivo et al., 2011; Simone, Anderson, & Emmert, 2006; Simone et al., 2009). Removing these two measurements results in an enantiomeric ratio on the 1,4-DMAA standard of 50.3 ± 0.5 : 49.8 ± 0.5, which is racemic within the margin of error. When considering the calibration curve, all concentrations result in an enantiomeric ratio of 51 ± 2 : 49 ± 2; however, at concentrations above 5 µg L^{-1}, the stereoisomer ratio of 1,4-DMAA is 50 : 50. Measurements near the MDL are expected to have high errors, and as the concentrations

increase to factors greater than five of the MDL, the measurement error greatly decreases (Ranaivo et al., 2011; Simone et al., 2006; Simone et al., 2009; USEPA, 1996). This high error effect can be observed in the check standard analysis where the measured enantiomeric ratio of 1,4-DMAA was 52 ± 3 : 48 ± 3.

An analysis of the enantiomeric ratio of 1,4-DMAA species in the Changzhou samples shows a 52 ± 0.7 : 48 ± 0.7 ratio, based on reported concentrations (Table 7). Since the 1,4-DMAA concentrations presumably include 1,3-DMAA diastereomers, there is a larger potential error beyond the ± 0.7% in the analysis. In the Memphis geranium sample, the 1,3-DMAA concentrations are less than the MDL of the (-)-FLEC analysis, and thus provides an excellent opportunity to measure the enantiomeric ratio of 1,4-DMAA in a geranium plant. The average concentration of 1,4-DMAA reported based on enantiomer 1 is 35.7 ± 3.8 ng g^{-1} and based on enantiomer 2 is 37.3 ± 4.4 ng g^{-1} (Table 7). Using these concentrations, the enantiomeric ratio averaged 49 ± 0.7: 51 ± 0.7. The standard deviations of analysis indicate that enantiomer 2 shows a slight enrichment over enantiomer 1 within the geranium plant. Using the 3% error on ratio from the check standard as a worst case estimate, then the enantiomeric ratios of 1,4-DMAA in the Memphis geranium could be considered 50:50 within the error of analysis.

Table 8
*1,3-DMAA-FLEC and 1,4-DMAA FLEC stereoisomer ratio measurements in the linearity study. *Diastereomer peaks co-elute.*

1,3-DMAA					1,4-DMAA		
Conc. ($\mu g\ L^{-1}$)	1,3-DIA 1	1,3-DIA 2	1,3-DIA 3	1,3-DIA 4	Conc. ($\mu g\ L^{-1}$)	1,4-DIA 1	1,4-DIA 2
2.5	23	25	24	28	1	31	69
5	25	23	25	27	2	55	45
10	32	21	31	16	5	51	49
20	28	19	32	21	10	50	50
25	27	19	35	19	25	50	50
50	41*	0*	43	16	50	50	50

Analysis of 1,3-DMAA and 1,4-DMAA by MTPA derivatization. The DMAA-FLEC derivatization chemistry proved to be useful for providing an independent chemistry providing confirmation of the presence of 1,3-DMAA and 1,4-DMAA in the geranium samples and estimating the stereoisomer ratios of the DMAA species present. However, the existence of both 1,3-DMAA and 1,4-DMAA in geranium plant made obtaining a high quality measurement of the stereoisomer ratios difficult at best. In addition, the instability of the DMAA-FLEC products made the analysis labor intensive, requiring individual derivatization and immediate analysis of each standard and sample. This means that each study required an analyst be present for the duration, and automation could not be used during the HPLC-MS/MS analysis. Thus, an alternative chiral derivatizing agent was explored to determine if better stability and separation was possible. Mosher's acid chloride [(-)-MTPA] was chosen based on its similarity in structure to (-)-FLEC. Like (-)-FLEC, (-)-MTPA also contains an acyl chloride functional group that will react with the amine group of DMAA under basic conditions.

DMAA-MTPA stereoisomer separation overview. The derivatization of the DMAA with the enantiomerically pure (-)-MTPA reagent was a drop-in replacement of

the FLEC reagent. No additional optimization of the reaction conditions was needed. Studies with (-)-MTPA analyzed DMAA stereoisomer ratios but did not determine DMAA concentrations; therefore, no MDL studies are reported for DMAA-MTPA. Figure 17-A is a chromatogram that demonstrates the separation of 1,3-DMAA-MTPA at a concentration of 15 µg L^{-1}. The four 1,3-DMAA-MTPA diastereomers could not be completely separated and as a result only two peaks (labeled 1,3-Peak 1 and 1,3-Peak 2) are obtained for 1,3-DMAA-MTPA. The retention time for the two 1,3-DMAA-MTPA peaks are 36.58 and 37.10 minutes. Figure 17-B demonstrates the separation of 15 µg L^{-1} of 1,4-DMAA-MTPA. The 1,4-DMAA-MTPA diastereomers are resolved into two peaks (1,4-DIA 1 and 1,4-DIA 2) that correspond to the two 1,4-DMAA enantiomers. The retention times for the two 1,4-DMAA-MTPA diastereomers are 37.44 and 38.13 minutes. Figure 17-C shows the co-elution of the 1,3-DMAA-MTPA diastereomers with the 1,4-DMAA-MTPA diastereomers at 15 µg L^{-1}. The 1,4-DIA 1 peak overlaps with the 1,3-Peak 2. Due to the lack of separation of all four 1,3-DMAA-MTPA stereoisomers by the analytical column alone, a preparatory scale HPLC (prep HPLC) was employed to separate the 1,3-DMAA diastereomer pairs prior to derivatization.

In the prep HPLC method a DMAA standard (or plant extract) is separated and the column effluent fractions are collected. The collected fractions are analyzed with the HPLC-MS/MS method from the previous research (Chapter 2, “HPLC-MS/MS instrumentation”) to determine DMAA content (Figure 18). The DMAA fractions, two 1,3-DMAA fractions and one 1,4-DMAA fraction (Figures 18-A1, -A4, and -A6), are then derivatized with (-)-MTPA. The derivatized fractions are analyzed with the analytical scale DMAA-MTPA HPLC-MS/MS method. The prep HPLC process with

fraction derivatization is illustrated in Figure 18. The derivatized fractions are then used to determine the stereoisomer ratios for 1,3-DMAA and 1,4-DMAA in the standard and plant extract

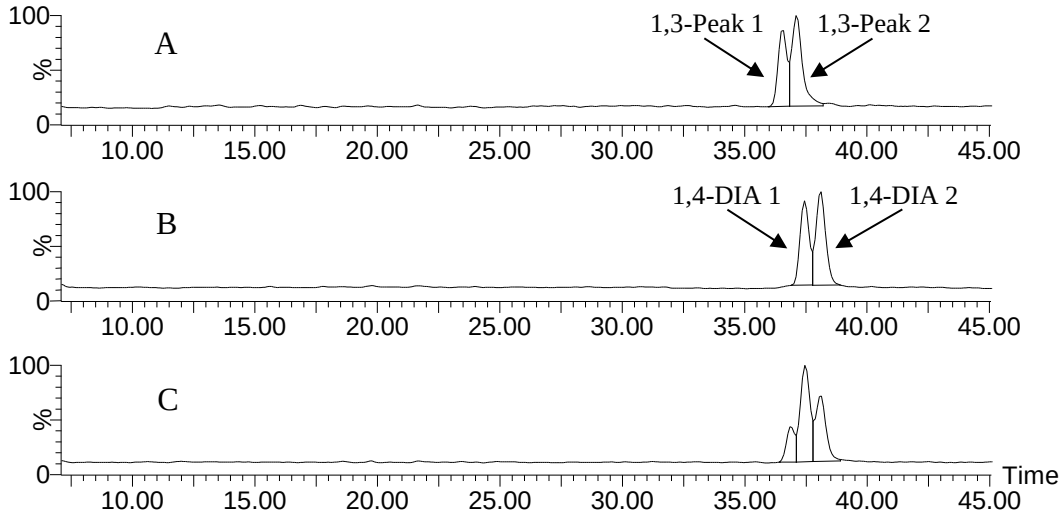

Figure 17. 1,3-DMAA-MTPA and 1,4-DMAA-MTPA derivatives separation. Each DMAA species has a concentration of 15 µg L^{-1}. Chromatogram A: 1,3-DMAA-MTPA derivative. Chromatogram B: 1,4-DMAA-MTPA derivative. Chromatogram C: Combined 1,3-DMAA- and 1,4-DMAA-MTPA derivatives.

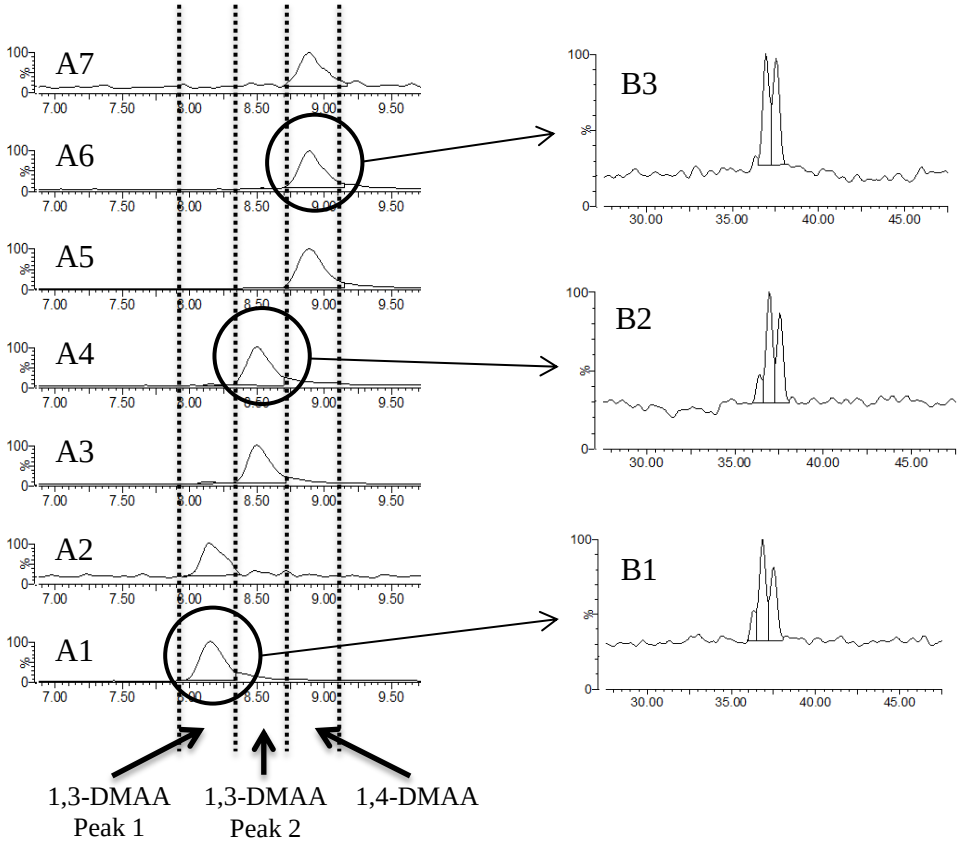

Figure 18. A1-A7: Chromatograms for each fraction collected on the prep HPLC. B1: Derivatized 1,3-DMAA Peak 1 fraction. B2: Derivatized 1,3-DMAA Peak 2 fraction. B3: Derivatized 1,4-DMAA fraction.

Stability studies of 1,3-DMAA-MTPA and 1,4-DMAA-MTPA derivatives. The derivatization of 1,3-DMAA and 1,4-DMAA with (-)-MTPAwas also carried out at room temperature and with a reaction time of 30 minutes. Stability studies for 1,3-DMAA-MTPA and 1,4-DMAA-MTPA (each with a concentration of 25 µg L^{-1}) derivatives were conducted for twenty hours. In the same manner as the DMAA-FLEC stability studies, the peak area of the DMAA-MTPA derivatives is plotted as a function of the elapsed time to ascertain their stability. As mentioned previously, the derivatization of 1,3-DMAA with (-)-MTPA only produces two peaks for all four diastereomers. Therefore,

two peaks (1,3-Peak 1 and 1,3-Peak 2) are plotted in the stability study graph. Both 1,4-DMAA diastereomers are plotted in the 1,4-DMAA-MTPA stability study graph (Figure 19). Both1,3-DMAA-MTPA and 1,4-DMAA-MTPA derivative products were stable for the entire length of the study (20 hours).

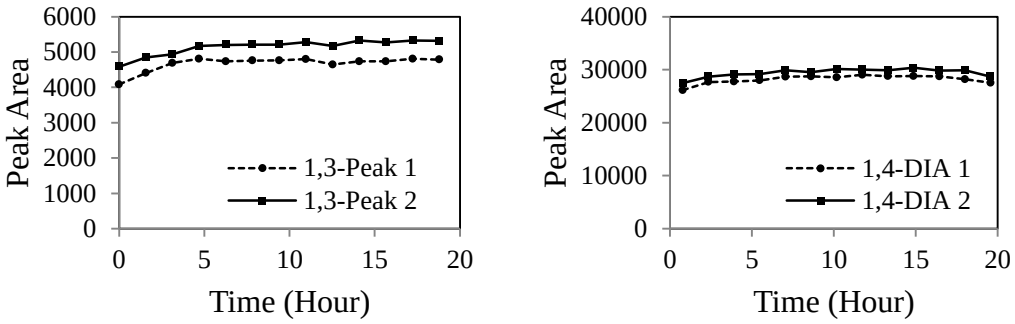

Figure 19. 1,3-DMAA-MTPA and 1,4-DMAA-MTPA derivative stability study. Left plot: 15 µg L^{-1} 1,3-DMAA-MTPA derivative. Right plot: 15 µg L^{-1} 1,4-DMAA-MTPA derivative.

DMAA-MTPA stereoisomer analysis. The stereoisomer ratios were calculated for the DMAA standard and for a new (harvest date of May 2013) Changzhou, China geranium sample using the DMAA-MTPA method. As mentioned previously, the standard (or plant extract) is first separated with the prep HPLC and the fractions are collected. Three fractions, corresponding to the two diastereomer pairs for 1,3-DMAA and one for the 1,4-DMAA enantiomers, are then derivatized with the (-)-MTPA. The derivatized products are analyzed with the HPLC-MS/MS. In this manner, each fraction should produce two diastereomer peaks in the fraction chromatogram (four peaks total for 1,3-DMAA and two peaks total for 1,4-DMAA). The chromatograms for the derivatized standard and Changzhou plant extract fractions are presented in Figure 20. The 1,4-

DMAA-MTPA chromatogram has two peaks corresponding to the two 1,4-DMAA diastereomers. The diastereomer ratios were calculated by dividing each peak area by the sum of both diastereomer peak areas. The ratios for the 1,4-DMAA enantiomers in the standard were 52 ± 1 (1,4-DIA 1) and 48 ± 1 (1,4-DIA 2). The ratios for the Changzhou sample 1,4-DMAA stereoisomers were 51 ± 1 and 49 ± 1 for 1,4-DIA 1 and 1,4-DIA 2, respectively (Table 9). The synthetic standard shows a slight enrichment of 1,4-DMAA enantiomer 1 over enantiomer 2. This trend is also apparent for the Changzhou sample with the possibility of a racemic mixture within the margin of error.

Upon inspection it was found that both of the derivatized 1,3-DMAA fraction chromatograms have three peaks instead of two. These chromatograms resemble the chromatogram of 1,3-DMAA-MTPA and 1,4-DMAA-MTPA combined (Figure 17-C) and it was concluded that some of the 1,4-DMAA fraction must be co-eluting with the 1,3-DMAA fractions. For example, in chromatogram Figure 20-A1, which is the derivatized fraction of 1,3-DMAA Peak 1(Figure 18-A1), it was found that both 1,3-DIA1and 1,3-DIA 2 diastereomers are present as well as contributions from 1,4-DIA 1 and 1,4-DIA 2. Due to this co-elution 1,3-DIA 2 has contributions from 1,4-DIA 1 and must be estimated through a series of calculations:

- First the 1,4-DMAA stereoisomer ratios were calculated using the two peak areas of the 1,4-DMAA-MTPA fraction chromatogram (Figure 20-A3).
- This ratio was then used to calculate the peak area of 1,4-DIA 1 in the peak with contributions from both 1,3-DIA 2 and 1,4-DIA 1 (Figure 21).
- The peak area for 1,3-DIA 2 was then calculated by subtracting the calculated peak area for 1,4-DIA 1 from the total peak area (1,3-DIA 2 + 1,4-DIA 1).

- The stereoisomer ratios are determined by dividing the peak area of each stereoisomer by the sum of the total peak area for all diastereomers.
- The error is then propagated for the subtraction (Equation 5) and division (Equation 6) operations used to calculate stereoisomer ratios.

$$e_y = \sqrt{e_{x1}^2 + e_{x2}^2} \quad \text{(Equation 5)}$$

$$\%e_y = \sqrt{\%e_{x1}^2 + \%e_{x2}^2} \quad \text{(Equation 6)}$$

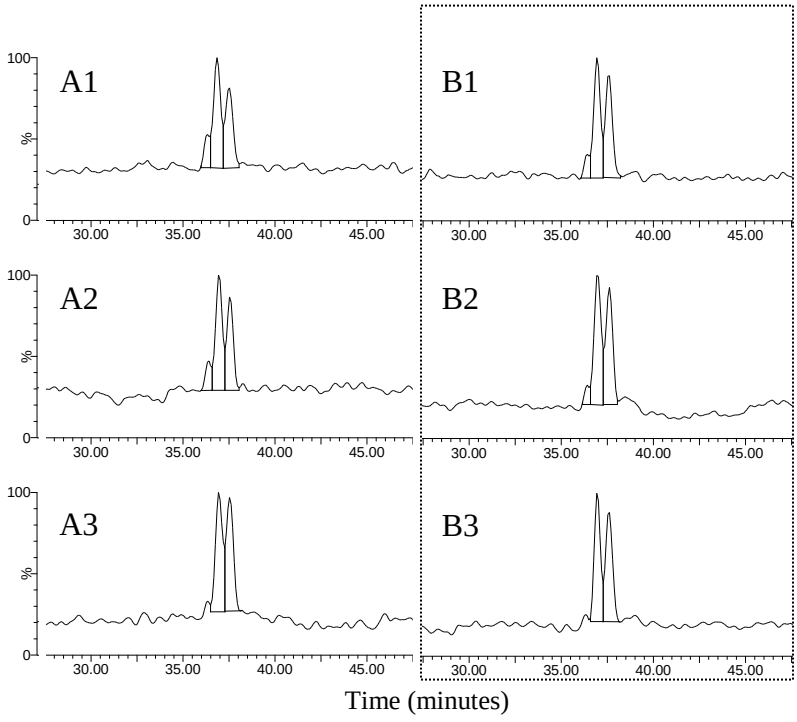

Figure 20. Derivatized fraction chromatograms for a DMAA standard (A1-A3) and the Changzhou sample (B1-B3). A1 & B1: 1,3-DMAA Peak 1 fraction. A2 & B2: 1,3-DMAA Peak 2 fraction. A3 & B3: 1,4-DMAA fraction.

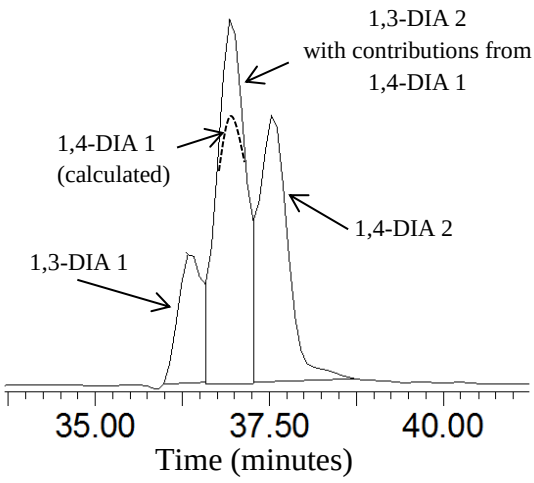

Figure 21. Close-up of derivatized 1,3-DMAA fraction chromatogram to illustrate co-elution of 1,4-DIA 1 with 1,3-DIA 2. The 1,4-DIA 1 peak area contribution is calculated in order to estimate the peak area of 1,3-DIA 2.

Stereoisomer ratios with propagation of error for a DMAA standard and the new Changzhou plant extract are presented in Table 9. The ratios for the 1,3-DMAA diastereomers in the synthetic standard were 33 ± 4, 20 ± 8, 26 ± 3, and 21 ± 8%. Although the error is high for the 1,3-DMAA-MTPA analysis, the ratio of DIA1+DIA2: DIA3+DIA4 is 53:47 (1.13) and matches both the DMAA-FLEC analysis of the standard and the previously reported (Chapter 2) diastereomer analysis of the 1,3-DMAA standard. The ratios for the Changzhou sample 1,3-DMAA diastereomers were 19 ± 3: 16 ± 3: 25 ± 4: 40 ± 14. This gives a ratio of 35:65 (0.54) for DIA1+DIA2: DIA3+DIA4. This ratio is not consistent with any of the diastereomer ratios reported for various Changzhou plant samples in Chapter 2 (1.10 ± 0.01, 1.02, 1.25 ± 0.03, and 1.16 ± 0.10). It is important to note that the error of the previously reported diastereomer ratios (calculated as % RSD) ranges from 0.9 to 8.6 % and ranges from 12 to 40 % for each 1,3-DMAA stereoisomer in the DMAA-MTPA analysis. The discrepancy between the two

reports of 1,3-DMAA diastereomer ratios is likely due to the increase in error present in the DMAA-MTPA analysis. This increased error is attributed to the presence of the 1,4-DMAA species in the preparatory HPLC fractions of 1,3-DMAA.

Table 9
1,3-DMAA-MTPA and 1,4-DMAA-MTPA stereoisomer ratio measurements for a DMAA standard and the Changzhou plant extract.

	1,3-DMAA-MTPA			1,4-DMAA-MTPA	
	Standard	Changzhou		Standard	Changzhou
1,3-DIA 1	33 ± 4	19 ± 3	1,4-DIA 1	52 ± 1	51 ± 1
1,3-DIA 2	20 ± 8	16 ± 3	1,4-DIA 2	48 ± 1	49 ± 1
1,3-DIA 3	26 ± 3	25 ± 4			
1,3-DIA 4	21 ± 8	40 ± 14			

Conclusions

Two enantiomerically-pure derivatizing reagents were used to derivatize and separate the four diastereomers of 1,3-DMAA and the two enantiomers of 1,4-DMAA. The extraction procedure combined with the FLEC-DMAA derivatization chemistry is able to determine concentrations of the stereoisomers at the 10-20 ng g^{-1} range in geranium plants. The (-)-FLEC analysis was applied to two geranium samples and compared to previously published research (Fleming et al., 2012) as well as a simple pre-concentration method implemented to analyze sub-ng g^{-1} levels of DMAA species. The (-)-FLEC analysis reported similar concentrations compared to the analysis by Fleming et al. (Chapter 2) of 1,3-DMAA and 1,4-DMAA for the Changzhou, China plant extract.

The results of the 1,3-DMAA-FLEC analysis provide the first, independent chemical analysis confirmation of 1,3-DMAA in the Changzhou, China *P. graveolens*

sample. In addition, the first reported concentrations of 1,4-DMAA in an American, *P. zonale* geranium plant were reported here by two, independent chemistries on the same plant extract. Concentrations of 1,3-DMAA were also detected in the American *P. zonale*; but less than the MDL for two of the three analysis methods. The enantiomeric ratio analysis of 1,4-DMAA in the Changzhou *P. graveolens* geranium sample showed a 2 – 4 % enrichment of one enantiomer, while the American *P. zonale* geranium sample had 1 – 2 % enrichment of one 1,4-DMAA enantiomer. An error analysis on both samples indicates the possibility that the enantiomeric ratio of 1,4-DMAA in both samples is racemic.

However, the instability of the DMAA-FLEC derivative is problematic and a more stable chemistry was desired. The DMAA-MTPA derivatization analysis provides another method for the stereoisomer analysis of 1,3-DMAA and 1,4-DMAA with a derivative that is stable for at least 20 hr. The DMAA-MTPA method requires an additional separation step with a preparatory scale HPLC in order to separate all four 1,3-DMAA-MTPA diastereomers. Unfortunately, the high error associated with the 1,3-DMAA-MTPA analysis does not allow for a direct comparison of the standard and plant sample stereoisomer ratios. A comparison was made for the 1,4-DMAA-MTPA analysis and it was found that the standard and the Changzhou sample diastereomers ratios could be the same within the error of the analysis. The DMAA-MTPA derivatization analysis also provides a third, qualitative approach for the confirmation of 1,3-DMAA and 1,4-DMAA in a geranium plant samples. This method was able to confirm the presence of 1,3-DMAA and 1,4-DMAA in a new Changzhou geranium plant sample. The MTPA

chemistry shows promise from a stability perspective, but may require a higher resolution separation such as GC or UPLC to improve the analysis.

Chapter 4

Confirmation of the Presence of Haloacetic Acids in Bulk Sodium Hypochlorite Disinfectant Solutions by HPLC-MS/MS

Introduction

Chlorination is the most common practice for disinfecting drinking water. Chlorine can be applied to drinking water via chlorine gas, calcium hypochlorite as a solid, or aqueous sodium hypochlorite solutions. Currently, about one third of water utilities use chlorine gas for disinfection; however, many utilities are moving toward sodium hypochlorite solutions due to homeland security concerns (Snyder, Stanford, Pisarenko, Gordon, & Asami, 2009). Regardless of how chlorine is applied, equilibrium of hypochlorous acid and hypochlorite ion will be established at the pH of drinking water. The chemical species chlorine (Cl_2), hypochlorous acid (HOCl) and hypochlorite ion (OCl^-) are termed free available chlorine (FAC). The ratio and specific FAC species present are dependent on pH. Figure 22 (adapted from Emmert, 1999) is a plot of FAC species percent abundance as a function of pH, and at the pH of drinking water (6.5 to 8.5) HOCl and OCl^- are the predominant species. (Greenberg, Clesceri, & Eaton, 1992). These free available chlorine species will then react with natural organic matter in the water to form halogenated disinfection by-products (DBPs) (Sadiq & Rodriguez, 2004).

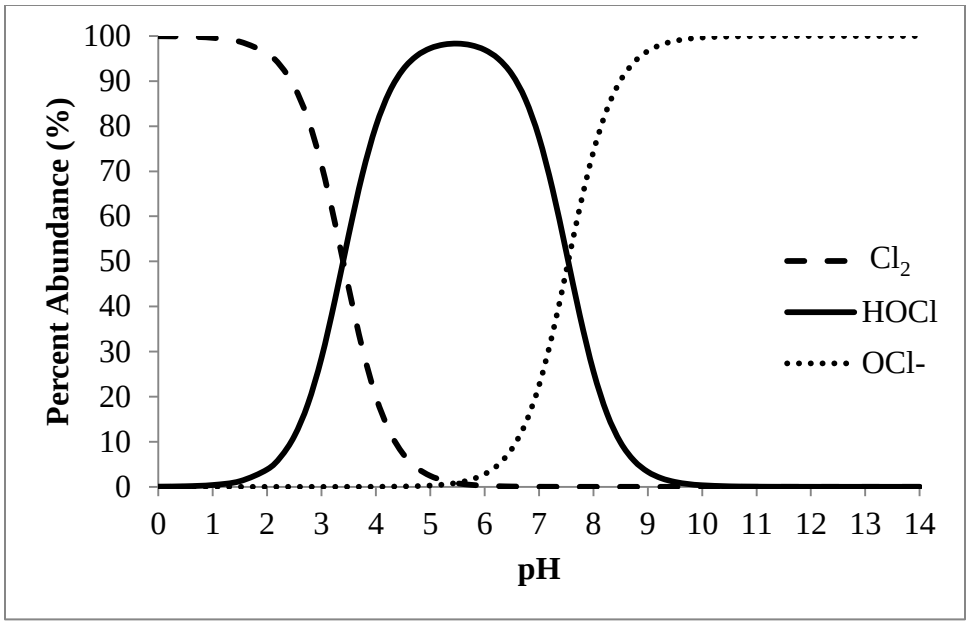

Figure 22. Percent abundance of FAC species vs. pH (adapted from Emmert, 1999).

Due to their carcinogenic properties, many DBPs pose adverse health effects (Richardson, Plewa, Wagner, Schoeny, & DeMarini, 2007). As a result the two major classes of DBPs, trihalomethanes (THMs) and haloacetic acids (HAAs), are regulated by the United States Environmental Protection Agency (USEPA). There are nine HAAs, termed HAA9, that can be found in drinking water but only five (HAA5) are regulated. The five regulated HAAs include: monochloroacetic acid (MCAA), dichloroacetic acid (DCAA), trichloroacetic acid (TCAA), monobromoacetic acid (MBAA), and dibromoacetic acid (DBAA). The four remaining unregulated HAAs include: bromochloroacetic acid (BCAA), bromodichloroacetic acid (BDCAA), dibromochloroacetic acid (DBCAA), and tribromoacetic acid (TBAA). Maximum contaminant levels have been set by the USEPA at 0.080 mg L^{-1} for THMs and 0.060 mg L^{-1} for HAA5 (USEPA, 2006).

It is well known that DBP formation, following water chlorination, largely takes place in the distribution system (Krasner, 2009; Richardson & Ternes, 2011; Richardson & Postigo, 2012). Some DBPs, thought to form primarily in the distribution systems (chlorate, bromate, and perchlorate ion), have been shown to come largely from bulk hypochlorite solutions (Gordon, Adam, & Bubnis, 1995; Gordon et al., 1993; Snyder et al., 2009; Stanford, Pisarenko, Snyder, & Gordon, 2011). Recently, there has been research conducted to determine if THMs and HAAs as well as hexavalent chromium can also be found in these hypochlorite solutions that are used for drinking water disinfection (Emmert et al., 2013).

Emmert and co-workers (2013) analyzed 30 bulk hypochlorite solutions for total chlorine, THMs, HAAs and hexavalent chromium. The bulk hypochlorite solutions were collected from a wide geographic region including Arkansas, Illinois, Missouri, New Jersey, New York, Oklahoma, Tennessee, and Texas. The THMs and hexavalent chromium were not detected at concentrations above the MDL value for their respective methods in any of the hypochlorite solutions. HAAs were detected in all of the hypochlorite solutions using a post-column reaction ion chromatography (PCR-IC) instrument. Following the detection of HAAs in the bulk sodium hypochlorite solutions, quantitative models were developed to predict the contribution of HAAs in the hypochlorite solutions to the concentration of HAAs in the finished drinking water. The three models were: a simple Dose-Dilution model (Emmert et al., 2013; Henson, 2014), a Kinetic model, and a Kinetic-Observed model (Brown, 2014; Emmert et al., 2013). It was determined that Kinetic-Observed provided the most conservative and potentially most realistic estimate of the contribution of HAAs in sodium hypochlorite solutions to HAAs

detected in the finished drinking water. This model determined that the detected concentrations of HAAs found in sodium hypochlorite solutions could contribute up to 30% to the MCL in the finished drinking water through dilution.

The PCR-IC employs two independent chemistries to separate and detect all nine HAAs in drinking water (Emmert, Brown, Simone, Geme, & Cao, 2007; Simone et al., 2009) and diluted hypochlorite solutions (Emmert et al., 2013). The HAAs are first separated in time using anion-exchange chromatography and are then reacted with post-column reagents, nicotinamide and potassium hydroxide, to yield a fluorescent product. The PCR-IC instrument has two primary advantages, minimal sample preparation and relatively inexpensive instrumentation cost, when compared to USEPA 552.3 (USEPA, 2003) which is the standard method for HAAs analysis in drinking water. Although the PCR-IC is selective for the detection of HAAs, an independent method is required to confirm the chemical identity of HAAs in bulk sodium hypochlorite solutions. A method based on liquid chromatography tandem mass spectrometry (HPLC-MS/MS) would be ideal for providing such confirmation.

Several research groups have developed methods for the detection of HAAs in drinking water using HPLC-MS/MS (Chen, Chang, & Wang, 2009; Meng, Wu, Ma, Jia, & Hu, 2010; Prieto-Blanco et al., 2012). Chen et al. (2009) separated ten HAAs using a hydrophilic interaction chromatography (HILIC) ultra-performance liquid chromatography (UPLC) column and a BetaMax Acid column. The HILIC UPLC column gave the lowest limits of detection (0.08—2.73 μg L^{-1}) for this study but required samples to be diluted in 90 % acetonitrile prior to injection resulting in a large dilution of the sample. Prieto-Blanco et al. (2012) separated five of the haloacetic acids with a

polycyclic aromatic hydrocarbon (PAH) phase column and an ion-pairing reagent dibutylamine (DBA). The water samples required a pre-concentration step employing solid-phase extraction (SPE) in order to achieve low limits of detection (0.04—0.3 µg L^{-1}). In addition to drinking water, Prieto-Blanco et al. (2012) also analyzed pool water and river water. Unfortunately, no recovery data was reported for the sample analysis. Quality control procedures, including recovery analysis, are important when monitoring the overall effects of different procedural steps such as SPE. Finally, Meng et al. (2010) separated and detected nine HAAs in drinking water with an UPLC C8 column and a HPLC-MS/MS. The limits of detection for all HAAs ranged from < 1 µg L^{-1} to 9 µg L^{-1} and the analysis time was under ten minutes. Mean percent recoveries ranged from 80-108% for spiked water samples. Each of these methods focused on natural waters or treated drinking water which offer a simpler matrix for analysis.

The focus of this research was to develop a HPLC-MS/MS method capable of detecting HAAs in bulk sodium hypochlorite solutions. The HPLC-MS/MS is operated in multiple reaction monitoring (MRM) mode to achieve the highest signal to noise ratio possible. In MRM mode the HPLC-MS/MS employs two stages of mass filtering to detect the target analyte, in this case the individual HAA9 species. This mode allows for a highly selective and sensitive measurement. Detailed MDL, accuracy, precision, and linearity studies are reported. The HPLC-MS/MS method was compared to USEPA 552.3 (USEPA, 2003) and PCR-IC methods in drinking water samples. This comparison study was done to establish agreement of the HPLC-MS/MS method prior to hypochlorite solution analysis. The HPLC-MS/MS method was then used to analyze seven individual bulk hypochlorite solutions in a side-by-side comparison with the PCR-IC. Multiple

matrix effects and mitigation strategies are presented for the analysis of HAAs in hypochlorite solutions and followed by an additional round of analysis. Mitigation strategies include chemical quenching and solid phase extraction (SPE).

Experimental

Chemicals and reagents. All chemicals and reagents had a purity of 97% or greater. All standards and eluents were prepared in reagent-grade water with a resistivity of 18.2 MΩ·cm produced by a Barnsted e-pure four cartridge system. Glassware was cleaned with a dilute nitric acid solution and rinsed with reagent-grade water three times. MCAA, MBAA, DCAA, BCAA, DBAA, TCAA, BDCAA, DBCAA, TBAA, and nicotinamide were obtained from Sigma Aldrich (St. Louis, MO, USA). Potassium hydroxide (KOH), sulfuric acid (H_2SO_4),sodium bicarbonate ($NaHCO_3$), 1,2,3-trichloropropane, 2-bromobutanoic acid (2-BBA), potassium iodide (KI), potassium iodate (KIO_3), sodium thiosulfate pentahydrate ($Na_2S_2O_3 \cdot 5\ H_2O$), methyl tert-butyl ether (MTBE), glacial acetic acid (CH_3COOH), di-n-butylamine (DBA), and Optima LC/MS acetonitrile (CH_3CN) were obtained from Fisher Scientific (Waltham, MA, USA).

A 1000 mg L^{-1} HAA9 stock standard was prepared by adding 25.0 mg of each HAA to a 25.0 mL volumetric flask and diluting with MTBE. The stock was diluted accordingly with reagent water to prepare calibration standards. The 5 mM DBA (adjusted to pH 5.2 with acetic acid) was prepared by adding 840 µL DBA to ~950 mL reagent water, adding acetic acid drop wise to adjust pH to 5.2, and then diluting to 1000 mL.

Solutions for the iodometric titrations were made as follows: The 0.2 M sulfuric acid solution was prepared by diluting 11.1 mL concentrated sulfuric acid into 1000 mL

reagent water. The 0.1 M sodium thiosulfate titrant was prepared by adding ~25.0 g $Na_2S_2O_3 \cdot 5\ H_2O$ to 1.0 L freshly boiled reagent water. The 0.017 M potassium iodate standard was made by adding 6.567 g of KIO_3 to 1.0 L of reagent water.

Instrumentation. Drinking water samples were analyzed with the USEPA 552.3, PCR-IC, and HPLC-MS/MS methods. Prior to sodium hypochlorite solution analysis for HAAs, the FAC concentration (mg L^{-1} Cl_2) was determined for each sodium hypochlorite solution by iodometric titration. The sodium hypochlorite solutions were then diluted appropriately to achieve a final concentration of 50 mg L^{-1} for the PCR-IC analysis and 100 mg L^{-1} for the HPLC-MS/MS analysis.

***Iodometric determination of hypochlorite by automated titration*.** The FAC concentration was determined for each sodium hypochlorite solution by an iodometric titration. This iodometric titration was performed with a VIT90 Video Titrator (Radiometer; Copenhagen, Denmark) that uses potenitometry to identify the endpoint. The titrator is equipped with a M231 Pt-9 Platinum electrode and alomel REF-421 electrode pair (Hach).

In this titration, the hypochlorite solution is first acidified with a 5 mL 0.2 M sulfuric acid solution. Next, an excess of iodide ion (I^-) is added and oxidized to the triiodide ion (I_3^-, Eq. 7). This solution is then titrated with a ~0.1 M sodium thiosulfate solution. The triiodide ion oxidizes thiosulfate ion ($S_2O_3^{2-}$) to tetrathionate ion ($S_4O_6^{2-}$, Eq. 8). The end-point, measured by potentiometry, is the volume of titrant needed to reach the inflection point of the titration curve. This process is automated by the VIT90 titrator. Equations 7 and 8 illustrate the net chemical reactions of this titration (Harris, 2007):

$$2HOCl + 3I^- + 2H^+ \rightarrow I_3^- + 2Cl^- + 2H_2O \quad \text{(Equation 7)}$$

$$I_3^- + 2S_2O_3^{2-} \rightarrow S_4O_6^{2-} + 3I^- \quad \text{(Equation 8)}$$

HAAs analysis by USEPA 552.3. Drinking water samples were analyzed with USEPA Method 552.3 (USEPA, 2003). In this method HAAs are determined using liquid-liquid micro-extraction, derivatization, and gas chromatography with electron capture detection (GC-ECD). A 40 mL drinking water sample is spiked with 20 µL of 20 µg mL^{-1} 2-bromobutanoic acid as a surrogate standard and then adjusted to a pH of 0.5 or less with concentrated sulfuric acid. The sample is then extracted with 4 mL of MTBE containing 1000 µg L^{-1} 1,2,3-trichloropropane, the internal standard. The HAAs in MTBE (~3 mL) are transferred to a conical derivatization vial. Then 3 mL of acidic methanol is added, followed by heating to 50 °C for 2 hr to convert the HAAs to their corresponding methyl esters. The acidic methanol and MTBE phase containing the methylated HAAs are then separated by the addition of a concentrated sodium sulfate solution. The lower aqueous/ methanol phase is discarded and the remaining phase is neutralized with 1 mL of a saturated solution of sodium bicarbonate. A 1 mL aliquot of the MTBE phase is then transferred to an auto-sampler vial and analyzed by GC-ECD.

The GC-ECD was a Varian CP-3800 equipped with a Varian CP-8400 auto-sampler. The separation was performed on a Zebron ZB-1MS capillary GC column (Phenomenex; Torrance, CA, USA) with a 2.0 µL injection volume. The temperature program for the column oven was held at 40 °C for 10 min, increased to 65 °C at 2.5 °C min^{-1}, increased to 75 °C at 10 °C min^{-1}, held at 75 °C for 2 min, increased to 205 °C at 20 °C min^{-1}, increased to 210 °C at 40 °C min^{-1}, and finally held at 210 °C for 7 min. The

total chromatographic run time was 36.63 min. Data integration was performed with Varian Star Workstation software (Version 6.41).

HAAs analysis by PCR-IC. Drinking water samples and sodium hypochlorite solutions were analyzed with the PCR-IC for the detection of HAAs. The PCR-IC measures HAA concentrations using anion-exchange chromatography followed by a selective post-column reaction that produces a fluorescent product (Emmert et al., 2007, Simone et al., 2009). The separation was performed on a Dionex AG-18 (50 x 4.6 mm) and AS-18 (250 x 4.6 mm) guard and analytical column, respectively. A gradient pump mixes and delivers two eluents, 200 mM KOH and reagent water, to the column. The sample, delivered via peristaltic pump, is then injected onto the anion-exchange column by a 6-port high-pressure valve. After separation, the HAAs in the column effluent are flowed together with two post column reagents, 3.07 M nicotinamide and 2.0 M KOH, delivered via peristaltic pump. The effluent and post column reagents are mixed using 40 m of KOT (knitted open tubular) reaction coils, heated to 98 °C using a water bath (Fisher Scientific; Pittsburgh, PA, USA). This reaction mixture is then cooled to ~4 °C in a 1 m KOT using a peltier device to enhance fluorescence. The fluorescent product is then detected using a Shimadzu RF-551 (Kyoto, Japan) with the excitation wavelength set at 365 nm and emission wavelength set at 455 nm.

Optimized HPLC-MS/MS method for analysis of HAA9 species. Drinking water samples and sodium hypochlorite solutions were analyzed with the HPLC-MS/MS method for the detection of HAAs. The HPLC-MS/MS instrument consists of an Agilent 1100 HPLC coupled (with a split flow) to a Waters Quattro Ultima MS (Figure 23). The Agilent 1100 HPLC was equipped with a degasser, binary pump with solvent cabinet, autosampler, and a thermostatted column compartment. The MS was operated in negative mode. HAA separation was performed with a Phenomenex Kinetex C18 phase column using di-butylamine (DBA) as an ion-pairing reagent in binary gradient mode. Eluent A was reagent water containing 5 mM DBA adjusted to a pH of 5.2 and eluent B was acetonitrile. The column compartment temperature was set at 30 °C and the gradient pump was set at a flow rate of 0.350 mL min^{-1}. The gradient elution program was as follows: initial conditions were 95% Eluent A and 5% Eluent B. Eluent B was increased to 40 % from zero to 5 min. From 5-7 min Eluent B was increased to 100 % and held for 1 min. From 8-9 min Eluent B was decreased back to 5 % (initial conditions) and held for 5 min. Total chromatographic analysis time was 15 min.

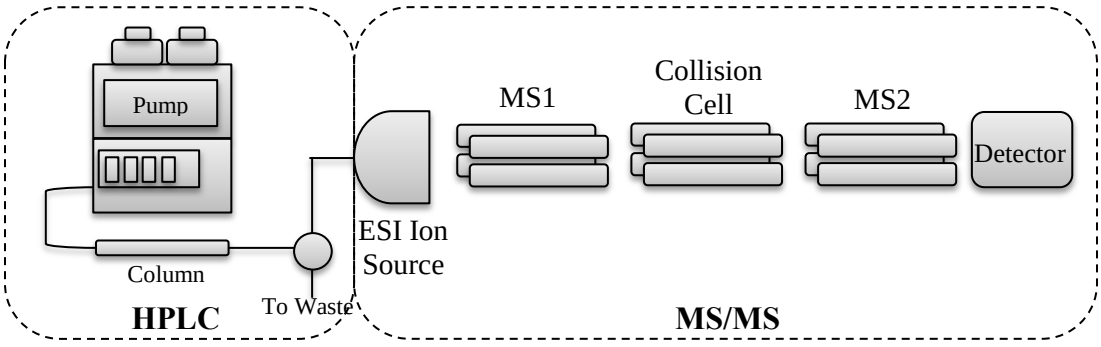

Figure 23. Schematic of HPLC-MS/MS.

The mass spectrometer operating conditions were as follows: the capillary voltage was 2.2 kV, the source temperature was 120 °C with a flow of 86 L hr^{-1} and the desolvation temperature was 350 °C with a flow of 635 L hr^{-1}. Mass transitions, dwell times, cone voltages, and collision energies for each individual HAA species were individually tuned and are presented in Table 10. Cone voltages and collision energies are optimized for individual compounds and vary due to differences in energy required to induce ionization and fragmentation. Figure 24 shows typical multiple reaction monitoring (MRM) HPLC-MS/MS chromatograms for individual HAA species at a concentration of 20 µg L^{-1}.

Table 10
Names, abbreviations, measured ions (m/z), and optimized cone voltage for each precursor ion and collision energies used in selected MRM transitions for each haloacetic acid.

HAAs	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
(Mono)chloroacetic acid (MCAA)	93	35.4	17	10
(Mono)bromoacetic acid (MBAA)	137	79.3	17	10
Bromochloroacetic acid (BCAA)	173	129.2	17	9
Dichloroacetic acid (DCAA)	127	83.2	17	10
Dibromoacetic acid (DBAA)	217	173	17	10
Trichloroacetic acid (TCAA)	161	117.2	15	9
Bromodichloroacetic acid (BDCAA)	163	81.3	17	10
Dibromochloroacetic acid (DBCAA)	207	79.3	15	15
Tribromoacetic acid (TBAA)	251	79.3	28	14

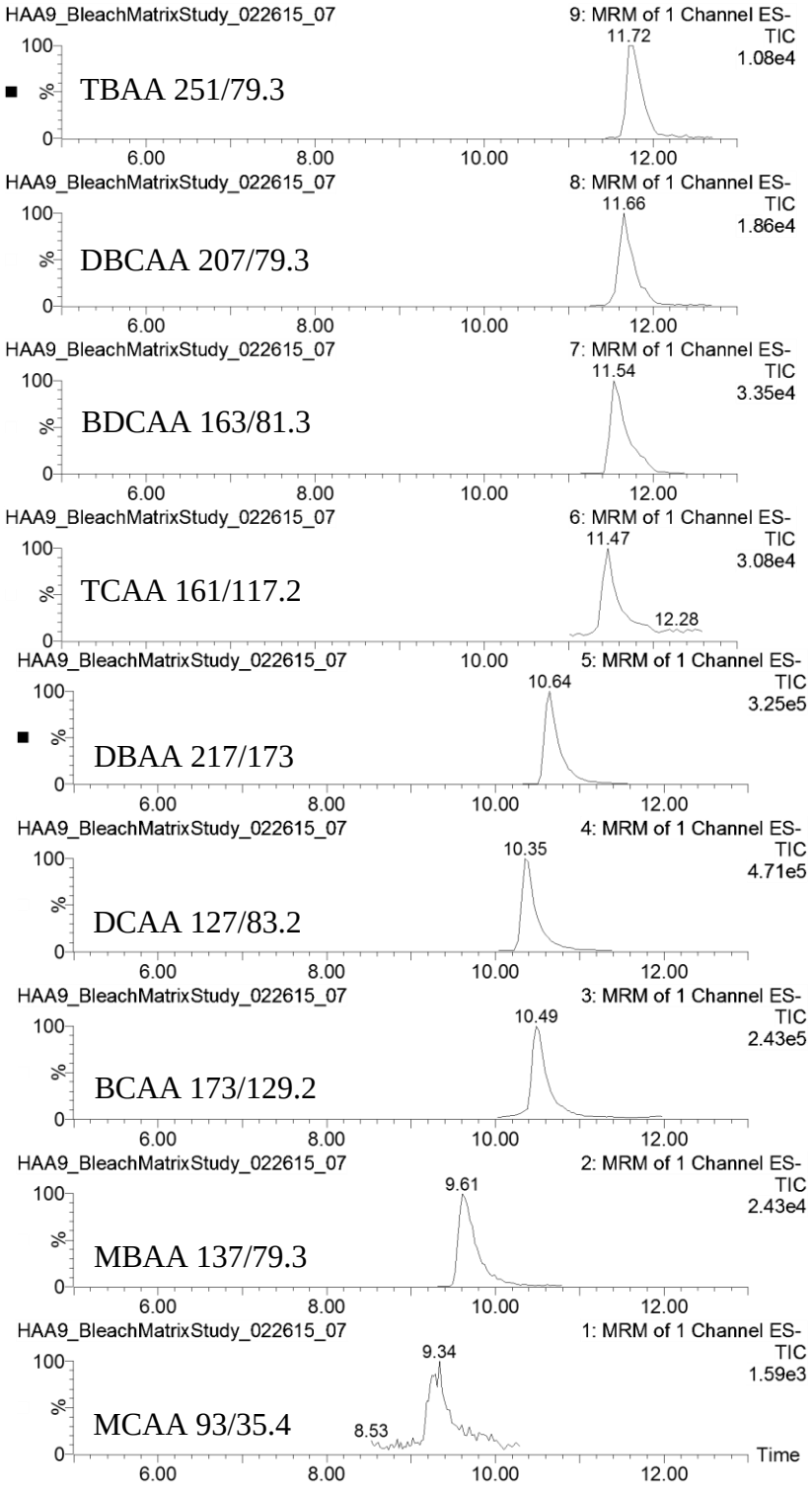

Figure 24. HPLC-MS/MS MRM chromatograms of nine HAAs in a 20 µg L^{-1} standard solution.

It is important to mention that the HPLC-MS/MS analysis suffers from the presence of "artifact peaks" in the chromatograms for MRM #3 and #6, corresponding to HAAs BCAA and TCAA. These artifact peaks are present in all blank (reagent water) runs, including blanks ran prior to any standard or sample runs. Additionally, artifact peaks were detected in MRM #3 (BCAA) and MRM #6 (TCAA) in all chromatograms for blanks of Optima LC-MS/MS grade water and Optima LC-MS/MS grade acetonitrile. This information indicates that the artifact peaks are not a result of carryover. Nevertheless, two blank runs are collected between each sample analysis. Also, integration of artifact peaks does not give rise to concentrations higher than the MDLs for those HAAs.

Optimized sample preparation and solid-phase extraction method for diluted hypochlorite solutions. The optimized sample preparation method employs a solid-phase extraction procedure; adapted from Henson, Emmert, and Simone (2014) and Barron and Paull (2004). The extraction was performed off-line with a Supelco Visiprep™ (Sigma Aldrich; St. Louis, MO, USA) SPE manifold and LiChrolut EN cartridges (Merck-Millipore; Darmstadt, Germany). This cartridge phase has been found to be suitable for HAA extraction and pre-concentration in previous work (Barron & Paull, 2004; Loos & Barcelo, 2001; Martinez, Borrull, & Calull, 1998; Sarzanini, Bruzzoniti, & Mentasti, 1999). Percent recoveries ranged from 26 to 91 % for MCAA, from 42 to 80 % for MBAA, from 45 to 104 % for DCAA, from 48 to 85 % for DBAA, from 62 to 101 % for TCAA, and from 33 to 78 % for TBAA for all four studies. HAAs BCAA, BDCAA, and CDBAA were not analyzed in these studies. Variations in method procedures, such as pH

values of solutions prior to SPE, are thought to be responsible for the disparity in percent recovery data reported between the studies (Barron & Paull, 2004).

The SPE method procedure is as follows: The diluted hypochlorite solutions were first adjusted to a pH ~1 with concentrated sulfuric acid. The sorbents were activated and conditioned with 5 mL of methanol at a flow rate of 2 mL min^{-1}. The sorbents were not allowed to dry, and subsequently 20 mL of diluted, acidified, hypochlorite solutions were passed through the cartridge at a rate of 2 mL/min. The cartridge was then washed with 2 mL reagent water. Finally, the HAAs were eluted with 10 mL of 10 mM NaOH resulting in a theoretical pre-concentration factor of 2.

Results and Discussion

HPLC-MS/MS linearity, MDL, accuracy and precision studies. Prior to any sample analysis, method detection limit, accuracy, precision, and linearity studies were conducted to establish instrument performance. The HPLC-MS/MS was calibrated with a set of low range (1—20 µg L^{-1}) and high range (5—60 µg L^{-1}) standards. Two separate check standards of 3 and 7 µg L^{-1} were analyzed seven times each. The accuracy of the analysis is estimated by mean percent recovery of the check standard analysis. The precision is estimated as the percent relative standard deviation.

The detection limit was estimated using three independent methods: the USEPA MDL (Glaser et al., 1981; USEPA, 1984), traditional MDL (Skoog et al., 2007), and uncertainty MDL (Harris, 2007). The USPEA MDL is estimated by multiplying the standard deviation of the experimental concentrations of the check standards with the t-value (for seven check standards) at the 98% confidence level (USEPA, 1996). The traditional MDL is calculated as three times the standard deviation of the peak-to-peak

noise divided by the slope of the linear regression line (Skoog et al., 2007). The uncertainty MDL is calculated using standard deviations of the slope, y-intercept, and signal of the linear regression. These standard deviations are used to propagate and determine the error of the concentration calculated using the linear regression line (Harris, 2007).

A separate linearity study, using calibration standards ranging from 1 to 150 µg L^{-1} of each HAA, was conducted to determine the dynamic range for each HAA species. The dynamic range is the range between the limit of quantitation (Skoog et al., 2007) and the limit of linearity (Skoog et al., 2007).

The results of a representative MDL, accuracy, precision, and linearity study are presented in Table 11. A typical external calibration plot of analyte peak area vs. concentration for the HPLC-MS/MS method is also presented (Figure 25). The USEPA MDLs for all HAAs were below 1.0 µg L^{-1} with the exception of MCAA at 2.37 µg L^{-1}, TCAA at 1.41 µg L^{-1} and TBAA at 1.19 µg L^{-1}. The traditional MDLs were also below 1.0 µg L^{-1} with the exception of MCAA at 2.28 µg L^{-1} and TCAA at 1.20 µg L^{-1}. The uncertainty MDLs ranged from 0.32 µg L^{-1} for MBAA to 1.95 µg L^{-1} for TCAA. All three estimates for the MDL for each HAA9 species are within an order of magnitude of each other which is ideal (Henson et al., 2014; Ranaivo et al., 2011; Simone et al., 2009).

Table 11

Analytical figures of merit of the individual HAA9 species for the HPLC-MS/MS method. Check standard concentrations were 3 µg L^{-1} for all HAAs with the exception of MCAA with a check standard concentration of 7 µg L^{-1}.

Analyte	Dynamic range (µg L^{-1})	MDL (µg L^{-1})			Mean % Rec. (%)	% RSD (%)
		USEPA	Trad.	Unc.		
MCAA	8-150	2.37	2.28	1.08	103	10.3
MBAA	1-150	0.36	0.12	0.32	112	3.3
BCAA	2−20	0.71	0.14	0.47	91	8.0
DCAA	1−50	0.21	0.07	0.36	107	2.1
DBAA	1−50	0.17	0.02	0.09	107	1.7
TCAA	5−20	1.41	1.20	1.95	57	25.1
BDCAA	2−20	0.51	0.06	0.83	98	5.7
DBCAA	3−50	0.80	0.29	0.40	93	9.0
TBAA	4−100	1.19	0.14	1.28	64	19.1

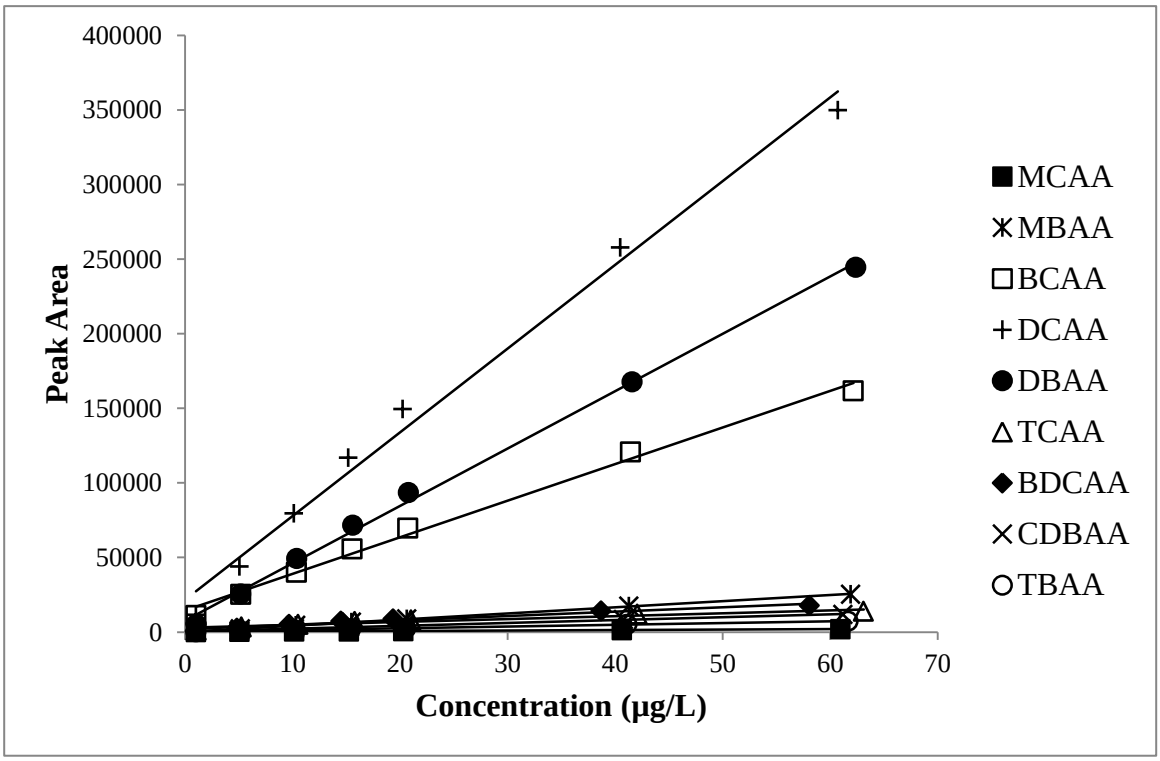

Figure 25. Typical calibration plot for HPLC-MS/MS analysis of HAAs.

Real world drinking water sample analysis. Real world drinking water samples were collected from two different regions and analyzed for HAA9. The two drinking water samples, labelled TN-1 and AR-1, were analyzed with HPLC-MS/MS, PCR-IC and USEPA 552.3. Typical MDL, accuracy, and precision studies for PCR-IC and USEPA 552.3 are presented in Table 12 and 13, respectively. The USEPA MDL values for the PCR-IC were less than 8 μg L^{-1} with the exception of TBAA and MBAA which were 10 and 19 μg L^{-1}, respectively. The PCR-IC mean % recovery values were 99 ± 7 % and % RSD values were less than 9% for all HAAs except MBAA at 19 %. For USEPA Method 552.3 the USEPA defined MDL values were less than 1 μg L^{-1}, ranging from 0.05 μg L^{-1} for BCAA and TCAA to 0.74 μg L^{-1} for MCAA. The mean % recovery values were 122 ± 20 % with the exception of DBCAA which was 235%. The % RSD values for USEPA Method 552.3 were all below 3%.

Table 12
PCR-IC MDL, accuracy, and precision. [a]The check standard concentration for all HAA9 species is 37.5 μg L^{-1}.

Analyte	USEPA MDL[a] (μg L^{-1})	Mean % Rec. (%)	% RSD (%)	r^2
MCAA	2.9	103	2.4	0.999
MBAA	19.2	81	19.4	0.946
BCAA	2.9	99	2.4	0.999
DCAA	7.5	105	6.0	0.994
DBAA	3.9	101	3.1	0.999
TCAA	6.2	100	5.0	0.994
BDCAA	2.6	100	2.2	0.999
DBCAA	4.3	103	3.5	1.000
TBAA	10.4	98	8.8	0.987

Table 13

USEPA Method 552.3 MDL, accuracy, and precision. [a] *The check standard concentration for all HAA9 species is 1.5 µg L^{-1} with the exception of MCAA which has a check standard concentration of 7.0 µg L^{-1}.*

HAA Species	USEPA MDL[a] (µg L^{-1})	Mean % Rec. (%)	% RSD (%)	r^2
MCAA	0.74	128	2.6	0.998
MBAA	0.16	125	2.7	0.996
BCAA	0.05	110	1.0	0.989
DCAA	0.10	139	1.6	0.996
DBAA	0.06	102	1.1	0.976
TCAA	0.05	98	1.1	0.985
BDCAA	0.06	114	1.1	0.992
DBCAA	0.06	235	0.6	0.990
TBAA	0.14	158	1.8	0.984

The drinking water samples were analyzed by all methods on the same day to prevent any bias due to continuing formation of HAAs in the sample bottles (Emmert et al., 2007; Emmert, Geme, Brown, & Simone, 2009; Hong et al., 2008; Simone et al., 2009). Percent recoveries for spiked water samples were used to evaluate the accuracy of each method. Then, concentrations were determined for a comparison of methods. Table 14 shows the concentrations of each HAA and percent recoveries of a spiked sample for the first drinking water sample, TN-1. Concentrations preceded by the less than symbol represent concentrations below the MDL for that particular analysis. USEPA method 552.3 reported concentrations ranging between 0.4 µg L^{-1} for TCAA up to 4.2 µg L^{-1} for DBAA with MCAA, DBCAA, with TBAA being less than the MDL. The HPLC-MS/MS reported concentrations that ranged between 0.7 µg L^{-1} for DCAA up to 2.9 µg L^{-1} for DBAA. MCAA, TCAA, BDCAA, DBCAA, and TBAA were all less than the MDL. The two methods agreed within 5 µg L^{-1} of each other for all HAAs. The PCR-IC method reported all HAAs concentrations as less than the MDL which was corroborated by

USEPA 552.3 and the HPLC-MS/MS analysis. This occurs here because the PCR-IC method MDLs are all higher than any of the HAA concentrations detected by the USEPA 552.3 and HPLC-MS/MS method. A 50 μg L^{-1} spike analysis was performed to gauge the effect of any potential matrix effects. The percent recoveries ranged from 98% to 132% for all HAAs for the USEPA 552.3 method, 81 to 127% for the HPLC-MS/MS method, and 53 to 114% for the PCR-IC method.

Table 14
Drinking water analysis of TN-1 with USEPA Method 552.3, HPLC-MS/MS (LC-MS) and PCR-IC. Percent recoveries calculated for a ~50 μg L^{-1} spiked sample.

	TN-1*					
	Sample (ug L^{-1})			Percent Recovery (%)		
HAA	EPA 552.3	LC-MS	PCR-IC	EPA 552.3	LC-MS	PCR-IC
MCAA	< 0.0	< 2.4	< 3.0	129	127	100
MBAA	0.9	0.8	< 19.2	116	115	53
BCAA	1.2	2.3	< 2.9	106	110	103
DCAA	1.3	0.7	< 7.5	114	96	114
DBAA	4.2	2.9	< 3.9	98	110	111
TCAA	0.4	< 1.4	< 6.2	104	81	104
BDCAA	1.0	< 0.5	< 2.6	106	101	97
CDBAA	< 0.1	< 0.8	< 4.3	115	100	102
TBAA	< 0.1	< 1.2	< 10.4	132	95	92

A second drinking water sample, labelled AR-1, was also analyzed with all three methods. The AR-1 sample is a more complex matrix with concentrations of HAAs expected to be higher than TN-1. The concentrations of each HAA and percent recoveries of a spiked sample for the second drinking water sample, AR-1, are shown in Table 15. USEPA method 552.3 reported concentrations ranging between 1.0 μg L^{-1} for MBAA up to 13.4 μg L^{-1} for DCAA with MCAA, DBAA, CDBAA, and TBAA as less than the

MDL. The HPLC-MS/MS reported concentrations ranging between 0.5 µg L^{-1} for BDCAA up to 8.4 µg L^{-1} for DCAA with MCAA,MBAA, DBAA, DBCAA, and TBAA as less than the MDL. The two methods agree within 5 µg L^{-1} of each other for all HAAs except BCAA which had a bias of -6.6 µg L^{-1}. The PCR-IC reported concentrations ranging between 3.5 µg L^{-1} for MCAA up to 32.1 µg L^{-1} for DCAA with MBAA, DBAA, BDCAA, CDBAA, and TBAA as less than the MDL. The DCAA concentration detected by the PCR-IC was about a factor of three times higher than the concentrations reported by the HPLC-MS/MS and USEPA 552.3. Previous work has shown that the PCR-IC reports DCAA concentrations, on average, 2.2 ± 0.9 times higher than that of USEPA 552.3 (Henson, Emmert, & Simone, 2014) and could be the case here as well. Percent recoveries (for a 50 µg L^{-1} spiked sample analysis) ranged from 103 to 140% for USEPA 552.3, 65 to 100 % for the HPLC-MS/MS, and 80 to 106 % for the PCR-IC. Overall, the three methods were found to be in agreement with the analysis of the two drinking water samples.

Table 15
Drinking water analysis of AR-1 with USEPA Method 552.3, HPLC-MS/MS and PCR-IC. Percent recoveries calculated for a ~40 µg L^{-1} spiked sample.

AR-1*						
	Sample (ug L^{-1})			Percent Recovery (%)		
HAA	EPA 552.3	LC-MS	PCR-IC	EPA 552.3	LC-MS	PCR-IC
MCAA	< 1.1	< 2.2	3.5	103	93	103
MBAA	1.0	< 0.6	< 17.7	122	100	80
BCAA	1.7	8.2	8.0	120	93	104
DCAA	13.4	8.4	32.1	108	85	106
DBAA	< 0.2	< 0.2	< 4.3	122	92	92
TCAA	8.4	6.9	8.7	110	65	94
BDCAA	1.6	0.5	< 4.2	123	86	89
CDBAA	< 0.1	< 0.8	< 4.3	130	79	94
TBAA	< 0.2	< 0.6	< 7.9	140	77	100

Preliminary bulk sodium hypochlorite solution analysis. A preliminary analysis of seven individual bulk sodium hypochlorite solutions from seven different utilities were analyzed for total chlorine using iodometric titration and for HAAs using the PCR-IC and HPLC-MS/MS method. All seven hypochlorite solutions were analyzed by both methods on the same day to prevent any bias due to continued formation and/or degradation of HAAs in the sampling bottles. The hypochlorite solutions were diluted to ~50 mg L^{-1} FAC for the PCR-IC analysis and ~100 mg L^{-1} FAC for the HPLC-MS/MS analysis. The concentrations of the HAAs (µg L^{-1}) in the diluted solutions (50 or 100 mg L^{-1} FAC) are multiplied by the dilution factor to calculate the concentration of HAAs (mg L^{-1}) in the undiluted bulk sodium hypochlorite solution (Table 16). A HAAs/FAC ratio is calculated to provide context of the contribution of the sodium hypochlorite solution to the HAAs concentrations found in the distribution system. This ratio (in units of µg L^{-1} HAAs/ mg L^{-1} FAC) normalizes the HAAs concentrations based on the hypochlorite ion concentration. It is calculated by dividing the concentration of HAAs by the FAC concentration in the undiluted hypochlorite solution.

The PCR-IC method detected concentrations of MCAA, DCAA, and TCAA in all seven samples as well as concentrations of DBAA in four of the samples (TN-2, NY-1, NJ-1, and MO-1). The MCAA concentrations ranged from 6 to 222 mg L^{-1}. DCAA concentrations ranged from 36 to 1208 mg L^{-1} and TCAA concentrations ranged from 3 to 84 mg L^{-1} for the PCR-IC method. The DBAA concentrations found in TN-2, NY-1, NJ-1, and MO-1 ranged from 2 to 136 mg L^{-1}.

Table 16
Bulk sodium hypochlorite solution analysis for HAAs by utility.

		HPLC-MS/MS		PCR-IC	
	Analyte	Conc. In NaOCl (mgL^{-1})	HAAs/FAC ratio (μgL^{-1} HAAs/ mgL^{-1} FAC)	Conc. In NaOCl (mgL^{-1})	HAAs/FAC ratio (μgL^{-1} HAAs/ mgL^{-1} FAC)
TN-1	MCAA	< MDL	–	163	1.3
	DCAA	19.9	0.16	543	4.4
	TCAA	24.9	0.20	74	0.6
IL-1	MCAA	< MDL	–	209	1.7
	DCAA	13.3	0.11	804	6.3
	TCAA	10.2	0.08	40	0.3
MO-2	MCAA	< MDL	–	10	1.2
	DCAA	0.8	0.10	41	4.9
	TCAA	1.4	0.17	3	0.4
TN-2	MCAA	< MDL	–	9	1.2
	DCAA	0.7	0.09	36	4.6
	DBAA	< MDL	–	2	0.3
	TCAA	0.8	0.10	3	0.3
NY-1	MCAA	< MDL	–	222	1.6
	DCAA	6.3	0.04	889	6.3
	DBAA	< MDL	–	82	0.6
	TCAA	2.4	0.02	53	0.4
NJ-1	MCAA	< MDL	–	189	1.3
	DCAA	6.3	0.04	1208	8.2
	DBAA	< MDL	–	136	0.9
	TCAA	3.1	0.02	84	0.6
MO-1	MCAA	< MDL	–	6	0.7
	DCAA	0.5	0.06	43	5.2
	DBAA	< MDL	–	4	0.4
	TCAA	0.5	0.06	3	0.3

The HPLC-MS/MS confirmed the presence of both DCAA and TCAA in all seven of the bulk hypochlorite solutions. DCAA concentrations ranged from 0.5 to 19.9 mg L^{-1} and TCAA concentrations ranged from 0.5 to 24.9 mg L^{-1}. These concentrations are much lower than those detected by the PCR-IC method. The PCR-IC TCAA

concentrations were a factor of 2 to 30 times higher than the concentrations reported by the HPLC-MS/MS. The PCR-IC DCAA concentrations ranged from 28 to 205 times higher than the HPLC-MS/MS. This large difference in concentrations between the two methods cannot be explained by the systematic bias reported by Henson et al. (2014). Additionally, the preliminary HPLC-MS/MS analysis was not able to confirm the presence of either MCAA or DBAA in any of the collected hypochlorite solutions. The considerably lower values for detected HAA concentrations indicate that HPLC-MS/MS analysis of HAAs may be suffering from ion suppression induced by matrix effects. Matrix effects are defined as the presence of interferents in the sample that influence the quantification of the target analyte (Bylda et al., 2014). Although the mass spectrometer is a highly selective and sensitive detector, the electrospray ionization process is prone to ion suppression effects (King et al., 2000) and the sodium hypochlorite solutions contain high concentrations of sodium, chloride, hypochlorite, and chlorate ions.

Quantification of matrix effects. Due to the possibility of ion suppression present in the HPLC-MS/MS analysis of HAAs, a study was conducted to quantify the matrix effects. In this study the ion suppression was evaluated by analyzing un-spiked and spiked (20 µg L^{-1}) diluted bleach samples. Percent recoveries were calculated using Equation 9 and are presented in Table 17.

$$\%\ Recovery = \frac{Spike\ conc.(\mu g\ L^{-1}) - Unspiked\ conc.(\mu g\ L^{-1})}{Theoretical\ spike\ conc.(\mu g\ L^{-1})}\ x\ 100 \qquad \text{(Equation 9)}$$

With the exception of MCAA, all of the HAAs had acceptable percent recoveries for all seven of the hypochlorite solutions. MCAA had low percent recoveries with an

average of 11 ± 7%. While the percent recoveries for MBAA are within the acceptable range, they are on the low side with an average of 69 ± 7%. The low recoveries for MCAA and MBAA were attributed to ion suppression likely occurring at the electrospray ionization source.

Table 17
Percent recoveries for a ~20 µg L^{-1} spiked hypochlorite solution for all seven bulk sodium hypochlorite samples.

	Percent Recovery (%) for 20 µg/L spike						
Analyte	MO-2	TN-2	TN-1	IL-1	MO-1	NY-1	NJ-1
MCAA	13	2	13	0	15	18	16
MBAA	61	69	74	57	77	74	69
BCAA	107	115	102	103	119	107	104
DCAA	91	103	111	101	107	108	115
DBAA	97	101	106	101	106	110	110
TCAA	80	95	102	102	96	101	93
BDCAA	102	102	97	97	115	117	111
CDBAA	88	94	92	87	98	112	98
TBAA	78	77	83	74	83	101	92

The *source* of the ion suppression in the sample matrix was investigated through pH and chloride ion studies. In the pH study, a set of HAA9 (30 µg L^{-1}) standard solutions (prepared in reagent water) were made to vary in alkalinity by the addition of increasing concentrations of 0.1 M NaOH. Eight solutions were prepared with pH values ranging from ~6 to ~12. These solutions were analyzed with the HPLC-MS/MS method and the peak area for each HAA was plotted against the varying pH values (Figure 26). There is no significant suppression or enhancement of signal intensity for any of the HAAs in the pH range between ~6 and ~11. The analytical signal for both MCAA and MBAA decrease at a pH value of ~12. A diluted bleach sample of ~100 mg L^{-1} FAC does

not exceed a pH of ~10 and therefore this suppression would not be present in a typical analysis of sodium hypochlorite solutions.

The second ion suppression study investigated changes in analytical signal caused by chloride ions. In this study a set of HAA9 standard solutions (prepared in reagent water) were made to vary in chloride ion concentration by the addition of sodium chloride. The sodium ion is not expected to affect the analysis because it does not participate in ion-paring with dibutylamine at pH 5.2 and will elute in the void volume of the chromatographic separation. Chloride ion was chosen because it is one of the anion species expected to be present in the sodium hypochlorite solutions along with hypochlorite, chlorate, and hydroxide ions. These anions arise from the bleach manufacturing process (Snyder et al., 2009). The diluted bleach samples analyzed with the HPLC-MS/MS method are expected to have a maximum chloride ion concentration of ~100 mg L^{-1}. Seven solutions were prepared with [Cl^-] ranging from 0 to 100 mg L^{-1}. The solutions were analyzed with the HPLC-MS/MS method and the peak area for each HAA was plotted against chloride ion concentration (Figure 27). As indicated by the graph there was no significant suppression of analytical signal apparent for any of the HAAs in this study. These two ion suppression studies indicated that the presence of chloride ion concentrations and pH do not play a role in the suppression of the MCAA signal in the HPLC-MS/MS analysis.

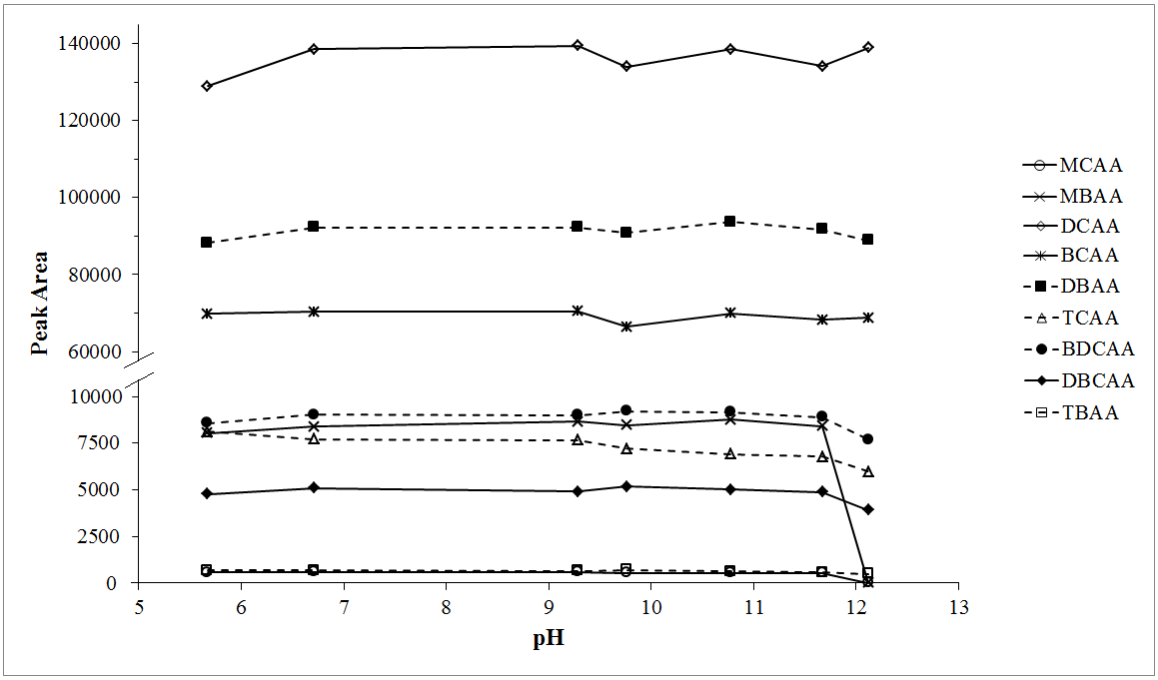

Figure 26. Matrix effects study of pH for each HAA species. Note: pH meter calibrated with 4,7, and 10 buffer solutions.

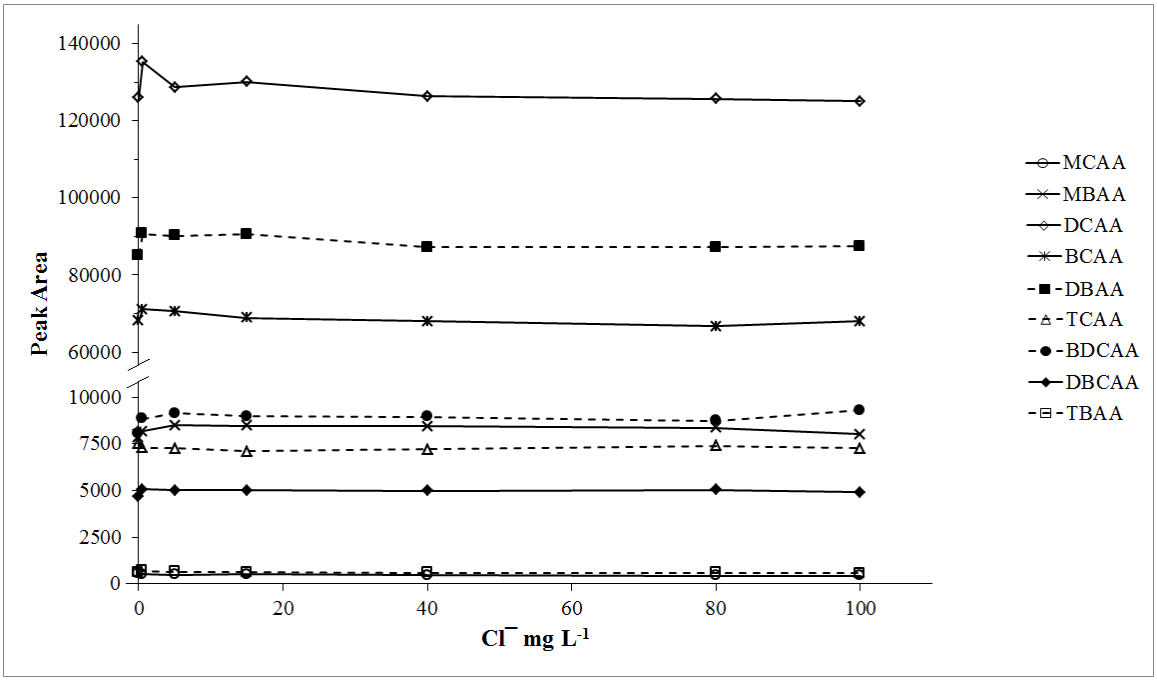

Figure 27. Matrix effects study of chloride ion for each HAA species.

The pH and chloride ion studies did not produce conclusive results for ion suppression of HAA9 analytical signal. A third study was conducted to evaluate the effects of increasing sodium hypochlorite concentrations on the HAA9 species. This study was performed by spiking a set of HAA9 standards with increasing volumes of sodium hypochlorite solution. Seven solutions were prepared with the FAC ranging from 0 to 486 mg L^{-1}. Again, the solutions were analyzed with the HPLC-MS/MS method and the peak area for each HAA was plotted against FAC concentration (Figure 28). The signal intensity (measured as peak area) for DCAA and TCAA increases with increasing FAC concentrations (up to ~360 mg L^{-1}). This is expected due to the presence of DCAA and TCAA in the sodium hypochlorite solutions confirmed in this report and based on the findings of Emmert and co-workers (2013). The signal intensity of BCAA is flat with increasing FAC concentrations. DBAA, BDCAA, DBCAA, and TBAA signal intensities decrease with increasing FAC concentrations. DBAA, BDCAA, and DBCAA experience an average signal loss of 29 ± 2% and TBAA has a signal loss of 45%. MCAA and MBAA experience the most loss of analytical signal at 81% and 42%, respectively, before complete loss of signal. Much like the quantification of matrix effects study (Table 17), MCAA and MBAA signals are the most susceptible to ion suppression in the presence of hypochlorite ion.

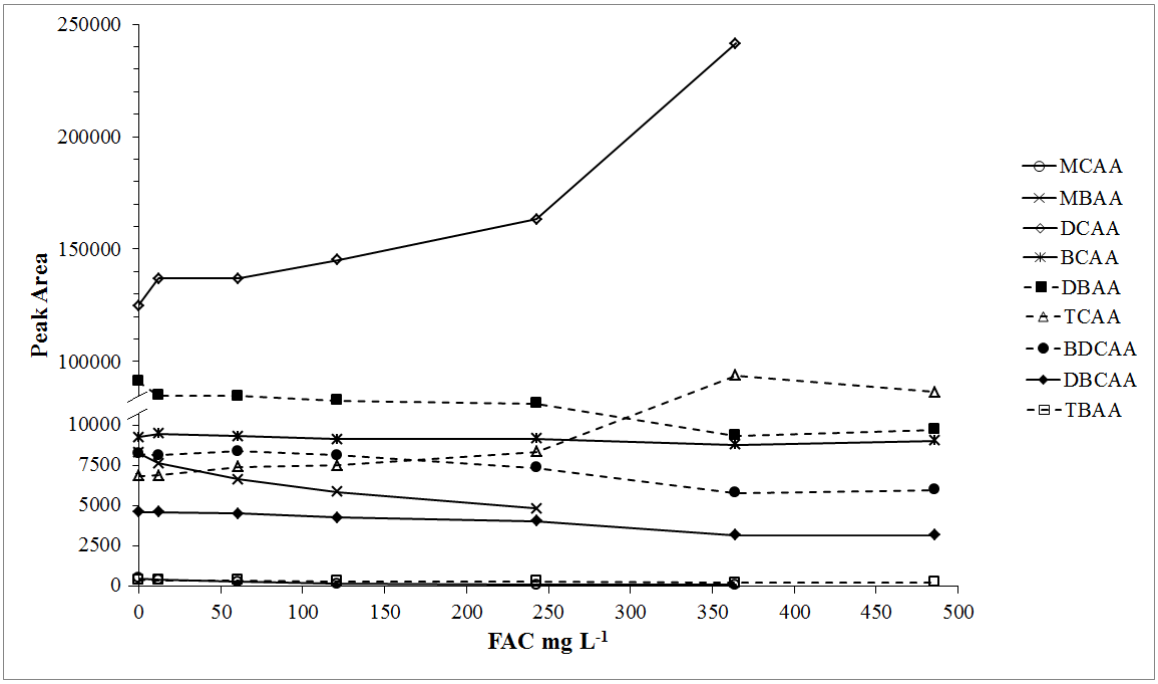

Figure 28. Evaluation of OCl^- concentration on HAA9 signal intensity.

Following the ion suppression studies, sample preparation studies were performed to mitigate or eliminate matrix effects present in the sodium hypochlorite solutions in order to determine the true concentrations of HAAs. Three sample preparation methods were explored to accomplish this. First, diluted bleach solutions (TN-1) were pre-treated with sodium thiosulfate and oxalic acid to chemically quench the hypochlorite ion in solution. Sodium thiosulfate is the titrant employed in the iodometric titration for FAC concentrations and oxalic acid has been shown in previous research to quench chlorine species (Choo, 2013). For the chemical quenching method with sodium thiosulfate, a diluted sodium hypochlorite solution was titrated using the iodometric method. This titration determines the volume of sodium thiosulfate titrant needed to "chemically quench" the solution. Subsequently, another diluted sodium hypochlorite solution was dosed with this pre-determined volume of sodium thiosulfate titrant. This solution was

further diluted to ~100 mg L^{-1} FAC and analyzed with the HPLC-MS/MS method. For the chemical quenching method with oxalic acid, 1 mL of a 100 g L^{-1} oxalic acid solution was added to the diluted hypochlorite solution (~100 mg L^{-1}) and analyzed with the HPLC-MS/MS method.

The final sample preparation method explored was SPE. The SPE step was performed on a LiChrolut EN SPE cartridge which has been found to be suitable for HAA extraction and pre-concentration in previous work (Barron & Paull, 2004; Loos & Barcelo, 2001; Martinez et al., 1998; Sarzanini et al., 1999). Previously reported percent recoveries ranged from 26 to 91 % for MCAA, 42 to 80 % for MBAA, 45 to 104 % for DCAA, 48 to 85 % for DBAA, 62 to 101 % for TCAA, and 33 to 78 % for TBAA for all four studies. BCAA, BDCAA, and DBCAA were not analyzed in these studies. Variations in method procedures, such as pH values of solutions prior to SPE, are thought to be responsible for the disparity in percent recovery data reported between the studies (Barron & Paull, 2004).

In this SPE study, a diluted bleach sample (TN-1) was acidified and then flowed through a LiChrolut EN cartridge. The protonated HAAs will adsorb to the styene-divinylbenzene polymer resin while allowing ionic species such as chloride ion and sulfate ion to pass through without adsorption. The HAAs are then ionized and subsequently eluted using sodium hydroxide. Using this approach, the HAAs can be separated from species present in the bulk hypochlorite solution that cause matrix effects.

To evaluate the efficacy of each sample preparation strategy, un-spiked and spiked (20 µg L^{-1}) hypochlorite solution samples were prepared with each sample preparation method and analyzed with the HPLC-MS/MS method. Percent recoveries for

each method were calculated (Equation 9) and are presented in Table 18. The hypochlorite solution treated with sodium thiosulfate produced excellent percent recoveries for all of the HAAs except for MCAA which had a percent recovery of 19 %. These results were similar to those of the spiking studies reported in Table 17. The diluted hypochlorite solution treated with oxalic acid produced low percent recoveries (ranging from 0 to 61 %) for all HAAs. These two sample pre-treatment methods were ruled out as viable methods for hypochlorite analysis based on their low percent recoveries for MCAA, DCAA and TCAA. The SPE method produced acceptable percent recoveries ranging between 49 % for TBAA and 138 % for MCAA. While the TBAA recovery is relatively low, it is uncommon in sodium hypochlorite solutions, and MCAA was the priority for this analysis. This SPE method was used in conjunction with the HPLC-MS/MS method to determine HAAs concentrations in the hypochlorite solutions.

Table 18
Sample preparation study: percent recoveries for a 20 µg L^{-1} spiked sample.

Analyte	Treated with $Na_2S_2O_3$	Treated with Oxalic Acid	LiChrolut EN SPE
MCAA	19	18	138
MBAA	76	54	82
BCAA	104	0	101
DCAA	112	0	87
DBAA	105	11	97
TCAA	141	0	85
BDCAA	118	57	81
CDBAA	107	61	65
TBAA	98	51	49

Bleach samples TN-2, NY-1, NJ-1, MO-1 and MO-2 were also analyzed with the optimized SPE sample preparation and HPLC-MS/MS method. The percent recoveries for the spiked (20 µg L^{-1}) samples are presented in Table 19. Averages of percent recoveries are also reported in order to summarize the success (or failure) of the SPE process for each individual HAA. The addition of the SPE step increased spike recoveries for MCAA in samples TN-2 and MO-1, giving recoveries of 113 and 129 %, respectively. The recoveries for MCAA in NY-1, NJ-1, and MO-2 also increased but were higher than typical recoveries (Barron & Paull, 2004; Loos & Barcelo, 2001; Martinez et al., 1998; Sarzanini et al., 1999). NY-1, NJ-1, and MO-2 bleach samples gave MCAA recoveries of 183, 155, and 160 %, respectively. All five bleach samples had low recoveries for TBAA ranging between 34 and 46 %. The remaining HAAs, with the exception of the MO-1 recovery for DBCAA, had acceptable percent recoveries for each bleach sample, ranging from 52 to 140 %.

Incorporating the SPE method with the HPLC-MS/MS analysis provided an additional comparison of the HPLC-MS/MS method with the PCR-IC method for the analysis of sodium hypochlorite solutions. The concentrations for sodium hypochlorite samples TN-2, NY-1, NJ-1, MO-1, and MO-2 for both methods are presented in Table 20. Qualitatively, the methods agree in the detection of MCAA, DCAA, and TCAA. The PCR-IC still reported higher HAA concentrations than the HPLC-MS/MS with the PCR-IC concentrations being a factor of 2-75 times higher. However, this over prediction was reduced for MCAA, DCAA and TCAA compared to the preliminary analysis. The PCR-IC method detected concentrations of DBAA (3 to 116 mg L^{-1}) in all five samples and the HPLC-MS/MS did not detect DBAA above the MDL in any sample.

Table 19
SPE percent recoveries for ~20 µg L^{-1} spiked hypochlorite solutions for TN-2, NY-1, NJ-1, MO-1, and MO-2.

	Percent Recoveries (%)					
Analyte	TN-2	NY-1	NJ-1	MO-1	MO-2	Average
MCAA	113	183	155	129	160	148 ± 27
MBAA	105	94	129	103	119	110 ± 14
BCAA	135	128	105	96	110	115 ± 16
DCAA	79	136	64	83	121	97 ± 30
DBAA	136	134	112	99	139	124 ± 18
TCAA	128	140	133	91	139	126 ± 20
BDCAA	118	130	82	52	76	92 ± 32
DBCAA	73	77	69	45	62	65 ± 13
TBAA	43	44	44	34	46	42 ± 5

Table 20
Bulk sodium hypochlorite solution analysis for HAAs by utility. HPLC-MS/MS analysis includes SPE step.

		HPLC-MS/MS		PCR-IC	
	Analyte	Conc. In NaOCl (mgL^{-1})	HAAs/FAC ratio (μgL^{-1} HAAs/ mgL^{-1} FAC)	Conc. In NaOCl (mgL^{-1})	HAAs/FAC ratio (μgL^{-1} HAAs/ mgL^{-1} FAC)
TN-2	MCAA	0.4	0.05	6.3	0.86
	DCAA	1.2	0.17	14.5	1.98
	TCAA	0.6	0.08	1.4	0.19
NY-1	MCAA	2.7	0.02	176.4	1.50
	DCAA	23.1	0.20	467.6	3.98
	TCAA	1.0	0.01	26.4	0.22
NJ-1	MCAA	2.5	0.02	143.0	1.03
	DCAA	23.6	0.17	987.7	7.09
	TCAA	1.5	0.01	91.0	0.65
MO-1	MCAA	0.5	0.05	2.4	0.22
	DCAA	1.5	0.14	27.3	2.55
	TCAA	0.5	0.04	2.6	0.24
MO-2	MCAA	0.6	0.06	7.1	0.80
	DCAA	1.2	0.14	24.4	2.76
	TCAA	1.0	0.11	3.3	0.37

Conclusions

A liquid-chromatography mass spectroscopy method with a solid-phase extraction step was developed for the analysis and confirmation of HAAs in bulk sodium hypochlorite solutions used for drinking water disinfection. Real world drinking water samples were first analyzed by the HPLC-MS/MS method alongside the PCR-IC and USEPA 552.3methods. All three methods were said to be in agreement for the analysis of HAAs in drinking water (with the exception of water samples with high concentrations of DCAA). Next, seven sodium hypochlorite feedstock solutions were analyzed for HAAs with the HPLC-MS/MS and PCR-IC method. These results confirmed the presence of both DCAA and TCAA in the hypochlorite solutions. The HPLC-MS/MS method suffered from matrix effects which prohibited the detection of MCAA. Studies were performed to determine the source of ion suppression in the analysis and ultimately a solid-phase extraction sample preparation technique was employed in order to mitigate matrix effects. Incorporating the SPE step into the HPLC-MS/MS analysis allowed for a qualitative confirmation of the presence of MCAA, DCAA, and TCAA in the sodium hypochlorite solutions.

Chapter 5

Conclusions and Recommendations for Future Research

The main goal of this research was to develop and implement HPLC-MS/MS methods for the detection of 1,3-DMAA and 1,4-DMAA in geranium plants and for the detection nine haloacetic acids in sodium hypochlorite solutions. The analysis of 1,3-DMAA and 1,4-DMAA in geranium plants encompassed two objectives: The first study was to determine the presence or absence of 1,3-DMAA and 1,4-DMAA in geranium plants from three different regions of China. The second study focused on determining stereoisomer ratios of 1,3-DMAA and 1,4-DMAA in the plant and the synthetic (standard) material. All methods were accomplished with reverse-phase high performance liquid chromatography and a triple quadrupole mass spectrometer capable of multiple reaction monitoring. Sample preparation techniques such as solid phase and liquid-liquid extractions were applied when needed. The conclusions for each body of work and recommendations for future research are discussed in this chapter.

Analysis of 1,3-DMAA and 1,4-DMAA in geranium plants conclusions.

Geranium plants from three regions of China were analyzed for the presence of 1,3-DMAA and 1,4-DMAA using an extraction and HPLC-MS/MS method. External calibration and standard addition analyses confirmed the presence of both DMAA species in the Changzhou region samples. Alternative HPLC-MS/MS methods were also developed, utilizing a more specific chemistry with chiral derivatizing agents (CDAs). 1,3-DMAA and 1,4-DMAA were reacted with two CDAs to form diastereomers that can be separated on a traditional achiral column. CDAs investigated include: (-)-1-(9-fluorenyl)ethyl chloroformate [(-)-FLEC] and (R)-(-)-α-methoxy-α-(trifluoromethyl)

phenylacetyl chloride [(-)-MTPA] or Mosher's acid chloride. The DMAA-FLEC analysis was able to provide a confirmation of the presence of both 1,3-DMAA and 1,4-DMAA in the Changzhou, China plant sample that was previously analyzed. Also, a local geranium sample was pre-concentrated (via liquid-liquid extraction) and analyzed with the original HPLC-MS/MS method and with the DMAA-FLEC analysis method. Both methods confirmed the presence of DMAA in this geranium plant sample. The DMAA-FLEC analysis also provided an enantiomeric ratio analysis of 1,4-DMAA. For both geranium samples, one 1,4-DMAA enantiomer was enriched 1 – 4 % over the other. Stability studies found that the DMAA-FLEC derivative product was unstable, producing a very labor intensive method. As a result, an alternative CDA [(-)-MTPA] was investigated. The DMAA-MTPA product was found to stable up to twenty hours but the chromatographic separation lacked resolution. To remedy this absence of resolution 1,3-DMAA and 1,4-DMAA were separated on a preparatory scale HPLC prior to derivatization. The DMAA-MTPA analysis was carried out for a new Changzhou, China geranium sample and was able to confirm the presence of both 1,3-DMAA and 1,4-DMAA. Unfortunately, the high error associated with the DMAA-MTPA analysis does not allow for a confident analysis of individual stereoisomers of DMAA.

1,3-DMAA and 1,4-DMAA in geranium plants analysis recommendations. The major shortcoming of the DMAA-FLEC and DMAA-MTPA analyses was the lack of chromatographic resolution of individual 1,3-DMAA and 1,4-DMAA stereoisomers. This deficiency in resolution prohibited a confident analysis of stereoisomer ratios that is needed to compare DMAA standards and plant samples. It is highly unlikely that adjusting the current chromatographic conditions would provide a better separation and

this would be a last resort since the compounds already require an analysis time of over thirty minutes with the current separation. Therefore, future work should focus on the analysis of DMAA stereoisomers with different analytical instrumentation. The most logical step would be to replace the liquid chromatography separation with a gas chromatography separation.

Preliminary work, done in collaboration with Dr. Patricia Ranaivo, using the DMAA-MTPA chemistry and a GC-MS instrument has already shown potential for the separation of both 1,3-DMAA and 1,4-DMAA stereoisomers. In this method the 1,3-DMAA and 1,4-DMAA derivative products were analyzed with a Shimadzu Ultra QP2010 GC-MS (Kyoto, Japan) operated in EI mode. The derivative products were separated on a ZB-5 fused silica column with a (5%-phenyl)-polymethyl siloxane stationary phase. Figure 29 is a chromatogram showing the separation of a mixed standard solution of 1,3-DMAA and 1,4-DMAA at 720 mg L^{-1}. The chromatogram illustrates acceptable resolution for all four 1,3-DMAA diastereomers (retention times 30.25, 30.47, 30.81, and 30.98 minutes) and the two 1,4-DMAA enantiomers (retention times 30.67 and 31.12 minutes). Future work should focus on obtaining MDL, accuracy, and precision data for this analysis and will likely require some method development in order to achieve MDLs in the range required for geranium plant analysis.

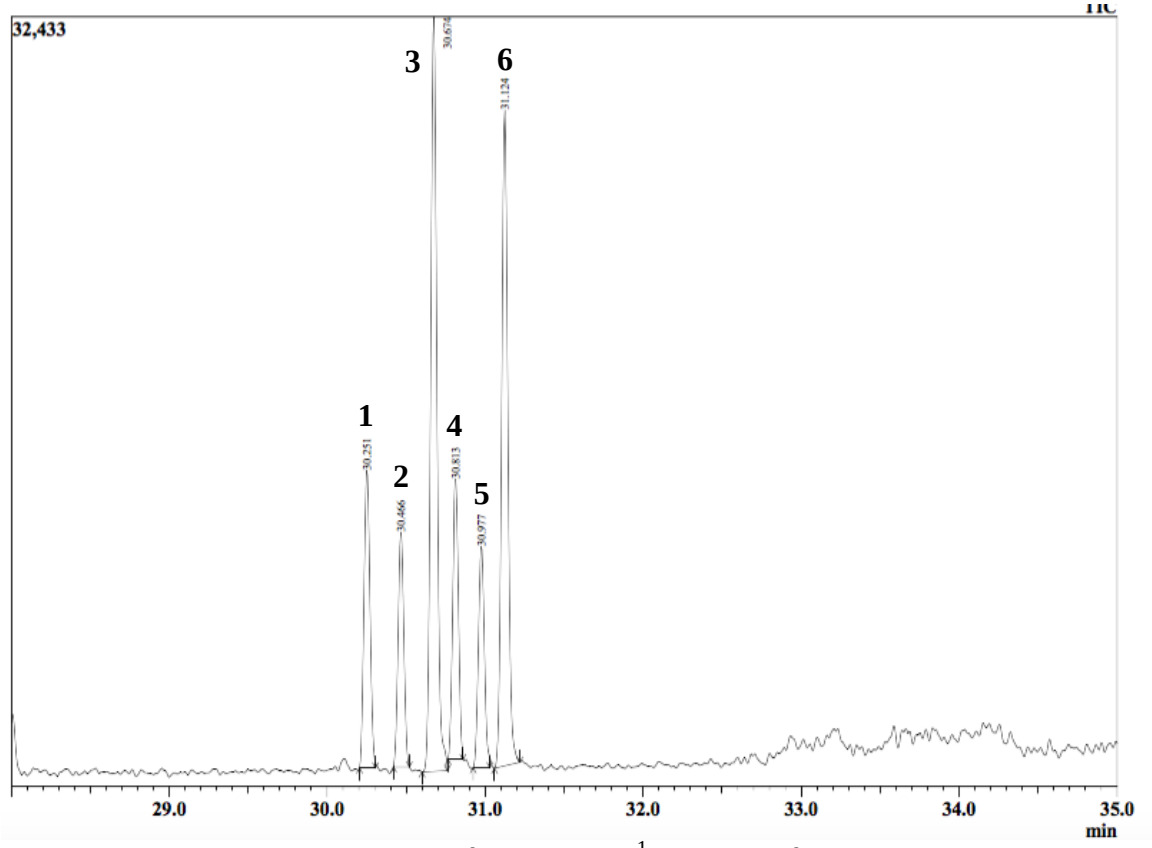

Figure 29. GC-MS chromatogram of a 720 mg L^{-1} standard of both 1,3-DMAA and 1,4-DMAA derivatives. Peaks 1, 2, 4, and 5 correspond to 1,3-DMAA stereoisomers and peaks 3 and 6 correspond to 1,4-DMAA stereoisomers.

Haloacetic acids in sodium hypochlorite analysis conclusions. A HPLC-MS/MS method was developed for the detection of nine haloacetic acids in bulk sodium hypochlorite solutions used for disinfection of drinking water. The HPLC-MS/MS method was validated in a comparison study of drinking water with the USEPA 552.3 method. The HPLC-MS/MS results for the analysis of drinking water were also consistent with the PCR-IC analysis with the exception of samples with high DCAA concentrations. Preliminary analysis of the sodium hypochlorite solutions with the HPLC-MS/MS method indicated the presence of ion suppression due to matrix effects. The presence of matrix effects resulted in the implementation of an additional solid phase

extraction step in order to separate the analyte from the high ionic strength matrix. Five sodium hypochlorite solutions were then analyzed for the presence of haloacetic acids with the SPE-HPLC-MS/MS method and the PCR-IC method. Both methods were able to confirm the presence of MCAA, DCAA, and TCAA in the sodium hypochlorite solutions. Unfortunately, the two methods reported very different concentrations for these HAAs. For example, the PCR-IC detects DCAA at a factor of 40 times higher than the HPLC-MS/MS in some cases. Also, the PCR-IC detected concentrations of DBAA while the SPE-HPLC-MS/MS method did not detect DBAA above the MDL of the analysis.

Haloacetic acids in sodium hypochlorite analysis recommendations. Future work for the HAAs in sodium hypochlorite solution studies should focus on defining the source or sources responsible for the large differences in concentrations reported between the SPE-HPLC-MS/MS method and the PCR-IC method. Discovering the origin of this bias is important because the HPLC-MS/MS was designed to offer a confirmation method for the PCR-IC. The PCR-IC detection chemistry is not as selective as MS detection therefore initial studies have focused on investigating the presence of interfering species in the PCR-IC analysis of sodium hypochlorite solutions. For example, chlorate ion and perchlorate ion have been shown to form in the bulk sodium hypochlorite solutions (Snyder et al., 2009). Further work, investigating whether the ions reported in the sodium hypochlorite solutions will produce interferences in the PCR-IC analysis, should be investigated as their presence could potentially skew the reported HAA concentrations. In addition, the presence of dalapon (an herbicide) as an interfering compound should be investigated further in drinking water samples and the sodium hypochlorite solutions. Dalapon (2,2-Dichloropropanoic acid) is currently regulated by

the USEPA and has a maximum contaminant level of 0.2 mg L^{-1} in drinking water (USEPA, 2006) and has a chemical structure similar to TCAA in which a chlorine atom has been replaced with a methyl group. This similarity in compound structure indicates that dalapon should react with nicotinamide, produce a fluorescent product and potentially co-elute with an HAA. In addition, dalapon is not stable and can degrade at temperatures reaching 25 − 30°C (USEPA, 2009). Thus, investigation of dalapon by establishing the PCR-IC MDL, determining whether it is commonly present in drinking water, and whether it is present in sodium hypochlorite solutions could lead to identification of the PCR-IC discrepancy with HPLC-MS/MS. Preliminary work using the HPLC-MS/MS for dalapon analysis determined the MRM transition was 141/97 *m/z* and it elutes between MBAA and BCAA. Further studies with the HPLC-MS/MS using SPE could provide additional information regarding the presence of other anionic and halogenated compounds that could potential react and produce signal in the PCR-IC analyzer.

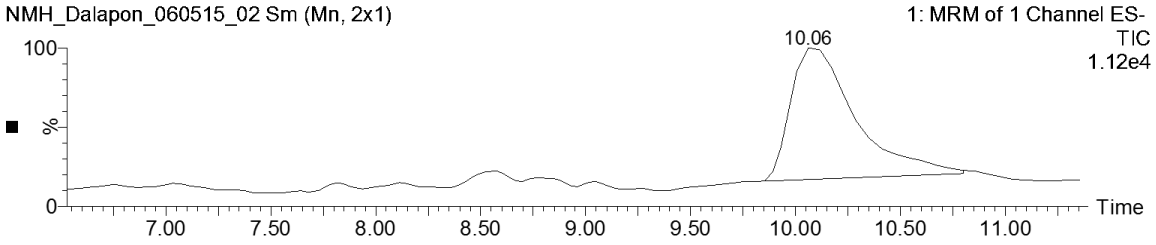

Figure 30. HPLC-MS/MS chromatogram of dalapon MRM transition in a sodium hypochlorite solution.

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