Development of a MALDI-TOF-MS Method for the Analysis of Cyanobacterial Neurotoxin β-N-Methylamino-L-alanine (BMAA) in Search of BMAA Incorporation in Biological Samples

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DEVELOPMENT OF A MALDI-TOF-MS METHOD FOR THE ANALYSIS OF
CYANOBACTERIAL NEUROTOXIN $\beta$-$N$-METHYLAMINO-L-ALANINE (BMAA)
IN SEARCH OF BMAA INCORPORATION IN BIOLOGICAL SAMPLES

A thesis submitted in partial fulfillment of
the requirements for the degree of
MASTER OF SCIENCE
in
FORENSIC SCIENCE
by
Laura M. Conklin

2015
To: Dean Michael R. Heithaus  
College of Arts and Sciences  

This thesis, written by Laura M. Conklin, and entitled Development of a MALDI-TOF-MS Method for the Analysis of Cyanobacterial Neurotoxin β-N-METHYLAMINO-L-ALANINE (BMAA) in Search of BMAA Incorporation in Biological Samples, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this thesis and recommend that it be approved.

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John Berry, Major Professor

Date of Defense: November 10, 2015

The thesis of Laura M. Conklin is approved.

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Dean Michael R. Heithaus  
College of Arts and Sciences

_______________________________________
Dean Lakshmi N. Reddi  
University Graduate School

Florida International University, 2015
DEDICATION

This thesis work is dedicated to my parents Robert and Susan Conklin for always encouraging me to follow my dreams and loving me unconditionally. Without their love, support and incredible patience, this thesis would not be possible. I would also like to thank my brothers and their wives for their words of encouragement and support throughout this program. I am truly thankful for having you all in my life.
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ABSTRACT OF THE THESIS

DEVELOPMENT OF A MALDI-TOF-MS METHOD FOR THE ANALYSIS OF CYANOBACTERIAL NEUROTOXIN β-N-METHYLAMINO-L-ALANINE (BMAA)

IN SEARCH OF BMAA INCORPORATION IN BIOLOGICAL SAMPLES

by

Laura M. Conklin

Florida International University, 2015

Miami, Florida

Professor John Berry, Major Professor

Beta-\(\text{N}\)-methylamino-L-alanine (BMAA) is a non-protein amino acid produced by many cyanobacteria, and thought to induce neurotoxic effects through excitotoxicity, contributing to neurodegenerative diseases such as Amyotrophic Lateral Sclerosis/Parkinsonism-dementia complex (ALS-PDC) and Alzheimer’s. The ubiquitous nature of cyanobacteria, and evidence of biomagnification through our food web, creates a dire need for the development of an analytical platform that will provide accurate identification and quantification of BMAA amounts in our ecosystem and potential food supply. The present study evaluated the ability of a MALDI-ToF-MS method to detect and quantify BMAA in a variety of biological matrices. Through validation procedures, it was demonstrated that this MALDI-ToF-MS method provided comparable data to currently accepted analytical methods, specifically LC-MS/MS. Further, the development of said method reduced sample preparation and data acquisition time (1-2 seconds per sample), while providing high throughput analysis and eliminating the need for derivatization, chromatographic separation, and modification of amino acids.
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1. INTRODUCTION AND LITERATURE REVIEW

The connection between β-N-methylamino-L-alanine (BMAA) and Amyotrophic Lateral Sclerosis/Parkinsonism-dementia complex (ALS-PDC) came to light in 1953 when a medical report was released stating that on the island of Guam, the incidence rate of ALS-PDC among the native Chamorro people was 100-fold higher than anywhere else in the world (Arnold et al., 1953). In this report, however, it was thought that genetic variations among the Chamorro people was the causative factor for the increased incidence of ALS-PDC (Arnold et al., 1953). Once researchers failed to correlate a genetic causation to the increased incidence of ALS-PDC, they turned to possibility of environmental factors being the cause (Spencer et al., 1986). The Chamorro people consume a diet that relies heavily the seeds of the native Cycad tree to produce flour (Murch et al., 2004). It was then suggested that the consumption of these seeds from the Cycad tree was the cause of the increased rate of this specific neurodegeneration among the Chamorro people, deeming this hypothesis as, “The Cycad Hypothesis” (Murch et al., 2004).

It was not until 1967 in a study conducted by Vega et al, that BMAA was isolated from the seeds of the cycad tree and found to have neurotoxic effects after being injected into chicks and rats, causing symptoms that paralleled that of patients suffering from neurodegenerative diseases such as ALS-PDC and Alzheimer’s (Vega et al., 1967). Various studies done on rats, chicks, macaques, and mice, further supported a new hypothesis that BMAA was the component of the cycad seeds that was the causative agent of neurodegeneration. At this point in time, the origin of BMAA was still unknown. In 2003, it was found that BMAA was a non-protein amino acid that was
being produced by the cyanobacterial genus *Nostoc* as a symbiont to the coralloidal roots of the cycad tree (Murch et al., 2004). Figure 1 depicts the chemical structure of BMAA. In this study, by Murch, et al. (2004), not only was BMAA identified as a product of this cyanobacteria, but evidence of biomagnification through the cycad tree and further into the food web to the Chamorro people was seen. By analyzing various portions of the cycad tree (i.e., roots, leaves, and seeds) and the hair and skin of a species of Flying Fox, a type of fruit bat, that forages on the seeds, levels of BMAA were seen to have increased along the food web, accumulating along the way, until finally being consumed by the Chamorro people either through flour produced from the seeds of the cycad, or by the Flying Foxes who forage on the fruit and seeds of the Cycad tree (Murch et al., 2004). Further, this study found the presence of BMAA in brain tissues of patients who had suffered not only from ALS-PDS, but Alzheimer’s as well (Murch et al., 2004). These findings provided evidence for the theory of the biomagnification of BMAA among food webs and also pushed for further research as to the role of BMAA in neurodegenerative diseases (Murch et al., 2004). Other subsequent studies found that 90% of cyanobacterial species produced BMAA, suggesting that human exposure to this neurotoxin might be much more prevalent than previously thought (Cox et al., 2005). Recent studies showing BMAA present in tissues of some of the top predators such as mussels and shark fins, suggest that the ability of this amino acid to biomagnify, could prove to be a serious risk to the entire human population, if the mode of action of the neurotoxin is the cause of many neurodegenerative diseases (Mondo et al., 2012).
Currently, there is very little consensus as to a method of detection as well as an accurate technique for the analysis of BMAA. The only true consensus regarding BMAA are the neurotoxic effects that it has on a cellular level based on \textit{in vivo} studies, however, the link between BMAA and ALS has yet to be effectively demonstrated in an animal model (Karamyan et al., 2008; Duncan et al., 1991). The ubiquitous nature of cyanobacteria and evidence of biomagnification through our food web, creates a need for researchers to clearly understand the mode of action by which BMAA exerts itself on primary motor neurons, and the development of an analytical platform that will provide accurate identification and quantification of BMAA amounts in our ecosystem and potential food supply. The present project entails the development of an analytical method using MALDI-ToF/ToF-MS/MS that ensures the accurate identification and quantification of BMAA with minimal sample preparation. By method development, clear reporting, validation and inter-laboratory comparisons, the discrepancies that currently exist regarding previous studies are diminished. The development of such analytical platform aims to give researchers a better understanding of the extent or magnitude of human exposure to BMAA.
The aim of the current research is to provide an evaluation of MALDI-ToF/ToF-MS/MS as an analytical platform for the identification and quantification of BMAA in a variety of matrices. The research involves method development, validation studies, and subsequent employment to evaluate relevant biological samples from South Florida waters, where BMAA accumulation has been previously reported. The MALDI-TOF technique was found to effectively measure BMAA in a range of biological matrices, and moreover, was notably able to discriminate, specifically by means of so-called MALDI-TOF/TOF, BMAA from diaminobutyric acid (DAB), a structural isomer also produced by cyanobacteria, which has been suggested to potentially confound analytical results. The details of the techniques, including analytical results obtained from application to biological samples from South Florida waters, comparison to conventional (e.g. liquid chromatography/mass spectrometry) techniques and potential for understanding toxicity of BMAA will be discussed.

The goal of developing a MALDI-TOF/TOF-MS/MS method is to offer another analytical platform that allows for minimal sample preparation, rapid analysis, while eliminating the need for derivatization, chromatographic separation and modification of amino acids. If the proposed method were to be coupled to MALDI imaging mass spectrometry (IMS), accurate quantitation of BMAA along with visual spatial localization would be possible.
1.1. SUGGESTED MECHANISM OF BMAA – INDUCED NEURODEGENERATIVE EFFECTS

On the basis of the current literature, there are many differences in opinion as to what the mode of activity BMAA puts forth on neuronal cells, however, many studies have shown significant evidence that BMAA has the capability of crossing the blood-brain barrier, competitively binding to glutamate receptors, causing chemical and cellular ion shifts that lead to depolarization, excitotoxicity, and eventually neuronal cell apoptosis (Duncan et al., 1991; Lobner et al., 2007; Cucchiaroni et al., 2010). In 1991, Duncan, et al. (1991) studied the pharmacokinetics and the permeability of the blood-brain barrier, and were able to show that in a rat model, BMAA can cross the blood-brain barrier by an L-type amino acid carrier. Lobner, et al. (2007) conducted a study in 2007 in which they looked that the synergistic effects of low doses of BMAA with amyloid-β and MPP+, both known neurotoxins. Their study provided data suggesting that BMAA acts on NMDA receptors and another particular glutamate receptor (mGluR5) (Lobner et al., 2007). One other notable study was done by Cucchiaroni, et al. (2010) in which they found that an injection of BMAA caused an increase in Ca+, increased production of reactive oxygen species (ROS), and the heavy release of cytochrome-c into the cytosol in a rat neuron model. The release of cytochrome-c could account for diminished mitochondrial activity, excitotoxicity, and cellular apoptosis. In conclusion, after BMAA is consumed or ingested, it passes through the blood-brain barrier via L-type amino acid carriers, and then competitively binds to NMDA receptors along with other glutamate receptors (Cucchiaroni et al., 2010). The binding of BMAA to NMDA and other glutamate receptors then causes cellular ion shifts of Ca+, Na+, and K+, an increase in
ROS production, depolarization of the cell, and a heavy release of cytochrome-c (Cucchiaroni et al., 2010). This so called “excitotoxicity” mechanism could be the causative mode of activity of BMAA on neuronal cells, leading to many neurodegenerative diseases.

More recently, and perhaps more notable, in a study done by Dunlop, et al. (2013), researchers incubated multiple types of human cells with $^3$H-BMAA and unlabeled BMAA and were able to determine that not only does BMAA become misincorporated into human proteins, but binding of BMAA is inhibited by L-serine. Further, the study showed that the formation of autofluorescent bodies, that are characteristic of protein aggregation, was diminished by the co-incubation of BMAA and L-serine with human cells (Dunlop et al., 2013). The diminished presence of autofluorescent bodies with the co-incubation of BMAA and L-serine, suggests that the misincorporation of BMAA into human cells is a protein-synthesis dependent process (Dunlop et al., 2013). Figure 2 shows the possible mechanism of BMAA misincorporation via a protein-synthesis dependent process (Dunlop et al., 2013). It must be noted that the mechanism by which BMAA becomes incorporated into proteins and crosses the blood-brain barrier is still debated, however L-type amino acid transport has been suggested, and the studies described above are progressive steps to understanding the true mechanism by which BMAA acts.
1.2. COMMON METHODS OF ANALYSIS FOR BMAA

Current methodology for the analysis of BMAA in a variety of biological matrices and mixtures of amino acids prepared by splitting proteins by means of acid hydrolysis, commonly referred to as protein hydrolysates, primarily include, high performance liquid chromatography with postcolumn fluorescence detection (HPLC-FLD), liquid chromatography mass spectrometry (LC-MS), and liquid chromatography tandem mass spectrometry (LC-MS/MS) using both derivatized and underivatized BMAA samples.
The reported incorporation of BMAA into proteins, in protein hydrolysates, led to the universal use of hydrolysis procedures to account for all BMAA in a sample (bound and free). All of these methods employ liquid chromatography, separating a mixture using a liquid mobile phase that carries the mixture through a stationary phase within a chromatographic column and further into a detector or mass spectrometer. Researchers over the past decade have utilized a variety of methods and protocols regarding which analytical method produces data to best quantify BMAA in biological samples, and therefore, the published data has suggested to potentially overestimate and/or fail to detect BMAA in a sample. Table 2 depicts the variety of analytical techniques that some research groups have used; the majority of research groups have chosen LC-MS/MS (Table 2).

Table 1 describes the reported concentrations of BMAA in various matrices. Over the last fifteen years, researchers have analyzed multiple samples that vary from cycad seeds to very complex matrices such as lobsters and brain tissues. The amount of BMAA found in these matrices, varies from 0μg/g to 2,286μg/g (Table 1) (Banack et al., 2014; Brand et al., 2010; Christensen et al., 2012; Faasen et al., 2009; Karlsson et al., 2014; Mondo et al., 2014; Spacil et al., 2010).
Table 1. Concentrations of BMAA in different matrices from current literature.

<table>
<thead>
<tr>
<th>CITATION</th>
<th>METHOD</th>
<th>BMAA (μg/g)</th>
<th>SAMPLE TYPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fassen 2009</td>
<td>LC-MS/MS</td>
<td>4-42</td>
<td>Cyanobacterial blooms in Dutch urban waters</td>
</tr>
<tr>
<td>Brand 2010</td>
<td>LC-MS/MS</td>
<td>275-2,286</td>
<td>Blue Crab, oyster, Puffer Fish, shrimp, White Perch</td>
</tr>
<tr>
<td>Spacil 2010</td>
<td>HPLC-MS/MS</td>
<td>0-109.4</td>
<td>Prokaryotic cyanobacterium to Eukaryotic fish</td>
</tr>
<tr>
<td>Christensen 2012</td>
<td>LC-MS/MS</td>
<td>19.1-50.1</td>
<td>Cycad, oyster, Blue Crab</td>
</tr>
<tr>
<td>Mondo 2014</td>
<td>HPLC-FD</td>
<td>86-265</td>
<td>Shark cartilage dietary supplements</td>
</tr>
<tr>
<td></td>
<td>HPLC-MS/MS</td>
<td>75-352</td>
<td></td>
</tr>
<tr>
<td>Karlsson 2014</td>
<td>HPLC-MS/MS</td>
<td>0.023-245.978</td>
<td>Liver and brain samples of rats</td>
</tr>
<tr>
<td>Banack 2014</td>
<td>LC-MS/MS</td>
<td>0.4-27</td>
<td>Lobsters from Florida</td>
</tr>
</tbody>
</table>

In most of the first studies in the early 2000’s, researchers began using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatization prior to HPLC-FLD (Banack et al., 2003; Cox et al., 2003; Murch et al., 2004). Derivatizing agent, AQC adds a fluorescent “tag” to BMAA, making it detectable HPLC-FLD methods (Banack et al., 2003; Cox et al., 2003; Murch et al., 2004; Faassen et al., 2012; Mondo et al., 2012; Mondo et al., 2014). Similarly, Esterhuizen-Londt (2011) used another derivatization method using propyl chloroformate, EZ-faast™. The HPLC-FLD method has its drawbacks, which are discussed below. Murch et al. (2004) discovered that BMAA can also be incorporated into proteins in samples, and requires hydrolysis to release the amino acid from its bound form. The discovery of protein incorporation accounted for an underestimation of BMAA in biological samples in previous studies.
Faasen, et al. (2012) compared three analytical techniques, HPLC-FLD and LC-MS/MS using derivatized and underivatized samples (Faasen et al., 2012). Following the literature of comparative analytical methods of BMAA analysis in the present study, LC-MS/MS using derivatized samples (to reduce polarity), or underivatized samples, proved to be more accurate than HPLC-FLD, on the basis of its higher selectivity: four different selective criteria are required rather than the two required by HPLC-FLD (Faasen et al., 2012). The two selectivity criteria required for HPLC include a fluorescent signal by derivatization with AQC (AccQ-Taq) (the derivatization method that makes the derivatives more stable than the original amino acids and adds a fluorescent tag for analysis), and retention time, whereas the four selective criteria of LC-MS/MS are retention time, two separate mass to charge ratios (m/z) based on precursor and product ions, and product ion abundance ratio (Faasen et al., 2012). The lower selectivity of HPLC-FLD and LC-MS led to the detection of higher concentrations of BMAA than LC-MS/MS, however, this was likely a result of lower selectivity and the false positives of other amino groups that fluoresced with AQC or other derivatization methods (Faasen et al., 2012). There has been little research done between labs to use different published methods to analyze the same set of samples, therefore, creating many discrepancies between data sets. There has yet to be a single method or interlaboratory evaluations of methods. The magnitude of potential human exposure is still unknown therefore, because of the wide range of data.

In a study done by Karlsson, et al. (2012) researchers used Matrix Assisted Laser Desorption Ionization – imaging mass spectrometry (MALDI-IMS) in an effort to image and study protein changes in the brains of adult rats after exposure to BMAA during the
neonatal period. The instrumental method MALDI/IMS works by using dyes to accentuate proteins of interest, and by taking images at different stages, MALDI allows researchers to see protein changes (Karlsson et al., 2012). Not only does MALDI-IMS provide spatial localization of multiple proteins, but this instrument has the capability to provide a rough estimate of the quantity of protein or analyte of interest in one location. Karlsson, et al. (2012) looked at different sections of brain tissue and saw multiple changes in proteins using this method. The study by Karlsson, et al. (2012) in particular provided data suggesting that BMAA changes various proteins in the hippocampus along with neurodegenerative symptoms and neuronal cell apoptosis.

Thus far, matrix assisted laser desorption time of flight mass spectrometry (MALDI-ToF/ToF-MS/MS), has not been used for the quantitative analysis of BMAA, however, research has been done using MALDI-ToF/ToF-MS/MS to quantify other small molecules, including other amino acids (Alterman et al., 2004; Cohen et al., 2014; Dong et al., 2010; Gobey et al., 2005; Gogichaeva et al., 2006). Both HPLC-FLD and LC-MS/MS require extensive sample preparation, derivatization, acid hydrolysis, reverse-phase elution for separation, and a data acquisition time of 27 minutes (Faasen et al., 2012). If MALDI-ToF-MS proves to be a successful method for the quantification and qualification of BMAA, it would reduce the time for sample preparation and data acquisition to only a few seconds per sample, provide high throughput analysis, and would eliminate the need for derivatization, chromatographic separation, and modification of amino acids (Gogichaeva et al., 2012). Furthermore, coupling MALDI-ToF-MS to MALDI-IMS could be innovative in terms of imaging the location of BMAA in biological systems while quantifying the concentrations in these locations.
1.3. BMAA AND ITS STRUCTURAL ISOMER DAB

Banack et al. (2010) published a paper discussing a structural isomer of BMAA that often co-exists in nature. This structural isomer, 2-4-diaminobutyric acid (DAB) has the same mass as BMAA (118.13 m/z), but has a different structure and fragment ions (Figure 3) (Banack et al., 2010). The fragment ions of BMAA are 119 m/z to 102 m/z and 76 m/z, whereas the fragment ions for DAB are 119 m/z to 101 m/z and 74 m/z, respectively. The co-occurrence of BMAA and DAB, and the inability for HPLC-FLD and LC-MS to distinguish the structural isomers could lead to overestimation of BMAA in a biological sample. The amino acid DAB, is another non-protein amino acid that was first discovered, in a variety of antibiotics, as a bacterial metabolic product, and is commonly found in both prokaryotes and eukaryotes (Banack et al., 2010). Since 2010, many research groups have successfully separated BMAA from DAB to provide more accurate data on the quantitation of BMAA in biological samples.

Figure 3. Chemical structures of β-N-methylamino-L-alanine (BMAA) and its structural isomer 2-4-diaminobutyric acid (DAB)
1.4. LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

Chromatography, whether it be liquid or gas, is the separation of a mixture or solution based on three interactions: the mobile phase, the stationary phase, and the analytes within the mixture. Liquid chromatography uses a liquid mobile phase, as the name suggests, and carries the mixture through a column that contains a stationary phase. Within this column, the analytes in the mixture interact with the stationary phase, passing through the column at different times. The relative affinity of the analyte to the stationary phase determines the time in which it will elute, or its retention time. On the basis of an analytes chemical structure and polarity, it will interact differently with the mobile phase and surface of the stationary phase. These differences in interaction, result in different retention times of components in a mixture. Liquid chromatography is commonly used in the study of natural products and toxins.

The type of mobile phase must be carefully selected. Common mobile phases used in liquid chromatography are acetonitrile, dioxane, ethanol, methanol, isopropanol, tetrahydrofuran, and water (Buszewski et al., 2012). Additives such as formic acid may be mixed into water or acetonitrile to shift the polarity of the mobile phase (Buszewski et al., 2012). For the analysis of BMAA, multiple mobile phases have been used, but are also dependent on the stationary phase of the column (Table 2). Table 2, illustrates common mobile phases that have been used for the study of BMAA in biological samples, along with chromatographic columns that have been used for each mobile phase. For the study of amino acids, particularly BMAA, a combination of acetonitrile, water, and a dilute acid is typically used (Murch et al., 2004).
The column used for amino acid analysis is dependent on the mobile phase, composition and polarity of the analyte, derivatization, and whether positive or negative mode is used. Research groups who have used AQC derivatization, typically use a C18 column, whereas groups who analyze BMAA in its natural form, or underivatized, typically use a hydrophilic Interaction liquid chromatography (HILIC) column.
Table 2. Methods of detection and quantitation of BMAA in biological samples in current literature.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Analytical Quantitation Method</th>
<th>Derivatization Method</th>
<th>Column ID</th>
<th>Mobile Phase</th>
<th>Instrument</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murch (2004)</td>
<td>LC-MS &amp; HPLC-FLD</td>
<td>AQC</td>
<td>Waters Nova-Pak C18</td>
<td>140mM sodium acetate, 5.6mM trimethylamine pH 5.2 (mobile phase A), 60% ACN in water (mobile phase B)</td>
<td>Agilent liquid chromatography mass spectrometer, single quad, DAD</td>
</tr>
<tr>
<td>Rosen (2008)</td>
<td>LC-MS/MS</td>
<td>NONE</td>
<td>Zic®-HILIC (5μm, 50x2.1mm)</td>
<td>ACN and 60mM FA in water</td>
<td>Micromass Quattro Ultima or API 4000 QTRAP and Waters Alliance 2690 or Agilent 1100</td>
</tr>
<tr>
<td>Faassen (2009)</td>
<td>LC-MS/MS</td>
<td>NONE</td>
<td>Zic®-HILIC (5μm, 2.1x150mm)</td>
<td>65% ACN, 35% water and 0.1% FA</td>
<td>Agilent 1200 LC and an Agilent G6410A QQQ</td>
</tr>
<tr>
<td>Spacil (2010)</td>
<td>LC-MS/MS</td>
<td>AQC</td>
<td>Hypersil GOLD C18</td>
<td>0.1% FA in water and ACN (93:7) (mobile phase A), 0.1% FA in ACN (mobile phase B)</td>
<td>API 2000™ LC-MS/MS QQQ</td>
</tr>
<tr>
<td>Brand (2010)</td>
<td>LC-MS/MS</td>
<td>AQC</td>
<td>Thermo Hypersil GOLD (100mmx2.1mm, 3μm particle column)</td>
<td>0.1% FA in water (mobile phase A), 0.1% FA in ACN (mobile phase B)</td>
<td>QQQ, Waters 2475 Multi-λ-Fluorescence Detector</td>
</tr>
<tr>
<td>Esterhuizen-Londt (2011)</td>
<td>LC-MS</td>
<td>EZ: faast™ amino acid analysis kit</td>
<td>Phenomenex AAA-MS 250x2.0mm</td>
<td>10mM ammonium formate in water (mobile phase A), 10mM ammonium formate in methanol (mobile phase B)</td>
<td>Shimadzu LC 20AB and Shimadzu 2010 EV</td>
</tr>
<tr>
<td>Christensen (2012)</td>
<td>LC-MS/MS</td>
<td>AQC</td>
<td>Waters Xbridge 4.6x150mm C18 column</td>
<td>Proprietary Waters AccQ-Tag Ultra Eluents</td>
<td>Agilent Technologies 1200 LC with an Agilent Technologies G6410A QQQ</td>
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<tr>
<td>Glover (2012)</td>
<td>LC-TOF-MS</td>
<td>AQC</td>
<td>Waters amino acid analysis column (100x2.1mm; 1.7μm, C18)</td>
<td>0.1% FA in water (mobile phase A), ACN (mobile phase B)</td>
<td>Single quadrapole mass spectrometer (Waters EMD 1000) TOF-MS; Waters LCT Premier</td>
</tr>
<tr>
<td>Faassen (2012)</td>
<td>LC-MS/MS (derivatized and underivatized) and HPLC-FLD</td>
<td>NONE and AQC</td>
<td><strong>LC-MS/MS</strong>: (derivatized) Zorbax Eclipse AAA (4.6x75mm, 3.5μm column) (underivatized) Zic®-HILIC (2.1x150mm, 5μm diameter column) <strong>HPLC-FLD</strong>: Nova-Pak C18 (3.9x300mm, 4μm column)</td>
<td><strong>LC-MS/MS</strong>: ACN with 0.1% FA (mobile phase A), Millipore water and 0.1% FA (mobile phase B) <strong>HPLC-FLD</strong>: 140mM sodium acetate and 5.6mM trietylamine in Millipore water pH 5.2 (mobile phase A), ACN (mobile phase B), Millipore water (mobile phase C)</td>
<td>LC-MS/MS: Agilent 1200LC and Agilent 6401A QQQ <strong>HPLC-FLD</strong>: Agilent 1100 LC-FLD</td>
</tr>
<tr>
<td>Mondo (2012)</td>
<td>LC-MS/MS and HPLD-FLD</td>
<td>AQC</td>
<td>Waters Nova-Pak C18 column (3.9mmx300mm)</td>
<td><strong>LC-MS/MS</strong>: Aqueous 0.1% FA (mobile phase A), 0.1% FA in ACN (mobile phase B) <strong>HPLC-FLD</strong>: 140mM sodium acetate, 5.6mM trietylamine, pH 5.2 (mobile phase A), 52% ACN in water (mobile phase B)</td>
<td>LC-MS/MS: Triple quadrapole system <strong>HPLC-FLD</strong>: Waters 2475 Multi λ-Fluorescence Detector</td>
</tr>
<tr>
<td>Author (Year)</td>
<td>Methodology</td>
<td>Columns</td>
<td>Mobile Phases</td>
<td>Instrumentation</td>
<td></td>
</tr>
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</tr>
<tr>
<td>Jiang (2012)</td>
<td>LC-MS/MS</td>
<td>AQC</td>
<td><strong>HPLC:</strong> Hypersil GOLD C18 column (100x2.1mm, 3μm particle size) <strong>UHPLC:</strong> Agilent Bonus RP Rapid Resolution High Throughput (RRHT) column (100x2.1mm, 1.8μm particle size)</td>
<td>Triple quadrupole system</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>HPLC:</strong> 5% ACN in water with 0.3% acetic acid and 0.0005% TFA (mobile phase A), ACN with 0.3% acetic acid and 0.0005% TFA (mobile phase B) <strong>UHPLC:</strong> 0.1% FA in water (mobile phase A), 0.1% FA in ACN (mobile phase B)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jiang (2013)</td>
<td>LC-MS/MS</td>
<td>AQC</td>
<td>ACCQ-TAG™ ULTRA C18 column (100x2.1mm, 1.7μm particle size)</td>
<td>TSQ Vantage triple quadrupole mass spectrometer</td>
<td></td>
</tr>
<tr>
<td>Dunlop (2013)</td>
<td>LC-MS/MS</td>
<td>AQC</td>
<td>Waters AccQTag Ultra C18 column (2.1x100mm)</td>
<td>Thermo Scientific Finnigan TSQ Quantum Ultra AM tandem mass spectrometer QQQ</td>
<td></td>
</tr>
<tr>
<td>Masseret (2013)</td>
<td>LC-MS/MS</td>
<td>AQC</td>
<td>Waters AccQTag Ultra column (2.1x100mm)</td>
<td>Thermo Scientific Finnigan TSQ Quantum Ultra AM tandem mass spectrometer QQQ</td>
<td></td>
</tr>
<tr>
<td>Faassen (2013)</td>
<td>LC-MS/MS</td>
<td>NONE</td>
<td>ZIC®-HILIC column (2.1x150mm, 5μm diameter)</td>
<td>Agilent 1200 LC and an Agilent 6410A QQQ</td>
<td></td>
</tr>
<tr>
<td>Author</td>
<td>Methodology</td>
<td>AQC</td>
<td>Mobile Phase A</td>
<td>Mobile Phase B</td>
<td>Instrumentation</td>
</tr>
<tr>
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</tr>
<tr>
<td>Karlsson</td>
<td>LC-MS/MS</td>
<td>AQC</td>
<td>5% ACN in water with 0.3% acetic acid (mobile phase A), ACN with 0.3% acetic acid (mobile phase B)</td>
<td>TSQ Vantage QQQ</td>
<td></td>
</tr>
<tr>
<td>Mondo (2014)</td>
<td>LC-MS/MS and HPLC-FLD</td>
<td>AQC</td>
<td>LC-MS/MS: BEH C18 column (150x2.1mm, 1.7μm) HPLC-FLD: Waters Nova-Pak C18 column (3.9x300mm)</td>
<td>LC-MS/MS: 20mM ammonium formate with 0.2% FA (mobile phase A), 0.1% FA in ACN (mobile phase B) HPLD-FLD: 140mM sodium acetate, 5.6mM trimethylamine, pH 5.7 (mobile phase A), 52@ aqueous ACN (mobile phase B)</td>
<td>LC-MS/MS: Waters Acquity I-Class UPLC system HPLC-FLD: Waters 1525 Binary HPLC, Waters 2475 Multi λ-Fluorescence detector</td>
</tr>
<tr>
<td>Banack (2014)</td>
<td>LC-MS/MS</td>
<td>AQC</td>
<td>0.1% FA in water (mobile phase A), 0.1% FA in ACN (mobile phase B)</td>
<td>Waters Acquity-UHPLC and Thermo TSQ QQQ</td>
<td></td>
</tr>
<tr>
<td>Jiao (2014)</td>
<td>LC-MS/MS</td>
<td>AQC</td>
<td>28% ACN in water with 0.1% NH₄COOH</td>
<td>Agilent 1290/6460 HPLC/MS/MS spectrometry system with ESI</td>
<td></td>
</tr>
<tr>
<td>Baptista</td>
<td>LC-MS/MS</td>
<td>NONE</td>
<td>ACN with 0.1% FA (mobile phase A), Deionized water with 0.1% FA (mobile phase B)</td>
<td>Thermo LCQ Fleet Ion Trap LC-MS system</td>
<td></td>
</tr>
</tbody>
</table>
1.5. HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY (HILIC)

The HILIC column was first developed and published in by Linden et al. (1975). Hydrophilic interaction liquid chromatography (HILIC), is typically described as a variant on normal phase liquid chromatography in which the mobile phase used is less polar than the stationary phase (Buszewski et al., 2012). Common stationary phases are similar to those utilized in normal phase liquid chromatography, such as, silica. In contrast to normal phase liquid chromatography, HILIC employs a mobile phase that is often used in reverse phase liquid chromatography (Buszewski et al., 2012). The use of HILIC has become more and more popular as this development allows for the analysis of small molecules in complex matrices and can be used for ion chromatography to analyze analytes that are positively or negatively charged (Buszewski et al., 2012). There is some debate as to how retention is achieved using HILIC, however, the most current explanation described by Buszewski et al. (2012), is depicted below (Figure 4). On the basis of Buszewski’s theory, separation is achieved by partitioning, or liquid/liquid extraction, per say (Buszewski et al., 2012). With a mobile phase composed of acetonitrile and water, the water forms a layer on the surface of the very hydrophilic stationary phase (silica) (Buszewski et al., 2012). The acetonitrile forms an additional layer on top of the aqueous layer (Buszewski et al., 2012). This interaction creates a type of liquid/liquid attraction in which the analyte moves towards the hydrophilic stationary phase, being retained within the aqueous layer (Buszewski et al., 2012). On the basis of the increasing hydrophilicity of the analyte, elution off of the stationary phase of the column will occur later, separating the analyte from the matrix.
1.6. MATRIX ASSISTED LASER DESORPTION IONIZATION (MALDI)

Matrix assisted laser desorption ionization (MALDI), is an ionization technique that requires the sample to be placed in a matrix that protects the sample from being destroyed by a laser that is applied to the sample/matrix. With MALDI-ToF-MS, a laser is applied to the sample/matrix, ionizing the sample and allowing the secondary ions to travel through the flight tube, separating them by mass and charge, and finally analyzing them by the ToF analyzer (Figure 5). Quantitative amino acid analysis has been done using MALDI-ToF-MS/MS, however, no research has been published using these techniques.
for the analysis of BMAA, and therefore, operating parameters had to be optimized for this particular amino acid. Previous research has suggested that BMAA becomes misincorporated into human proteins by replacing L-serine, and in a study done by Dunlop, et al. (2013), data provided evidence for this misincorporation. In a study done by Alterman, et al. (2004) at the University of Kansas, MALDI-ToF-MS/MS proved to be successful in the quantification and qualification of nineteen (19) amino acids using α-cyano-4-hydroxycinnamic acid (CHCA) as the matrix. Other matrices used in this study included meso-tetra (pentafluorophenyl) porphine (F20TTP), and 2,5-dihydroxybenzoic acid (DHB), however, with F20TTP and DHB, only 13 and 14 of the amino acids were observed, respectively (Alterman et al., 2004). Serine was not observed using DHB. On the basis of the success of this previous study, CHCA was the matrix of choice for the analysis of BMAA using MALDI-ToF-MS/MS, in addition to the data showing strong evidence that BMAA is capable of replacing L-serine (Alterman et al., 2004). For the analysis of amino acids, 1μL of CHCA is typically spotted onto each sample well, and allowed to dry. Once dry, 1μL of sample is spotted onto each sample well on top of the dried matrix. The plate is then allowed to air dry, and is then placed into the holder of the MALDI instrument.

As discussed by Gogichaeva, et al. (2012), the use of MALDI-ToF/ToF-MS/MS as a qualitative and quantitative method for amino acid analysis, has many advantages over liquid chromatography. The MALDI-ToF/ToF-MS/MS decreases the data acquisition time to only a few seconds as opposed to the data acquisition time using LC-MS/MS of 30-45 minutes. This research group also determined that MALDI-ToF/ToF-MS/MS is capable of distinguishing structural isomers (Gogichaeva et al., 2012). Derivatization and
chromatographic separation is not necessary with MALDI-ToF-MS, however, many methods using HPLC and LC-MS/MS require both.

Figure 5. Schematic diagram of MALDI-ToF-MS/MS instrument in reflector mode.

Figure 5 is a rudimentary diagram illustrating a MALDI-ToF-MS instrument used in reflector mode. Using reflector mode, the sample has more time in the flight tube, allowing for greater separation of a mixture, to differentiate between isobaric and isomeric isotopes. In linear mode, isomers cannot be distinguished from each other, and can lead to an overestimation of an analyte if an isomer is present in the sample.
1.7. SPECIFIC AIMS

The main objective of this research included laboratory experiments to develop a quantitative MALDI-TOF/TOF-MS/MS method to detect and quantify BMAA in a variety of matrices. Through a series of aims, the main objective was achieved. The aims presented in this study are described as follows:

i. Adapt/develop an LC-MS/MS method to detect and quantify BMAA in a variety of matrices

ii. Develop a MALDI-TOF/TOF-MS/MS method to detect and quantify BMAA in a variety of matrices

iii. Synthesize $^{13}$C-BMAA to use as an internal standard for both the MALDI-TOF/TOF-MS/MS and LC-MS/MS method

iv. Compare quantitative results from LC-MS/MS to that of MALDI-TOF/TOF-MS/MS

v. Apply MALDI-TOF/TOF-MS/MS method to biological samples in search of BMAA incorporation

2. MATERIALS AND SAMPLE PREPARATION

2.1. MATERIALS

Reference standards of L-BMAA hydrochloride was purchased from Sigma Aldrich (St. Louis, MO), and DL-2,4-DAB dihydrochloride was purchased from Alfa Aesar (Ward Hill, MA). Amino Acid Standard (AAS) was purchased from Sigma Aldrich (St. Louis, MO) in a solution of 0.1 N HCl (Table 2). Methyltyrosine was purchased from Sigma Aldrich (St. Louis, MO). Bovine Serum Albumin (BSA) was
purchased from Sigma Aldrich (St. Louis, MO). The $^{13}$C-BMAA was prepared in house according to a D$_3$-BMAA synthesis by Jiang, et al. (2013) with some modifications (see below). Acetonitrile, HCl, HPLC grade water, and chloroform were purchased from Fisher Scientific (Fair Lawn, NJ). Biological tissue samples were provided by Dr. Larry Brand (Division of Marine Biology and Fisheries, Rosentiel School of Marine and Atmospheric Science) in 2012, from the Johnson Key Basin, Florida. The standard stock solutions of both BMAA and DAB in powder form were prepared with HPLC grade water at 10mg/mL for BMAA and 1mg/mL for DAB, respectively. These stock solutions were diluted to varying concentrations dependent on sample. For methodology, please see Chapters 3 and 4.

The matrices, AAS, BSA, and biological tissue samples, were chosen for this research to demonstrate the validity of a MALDI-TOF/TOF-MS/MS method when spiking samples in increasingly complex matrices. The biological tissue samples (shrimp) provide an example of biological tissues from an aquatic system where the presence of BMAA has been seen in previous studies.
Table 3. Amino Acid Standard Composition

<table>
<thead>
<tr>
<th>AMINO ACID</th>
<th>MOLECULAR FORMULA</th>
<th>CONCENTRATION (μMoles/mL)</th>
<th>MOLECULAR WEIGHT</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Alanine</td>
<td>C₃H₇NO₂</td>
<td>2.50</td>
<td>89.09</td>
</tr>
<tr>
<td>Ammonium Chloride</td>
<td>NH₄Cl</td>
<td>2.50</td>
<td>53.49</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>C₆H₁₄N₄O₂</td>
<td>2.50</td>
<td>174.2</td>
</tr>
<tr>
<td>L-Aspartic Acid</td>
<td>C₄H₇NO₄</td>
<td>2.50</td>
<td>133.1</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>C₆H₁₂N₂O₄S₂</td>
<td>1.25</td>
<td>240.3</td>
</tr>
<tr>
<td>L-Glutamic Acid</td>
<td>C₅H₉NO₄</td>
<td>2.50</td>
<td>147.1</td>
</tr>
<tr>
<td>Glycine</td>
<td>C₂H₇NO₂</td>
<td>2.50</td>
<td>75.07</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>C₆H₉N₃O₂</td>
<td>2.50</td>
<td>155.2</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>C₆H₁₃NO₂</td>
<td>2.50</td>
<td>131.2</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>C₆H₁₃NO₂</td>
<td>2.50</td>
<td>131.2</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>C₆H₁₄N₂O₂</td>
<td>2.50</td>
<td>146.2</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>C₅H₁₁NO₂S</td>
<td>2.50</td>
<td>149.2</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>C₉H₁₁NO₂</td>
<td>2.50</td>
<td>165.2</td>
</tr>
<tr>
<td>L-Proline</td>
<td>C₅H₉NO₂</td>
<td>2.50</td>
<td>115.1</td>
</tr>
<tr>
<td>L-Serine</td>
<td>C₃H₇NO₃</td>
<td>2.50</td>
<td>105.1</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>C₄H₉NO₃</td>
<td>2.50</td>
<td>119.1</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>C₉H₁₁NO₃</td>
<td>2.50</td>
<td>181.2</td>
</tr>
<tr>
<td>L-Valine</td>
<td>C₅H₁₁NO₂</td>
<td>2.50</td>
<td>117.2</td>
</tr>
</tbody>
</table>
2.2. SAMPLE PREPARATION

2.2.1. SYNTHESIS OF $^{13}$C-BMAA

The synthesis of $^{13}$C-BMAA was performed according to a D$_3$-BMAA synthesis by Jiang (2013) with some modifications. Sodium hydroxide (0.22g) was added to a room temperature solution of (0.25g) $^{13}$C-methylamine hydrochloride in 1mL HPLC grade water and stirred until dissolved. Two-Acetamidoacrylic acid (0.0925g) was then added to the solution and stirred at room temperature for 10 minutes. The mixture was then stirred for 22 hours at 35°C. The mixture was cooled back down to room temperature and the solution was placed in a rotary evaporator to remove methylamine. The crude product was dissolved in excess 3M HCl (2.0mL), and refluxed for 2 hours at 100°C. After refluxing, the sample was placed in a vacuum centrifuge to remove HCl in vacuo. The product was recrystallized in an ice bath (~4°C) using ethanol and water. The crystals were then filtered and washed with cold ethanol four times, and then dried under vacuum. The final yield of $^{13}$C-BMAA was 169.5mg. The NMR spectral data is consistent with the desired outcome (Figure 7).

![Proposed mechanism of $^{13}$C-BMAA](image)

Figure 6. Proposed mechanism of $^{13}$C-BMAA

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Figure 7. $^1$H-NMR spectra of unlabeled BMAA (top figure) and $^{13}$C-BMAA (bottom figure). The singlet peak at 3.083 ppm is absent in the unlabeled BMAA.
2.2.2. MALDI MATRIX

The α-Cyano-4-hydroxycinnamic acid (CHCA) matrix was prepared according to Gogichaeva et al. (2012) without recrystallization, because of a higher purity compound that has become available since the publication of the paper. A solution of 8.5mL ACN, 2.47mL water, and 30μL of 10% trifluoroacetic acid was prepared. A 1mL aliquot of this solution was placed in a 1.5mL vial and CHCA (powdered form) was added until saturation. The solution was prepared fresh each day of analysis to ensure no degradation of the matrix.

2.2.3. STANDARDS FOR METHOD DEVELOPMENT

Stock solutions with concentrations of 1mg/mL (1000ppm) of BMAA and DAB were prepared in HPLC grade water and used for the standard calibration curve, and to spike AAS, BSA, and biological tissue homogenates for method development. The stock solutions were diluted to different concentrations depending on the analysis. The structural isomer DAB, was used as an internal standard for MALDI-TOF/TOF-MS/MS. Validation procedures were done using BMAA and DAB standards.

2.2.4. BMAA AND AMINO ACID STANDARDS

Stock solutions, 1mL of 100μM AAS were prepared and spiked with 200μM of methyltyrosine as an internal standard (IS) and increasing concentrations of BMAA (0, 20, 50, 100, and 300μM). The solutions were prepared using HPLC grade water and stored in a refrigerator at 4°C until analysis.
2.2.5. BMAA AND BOVINE SERUM ALBUMIN

Two methods of hydrolysis were employed for the analysis of BSA as part of methods development, and subsequent analysis of tissue samples. In the first method, a microwave-assisted acid hydrolysis was used. According to previous literature, this method of hydrolysis vastly reduces the hydrolysis time, while still providing comparable digestion of proteins (Zhong et al., 2004). For development of this method, 10μL (1μg/1μL) of aqueous BSA solution was combined with 10μL of 20mM Dithiothreitol (DTT), varying concentrations of BMAA (0, 20, 50, 100, 120, and 300μM) and constant concentration of DAB (IS) (100μM) in 1.5mL polypropylene Eppendorf tubes (6 tubes). The tube was then capped, sealed with tephlon tape and incubated for 20 minutes at 60°C. Once this was complete, the tape was removed and 20μL of 50% (vol/vol) trifluoroacetic acid (TFA) was added. The Eppendorf tubes were resealed, and placed in a round bubble rack floating in at least 100mL of distilled water in a round polypropylene container (Figure 8). The samples were then irradiated for 7.5 minutes in 900 watt microwave. Once irradiation was complete, samples were dried in vacuo using a Speedvac at 45°C, and then resuspended in 50μL of 0.1% aqueous formic acid. The samples were centrifuged at 14,000rpm for 5 minutes to remove particulate matter and the supernatant was stored at 4°C until analysis with MALDI-TOF/TOF-MS/MS. For analysis, 1μL of CHCA matrix was spotted onto the MALDI plate, allowed to dry, and then 1μL of BSA sample was spotted on top of the dried matrix. Each sample was analyzed and assayed in duplicate.
For the second method of hydrolysis, a more traditional hydrolysis was employed. Bovine serum albumin (BSA) (0.5g) was weighed out and transferred to 10mL of 6M HCl and hydrolyzed for 24 hours at 110°C. After hydrolysis, the sample was filtered using vacuum filtration (Whatman 42.5mm 0 circles, 11μm porosity), and further filtered with a 25mm syringe filter (Fisherbrand, 0.2μm, PTFE, Non-sterile). The filtered extract was then transferred to a 25 mL round bottom flask and attached to a rotary evaporator (BUCHI Rotovapor R-3, Recirculating Chiller F-100, and Vacuum Pump V-700) with the water bath set at 65°C until dry. The residue was resuspended in 10 mL of HPLC grade water, and washed with 10 mL of chloroform. The organic layer was discarded, and the aqueous layer was placed into a 25 mL glass vial and stored at 4°C until analysis. Samples (40μL) were prepared containing 20μL of CHCA matrix, BSA solution, and varying concentrations of BMAA and 50ppm DAB (IS). The concentrations for BMAA
were 0, 10, 25, 50, 75, and 125ppm. Each sample (2μL) was spotted onto a MALDI plate with two spots for each concentration. All BSA samples were analyzed in duplicate and quantitation of BMAA was done using MALDI-TOF/TOF-MS/MS.

2.2.6. ANALYSIS OF BIOLOGICAL TISSUES

Biological tissues (shrimp), were collected by Larry Brand in 2011 from the Johnson Key Basin, ENP, Florida. The tissues were stored in ethyl alcohol prior to extraction procedures. Tissues were soaked in 500mL MilliQ water for an hour to wash. Tissue samples were then submerged in liquid nitrogen and homogenized using a sterilized food processor. Tissue homogenates (1g) were hydrolyzed in 10mL of 6M HCl for 24 hours at 110°C (Figure 9). After hydrolysis, particulate matter was removed using vacuum filtration (Whatman 42.5mm 0 circles, 11μm porosity) and further filtered with 25mm syringe filters (Fisherbrand, 0.2μm, PTFE, Non-sterile). Filtered extract was transferred into ten 1.5mL Eppendorf tubes, 1mL aliquots per tube. The samples were dried in vacuo using a Speedvac at 45°C. The samples were then spiked with increasing concentrations of BMAA (0, 1, 10, 75, and 200ppm) and resuspended to 1mL with 0.1% aqueous formic acid. To remove any residual lipids prior to analysis, the resuspended extract was washed with 500μL of chloroform. The chloroform layer was then discarded. Further, a solid-phase extraction (SPE) method developed by Li et al., in 2012 was adapted for these biological samples (Li et al., 2012). Phenomenex Strata-X-C SPE 33μm Polymeric Strong Cation, 200mg/6mL cartridges were used for an SPE clean-up. The columns were activated with 5mL of methanol, followed by 5mL of 0.1% formic acid. The samples (1mL) were then applied to the columns. The samples were washed
again with 5mL of 1% formic acid, and then 5mL of methanol. Finally, the analyte was eluted with 5mL of NH₄OH v/v in methanol and collected in clean, 8mL glass vials. The samples were then dried under nitrogen and resuspended in 1mL of HPLC grade water. Each sample was spiked with 25ppm of DAB as an internal standard (IS). The samples were stored at -4°C until analysis. A schematic of the sample preparation can be seen in Figure 10. All biological tissue samples were analyzed and assayed in triplicate. Quantitation of BMAA was performed using MALDI-TOF/TOF-MS/MS and the method of standard addition.

Figure 9. Acid Hydrolysis Apparatus
Figure 10. Sample preparation schematic of biological tissues with a solid phase extraction clean-up for the quantitative analysis of BMAA by MALDI-TOF/TOF-MS/MS.

3. ANALYSIS BY LC-MS/MS

3.1. INSTRUMENTAL PARAMETERS

The LC-MS/MS analysis was performed using a Shimadzu Prominence LC-20AD Ultra-Fast Liquid Chromatograph combined with an AB Sciex QTRAP 5500 Triple-Quadrupole mass spectrometer, equipped with a Turbospray ESI source supplied
with nitrogen gas. The BMAA was separated from other amino acids using hydrophilic interaction chromatography (HILIC). A binary gradient system was used with 0.1% formic acid dissolved in HPLC grade water (mobile phase A), and 0.1% formic acid in acetonitrile (mobile phase B) with a flow rate of 0.5mL/min. The column temperature was set at 40°C, while the auto sampler temperature was set at 15°C. The elution gradient (10 minutes) was as follows: time 0 = 30% A; 0.5min. = 30% A; 5min. = 95% A; 6.25min. = 95% A; 6.5min. = 5% A; 8min. = 5% A; 8.25min. = 30% A; 10min. = 30% A. Separation was achieved using a Phenomenex Kinetex HILIC 100 x 4.6mm, 2.6μm particle size column. The mass spectrometer was operated in positive mode. The source temperature was 600°C and the ESI spray voltage was set at 5,500V. Each of the following values are arbitrary, however, the auxiliary gas setting was 40, the nebulizer gas setting was 60, and the curtain gas setting was 30.

The protonated molecular ion of BMAA (119.033 m/z) was used as the parent ion for multiple reaction monitoring (MRM) analysis. The following transitions were monitored: 119>102 (collision energy of 15V) and 119>76 (collision energy 17V). The analyte BMAA, was successfully separated from its isomer DAB and 13C-BMAA. As seen in figure 11, with this LC-MS/MS method, for the first transition (BMAA 119>102, DAB 119>101, and 13C-BMAA 120>103), BMAA elutes at 5.6 minutes, whereas DAB elutes at 5.54 minutes, and 13C-BMAA elutes at 5.49 minutes. Peak area and intensity values for each analysis were exported to Microsoft Excel and processed. Analyses were done at the Advanced Mass Spectrometry Facility at Florida International University.
Figure 11. LC-MS/MS spectra of BMAA, DAB, and $^{13}$C-BMAA standards.
3.2. RESULTS/DISCUSSION

3.2.1. ANALYSIS OF STANDARDS

Varying concentrations of BMAA (0, 0.05, 0.1, 0.25, 0.5, and 1.0ppm) were spiked with 1ppm of $^{13}$C-BMAA and 1ppm of DAB. Both DAB and $^{13}$C-BMAA were used as internal standards to determine which would provide a better linearity. Multiple linear regressions were performed and can be seen in Figures 12-15. Figure 12 describes the linear regression of BMAA with $^{13}$C-BMAA as the internal standard from the first transition of 119.033>102.2 m/z for BMAA and 120.062>103.1 m/z. The analyte, BMAA, was expected to elute at 5.49 minutes, whereas $^{13}$C-BMAA was expected to elute at 5.47 minutes. Further, Figure 13 describes the linear regression of BMAA again with $^{13}$C-BMAA as the internal standard with a different transition. The transition used for BMAA was 119.033>76.1 m/z, whereas the transition for $^{13}$C-BMAA was 120.062>103.1 m/z (Figure 13).

Figure 14 shows the linear relationship between BMAA with DAB as the internal standard from the first transition of 119.033>102.2 m/z for BMAA and 119.033>101.2 m/z for DAB. The analyte, BMAA, was expected to elute at 5.49 minutes, whereas DAB was expected to elute at 5.52 minutes for this particular transition. Figure 15 also shows the linear relationship of BMAA with DAB as the internal standard, but with the transitions of 119.033>76.1 m/z for BMAA and 119.033>101.2 m/z for DAB. The particular transition just described, provided the greatest linearity with an R$^2$ value of 0.9999. The chromatograms are shown in Figures 16-23 for the calibration standards and amino acid mixtures.
Figure 12. Representative LC-MS/MS calibration curve for the concentration ratios of BMAA to $^{13}$C-BMAA (IS) in ppm against the peak area ratios of BMAA to $^{13}$C-BMAA (IS). The transitions for BMAA and $^{13}$C-BMAA are 119.03>102.2 m/z and 120.062>103.1 m/z, respectively.

$$y = 3.856x + 6.0096$$
$$R^2 = 0.9796$$

Figure 13. Representative LC-MS/MS calibration curve for the concentration ratios of BMAA to $^{13}$C-BMAA (IS) in ppm against the peak area ratios of BMAA to $^{13}$C-BMAA (IS). The transitions for BMAA and $^{13}$C-BMAA are 119.03>76.1 m/z and 120.062>103.1 m/z, respectively.

$$y = -1.7656x^2 + 4.4781x + 0.0332$$
$$R^2 = 0.9995$$
Figure 14. Representative LC-MS/MS calibration curve for the concentration ratios of BMAA to DAB (IS) in ppm against the peak area ratios of BMAA to DAB (IS). The transitions for BMAA and DAB are 119.033>102.2 m/z and 119.033>101.2 m/z, respectively.

Figure 15. Representative LC-MS/MS calibration curve for the concentration ratios of BMAA to DAB (IS) in ppm against the peak area ratios of BMAA to DAB (IS). The transitions for BMAA and DAB are 119.033>76.1 m/z and 119.033>101.2 m/z, respectively.
3.2.2. ANALYSIS OF BMAA IN AMINO ACID MATRIX

Varying concentrations of BMAA (0, 0.1, and 0.25) were spiked with 1ppm of $^{13}$C-BMAA and 1ppm of DAB in a 50ppm amino acid solution. Using the calibration curves above, the fourth linear regression was used to calculate the concentration of BMAA in each amino acid mixture. The peak areas from the BMAA transition of 119.003>76.1 m/z and DAB transition of 119>101.2 m/z of each spiked sample were exported to Microsoft Excel where they were processed. The peak area ratios of BMAA to DAB were calculated for each sample and then substituted into the linear regression equation from Figure 15. From the calculated value, percent recovery was then calculated using the equation.

$$
\text{%Recovery} = \frac{C_{\text{Calculated}} - C_{\text{from blank}}}{C_{\text{spiked}}} 
$$

The calculated concentration from the 0.1ppm spike was 0.102ppm which led to a % recovery of 102%. The calculated concentration from the 0.25ppm spike was 0.248ppm which led to a percent recovery of 99.2%. The calibration using the isotopically-labeled standard $^{13}$C-BMAA was not successful and those transitions could not be calculated with the amino acid mixture samples. The chromatograms are shown in Figures 16-23 for the calibration standards and the amino acid mixtures.
Figure 16. Chromatograms of water blanks, 0ppm BMAA and 0.05ppm BMAA standards with 1ppm spikes of $^{13}$C-BMAA and DAB as internal standards. BMAA transitions 119.033$\rightarrow$102.2 m/z and 119.033$\rightarrow$76.1 m/z and $^{13}$C-BMAA transition 120.062$\rightarrow$103.1 m/z are shown above. Compliments of the Advanced Mass Spectrometry Facility, FIU.
Figure 17. Chromatograms of 0.1ppm, 0.25ppm, and 0.5ppm BMAA standards with 1ppm spikes of $^{13}$C-BMAA and DAB as internal standards. BMAA transitions 119.033>102.2 m/z and 119.033>76.1 m/z and $^{13}$C-BMAA transition 120.062>103.1 m/z are shown above. Compliments of the Advanced Mass Spectrometry Facility, FIU.
Figure 18. Chromatograms of 0ppm and 1ppm BMAA standards with 1ppm spikes of $^{13}\text{C}$-BMAA and DAB as internal standards and 0ppm BMAA in an amino acid matrix with the same concentrations of internal standards ($^{13}\text{C}$-BMAA and DAB). BMAA transitions 119.033>102.2 m/z and 119.033>76.1 m/z and $^{13}\text{C}$-BMAA transition 120.062>103.1 m/z are shown above. Compliments of the Advanced Mass Spectrometry Facility, FIU.
Figure 19. Chromatograms of 0.1ppm, 0.25, and 0.5ppm BMAA in an amino acid matrix with 1ppm spikes of both internal standards (\(^{13}\)C-BMAA and DAB). BMAA transitions 119.033>102.2 m/z and 119.033>76.1 m/z and \(^{13}\)C-BMAA transition 120.062>103.1 m/z are shown above. Compliments of the Advanced Mass Spectrometry Facility, FIU.
Figure 20. Chromatograms of water blank, 0.0ppm and 0.05ppm BMAA standards with 1ppm spikes of $^{13}$C-BMAA and DAB as internal standards. BMAA transitions 119.033>102.2 m/z and 119.033>76.1 m/z and DAB transition 119.033>101.2 m/z are shown above. Compliments of the Advanced Mass Spectrometry Facility, FIU.
Figure 21. Chromatograms of 0.1ppm, 0.25ppm and 0.5ppm BMAA standards with 1ppm spikes of $^{13}$C-BMAA and DAB as internal standards. BMAA transitions 119.033>102.2 m/z and 119.033>76.1 m/z and DAB transition 119.033>101.2 m/z are shown above. Compliments of the Advanced Mass Spectrometry Facility, FIU.
Figure 22. Chromatograms of 0ppm and 1ppm BMAA standards with 1ppm spikes of $^{13}$C-BMAA and DAB as internal standards and 0ppm BMAA in an amino acid matrix with the same concentrations of internal standards ($^{13}$C-BMAA and DAB). BMAA transitions 119.033>102.2 m/z and 119.033>76.1 m/z and DAB transition 119.033>101.2 m/z are shown above. Compliments of the Advanced Mass Spectrometry Facility, FIU.
Figure 23. Chromatograms of 0.1ppm, 0.25, and 0.5ppm BMAA in an amino acid matrix with 1ppm spikes of both internal standards ($^{13}$C-BMAA and DAB). BMAA transitions 119.033>$102.2$ m/z and 119.033>$76.1$ m/z and DAB transition 119.033>$101.2$ m/z are shown above. Compliments of the Advanced Mass Spectrometry Facility, FIU.
3.3. CONCLUSIONS

The LC-MS/MS data in chapter 3 are in agreement with current literature using a HILIC column and underivatized BMAA. The developed method on the AB Sciex QTRAP 5500 Triple-Quadrupole mass spectrometer successfully separated BMAA from its structural isomer DAB. By using DAB as an internal standard, exceptional linearity was achieved. As previously reported in literature, DAB tends to co-occur with BMAA in nature. With successful separation of the isomers, using DAB as an internal standard proves to be a valuable internal standard for the quantitation of BMAA. To date, I am unaware of DAB being used as an internal standard.

The use of $^{13}$C-BMAA as an internal standard was unsuccessful. In the chromatogram for 0ppm BMAA in Figure 16, a peak for BMAA still appeared, however, this peak is not present in the water blank that was injected prior to and after the samples. This peak indicates that the $^{13}$C-BMAA that was synthesized is not isotopically pure and did not get completely labeled during the synthesis.

4. ANALYSIS BY MALDI-TOF/TOF-MS/MS

4.1. INSTRUMENTAL PARAMETERS

The MALDI-TOF/TOF-MS/MS analysis was performed using a Bruker Autoflex III Smartbeam MALDI-TOF/TOF-MS/MS. All spectra were acquired using reflector mode and operated in positive ion mode. The laser was set at 85% with a detector gain of 25x (1,890V). In order to cover as much of the target area as possible, the random walk mode was set to complete sample with 300 firing positions per spot, and 900 shots total per spectra. The frequency of the laser was 200Hz which describes how many shots per
second occurred. The sample rate was set to 2.00 GS/s for the highest resolution. LIFT mode was used with the parent mass set to 119.14 Da. The acquisition mode was set to fragments in order to differentiate between isomers. Data was collected over the mass range of 80-143. Two to three spots were prepared for each sample. Analyses were done at the Advanced Mass Spectrometry Facility at Florida International University.

Two methods of matrix deposition were utilized: a dried droplet approach, and a mixed droplet approach. With the dried droplet approach, 1μL of CHCA matrix solution was deposited onto a MALDI plate in each individual wells (MTP 384 ground steel, ID 210) (Figure 24). Once dry, a 1μL aliquot of sample is deposited on top of the dried CHCA spot. With the mixed droplet approach, equal parts of CHCA matrix and sample were combined and vortexed. Once completely mixed, a 2μL aliquot of matrix/sample were deposed in each well.

Figure 24. Image of 384 well MALDI Plate.
Spectral data was exported to flexAnalysis Version 3.4 (Build 70) (Copyright © Bruker Daltonik GmbH) where the raw data was processed and peak height (intensity) and peak area values were determined. The mass list values were then exported to Microsoft Excel where peak intensity or peak area was plotted against that of methytyrosine (IS) or DAB (IS). Calibration curves were created for each of the different analyses (standards, amino acid matrix, BSA matrix, and biological tissue matrix).

4.2. BMAA FULL SCAN ANALYSES

4.2.1. FULL SCAN BMAA ANALYSIS

Varying concentrations of BMAA (0, 20, 60, 800, 100, 200, and 300μM) were spiked with 200μM of methytyrosine (IS). The parent ion of BMAA is seen at 119 m/z and the parent ion of the IS is seen at 196 m/z. The spectra can be seen in figure 25. The mass list values for the peak intensity were exported to Microsoft Excel and the representative calibration curve can be seen in figure 26. In full scan mode, successful separation of the BMAA standard and methytyrosine was accomplished. The representative calibration curve gives an $R^2$ value of 0.9881. A higher $R^2$ value could be obtained with more replicates.
Figure 25. Positive Ion MALDI-TOF-MS spectrum of BMAA and methylyrosine (IS) with CHCA as the matrix. BMAA Concentrations ranged from 0-300μM and methylyrosine was at 200μM. The BMAA parent ion is seen at 119 m/z and the methylyrosine parent ion at 196 m/z.
4.2.2. FULL SCAN BMAA ANALYSIS IN AMINO ACID MATRIX

An amino acid matrix was spiked with 200μM methyltyrosine (IS) and varying amounts of BMAA (0, 20, 50, 100, and 250μM). All spectra were obtained using the same parameters. Peak intensities and areas were exported to Microsoft Excel and processed. This set of experiments was performed to determine if the developed MALDI-TOF-MS method could still successfully quantify BMAA in a more complex matrix. The linear regression (Figure 27) of peak area intensities of BMAA vs. methyltyrosine (IS) provide good linearity, with a R² value of 0.9997. At the completion of this set of experiments, it was determined to continue with a MALDI-TOF/TOF-MS/MS method to attempt to discriminate and quantify BMAA with the presence of one of its structural isomers, DAB.

Figure 26. Representative calibration curve for the concentrations of BMAA in μM against the peak intensity ratios of BMAA to methyltyrosine (IS) with CHCA as the MALDI matrix. Parent ions for BMAA and methyltyrosine were 119 and 196, respectively.
**4.3. MALDI-TOF/TOF-MS/MS ANALYSES**

**4.3.1. ANALYSIS OF STANDARDS**

Varying concentrations of BMAA (0, 2, 5, 10, 15, 25 ppm) were spiked with 25 ppm DAB as an internal standard. Methyltyrosine was no longer used as an internal standard once MALDI-TOF/TOF-MS/MS was used, due to issues with ionizing two different parent ions at the same time. Structural isomer, DAB, has the same molecular mass as BMAA, however, has different fragment ions. It was therefore decided that if samples were spiked with the same concentration of DAB regardless of prior presence of DAB in a sample, it could be used as an internal standard. These analyses were run in triplicate for validation procedures. Figure 28 represents the spectra for one replicate of each concentration. As the concentration of BMAA increases, the intensity of the signal increases.

![Concentration (µM) vs. Peak Intensity Ratio (BMAA/IS)](image)

\[ y = 0.0001x^2 + 0.0136x - 0.0808 \]

\[ R^2 = 0.9997 \]
at 102 m/z increases. The intensity of the signal of DAB, remains constant. Following current literature, these fragment ions are specific to each analyte. Figure 29 represents the representative calibration curve for the average ratio of peak intensities of BMAA and DAB (IS). The error bars were calculated using the standard deviation. In this linear regression, a $R^2$ value of 0.9983 is seen, showing good linearity with DAB as an internal standard. The same parameters were used for each spectra, allowing for consistency and reproducibility.

Figure 28. Positive Ion MALDI-TOF/TOF-MS/MS spectrum of BMAA and DAB (IS) with CHCA as the matrix. BMAA Concentrations ranged from 0-25ppm and the concentration of DAB was at 25ppm. The BMAA fragment ion is seen at 102 m/z and the DAB fragment ion at 101 m/z.
4.3.2. ANALYSIS OF $^{13}$C-BMAA

Within this set of experiments, the use of $^{13}$C-BMAA as an internal standard was explored. As described in chapter 2, $^{13}$C-BMAA was synthesized in our laboratory at Florida International University. In current literature, deuterated BMAA is often used as an internal standard. The use of $^{13}$C-BMAA as an internal standard could have multiple benefits: visualization with NMR, more stability than deuterated BMAA, and similar fractionation to BMAA. In this set of experiments, $^{13}$C-BMAA solution was spotted directly on top of air-dried CHCA matrix and compared to the spectra of a mixture of 300μM BMAA with 100μM DAB. Spectra of the fragment ions of BMAA, DAB, and $^{13}$C-BMAA for the transitions 119>102, 119>101, and 120>103, respectively, can be
seen in Figure 30. Note that each analyte shows fragmentation peaks that differ by 1 m/z for this transition. The transitions 119>73 and 119>43.8 for BMAA and 120>74 and 120>44.7 are seen in the spectra of Figure 31.

The percent yield of $^{13}$C labelled BMAA was questioned with the LC-MS/MS data, and was therefore not used as an internal standard for the remainder of the experiments.

Figure 30. Positive Ion MALDI-TOF/TOF-MS/MS spectrum of BMAA and DAB (top), and $^{13}$C-BMAA (bottom) with CHCA as the matrix. BMAA Concentration was at 300μM, the concentration of DAB was 100μM, and the concentration of $^{13}$C-BMAA was at 300μM. The BMAA fragment ion is seen at 102 m/z, DAB at 101 m/z, and $^{13}$C-BMAA at 103 m/z.
Figure 31. Positive Ion MALDI-TOF/TOF-MS/MS spectrum of BMAA (top) and $^{13}$C-BMAA (bottom) with CHCA as the matrix. BMAA Concentration was at 300μM and the concentration of $^{13}$C-BMAA was at 300μM. The BMAA fragment ions are seen at 73 m/z and 43.8 m/z, and $^{13}$C-BMAA at 74 m/z and 44.7 m/z.

4.3.3. ANALYSIS OF BMAA IN BSA MATRIX

Bovine serum albumin was hydrolyzed overnight as described in the methodology section. The hydrolyzed solution was separated into five samples and spiked with 50ppm of DAB (IS) and increasing concentrations of BMAA (0, 10, 50, 75, and 125ppm). With this matrix, and this method of hydrolysis, the amount of noise and matrix effects increased the limit of detection to over 100ppm. The spectra in Figure 32 illustrate the
amount of noise. The peak shape and resolution rapidly decrease below the BMAA concentration of 125ppm.

Figure 32. Positive Ion MALDI-TOF/TOF-MS/MS spectrum of BMAA and DAB (IS) in a hydrolyzed BSA solution with CHCA as the matrix. BMAA concentrations ranged from 0-125ppm, and the concentration of DAB in each spectra was 50ppm. The fragment ions are seen at 102 m/z for BMAA and 101 m/z for DAB. Note that below 125ppm, the peak for BMAA at 102 m/z is split into two peaks at 102 m/z and 103 m/z and the signal intensity is greatly reduced.
4.3.4. BMAA ANALYSIS IN BIOLOGICAL TISSUES

Hydrolyzed biological tissue solution was separated into five samples and spiked with 50ppm DAB (IS) and increasing amounts of BMAA (10, 25, 50, 75, and 100ppm). Two spots of each concentration were deposited onto the MALDI plate. MALDI-TOF/TOF-MS/MS analysis was performed on the sample with the highest concentration of BMAA (100ppm). Ionization of the analyte was not possible. The data and spectra are not included in my thesis, because of the inability to qualify or quantify the analyte of interest (BMAA) or the internal standard (DAB).

As a result of the inability to ionize the analyte of interest (BMAA) and the internal standard (DAB), additional biological samples (shrimp) were prepared using a solid-phase extraction clean-up method that was described in chapter 2. Post SPE clean-up, five samples, comprised of hydrolyzed tissue, spiked with 25ppm DAB (IS) and increasing concentrations of BMAA (0, 1, 10, 75, and 200ppm) were spotted onto the MALDI plate. Two spots of each concentration were deposited, using the dried droplet method. MALDI-TOF/TOF-MS/MS analysis was performed for each concentration in triplicate.

Figure 33 represents the spectra for one replicate of each concentration. As the concentration of BMAA increases, the intensity of the signal at 102 m/z increases. The intensity of the signal of DAB, remains constant. Based on current literature, these fragment ions are specific to each analyte. Figure 34 represents the representative calibration curve for the average ratio of peak intensities of BMAA and DAB (IS). The error bars were calculated using the standard deviation. In this linear regression, a $R^2$
value of 0.9999 is seen, showing good linearity with DAB as an internal standard. The same parameters were used for each spectra, allowing for consistency and reproducibility. The method of standard addition was used to calculate the concentration of BMAA in the unspiked sample. Based on the linear regression, the unspiked sample contains ~234ppm BMAA. To relate this back to the original sample, in 1g of biological tissue (shrimp) collected from the Florida Bay, there is approximately 234µg/g of BMAA. This value falls within the reported ranges of other studies seen in table 1.

Figure 33. Positive Ion MALDI-TOF/TOF-MS/MS spectrum of BMAA and DAB (IS) in a hydrolyzed biological tissue (shrimp) with CHCA as the matrix. Spiked BMAA concentrations ranged from 0-200ppm, and the concentration of DAB in each spectra was 25ppm. The fragment ions are seen at 102 m/z for BMAA and 101 m/z for DAB.
Figure 34. Representative standard addition calibration curve for the concentrations of BMAA in ppm against the peak intensity ratios of BMAA to DAB (IS) with CHCA as the MALDI matrix. Parent ions for BMAA and DAB are 119 and their respective fragments are 102 and 101. Spiked concentrations of BMAA ranged from (0-200ppm) and the concentration of DAB was 25ppm. Error bars were calculated using the standard deviation. The calculated concentration of BMAA in the unspiked sample is 234.52ppm.

4.4. VALIDATION PROCEDURES

As a result of complications with matrix effects that resulted in increased limits of detection, validation procedures of the MALDI-TOF/TOF-MS/MS method was performed using BMAA and DAB (IS) standards in an aqueous solution. The following validation procedures show proof of concept and proof of method. Varying concentrations of BMAA (0, 2, 5, 15, and 25ppm) were spiked with 25ppm of DAB as an internal standard. Each concentration was spotted in triplicate, analyzed, and the data was exported to Microsoft Excel for processing. Method validation followed protocols published by the International Conference on Harmonisation of Technical Requirements
4.4.1. LIMIT OF DETECTION

The determination of the limit of detection was derived from the standard deviation of the blank. Five replicates of a blank, air-dried CHCA matrix, were analyzed using the optimized MALDI-TOF/TOF-MS/MS method with the same parameters as the other samples. The peak intensities of the analytical background at 102 m/z was exported to Microsoft Excel (Table 4). These values were used to calculate the mean and the standard deviation. The standard deviation, in accordance to the analytical background response of the blank and the mean were used to calculate the limit of detection using the equation (Figure 35), where $\sigma$ is the standard deviation, and $m$ is the mean of the peak intensities of the five blanks at 102 m/z.

$$LOD = 3 \left( \frac{\sigma}{m} \right)$$

<table>
<thead>
<tr>
<th>BLANK SAMPLE #</th>
<th>PEAK INTENSITY AT 102 m/z</th>
<th>PEAK INTENSITY AT 101 m/z</th>
<th>Ratio BMAA/DAB</th>
<th>MEAN</th>
<th>STD DEV</th>
<th>LOD (ppm)</th>
<th>LOQ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLANK 1</td>
<td>4.236</td>
<td>31.682</td>
<td>0.134</td>
<td>0.149</td>
<td>0.081</td>
<td>10.318</td>
<td>34.393</td>
</tr>
<tr>
<td>BLANK 2</td>
<td>4.139</td>
<td>34.250</td>
<td>0.121</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BLANK 3</td>
<td>1.400</td>
<td>21.583</td>
<td>0.065</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BLANK 4</td>
<td>4.847</td>
<td>17.019</td>
<td>0.285</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BLANK 5</td>
<td>2.084</td>
<td>14.559</td>
<td>0.143</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 35. Representative calibration curve for the concentrations of BMAA in ppm against the peak intensity ratios of BMAA to DAB (IS) with CHCA as the MALDI matrix. Parent ions for BMAA and DAB are 119 and their respective fragments are 102 and 101. Concentrations of BMAA ranged from (0-25ppm) and the concentration of DAB was 25ppm. Error bars were calculated using the standard deviation. Limit of detection and limit of quantitation were calculated by the measurement of the ratios of peak intensities of fragment ion 102 m/z over 101 m/z in the background response of the blank (5 replicates).

4.4.2. LIMIT OF QUANTITATION

The determination of the limit of quantitation was also based on the mean and standard deviation of the blank. The following equation was used to calculate the limit of detection:

\[
LOQ = 10 \left( \frac{\sigma}{m} \right)
\]

The value for the limit of quantitation is shown in figure 35.
4.4.3. ACCURACY

Accuracy was determined using the linear regression. Three concentration values were chosen (high, medium, and low) and were analyzed in triplicate. The calculated experimental concentration was found using the equation for the linear regression line (Figure 35). The mean was then calculated for each concentration and the percent recovery was calculated using the equation.

\[
\% \text{ Recovery} = \left( \frac{\text{Average } C_{\text{measured}} - \text{Average } C_{\text{blank}}}{C_{\text{theoretical}}} \right) \times 100
\]

The calculated concentrations for each replicate and their respective means can be seen in Table 5. The percent recovery values for BMAA using MALDI-TOF/TOF-MS/MS are 75.2 - 99.21%.

<table>
<thead>
<tr>
<th>Concentration (ppm)</th>
<th>Calculated Concentration #1 (ppm)</th>
<th>Calculated Concentration #2 (ppm)</th>
<th>Calculated Concentration #3 (ppm)</th>
<th>MEAN (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOW (2ppm)</td>
<td>2.36</td>
<td>2.65</td>
<td>0.89</td>
<td>1.96</td>
</tr>
<tr>
<td>MEDIUM (15ppm)</td>
<td>15.23</td>
<td>12.76</td>
<td>15.88</td>
<td>14.62</td>
</tr>
<tr>
<td>HIGH (25ppm)</td>
<td>21.27</td>
<td>30.79</td>
<td>23.73</td>
<td>25.26</td>
</tr>
</tbody>
</table>

4.4.4. PRECISION

The precision of the MALDI-TOF/TOF-MS/MS method was calculated using the standard deviation of the above mentioned series of concentrations. The same concentrations and replicates were used for the calculation of percent of relative standard
deviation. The standard deviation of each concentration was calculated as well as the mean. The percent of relative standard deviation was calculated using the equation.

\[ %RSD = \left( \frac{\sigma}{\bar{x}} \right) \times 100 \]

The percent relative standard deviation values for BMAA with MALDI-TOF/TOF-MS/MS are 11.26-48.14%.

4.4.5. RANGE AND LINEARITY

The linear range of the calibration is from 2ppm to 25ppm as seen in Figure 35. Linearity was shown to have a coefficient of determination of 0.9983 (Figure 35). Non-linearity was observed when concentrations went below 0.25ppm, which results in a decrease of the R² value.

4.5. CONCLUSIONS

The MALDI-TOF/TOF-MS/MS method proved to be a viable alternative to the traditional methods such as LC-MS/MS and HPLC of BMAA quantitation and separation. As shown with standards, an amino acid matrix, and a biological tissue matrix with SPE clean-up, this method is possible. With standards, detection was possible down to 0.2ppm and validation methods showed an acceptable percent recovery range of 75.2-99.21% that would have been increased if a concentration higher than 2ppm would have been used for the lowest concentration. The percent relative standard deviation again was outside of the acceptable range due to the concentration of the lower limit. If this concentration would have been increased, the percent relative standard deviation would have been better.
The use of methyltyrosine as an internal standard was successful in full scan, however, in TOF/TOF-MS/MS, instrumental limitations did not enable both parent ions (BMAA and methyltyrosine) to be ionized at the same time. As seen in the LC-MS/MS data, $^{13}$C-BMAA was not successful as an internal standard for MALDI for the same reasons it failed for LC-MS/MS, it proved to not be isotopically pure and/or completely labeled, resulting in an overestimate of BMAA in a sample. The use of DAB as an internal standard, however, proved to be a viable option. Since both BMAA and DAB share the same parent ion of 118.14, the MALDI laser was able to ionize both amino acids at the same time. As shown in previous literature, common fragment ions of BMAA and DAB are 102 m/z and 101 m/z, respectively. The data produced using MALDI-TOF/TOF-MS/MS also shows these fragment ions, and with these fragments, successful quantitation of BMAA is possible using MALDI-TOF/TOF-MS/MS. The discrimination capabilities of this method were shown as the method was able to discriminate between two structural isomers whose fragmentation ions differ by only 1 m/z.

Literature reports levels of BMAA in different matrices to be between 4-350μg/g, or approximately 4-350ppm (Table 1). With these reported values, the use of MALDI-TOF/TOF-MS/MS would be a viable option to analyze and quantify BMAA in a variety of samples. In sections 4.3.3 and 4.3.4, the MALDI-TOF/TOF-MS/MS method was unable to ionize the analyte in these matrices, until an SPE clean-up method was used. This proved that sample preparation, including the method of hydrolysis, needed to be optimized in order to analyze BMAA in a more complex matrix. With the BSA matrix, the limit of detection for BMAA diminished below 125ppm, and with the biological
tissue matrix, no BMAA was detected without an SPE clean-up. After additional clean-up steps, the MALDI-TOF/TOF-MS/MS method was capable of ionizing both BMAA and DAB in a complex biological matrix. Additionally, the amount of BMAA in the biological sample was calculated with a high level of confidence, based on the $R^2$ value of 0.9999 from the linear regression in figure 34.

5. DISCUSSION

Currently on any given day, there are 30,000 cases of ALS, 5.4 million cases of Alzheimer’s, and 500,000 Parkinson’s patients in the United States alone, and it is thought that out of all of these cases, only 10% are inherited (National Institute of Neurological Disorders and Stroke, 2014). The role of BMAA in neurodegenerative diseases is still unknown, however, the consumption of water or food sources that contain this amino acid could account for part of the 90% of unknown causes of these diseases. With that being said, the development of an analytical platform that is capable of analyzing and quantifying BMAA in a variety of matrices is essential to understand the extent of human exposure and toxicity. As the prevalence of neurodegenerative diseases increases, so does the interest for the study of what might cause these debilitating diseases.

This study has shown the usefulness and potential of MALDI-TOF/TOF-MS/MS for the analysis of BMAA and its structural isomer DAB. This method has proven to be a rapid analysis technique with minimal data processing. In this study, MALDI-TOF/TOF-MS/MS instrument parameters were optimized for the analysis of BMAA with a sample analysis time of seconds. However, there are limitations of MALDI-TOF/TOF-MS/MS
in the analysis of small molecules. With increasing complexity of matrices, instrument noise made it difficult and in some cases (biological tissue samples), impossible to detect the analyte of interest (BMAA) without additional clean-up steps. The possibility of MALDI-TOF/TOF-MS/MS to separate and resolve mixtures that contain structural isomers of BMAA with high accuracy and precision is there, however, it is important to state that additional research is essential for the use of this method as an analytical tool for the analysis of BMAA and other small molecules.

In terms of LC-MS/MS, although this method is much more sensitive, literature reports concentrations of BMAA in complex matrices between 0-2,286μg/g, this sensitive of a method could be somewhat unnecessary. Although LC-MS/MS has a much longer analysis time, 10-45 minutes per sample, this method has provided significant data in the study of BMAA. The developed MALDI-TOF/TOF-MS/MS method was able to detect BMAA down to 0.2ppm. In a complex matrix, this limit of detection would obviously increase, however, with the concentrations of BMAA reported in current literature, anything below 10ppm could be useful for the study of BMAA in biological samples.

With further research, MALDI-TOF/TOF-MS/MS could compete with traditional instrumentation such as LC-MS/MS. This study shows a proof of concept that a MALDI-TOF/TOF-MS/MS method is possible and application to a real world biological tissue sample. To combine MALDI-TOF/TOF-MS/MS with MALDI imaging would result in an analytical platform that would allow for minimal sample preparation, rapid analysis, and visualization of an analyte in a tissue sample, while also eliminating the need for derivatization, chromatographic separation, and modification of amino acids.
All of these attributes cannot be obtained with other instrumentation currently used. All of these qualities would greatly benefit the study of BMAA in neurodegenerative diseases and further the current understanding of the mechanism by which BMAA acts, its ability to biomagnify through the food web, and its role in neurodegenerative diseases.
LIST OF REFERENCES


Sigma Aldrich. β-N-methylamino-L-alanine. 


