N-β-methylamino-L-alanine: A Non-Protein Amino Acid Incorporated into Protein

by

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Abstract

N-β-methylamino-L-alanine (BMAA) is a naturally-occurring toxin produced in cyanobacteria that has been linked to neurological degeneration. Efforts to study and quantify BMAA in the environment are hampered by two biologically-occurring isomers, N-(2-aminoethyl)glycine (AEG) and 2,4-diaminobutyric acid (DAB), that exist in very low concentrations in many sample matrices, indicating a need for improved analytical methods.

The first objective of this thesis is to develop an accurate, precise and sensitive method for the analysis of BMAA and the isomers AEG and DAB that is applicable to a wide variety of sample matrices. A method for complete chromatographic separation of the isomers was developed using chemical derivatization, reversed phase chromatography and tandem mass spectrometry, and was validated with cyanobacteria-containing natural health products.

The mechanism of toxicity of BMAA is not fully understood, and previous studies have shown an association between BMAA and protein. The second objective of this thesis was to determine whether BMAA is incorporated into protein via a modified proteomics approach used to identify peptides containing BMAA in tissue from post-mortem human patients. This approach identified BMAA in proteins isolated from the frontal lobe of Amyotrophic Lateral Sclerosis (ALS), Alzheimer’s disease (AD), and control patients. To determine whether BMAA incorporation into protein occurs as an error in synthesis, a cell-free expression system was challenged with a deficiency of specific amino acids and supplemented with BMAA. BMAA was incorporated into protein via an error in synthesis. Together, these data demonstrate accurate methods for quantification of BMAA, a route of exposure through dietary products, and a potential mechanism for storage of BMAA in proteins.
Preface

A version of Chapter 1 has been published in the following manuscript:

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Chapter 2 is based on work I conducted in Dr. Susan Murch’s lab at UBC Okanagan. I completed all experimental work and data analysis.

Chapter 3 is based on work I conducted in Dr. Susan Murch’s lab at UBC Okanagan and Dr. Christoph Borchers’ lab at the University of Victoria-Genome BC Proteomics Centre. Patient tissue samples were received from Dr. Deborah Mash at the University of Miami. I carried out the experimental design, sample preparation and data analysis. Darryl Hardy completed the data acquisition.

Chapter 4 is based on work I conducted in Dr. Susan Murch’s lab at UBC Okanagan. I completed all experimental work and data analysis.
Table of Contents

Abstract ............................................................................................................................................ ii

Preface ............................................................................................................................................ iii

List of Tables ................................................................................................................................... ix

List of Figures .................................................................................................................................... x

Table of Symbols and Abbreviations ............................................................................................ xiii

Acknowledgements ....................................................................................................................... xvi

Chapter 1: Introduction and Literature Review .............................................................................. 1

1.1 Relevance and Historical Importance ................................................................................... 1

1.2 Discovery of N-β-methylamino-L-alanine (BMAA) ............................................................... 2

1.3 The BMAA Hypothesis .......................................................................................................... 3

1.4 The Importance of Methods for Analysis of BMAA .............................................................. 5

1.5 Methods of Analysis for BMAA ............................................................................................. 6

1.5.1 Sample Preparation ........................................................................................................ 7

1.5.2 Derivatization ................................................................................................................. 8

1.5.3 Chromatographic Separation ....................................................................................... 12

1.5.4 Mass Spectrometry....................................................................................................... 29

1.6 Method Validation Parameters .............................................................................................. 30
3.2.4 Protein Concentration by Bicinchinonic Acid (BCA) Assay ........................................... 69
3.2.5 Protein Denaturation .................................................................................................... 71
3.2.6 Protein Digestion .......................................................................................................... 71
3.2.7 Proteomics Analysis ...................................................................................................... 72
3.2.8 Data Analysis ................................................................................................................ 72
3.3 Results ................................................................................................................................. 73
3.3.1 BMAA, AEG, and DAB measured in Human Brain Samples .......................................... 73
3.3.2 De Novo Sequencing Output Files ................................................................................ 73
3.3.3 ALC Confidence Scores ................................................................................................. 75
3.3.4 Occurrence of BMAA in Peptides ................................................................................. 77
3.3.5 Relative Number of Peptides Containing BMAA .......................................................... 81
3.4 Discussion ............................................................................................................................ 83
3.4.1 Identification of a Non-Protein Amino Acid in Protein ................................................ 83
3.4.2 Occurrence of BMAA in Peptides ................................................................................. 84
3.4.3 Potential Limitations of this Approach ......................................................................... 86

Chapter 4: Cell-Free Expression as a Model for the Study of Incorporation of BMAA into Protein ........................................................................................................................................... 88
4.1 Synopsis ............................................................................................................................... 88
4.2 Methods ................................................................................................................................ 88
List of Tables

Table 1.1   Methods of detection and quantification of BMAA in biological samples ................ 14
Table 2.1   MRM transitions monitored in the analysis of BMAA, AEG and DAB ......................... 39
Table 2.2   Intra- and inter-day variability of instrument response and retention time ............. 57
Table 2.3   Instrument and method limits of detection by USP, IUPAC, and FLDA methods ...... 58
Table 2.4   Precision and accuracy in a biological matrix ............................................................ 59
Table 2.5   Quantification of BMAA, AEG and DAB in natural health products ....................... 61
Table 2.6   Estimated daily intake of BMAA, AEG and DAB in natural health products .......... 62
Table 3.1   Patient Information for Analysis of Protein Containing BMAA ................................. 70
Table 3.2   Measured concentrations (wet weight) of BMAA, AEG and DAB in human
            patient brain tissue ........................................................................................................... 73
Table 3.3   Peptides Identified As Containing BMAA in Each Subsample ................................. 76
List of Figures

Figure 1.1 *Cycas micronesica* and isolated cyanobacteria on Guam................................. 4

Figure 1.2 The hypothesis that BMAA accumulates through food webs and can be consumed from many different sources in the diet ................................................. 5

Figure 1.3 L-BMAA and its biologically-occurring isomers AEG and L-DAB ......................... 13

Figure 2.1 Sample preparation scheme for BMAA and its isomers ......................................... 37

Figure 2.2 Chromatographic separation using ammonium acetate and methanol of AEG, BMAA, and DAB after derivatization with AccQ-Fluor reagent ................................. 41

Figure 2.3 Chromatographic separation using ammonium formate and acetonitrile of BMAA, AEG, and DAB after derivatization with AccQ-Fluor reagent ........ 42

Figure 2.4 Capillary voltage optimization for AQC-derivatized N-β-methylamino-L-alanine..... 44

Figure 2.5 Capillary voltage optimization for AQC-derivatized N-(2-aminoethyl)glycine. .... 44

Figure 2.6 Capillary voltage optimization for AQC-derivatized 2,4-diaminobutyric acid .......... 45

Figure 2.7 Cone voltage optimization for AQC-derivatized N-β-methylamino-L-alanine. ....... 45

Figure 2.8 Cone voltage optimization for AQC-derivatized N-(2-aminoethyl)glycine ............... 46

Figure 2.9 Cone voltage optimization for AQC-derivatized 2,4-diaminobutyric acid.............. 46

Figure 2.11 Source offset optimization for AQC-derivatized N-β-methylamino-L-alanine. ...... 47

Figure 2.10 Source offset optimization for AQC-derivatized N-(2-aminoethyl)glycine .......... 47

Figure 2.12 Source offset optimization for AQC-derivatized 2,4-diaminobutyric acid .......... 48

Figure 2.13 Collision gas flow optimization for AQC-derivatized N-β-methylamino-L-alanine.... 48

Figure 2.14 Collision gas flow optimization for AQC-derivatized N-(2-aminoethyl)glycine .... 49
Figure 2.14 Collision gas flow optimization for AQC-derivatized 2,4-diaminobutyric acid ....... 49
Figure 2.16 Collision energy optimization for AQC-derivatized N-β-methylamino-L-alanine..... 50
Figure 2.17 Collision energy optimization for AQC-derivatized N-(2-aminoethyl)glycine ........ 51
Figure 2.18 Collision energy optimization for AQC-derivatized 2,4-diaminobutyric acid ........ 52
Figure 2.19 Dwell time optimization for AQC-derivatized N-β-methylamino-L-alanine. ........ 53
Figure 2.20 Dwell time optimization for AQC-derivatized N-(2-aminoethyl)glycine............. 53
Figure 2.21 Dwell time optimization for AQC-derivatized 2,4-diaminobutyric acid. .......... 54
Figure 2.22 Fluorescence image of intact spirulina in a commercial natural health product ...... 60

Figure 3.1 PEAKS user interface showing determined peptides sequences, MS/MS spectra, 
and sorting parameter options. .......................................................................................... 74

Figure 3.2 Typical ALC score diagram for samples obtained in this study; samples 
possessing an ALC lower than 30 were discarded........................................... 75

Figure 3.3 Low confidence score (ALC < 40%) MS/MS spectrum of a peptide identified as 
containing BMAA. ......................................................................................................... 78

Figure 3.4 High confidence score (ALC > 70%) MS/MS spectrum of a peptide identified as 
containing BMAA. ......................................................................................................... 79

Figure 3.5 High confidence score (ALC > 70%) MS/MS spectrum of peptide identified 
without BMAA........................................................................................................... 80

Figure 3.6 Relative number of peptides identified in each subcellular fraction as containing 
BMAA ....................................................................................................................... 82

Figure 4.1 Total protein in each cell-free synthesis reaction.......................................... 93
Figure 4.2  Percent of BMAA incorporated into de novo protein as measured by LC-MS/MS. ................................................................. 95

Figure 4.3  BMAA recovered after denaturation of the protein and hydrolysis of the protein pellet after denaturation ................................................................. 96

Figure 4.4  Incorporation of AEG and DAB into protein as measured by LC-MS/MS. ................. 97
Table of Symbols and Abbreviations

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
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<td>AEG</td>
<td>N-(2-aminoethyl)glycine (AEG)</td>
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<tr>
<td>ALC</td>
<td>Average Local Confidence</td>
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<td>ALS</td>
<td>Amyotrophic Lateral Sclerosis</td>
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<tr>
<td>ALS/PDC</td>
<td>Amyotrophic Lateral Sclerosis/Parkinson's Dementia Complex</td>
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<tr>
<td>AQC</td>
<td>6-aminoquinolyl-N-hydroxysuccinimidyl carbamate</td>
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<tr>
<td>BCA</td>
<td>Bicinchinonic Acid</td>
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<tr>
<td>BEH</td>
<td>Bridged ethyl hybrid</td>
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<tr>
<td>BMMA</td>
<td>N-β-methylamino-L-alanine</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>DAB</td>
<td>2,4-diaminobutyric acid</td>
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<td>DAD</td>
<td>Diode array detector</td>
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<td>DNA</td>
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<td>ECF</td>
<td>Ethyl chloroformate</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>EI-MS</td>
<td>Electron impact mass spectrometry</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>FLD</td>
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FMOC – Fluorenylmethyloxycarbonyl chloride
GC-MS – Gas chromatography-mass spectrometry
HCl – Hydrochloric acid
HILIC – Hydrophilic liquid interaction chromatography
HPLC – High-performance liquid chromatography
IEX – Ion Exchange
IUPAC – International Union of Pure and Applied Chemistry
L-DOPA – L-3,4-dihydroxyphenylalanine
LC-MS – Liquid chromatography – mass spectrometry
LLOQ – Lower limit of quantification
LOD – Limit of Detection
MALDI – Matrix assisted laser desorption ionization
MRM – Multiple reaction monitoring
MS – Mass spectrometry
MS/MS – Tandem mass spectrometry
NaOAc – Sodium acetate
PBS – Phosphate buffered saline
PCF – Propyl chloroformate
RPLC – Reversed-phase liquid chromatography
RSD – Relative standard deviation
SPE – Solid phase extraction
TCA – Trichloroacetic acid
TEA – Triethylamine

UPLC – Ultra-performance liquid chromatography

USP – United States Pharmacopeia

UV – Ultraviolet detection
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1.1 Relevance and Historical Importance

In 1952 and 1953, reports appeared in the medical literature describing an epidemic of neurodegenerative disease consistent with amyotrophic lateral sclerosis (ALS) on the island of Guam (Arnold et al., 1953; Korner, 1952; Tillema et al., 1953). The earliest documented case of the disease that could be confirmed by a death certificate occurred in 1904 but the indigenous Chamorro people had a long memory of the disease that they called “lytico” (Kurland et al., 1954). A “house-to-house” survey of three villages in 1953 concluded that the prevalence of ALS among the Chamorro on Guam and Rota was about 420 per 100,000 population as compared to ca. 4/100,000 in North America and Europe (Kurland et al., 1954a; Kurland et al., 1954b). The disease accounted for 8-10% of the adult Chamorro deaths per year and the highest incidence was found among the most traditional families (Kurland et al., 1954b). Further studies diagnosed a second clinical manifestation of the disease that was identified as “parkinsonism dementia” (PD) and the disease became broadly known as amyotrophic lateral sclerosis / parkinsonism dementia complex (ALS/PDC) (Reed et al., 1966).

In 1960, the indigenous Chamorro population of Guam was 34,762, including approximately 40,000 American military personnel and related staff or family, 6,000 Filipino contract workers and 600 Caroline Islanders (Reed et al., 1966). Initial observations indicated that the disease incidence was limited to the Chamorro in Guam and Chamorro who had moved to California, suggesting a genetic or hereditary basis for the disease (reviewed in Reed et al., 1966). From 1962-1964 two cases of the disease were reported among non-Chamorro (Reed et
al.; 1966; Reed et al., 1975; Plato et al., 1967). These cases, along with detailed statistical analysis of the Chamorro population, led to the conclusion that the risk of the disease is clustered among families but not genetically determined (Reed et al., 1975; Kurland et al., 1955a; Kurland et al., 1955b). A non-Chamorro living in the Chamorro community had an equal chance of becoming ill (Reed et al., 1966). Likewise, the possibilities of viral infections, transmissible agents from animals, micronutrient deficiencies, and calcium deficiency were all eliminated, leading to the conclusion that the disease was most likely the result of “long-term exposure to the environment” (reviewed in Reed et al., 1966; Reed et al., 1987). Over more than 40 years of study, the only factor that was positively correlated with the disease incidence was a preference for traditional Chamorro foods (Reed et al., 1987).

1.2 Discovery of N-β-methylamino-L-alanine (BMAA)

The ethnobotanist Marjory Whiting conducted extensive field studies in Guam to document the Chamorro culture and to determine whether traditional foods might be implicated in ALS/PDC (Whiting, 1963). Whiting identified flour made from the seeds of the indigenous cycad (Cycas micronesica Hill.; Figure 1.1 A) as a potentially toxic food source (Whiting, 1963), tested the flour on rats (Laquer et al., 1963) and submitted samples of cycad seeds to the London School of Pharmacy for analysis (Whiting, 1963). Analysis of these cycad seeds identified an extract that was neurotoxic to chicks and mice (Vega et al., 1967; Vega et al., 1968) and found to contain relatively large concentrations of N-β-methylamino-L-alanine (BMAA; Figure 1.2). Throughout the 1970s and 1980s, the potential neurotoxicity of BMAA was extensively investigated in many different model systems and, most significantly, BMAA was found to be neurotoxic in primates when administered at high doses (Spencer et al., 1987).
However, Duncan et al. (1989; 1991; 1992) argued that the traditional process of washing the cycad seeds in making flour removed a significant proportion of the BMAA and that the remaining dose was not sufficient to cause the disease (Duncan et al., 1988; Duncan et al., 1990) or that disease might be caused by zinc leaching from the wash buckets (Duncan et al., 1992). Research into the role of BMAA in progressive neurodegeneration was effectively suspended following these observations.

1.3 The BMAA Hypothesis

In 2002, Cox and Sacks (2002) proposed a hypothesis that re-invigorated research into the neurotoxicity of BMAA. They hypothesized that BMAA could accumulate through different trophic levels of the ecosystem resulting in different routes of exposure and biomagnification of the compound that would have increased the exposure of the Chamorro people (Cox and Sacks, 2002). Research in Guam demonstrated that BMAA could be accumulated through the traditional Chamorro food web from cycads to animals including flying foxes, deer and wild pigs (Banack et al., 2003; Cox et al., 2003; Murch et al., 2004a; Banack et al., 2006; Banack et al., 2009). In addition, BMAA was identified and quantified in brain autopsy samples of both Chamorro patients who died of ALS/PDC and Canadian Alzheimer’s patients (Murch et al., 2004b). Additionally, BMAA has been identified and quantified in brain tissues of ALS and AD patients in Miami (Pablo et al., 2009).
Figure 1.1: *Cycas micronesica* and isolated cyanobacteria on Guam. *Cycas micronesica* on Guam (A). Coralloid roots of *C. micronesica* with cyanobacteria infections (B). Cyanobacteria isolated from roots of *Cycas micronesica* Hill on Guam (C) and sequential isolate subcultures (D). A collection of cyanobacteria cultures from cycads at different locations on the island of Guam (E).

Together, these data suggested that BMAA is produced by cyanobacteria and accumulates in plants and animals that consume the plants, including humans who are thus exposed to unknown doses from multiple sources (Figure 1.2). In other parts of the world, BMAA accumulation from cyanobacteria through food webs has been shown in blue crab, shrimp and oysters in South Florida (Brandt *et al.*, 2010), mussels, oysters and bottom dwelling fish of the Baltic (Jonasson *et al.*, 2010) and traditional Chinese foods such as noodles (Roney *et al.*, 2009) and shark fins (Mondo *et al.*, 2010; Mondo *et al.*, 2014).
Figure 1.2: The hypothesis that BMAA accumulates through food webs and can be consumed from many different sources in the diet.

1.4 The Importance of Methods for Analysis of BMAA

Evaluating the BMAA hypothesis requires accurate analytical methods for detection, identification and quantification of BMAA (Cohen, 2012; Duncan, 2012). Montine et al. (2005) reported that they were unable to detect BMAA in autopsy tissues of Chamorro ALS/PDC patients (Montine et al., 2005). Several groups were unable to detect or quantify BMAA in cyanobacteria isolates (Rosen et al., 2008; Kruger et al., 2010; Li et al., 2012). To date, more than a dozen research groups have published 69 reports, with 60 confirming detection of BMAA and 9 publications reporting a failure to detect BMAA (Table 1.1). Duncan (2012) stated “the
role of BMAA in neurodegenerative disorders is fiercely debated. In large part, the controversy arises because of conflicting findings relating to the analytical work underpinning this complex web of observation and chemical analysis” (Duncan, 2012, pg. 804).

1.5 Methods of Analysis for BMAA

The majority of analytical methods for detection and quantification of BMAA have used standard amino acid analysis techniques modified for low concentrations in complex samples (Table 1.1). Almost 70% of the published literature relied on detection by MS (29%) or MS/MS (40%), while fluorescence detection (27%) and diode array detection (4%) proved less popular. The early approaches used GC-MS (Duncan et al., 1988; Duncan et al., 1990) or 9-fluorenylmethyl chloroformate (FMOC) derivatization followed by HPLC separation and fluorescence detection (Kisby et al., 1988). Work beginning in 2003 used a similar approach with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatization prior to reverse phase chromatographic separation and fluorescence detection (Banack et al., 2003; Cox et al., 2003; Murch et al., 2004a; Murch et al., 2004b). The BMAA peak assignment was confirmed by LC-MS using an orthogonal method (Cox et al., 2003; Murch et al., 2004a; Murch et al., 2004b). Similar results have been found with a third derivatization technique using propyl chloroformate derivatization (EZ:faast) with fluorescence and MS detection of the derivatized compound at m/z 333 (Esterhuizen et al., 2008; Esterhuizen et al., 2011). The majority of the methods that have failed to detect BMAA in biological samples have used LC-MS approaches for the detection of an underivatized [M+H]+ or MS/MS for associated daughter ions, with separation by a HILIC column rather than reverse phase or GC/GC-time of flight MS (Table 1.1; Rosen et al., 2008; Kruger et al., 2010; Li et al., 2012; Snyder et al., 2010; Faassen et al., 2009).
Recently, a study of the analysis of underivatized BMAA standards by MS found that the molecule is highly reactive and readily forms metal ion adducts and complexes in solution that complicate the analysis (Glover et al., 2012). No study has been done that compared the different approaches of different labs on a common set of samples or extracts.

1.5.1 Sample Preparation

Preparation of the samples is the critical first step to ensure accurate analysis of BMAA. Many published methods begin with precipitation of the protein from samples using either 0.1 N trichloroacetic acid (Cox et al., 2003; Murch et al., 2004a; Murch et al., 2004b), water (Khabazian et al., 2002) or aqueous ethanol (50%-80%)/ dilute HCl (Vega et al., 1967; Rosen et al., 2008; Kisby et al., 1988; Kubo et al., 2008). Other researchers subjected samples to freeze/thaw treatments in 80% methanol followed by ultrasonication (Jiang et al., 2013). The complete precipitation of proteins is essential since hydrolysis of the protein pellet with 6 N HCl at 110 °C increased the detectable concentrations of BMAA in cycad flour samples from Guam and could account for differences in the reported values of nearly six orders of magnitude (Murch et al., 2004a). Additional considerations include the need to remove excess lipids that may be present in some sample matrices. A liquid-liquid solvent partition using chloroform has been used by some researchers to remove excess lipids following acid hydrolysis (Jiang et al., 2013).

Solid phase extraction has been used in six reports of BMAA analysis with three of the manuscripts providing detailed procedures. Kubo et al. (2008) reported that BMAA, a highly polar molecule, is not effectively retained on C18 (Waters Sep-pak) or a lipophilic polymer (Waters Oasis HLB), but a polymeric cation exchange sorbent (Waters Oasis MCX), which
contains a γ-lactam moiety to aid in the retention of polar analytes, readily retained BMAA (Kubo et al., 2008). Jiang et al. (2013) used an Isolute HCX SPE column for sample clean-up, added D$_3$-BMAA as an internal standard and reported a recovery of the D3-BMAA of 63.3% (Jiang et al., 2013). Downing et al. (2011) reported isolation and concentration of BMAA from drinking water using a polymeric sorbent containing a phenylsulphonic acid moiety (Phenomenex Strata X-C) (Downing et al., 2011). Recovery of BMAA with this method was shown to be near 100% for analyte spiked into distilled water, but much lower recoveries of 40-60% were measured for other sample matrices. The authors proposed that competition for the cation exchange sites between basic amino acids may have reduced recovery (Downing et al., 2011). Li et al. (2012) compared the relative recoveries of the Strata X-C and Oasis MCX systems (Li et al., 2012). Slightly improved recoveries (2-6%) were reported for complex cyanobacterial samples with the Oasis cartridge despite using a much lower sorbent load (200 mg vs. 60 mg, respectively) (Li et al., 2012). Combined, these results suggest that SPE may be a highly effective cleanup and concentration procedure for BMAA analysis, but the type of cartridge must be carefully chosen for individual matrices; it is important to include an internal standard and the recovery of these methods seems to be highly variable.

1.5.2 Derivatization

Derivatization facilitates detection of amino acids in low concentrations in complex sample matrices, improves chromatographic separation, and improves the selectivity of the method (Banack et al., 2011). During derivatization, the samples are often dissolved in a buffer at pH as high as 9 and the buffer may also contain EDTA or similar compounds that chelate metal ions (Rebane et al., 2012). There are three main methods of amino acid derivatization
that have been used for identification and quantification of BMAA. Fluorenylmethyloxycarbonyl chloride (FMOC, Figure 1.3A) derivatization procedure effectively detected BMAA in trial animals, cycads and cycad flour (Kisby et al., 1998). It is possible to have two potential products of the derivatization reaction, either a single or double derivative, but only one BMAA peak has been reported in the fluorescence chromatogram and no MS data have been reported for this BMAA reaction (Cohen, 2012). In two recent studies, BMAA was not detected in FMOC-derivatized preparations of human brain tissue, cyanobacteria, water samples or supplements (Montine et al., 2005; Scott et al., 2009).
Figure 1.3: Published derivatization schemes for N-β–methylamino-L-alanine. Fluorenylmethyloxycarbonyl (FMOC) derivative (A), propylchloroformate derivative (B), and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivative (C).
The second derivatization technique uses the chloroformate derivative EZ:Faast (Esterhuizen et al., 2011; Snyder et al., 2010; Downing et al., 2011, Figure 1.3B). In this method, propylchloroformate is reacted with not only primary and secondary amines, but also with carboxyl groups in amino acids, with the derivative detectable by MS (m/z 333). When reacted with BMAA, this reagent generates a triply derivatized product with very different selectivity on C18 columns than other methods, making it an ideal system for orthogonal method comparisons (Downing et al., 2011).

The third method uses 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC, Figure 1.3C) derivatization for quantification of BMAA and has been verified by several orthogonal techniques (Murch et al., 2004a; Banack et al., 2011) including MS detection of the double derivative m/z 459 and monitoring for the single derivative peak (Banack et al., 2010; Banack et al., 2011). Successful AQC derivatization requires a pH of approximately 9, and acidic samples can fail to complete the reaction, leading to false negative results. Additionally, the borate buffer (Waters AccQFluor Reagent) provided as part of the derivatization protocol kit contains a chelating agent that removes metal ions from the solution which may aid in derivatization and sample detection.

One of the largest challenges with all derivatization methods, including AQC, is ensuring that the reaction goes to completion and that there is sufficient fluorescent tag to label all of the amino acids in a sample, especially when more than one reaction product is possible or when quantification of a minor metabolite in the presence of large excess of common reactants is required. BMAA is present in relatively small quantities in samples containing much larger amounts of the 20 protein amino acids, and the AQC derivatization reaction can result in two
products with insufficient amounts of the reagent (Glover et al., 2012). Lysine also forms both single and double derivatized forms in the same reaction and can be used as a secondary internal standard to determine the completeness of the derivatization for each individual sample (personal communication, Steven A. Cohen, Waters Corp.). The single derivatized form of lysine can be monitored by SIR of m/z 317.3 in MS systems or by the MRM transition m/z 317.3 > 171.0 in MS/MS instruments. The fragment m/z 171.0 results from loss of the AQC fluorophore tag from the parent ion and is a universal daughter ion in this method. When combined with its high ionization efficiency, this ubiquity makes it a good choice for MRM measurements for amino acids using this method. These data allow for monitoring and balancing of the derivatization reaction that is crucial to accuracy.

1.5.3 Chromatographic Separation

The choice of the chromatographic column and elution conditions are important for accurate BMAA analysis. Most separation systems used standard C18 columns and conditions but eight out of the nine reports that failed to detect BMAA in cyanobacteria used a HILIC column for separation and elution with formate / acetonitrile either isocratically or with a gradient (Table 1; reviewed in Cohen, 2012). One of the most difficult problems of analysis of BMAA in complex samples is the presence of isomers with identical molecular mass that need to be separated by chromatography (Banack et al., 2003; Banack et al., 2010; Jiang et al., 2012, Fig. 1.2). From a database search of all the theoretical isomers of BMAA, a subset of seven possible interfering compounds were identified (Jiang et al., 2012). Two of these isomers, 2,4-diaminobutyric acid (DAB) and N-(2-aminoethyl) glycine (AEG) (Figure 1.4), appear in biological matrices and have been studied in detail since 2003 (Banack et al., 2003; Rosen et al., 2008;
Separation and identification of BMAA from DAB and AEG is achieved with derivatized samples through chromatographic separation (Jiang et al., 2012; Banack et al., 2012; Faassen et al., 2012).

A second approach uses hydrophilic interaction liquid chromatography (HILIC) to separate underivatized BMAA from the sample matrix (Rosen et al., 2008; Kruger et al., 2010; Li et al., 2012; Kubo et al., 2008). HILIC separations rely on the interactions between the polar stationary phase, liquid-liquid partitioning, ionic and hydrophobic interactions with an initial elution in an organic solvent such as acetonitrile. One of the leading causes of peak distortion and signal loss in HILIC separations is the difference in viscosity and polarity between the sample and the organic elution buffer (Grumbach et al., 2010). Samples for BMAA analysis were dissolved in acid (Li et al., 2012), water (Rosen et al., 2008; Kruger et al., 2010) or a solution of acetonitrile and water (Kubo et al., 2008). The interaction of the polar sample diluents with the organic elution buffer may have reduced the solubility and separation of BMAA from the sample matrix (Grumbach et al., 2010).
<table>
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<tr>
<th>Year</th>
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<td>2003</td>
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<td>2003</td>
<td>Cox et al., 2002</td>
<td>Yes</td>
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<td>Waters Nova-Pak C18 column, 300 mm x 3.9 mm</td>
<td>140 mM NaOAc, 5.6 mM TEA, pH 5.2 and 60% Acetonitrile</td>
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<td>FLD</td>
<td>Waters 2487 Dual-L FLD, Waters 2488 UV Detector</td>
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<td>2003</td>
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Table 1.1: Methods of detection and quantification of BMAA in biological samples since 2003.

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<td>2005</td>
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<td>2007</td>
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<td>AQC</td>
<td>MS/MS</td>
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Table 1.1: Methods of detection and quantification of BMAA in biological samples since 2003.

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<td>Waters Nova-Pak C18 column, 300 mm x 3.9 mm</td>
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19
Table 1.1: Methods of detection and quantification of BMAA in biological samples since 2003.

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<td>AQC</td>
<td>MS, Waters EMD 1000 Single Quad</td>
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<td>2009</td>
<td>Cox et al., 2009</td>
<td>Yes</td>
<td>RPLC, Waters Nova-Pak C18 column, 300 mm x 3.9 mm</td>
<td>140 mM NaOAc, 5.6 mM TEA, pH 5.2 and 60% Acetonitrile</td>
<td>AQC</td>
<td>FLD, Waters 2487 Dual-L Dual Fluorescence Detector, Waters 2488 UV detector</td>
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<td>2009</td>
<td>Pablo et al., 2009</td>
<td>Yes</td>
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<td>0.1% Formic Acid in Water / 0.1% Formic Acid in Acetonitrile</td>
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<td>MS/MS, Thermo TSQ Quantum Discovery Max</td>
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<td>2009</td>
<td>Pablo et al., 2009</td>
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<td>Waters Nova-Pak C18 column, 300 mm x 3.9 mm</td>
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<td>2009</td>
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<td>2010</td>
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<td>Thermo Hypersil GOLD 100 mm x 2.1 mm, 3 um column</td>
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<td>Waters Nova-Pak C18 column, 3.9 mm x 300 mm</td>
<td>140 mM NaOAc, 5.6 mM TEA, pH 5.2 and 60% Acetonitrile</td>
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<td>2010</td>
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<td>Yes</td>
<td>RPLC</td>
<td>Agilent Zorbax Eclipse AAA 4.6 x 75 mm, 3.5 um Acetonitrile / Formic Acid</td>
<td>AQC</td>
<td>MS/MS</td>
<td>Agilent 6401A</td>
</tr>
<tr>
<td>2012</td>
<td>Glover et al., 2012</td>
<td>Yes</td>
<td>RPLC</td>
<td>Waters C18 AccQ-Tag Ultra 2.1 x 100 mm column Acetonitrile / Formic Acid</td>
<td>AQC</td>
<td>MS</td>
<td>Waters LCT Premier XE TOF</td>
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<tr>
<td>2013</td>
<td>Dunlop et al., 2013</td>
<td>Yes</td>
<td>RPLC</td>
<td>Waters AccQTag Ultra column 2.1x100 mm 0.1% Formic Acid in water / 0.1% Formic Acid in Acetonitrile</td>
<td>AQC</td>
<td>MS/MS</td>
<td>Thermo TSQ Quantum Discovery Max</td>
</tr>
<tr>
<td>2013</td>
<td>Jiang et al., 2013</td>
<td>Yes</td>
<td>RPLC</td>
<td>Agilent Bonus RP Rapid Resolution High Throughput, 100 x 2.1 mm, 1.8 μm Acetonitrile / Formic Acid</td>
<td>AQC</td>
<td>MS/MS</td>
<td>Thermo TSQ Vantage</td>
</tr>
</tbody>
</table>
Table 1.1: Methods of detection and quantification of BMAA in biological samples since 2003.

<table>
<thead>
<tr>
<th>Year</th>
<th>Authors, Year</th>
<th>Identification</th>
<th>LC Method</th>
<th>Column</th>
<th>Mobile Phase</th>
<th>MS Method</th>
<th>MS/MS</th>
<th>Instrument</th>
</tr>
</thead>
<tbody>
<tr>
<td>2014</td>
<td>Lage et al., 2014</td>
<td>Yes</td>
<td>RPLC</td>
<td>Waters AccQTag Ultra column 2.1x100 mm</td>
<td>0.01% formic acid in 0.05% ammonia / 0.01% formic acid in methanol</td>
<td>AQC</td>
<td>MS/MS</td>
<td>Waters Xevo TQ-MS</td>
</tr>
<tr>
<td>2014</td>
<td>Downing et al., 2014</td>
<td>Yes</td>
<td>RPLC</td>
<td>Phenomenex AAA-MS 250 2.0 mm, 4 μm</td>
<td>10 mM Ammonium Formate in Methanol</td>
<td>Propylchrlorfomate (EZ:faast)</td>
<td>MS/MS</td>
<td>Waters Micromass Quattro Micro</td>
</tr>
<tr>
<td>2014</td>
<td>Karlsson et al., 2014b</td>
<td>Yes</td>
<td>RPLC</td>
<td>Agilent Bonus RP Rapid Resolution High Throughput, 100 × 2.1 mm, 1.8 μm</td>
<td>Acetonitrile / Formic Acid</td>
<td>AQC</td>
<td>MS/MS</td>
<td>Thermo TSQ Vantage</td>
</tr>
<tr>
<td>2014</td>
<td>Jiao et al., 2014</td>
<td>Yes</td>
<td>RPLC</td>
<td>Agilent Eclipse XDB-C18 5 μm, 4.6 × 100 mm</td>
<td>28% acetonitrile in water with 0.1% ammonium acetate</td>
<td>AQC</td>
<td>MS/MS</td>
<td>Agilent 6460</td>
</tr>
</tbody>
</table>
Table 1.1: Methods of detection and quantification of BMAA in biological samples since 2003.

<table>
<thead>
<tr>
<th>Year</th>
<th>Authors, Year</th>
<th>Method</th>
<th>Column/Conditions</th>
<th>Solvent/Mobile Phase</th>
<th>Detection</th>
<th>MS</th>
<th>Instrument</th>
</tr>
</thead>
<tbody>
<tr>
<td>2012</td>
<td>Metcalf et al., 2012</td>
<td>Yes</td>
<td>RPLC</td>
<td>Waters C18 AccQ-Tag Ultra 2.1 x 100 mm column</td>
<td>Acetonitrile and 0.05% TFA; 70/30 (v/v), isocratic flow</td>
<td>AQC</td>
<td>MS/MS</td>
</tr>
<tr>
<td>2005</td>
<td>Montine et al., 2005</td>
<td>No</td>
<td>RPLC</td>
<td>Unspecified</td>
<td>Unspecified</td>
<td>FMOC</td>
<td>FLD</td>
</tr>
<tr>
<td>2008</td>
<td>Kubo et al., 2008</td>
<td>No</td>
<td>HILIC</td>
<td>Tosoh TSK-gel Amide- 80 column (250 x 2.0 mm, 5 um)</td>
<td>Acetonitrile / Water</td>
<td>None</td>
<td>MS</td>
</tr>
<tr>
<td>2008</td>
<td>Rosen et al., 2008</td>
<td>No</td>
<td>HILIC</td>
<td>SeQuant ZIC-HILIC, 5 um, 50 x 2.1 mm or 150 x 2.1 mm</td>
<td>Acetonitrile and 60 mM Formic Acid</td>
<td>None</td>
<td>MS/MS</td>
</tr>
<tr>
<td>2010</td>
<td>Snyder et al., 2010</td>
<td>No</td>
<td>GCxGC</td>
<td>60 m x 0.25 mm i.d. x 0.25 μm Restek RTX- 5MS</td>
<td>Helium Gas</td>
<td>ECF</td>
<td>MS</td>
</tr>
<tr>
<td>2010</td>
<td>Kruger et al., 2010</td>
<td>No</td>
<td>HILIC</td>
<td>Phenomenex Luna C18(2), 5 mm, 250 x 4.60 mm</td>
<td>2 mM Ammonium Formate (pH 3) in (A) Aater and (B) Acetonitrile/Water (90/10; v/v)</td>
<td>None</td>
<td>MS/MS</td>
</tr>
<tr>
<td>Year</td>
<td>Authors</td>
<td>HILIC</td>
<td>Column</td>
<td>Mobile Phase</td>
<td>Gradient</td>
<td>Eluent</td>
<td>MS/MS Type</td>
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<tr>
<td>------</td>
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<td>-------------</td>
<td>----------</td>
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<td>------------</td>
</tr>
<tr>
<td>2010</td>
<td>Li et al., 2010</td>
<td>No</td>
<td>HILIC</td>
<td>SeQuant ZIC-HILIC, 5 um, 150 x 2.1 mm</td>
<td>Acetonitrile and 60 mM FA; 60/40 (v/v), isocratic flow</td>
<td>None</td>
<td>MS/MS</td>
</tr>
<tr>
<td>2010</td>
<td>Spacil et al., 2010</td>
<td>No</td>
<td>HILIC</td>
<td>Tosoh TSK-gel Amide- 80 column (250 x 2.0 mm, 5 um)</td>
<td>Acetonitrile and 0.05% TFA; 70/30 (v/v), Isocratic Flow</td>
<td>None</td>
<td>MS/MS</td>
</tr>
<tr>
<td>2012</td>
<td>Faassen et al., 2012</td>
<td>No</td>
<td>HILIC</td>
<td>SeQuant ZIC-HILIC, 5 um, 150 x 2.1 mm</td>
<td>Acetonitrile / Formic Acid</td>
<td>None</td>
<td>MS/MS</td>
</tr>
<tr>
<td>2012</td>
<td>Li et al., 2012</td>
<td>No</td>
<td>HILIC</td>
<td>Tosoh TSK-gel Amide- 80, 250 x 2.0 mm, 5 um</td>
<td>Acetonitrile / Formic Acid</td>
<td>None</td>
<td>MS/MS</td>
</tr>
<tr>
<td>2013</td>
<td>Combes et al., 2013</td>
<td>No</td>
<td>HILIC</td>
<td>Merck Sequant ZIC-HILIC 3.5 um, 150 x 2.1 mm</td>
<td>0.1% Formic Acid in Water / 0.1% Formic Acid in Acetonitrile</td>
<td>None</td>
<td>MS</td>
</tr>
</tbody>
</table>
1.5.4 Mass Spectrometry

Most LC-MS/MS methods have identified BMAA as a single underivatized parent m/z 119 > 102 (loss of OH-) (Kruger et al., 2010; Faassen et al., 2009; Faassen et al., 2012) or a fragmentation pattern m/z 119, 102, 88, 76, 73 and 44 (Rosen et al., 2008). Using a GC/GC-time of flight MS, Snyder et al. (2010) created a proprietary algorithm to combine signals of m/z 100, 101, 102, 103, 114, 115, 116, 117, 118, 129, 131, 132, 133, 291, and 292 to identify and quantify BMAA. These approaches may have reduced accuracy due to ion suppression that can arise from competition for available charge, surface saturation of ESI droplets with analytes, increase in droplet viscosity and analyte hydrophobicity (Cohen, 2012). Recently, we demonstrated that overreliance on the [M+H]+ (m/z 119) can lead to false negative results and that at least 30 BMAA adducts may be common in MS analysis (Glover et al., 2012). The composition of the eluents and sample matrix contribute to the complexity of the adduct profile, and only 0-10% of the BMAA was represented by the [M+H]+ peak. Other authors have argued that a D3-BMAA internal standard “should be chelated with the metal to the same degree as BMAA, thus accounting efficiently for this effect” but this argument assumes that all reactions occur within the timeframe of the sample preparation from the addition of the D3-BMAA through the extraction and injection (Jiang et al., 2013). Interestingly, a recent publication compared the concentrations obtained with AQC derivatization and fluorescence detection to those obtained by MS/MS analysis of the underivatized parent concluded “HPLC-FLD overestimated BMAA concentrations in some cyanobacterial samples” (Faassen et al., 2012). It is much more likely that the MS/MS analysis of the [M+H]+ and daughter ions underestimated BMAA as a result of adduct formation and ion suppression (Cohen, 2012; Glover et al., 2012). Mass spectrometry
can also be used to partially solve the problems of confounding results created by isomers of BMAA. Jiang et al. (2012) reported that only one of the possible BMAA isomers, β-amino-N-methylalanine (BAMA), produced the identical daughter ions as BMAA in tandem MS analysis of the AQC derivative but that the relative ratio of the daughters varied significantly.

1.6 Method Validation Parameters

1.6.1 Limit of Detection

The limit of detection (LOD) is the lowest concentration at which a compound can be reliably identified using a given detection technique (Currie, 1997); accurate determination of the LOD is paramount in analytical chemistry to determine the suitability or appropriateness of a method. The United States Pharmacopeia (USP) method for determination of LOD is calculated as simply two or three times the detector noise at, for a chromatographic detector, the appropriate retention time (USP, 2009). The International Union of Pure and Applied Chemistry (IUPAC), however, calculates LOD based on average noise levels in matrix blanks; how much greater the signal must be than observed noise depends upon the desired confidence levels (Thompson et al., 2002). The Food and Drug Administration (FLDA) disagrees with these practices, defines the LOD as calculated from serial dilution of samples containing the analyte of interest and evaluation of signal to noise at each concentration to determine a value that accounts for sample matrix effects (FDA, 1994).

1.6.2 Lower Limit of Quantitation

The lower limit of quantitation (LLOQ) is the lowest concentration of an analyte that can be assigned a measurable concentration in a given matrix, and must be considered along with
the LOD. The USP defines LLOQ as 10 times the detector noise at the appropriate retention
time (USP, 2009), while IUPAC defines it as the concentration at which the detector
reproducibility increases to a relative standard deviation greater than 10% (Thompson et al.,
2002). As with LOD, the FLDA estimates LLOQ through visual examination of linearity at various
concentrations and calculation of relative standard deviation (RSD) at each point (must be less
than 15% at the lowest concentration on the calibration curve) (FLDA, 1994).

1.6.3 Linearity and Range

To ensure accurate quantitation, it is expected that a detector response be linear over
the quantifiable range (Betz et al., 2011). This is generally completed by examining a plot of
detector response as a function of concentration; the R² value is used to estimate linearity over
the region. R² values of 0.999 or better are considered acceptable (Betz et al., 2011), although
both the low- and high-concentration ends of the curve must be examined carefully, as a point-
of-high-leverage may have an large impact on linearity while maintaining a high R² value. As
linear calibration curves are most often used, analyte peak areas must fall within the linear
range to allow for proper quantification.

1.6.4 Accuracy vs. Precision

Accuracy is defined “as the measure of the closeness of the experimental value to the
actual amount of the substance in the matrix” (Betz et al., 2011), while precision is an indicator
of how closely repeat measurements match up to one another (Betz et al., 2011).

Determination of accuracy is usually done through recovery experiments in which different, but
known, amounts of an analyte are spiked into a sample prior to the extraction process; how
closely the spiked and unspiked samples vary can be used as a determination of the method
(Betz et al., 2011), as this will allow the operator to determine whether losses occur in the sample preparation process. Methods demonstrating high recovery are said to be accurate, as observed changes in concentration are close to actual changes in concentration, while those with highly reproducible measurements are said to be precise (Betz et al., 2011). Both precision and accuracy are critical components of any analytical method, and must be evaluated independently of each other.

1.7 Does BMAA Incorporate into Protein?

Following detection of BMAA in human tissues (Murch et al., 2005), it was hypothesized that proteins could act as a neurotoxic reservoir, storing and releasing BMAA with protein turnover, but the data were not sufficient to determine whether the BMAA was incorporated into the peptide backbone or whether the BMAA was associated with carrier proteins or attached through electrostatic interactions (Murch et al., 2005). This unknown state was termed “protein associated BMAA” and most subsequent researchers have included protein hydrolysis in BMAA analysis (Banack et al., 2011). Dunlop et al. (2013) recently described incorporation of $^3$H$_3$-labelled BMAA into protein in human cell cultures, and histological evidence indicated that the BMAA-containing proteins precipitated in the cells. Changes in neurological proteins, including changes in the structure of myelin basic protein, have been reported in rats fed BMAA (Karlsson et al., 2014a). If BMAA is incorporated into the protein primary structure via errors in protein synthesis, it may cause misfolding of proteins, as hydrophobic domains or regions of the protein could be exposed to an aqueous environment, thus leading to precipitation and aggregation, and therefore plaque formation observed in ALS patients from Guam (Murch et al., 2005).
1.8 Hypothesis and Objectives

The overall objective of the thesis was to determine whether BMAA is incorporated into the peptide backbone of proteins. I hypothesized that the non-protein amino acid is incorporated into protein via an error in synthesis. To investigate this hypothesis, I designed the following specific objectives:

1. To develop and validate a method for accurate detection and quantification of BMAA.
2. To determine whether BMAA is present in protein fragments isolated from human brain tissues using proteomics tools.
3. To determine whether BMAA can substitute for other amino acids in protein in an in vitro protein synthesis system.
Chapter 2: Accurate Methods for Detection and Quantification of BMAA

2.1 Synopsis

BMAA is a non-protein amino acid produced at low levels (μg/g range) by cyanobacteria in the environment (Banack et al., 2007). The strong correlation between BMAA and onset of neurological disease symptoms, including those of ALS and Alzheimer’s disease, indicate a need for proper quantification techniques to allow proper study (Glover et al., 2014). Analysis of BMAA has traditionally proven challenging; two biologically-occurring isomers exist and confound chromatographic separation prior to mass spectrometric detection (Glover et al., 2014). The objective of this work is to develop an accurate, precise and sensitive method for the analysis of BMAA and the isomers AEG and DAB that is applicable to a wide variety of sample matrices. For method development, the limit of detection and lower limit of quantification were calculated by the IUPAC, USP, and FLDA definitions. A method for complete chromatographic separation of the isomers was developed and tested on a series of natural health products containing cyanobacteria.

2.2 Methods

2.2.1 Chemical Sources

L-BMAA, hydrochloric acid (97%), and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO). Trichloroacetic acid (TCA), MS-grade methanol, glacial acetic acid, and hydrolysis vials were purchased from Fisher Scientific (Mississauga, ON). Centrifugal filters were purchased from Millipore (Mississauga, ON). The AccQ-Tag Ultra Amino Acid Analysis Kit was
purchased from Waters Corp (Milford, MA). The D₃,¹⁵N₂-BMAA internal standard was donated by the Institute for Ethnomedicine (Jackson, WY). An overview of the sample preparation scheme is shown in Figure 2.1.

2.2.2 Preparation of Standards

A 10 mg sample of each analyte (BMAA, AEG, and DAB) was weighed out; 1 mL of 20 mM HCl was added to each sample to create a 10 mg/mL (0.065 M) stock solution. A 100 μL aliquot of each was combined and further diluted with 700 μL of 20 mM HCl. This solution was then diluted ten-fold three times. To create a calibration curve, this solution was diluted by mixing a 250 μL aliquot in 750 μL of 20 mM HCl. This was then repeated an additional five times to create a six point set of calibration solutions ranging from 6.5 pmol/μL to 0.00063 pmol/μL.

2.2.3 Standard Curves

Derivatized calibration standards were prepared by combining a 20 μL aliquot of each standard with 40 μL of a 0.2 M sodium borate buffer, 20 μL of a 6.5 pmol/μL D₃,¹⁵N₂-BMAA solution, and then with 20 μL reconstituted AccQ-Fluor Reagent (Waters, P/N: 186003836) in a 1.5 mL microcentrifuge tube immediately before vortexing. Standards were heated for 10 minutes at 55 °C and transferred to a polypropylene autosampler vial. Injections were performed as described in section 2.2.6. All standards were run in triplicate on three different days.

2.2.4 Preparation of Samples

Approximately 50 mg of powdered sample, a natural health product containing spirulina and hypothesized to contain BMAA, was weighed out into a 4 mL glass vial. A stock solution of
10 N HCl was diluted down to 6 N using LCMS grade water. An aliquot of 980 μL of this stock solution was added to each vial, along with 20 μL of a 6.5 pmol/μL solution of the internal standard. Nitrogen gas was then blown over each for 30 seconds to displace atmospheric oxygen. All vials were sealed and placed in a heating block for 18 hours at 110 °C. After cooling, 400 μL of each solution were centrifuge filtered using a 0.22 μm PDVF membrane (Millipore Ultrafree MC) and stored at -20 °C until analysis.

A 10 μL aliquot of the filtrate was then dried down overnight in a speedvac (Labconco Centrivap). The following day, each sample was reconstituted in 200 μL of 20 mM HCl. A 20 μL aliquot was removed and combined with 60 μL of 0.2 M borate buffer and then with 20 μL reconstituted AccQ-Fluor Reagent in a 1.5 mL microcentrifuge tube immediately before vortexing. Samples were heated for 10 minutes at 55 °C and transferred to an autosampler vial.
2.2.5 Spiked Samples for Estimation of Recovery

To ensure proper recovery of BMAA and its isomers by this method, a mixture of all three was spiked into samples at two different concentrations. The volume of 6 N HCl was decreased to allow for a spike with final concentrations of 1.5 or 0.15 ng/mL in the hydrolysis solution.

2.2.6 Chromatography

A 10 μL aliquot from each sample was injected onto a reverse phase column (150 x 2.1 mm, 1.7 μm C18 BEH, Waters) using a Waters I-Class UPLC. Column temperature was set to 55 °C and the flow rate set to 0.650 mL/min. Mobile phase A was 20 mM ammonium acetate.
(adjusted to pH 5.0 using glacial acetic acid) and B 100% methanol. The gradient was set as follows: 0.0 min = 90.0% A; 5.0 min = 50% A curve 6; 5.1 min = 15% A curve 6; 5.5 min = 15% A curve 6; 5.6 min = 90% A curve 6; 7.0 min = 90% A curve 6.

The eluent was split so that only one-third of the eluent was directed to the ESI source of a Waters Xevo TQ-S triple quadrupole mass spectrometer. In order to control contamination buildup on the sample cone, largely resulting from involatile borate buffer salts in the derivatization mixture, a divert valve was used to modify the flow state throughout the run. From 0.85 to 5.6 minutes, eluent was sent to the mass spectrometer; for the remainder of the run, the eluent was shunted to waste.

2.2.7 Mass Spectrometry

The instrument was run in ES+ mode, with a cone voltage of 16 V, a capillary voltage of 750 V and a source offset of 20 V. The desolvation temperature was set to 550 ºC, with corresponding gas flow of 800 L/hr and a cone gas flow of 150 L/hr. Data were acquired in MRM mode, with ultra high purity argon, regulated to 7 psi, used as the collision gas. The instrument was configured such that a resolution of 0.75 amu was achieved across each quadrupole, with an applied span of 0.2 amu. A dwell time of 15 ms was used for all MRM transitions. All channels were monitored from 1.0 to 5.5 minutes. Data were acquired using the MRM transitions outlined in Table 2.1.

Lysine was used to monitor the derivatization reaction to ensure data integrity since lysine can accept up to two derivative tags with the AccQ-Fluor reagent used in these experiments. Incompletely derivatized lysine gives three distinct chromatographic peaks; the two mono-derivatized forms and the double derivatized lysine. Quantification of all three
peaks provides a measure of derivatization reaction completeness and ensured results are not influenced by incomplete derivatization.

Table 2.1: MRM transitions monitored in the analysis of BMAA, AEG and DAB.

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Precursor Ion (m/z)</th>
<th>Product Ion (m/z)</th>
<th>Collision Voltage (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoderivatized BMAA</td>
<td>230.0</td>
<td>171.0</td>
<td>30</td>
</tr>
<tr>
<td>Monoderivatized Lysine</td>
<td>317.3</td>
<td>171.0</td>
<td>18</td>
</tr>
<tr>
<td>AEG/BMAA/DAB</td>
<td>459.0</td>
<td>119.0</td>
<td>18</td>
</tr>
<tr>
<td>AEG/BMAA/DAB</td>
<td>459.0</td>
<td>171.0</td>
<td>30</td>
</tr>
<tr>
<td>DAB Qualifier</td>
<td>459.0</td>
<td>188.0</td>
<td>20</td>
</tr>
<tr>
<td>BMAA Qualifier</td>
<td>459.0</td>
<td>214.0</td>
<td>20</td>
</tr>
<tr>
<td>AEG Qualifier</td>
<td>459.0</td>
<td>258.0</td>
<td>20</td>
</tr>
<tr>
<td>AEG/BMAA/DAB</td>
<td>459.0</td>
<td>289.1</td>
<td>15</td>
</tr>
<tr>
<td>Derivatized Lysine</td>
<td>487.3</td>
<td>171.0</td>
<td>18</td>
</tr>
</tbody>
</table>

2.2.8 Data Analysis

Acquired data were processed using MassLynx V4.1 (Waters). Chromatograms were smoothed using a 3x2 Mean smooth. Detector Responses were recorded as the analyte peak area as a function of peak area of the internal standard.
2.3 Results

2.3.1 Chromatographic Separation of Isomers

I have developed a chromatographic separation for AQC-derivatized BMAA, AEG and DAB (Figure 2.2) that achieves a minimum of baseline separation of all analytes in less than five minutes, enabling rapid screening for BMAA in a variety of different matrices. My results show that complete separation of all three analytes was not possible in low-pH mobile phases, even using a long (150 mm) C18 column and high flow rates (>0.5 mL/min.) (Figure 2.3). Alternatively, using a buffered mobile phase with only slightly acidic conditions, in this case pH 5.0, was found to improve not only separation, but also the analyte signal-to-noise (Figure 2.2). Both ammonium formate and ammonium acetate (pH adjusted with their respective conjugate acids) were tested under a variety of different gradients, and ammonium acetate was determined to be a more appropriate buffer for this application, providing a better separation and improved analyte peak shape (Figures 2.2 and 2.3).
Figure 2.2: Chromatographic separation using ammonium acetate and methanol of AEG, BMAA, and DAB, respectively, after derivatization with AccQ-Fluor reagent.
Figure 2.3: Chromatographic separation using ammonium formate and acetonitrile of BMAA, AEG, and DAB, respectively, after derivatization with AccQ-Fluor reagent.
2.3.2 Optimization of Mass Spectrometry

All source parameters were carefully optimized in the mass spectrometer, and it was found that abnormally low capillary voltages produced a much higher instrument response (Figure 2.4 – Figure 2.6) than could be achieved at normal voltages. Other parameters were also tested, namely the cone voltage, source offset, collision gas flow, collision energy and dwell time (Figure 2.7 – Figure 2.21). Unless otherwise specified, all data shown is for the MRM channel 459>119, as this was found to be representative for all traces. The dwell time and source offset did not demonstrate large changes over the range tested, but both the cone voltages and dwell times were found to have slight maxima. Clear maxima were found for the collision gas flow and collision energies used to induce CID during chromatographic analysis (Figure 2.13 – Figure 2.18). MRM channels and associated collision voltages determined from this optimization step are listed in Table 2.1.
Figure 2.4: Capillary voltage optimization for AQC-derivatized N-β-methylamino-L-alanine.

Figure 2.5: Capillary voltage optimization for AQC-derivatized N-(2-aminoethyl)glycine.
Figure 2.6: Capillary voltage optimization for AQC-derivatized 2,4-diaminobutyric acid.

Figure 2.7: Cone voltage optimization for AQC-derivatized N-β-methylamino-L-alanine.
Figure 2.8: Cone voltage optimization for AQC-derivatized N-(2-aminoethyl)glycine.

Figure 2.9: Cone voltage optimization for AQC-derivatized 2,4-diaminobutyric acid.
Figure 2.11: Source offset optimization for AQC-derivatized N-β-methylamino-L-alanine.

Figure 2.10: Source offset optimization for AQC-derivatized N-(2-aminoethyl)glycine.
Figure 2.12: Source offset optimization for AQC-derivatized 2,4-diaminobutyric acid.

Figure 2.13: Collision gas flow optimization for AQC-derivatized N-β-methylamino-L-alanine.
Figure 2.15: Collision gas flow optimization for AQC-derivatized N-(2-aminoethyl)glycine.

Figure 2.14: Collision gas flow optimization for AQC-derivatized 2,4-diaminobutyric acid.
Figure 2.16: Collision energy optimization for AQC-derivatized N-β-methylamino-L-alanine for MRM channels A) m/z 459>289 B) m/z 459>258 C) m/z 459>171 and D) m/z 459>119.
Figure 2.17: Collision energy optimization for AQC-derivatized N-(2-aminoethyl)glycine for MRM channels A) m/z 459>289 B) m/z 459>214 C) m/z 459>171 and D) m/z 459>119.
Figure 2.18: Collision energy optimization for AQC-derivatized 2,4-diaminobutyric acid for MRM channels A) m/z 459>289 B) m/z 459>188 C) m/z 459>171 and D) m/z 459>119.
Figure 2.19: Dwell time optimization for AQC-derivatized N-β-methylamino-L-alanine.

Figure 2.20: Dwell time optimization for AQC-derivatized N-(2-aminoethyl)glycine.
Figure 2.21: Dwell time optimization for AQC-derivatized 2,4-diaminobutyric acid.
2.3.3 Limit of Detection by USP, IUPAC, and FDA Methods

Evaluation of the IUPAC, USP and FDA methods for determination of the LOD and LLOQ revealed that the USP method produces a value for each analyte that is several times higher than the LOD and LLOQ determined by the FDA (Table 2.3). Similarly, the IUPAC definition underestimated the LOD and LLOQ for BMAA and AEG, but overestimated it by a factor of two, relative to the FDA method, for DAB (Table 2.3).

2.3.4 Linearity

Calibration curves were visually evaluated and a statistical analysis performed at each of the six points, the correlation coefficient (R²) was calculated through linear regression; values of 0.9999 or better were reported in each case, indicating excellent linearity over the working range of the method. The %RSD was calculated at each point in the curve; the values presented in Table 2.2 are averages across all concentration levels, but individual calibration points all possessed %RSDs between 3-6%. While BMAA and AEG could be detected at values at least sixteen times lower in concentration, and DAB at four times, linearity was lost below the stated LLOQ, providing a reasonable value that matches with the FDA definition. For our analysis, the instrument LLOQ was determined to be 0.63 pmol/μL, with 0.13 pmol/μL used as the LOD (Table 2.3) to prevent any false identification of BMAA in a matrix despite the ability to obtain chromatographic peaks at concentrations lower than that level in calibration standards. Had the IUPAC or USP methods been used for the biological samples used in this study, a calibration curve demonstrating poor precision would be required, thus interfering with adequate quantification.
2.3.5 Measurement Precision and Accuracy in Spiked Samples

Low levels (1.5 and 0.15 ng of each analyte/50 mg of sample) were chosen due to the trace levels at which BMAA is often detected in many matrices. Precision was determined to be high at these levels (Table 2.4), with %RSDs lower than 5% in all cases. Accuracy was deemed adequate, with BMAA demonstrating the most variability, and recovery between 85 and 119%. AEG and DAB showed improved recoveries, with 88-100% and 83-107%, respectively. While these values do not provide outstanding recovery for all three analytes, they are well within the accepted values for analytes at this concentration level. Overall, this demonstrates that the method provided here is free of errors, both in the sample preparation and data analysis processes. Misidentification of the peaks based on retention time variability was not observed (%RSD = 0.5) (Table 2.2).
Table 2.2: Intra- and inter-day variability of instrument response and retention time (n=5).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Intra-day Variability – Detector Response (%RSD)</th>
<th>Inter-day Variability – Detector Response (%RSD)</th>
<th>Inter-day Variability – Retention Time (%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMAA</td>
<td>5.0</td>
<td>9.7</td>
<td>0.5</td>
</tr>
<tr>
<td>AEG</td>
<td>4.4</td>
<td>9.3</td>
<td>0.6</td>
</tr>
<tr>
<td>DAB</td>
<td>3.1</td>
<td>9.2</td>
<td>0.5</td>
</tr>
</tbody>
</table>
Table 2.3: Instrument and method limits of detection by USP, IUPAC, and FLDA methods.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>BMAA</td>
<td>0.52/1.70</td>
<td>0.10/0.12</td>
<td>0.16/0.63</td>
<td>0.03/0.13</td>
</tr>
<tr>
<td>AEG</td>
<td>0.46/1.53</td>
<td>0.12/1.43</td>
<td>0.16/0.63</td>
<td>0.03/0.13</td>
</tr>
<tr>
<td>DAB</td>
<td>1.42/4.68</td>
<td>0.30/1.56</td>
<td>0.16/0.63</td>
<td>0.03/0.13</td>
</tr>
</tbody>
</table>
Table 2.4: Precision and accuracy in a biological matrix.

<table>
<thead>
<tr>
<th>Amount Spiked Into 50 mg Sample (ng)</th>
<th>Accuracy – BMAA</th>
<th>Precision – BMAA (%RSD)</th>
<th>Accuracy – AEG</th>
<th>Precision - AEG (%RSD)</th>
<th>Accuracy – DAB</th>
<th>Precision – DAB (%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15</td>
<td>119%</td>
<td>4.3</td>
<td>100%</td>
<td>4.3</td>
<td>107%</td>
<td>4.2</td>
</tr>
<tr>
<td>1.5</td>
<td>85%</td>
<td>2.7</td>
<td>88%</td>
<td>3.1</td>
<td>83%</td>
<td>3.8</td>
</tr>
</tbody>
</table>
2.3.6 Application in Spirulina Samples

Application of this optimized method to natural health products containing cyanobacteria demonstrate that BMAA is present at low levels in many samples, ranging from 0.13 – 0.33 ug/g of dry powder (Table 2.5). Fluorescent imaging of these natural health products under a microscope demonstrates that much, if not all, of the spirulina present is left intact through processing (Figure 2.21). Both AEG and DAB were discovered in all samples tested. AEG was found to be present at much higher and more variable levels: concentrations between 0.14 and 6.5 ug/g were detected (Table 2.5). The samples were found to contain much higher levels of DAB than either of the other two isomers, ranging from 9.3 to 107 ug/g of dry powder (Table 2.5).

Figure 2.22: Fluorescence image of intact spirulina in a commercial natural health product, taken on a darkfield fluorescence microscope with a red filter applied.

When the recommended daily dosage on the packaging is taken into account, it becomes apparent that an individual consuming these products could ingest several micrograms of BMAA per day, and perhaps milligrams of DAB (Table 2.6).
Table 2.5: Quantification of BMAA, AEG and DAB in natural health products.

<table>
<thead>
<tr>
<th>Product Tested</th>
<th>BMAA (μg/g)</th>
<th>AEG (μg/g)</th>
<th>DAB (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural Health Product 1</td>
<td>&lt;LOQ</td>
<td>2.497 ± 0.075</td>
<td>18.811 ± 0.551</td>
</tr>
<tr>
<td>Natural Health Product 2</td>
<td>0.205 ± 0.005</td>
<td>0.139 ± 0.007</td>
<td>23.179 ± 0.556</td>
</tr>
<tr>
<td>Natural Health Product 3</td>
<td>0.134 ± 0.003</td>
<td>6.479 ± 0.315</td>
<td>9.324 ± 0.431</td>
</tr>
<tr>
<td>Natural Health Product 4</td>
<td>0.333 ± 0.022</td>
<td>2.790 ± 0.076</td>
<td>33.050 ± 1.081</td>
</tr>
<tr>
<td>Natural Health Product 5</td>
<td>0.193 ± 0.020</td>
<td>1.129 ± 0.044</td>
<td>107.065 ± 2.676</td>
</tr>
</tbody>
</table>
### Table 2.6: Estimated daily intake of BMAA, AEG and DAB in natural health products.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural Health Product 1</td>
<td>Bulk Powder</td>
<td>-</td>
<td>6.24</td>
<td>47.03</td>
</tr>
<tr>
<td>Natural Health Product 2</td>
<td>Bulk Powder</td>
<td>3.46</td>
<td>2.35</td>
<td>391.73</td>
</tr>
<tr>
<td>Natural Health Product 3</td>
<td>Bulk Powder</td>
<td>0.40</td>
<td>19.44</td>
<td>28.03</td>
</tr>
<tr>
<td>Natural Health Product 4</td>
<td>Tablet</td>
<td>1.00</td>
<td>8.37</td>
<td>99.15</td>
</tr>
<tr>
<td>Natural Health Product 5</td>
<td>Individual Powder Packet</td>
<td>2.32</td>
<td>13.55</td>
<td>1284.78</td>
</tr>
</tbody>
</table>
2.4 Discussion

2.4.1 Chromatographic Separation of Isomers

Many previous reports on the existence and concentration of BMAA in a variety of different matrices exist (Cohen, 2012), but there has been considerable contention in the literature with respect to the methods used for quantification. Much of the debate focuses on the ability, or lack thereof, to adequately distinguish BMAA, AEG and DAB, not only from each other, but from other amino acids and other background interferences as well (Banack et al., 2011). These three analytes have traditionally proven difficult to separate, with only recent literature demonstrating adequate chromatography (Jiang et al., 2012). The optimized method presented in this study demonstrates complete chromatographic separation on a short timescale (Figure 2.2), thus allowing for fast and efficient separation of the isomers.

2.4.2 Mass Spectrometry Parameters

Optimization of mass spectrometry parameters has proven valuable in this study; BMAA is often present at extremely low concentrations (Cohen, 2012), the importance of a sensitive method (i.e. a higher signal-to-noise) for use in quantitative analysis cannot be understated. These results demonstrate that the capillary voltage in ESI mass spectrometry can have significant impact on the ionization efficiency of BMAA, AEG and DAB. While AccQ-Fluor-derivatized analytes were examined in this study, these results are in accordance with earlier analyses (Glover et al.; 2012) reporting that source parameters can have a large impact on the observed and expected signals during analysis.
While not all source parameters were observed to impact the detector response, clear maxima exist for the collision energies used to induce CID. Figures 2.16 – 2.18 demonstrate that careful optimization of these parameters can lead to a large boost in sensitivity. Optimization of this particular parameter is common in mass spectrometry, and these data only reinforce the need. Overall, careful instrument optimization for compounds present at very low levels, such as BMAA, can lead to a drastic increase in sensitivity of the method, thus alleviating the need for increased sample clean up (Kubo et al., 2008) or extra equipment such as a post-column make-up pump to increase the organic content of the eluent to increase ionization efficiency (Jiang et al., 2012), thus lowering the chance of error in the analysis.

2.4.3 Limit of Detection by USP, IUPAC, and FLDA Methods

The FLDA method was determined to be the most appropriate for calculating LOD and LLOQ based upon manual examination of standard curves to determine loss of linearity and unacceptable signal-to-noise. The FLDA method relies on data from standards, and is not instead based on variations in noise level at a given point in the chromatogram (Betz et al., 2011). This is especially important for evaluating matrices such as a natural health products in which the raw materials containing BMAA may be highly diluted or adulterated with excipient products (Betz et al., 2011).

2.4.4 Linearity

Linearity is important for accurate quantification of small molecules in the sample matrix (Betz et al., 2011), and is highly dependent on instrument sensitivity and method selectivity. The optimized method provided a linear relationship between detector response and analyte concentration across a broad range of concentrations. This becomes especially
important in the analysis of BMAA, AEG and DAB in samples such as the natural health products
studied here: while BMAA was often found at concentrations less than $1 \mu g/g$, AEG was found
at higher concentrations, and DAB at concentrations exceeding $100 \mu g/g$. Given the large
concentration range in which these analytes are found, a linear detector response across the
region is critical for accurate quantification of these analytes.

2.4.5 Measurement Precision and Accuracy in Spiked Samples

The use of an isotopically-labelled internal standard helps to correct for minor losses
during the sample preparation process, but complete derivatization is still necessary to ensure a
high-quality method (Jiang, et al., 2012). The accuracy of the method was determined to be
within 20% of any given measurement; this falls within the FLDA guidelines to analytes present
at this concentration, and was accepted for this study (FLDA, 1994).

Precision was also extremely high for biological samples tested, with a %RSD of less than
five percent observed for all biological samples tested. This demonstrates that the analysis is
not prone to high between-sample variability, thus making the method both accurate and
precise.

2.4.6 Application in Spirulina Samples

With an ever-increasing number of reports demonstrating the potential toxicity of
BMAA upon ingestion (Cox et al., 2009), and the increasing use of algae in popular natural
health products (Mondo et al., 2014), it is becoming more and more important that both
human and animal foodstuffs are checked to ensure public safety in the event that raw
ingredients contain BMAA.
There are no reports of toxic action by AEG, but many publications have pointed to widespread human consumption of BMAA (Roney et al., 2009; Pablo et al., 2009; Brandt et al., 2010; Jonasson et al., 2010; Mondo et al., 2010), and while no clear value for a toxic dose by any mechanism can yet be determined, it cannot be said whether these amounts will or could contribute to neurodegenerative symptoms associated with chronic, low-level exposure to BMAA. As a result, these findings stress the need for a time- and cost-effective method, such as that presented here, to screen products containing spirulina for BMAA and its isomers.
Chapter 3: Determination of BMAA-Containing Proteins in Human Tissues By Bottom-Up Proteomics

3.1 Synopsis

The occurrence of BMAA in brain tissue that cannot be separated by any manner except hydrolysis to release individual amino acids (Murch et al., 2004b) is in accordance with more recent work (Dunlop et al., 2013) demonstrating that tritium-labeled BMAA can be incorporated into protein during synthesis. The objective of this investigation was to examine the occurrence of brain protein containing BMAA in tissue from post-mortem human patients. A strategy was developed to modify existing bottom-up proteomics protocols to include BMAA as a potential constituent in protein. This method was then applied to human tissues to determine if BMAA can be detected in human proteins and peptides.

3.2 Methods

3.2.1 Human Brain Tissues

Post-mortem human brain tissue samples were provided by Dr. Deborah Mash at the University of Miami Brain Bank. Samples were shipped and received on dry ice and stored at -80° C until analysis. Patient diagnosis and demographic information is summarized in Table 3.1. All samples were blinded, and all appropriate ethics protocols were followed. BMAA, AEG, and DAB were quantified in brain tissue using the method described in Chapter 2.

3.2.2 Protein Extraction

Approximately 100 mg of wet tissue were weighed, in triplicate, into microcentrifuge tubes immediately after storage at -80° C. One milliliter of ice-cold PBS was added to each tube
and vortexed for 30 seconds to remove any remaining blood. Samples were centrifuged (13,000 g x 4 minutes at 4°C), and the PBS supernatant discarded.

3.2.3 Separation of Protein by Subcellular Fractionation

The Pierce Subcellular Protein Fractionation Kit was used to fractionate the samples (P/N: 87790). A 1000 μL aliquot of the supplied cytoplasmic extract buffer and 10 μL of Halt Protease Inhibitor were added to each sample prior to Dounce homogenization to completely disrupt the tissue. After homogenization, the sample was transferred to a clean microcentrifuge tube. Each sample was centrifuged as before, and the supernatant transferred to a clean microcentrifuge tube. All collected fractions were kept on ice for the duration of the sample preparation process. The pellet remaining in each tube was treated with 650 μL of the supplied Membrane Extraction Buffer and 6.5 uL of the supplied Halt Protease Inhibitor and vortexed briefly. After incubation on ice for 10 minutes, samples were spun down as before, and the supernatant collected. Nuclear protein was removed with the use of 220 μL of the supplied Nuclear Extraction Buffer and 2.2 μL of Halt Protease Inhibitor. After vortexing, samples were gently rotated at room temperature for 30 minutes before centrifugation and collection of the supernatant as before. Extraction of chromatin-bound protein was completed with an additional 170 μL of Nuclear Extraction Buffer with 1.7 μL Halt Protease Inhibitor, 5 μL 100 mM CaCl2 and 3 μL of the supplied Micrococcal Nuclease. Each subsample was vortexed until the pellet was completely broken up, and gently rotated at room temperature for 30 minutes before centrifugation and supernatant removal. Removal of cytoskeletal protein was carried out with 125 μL of the supplied Pellet Extraction Buffer with 1.2 μL of Halt Protease Inhibitor. Samples were vortexed and incubated at room temperature for 10 minutes. After
centrifugation and collection of the supernatant containing solubilized protein, the remaining pellet from each sample was discarded.

3.2.4 Protein Concentration by Bicinchinonic Acid (BCA) Assay

The Pierce BCA Protein Assay Kit (PN 23227) was used to quantify total protein in each sample. A series of 10 calibration solutions were made using bovine serum albumin ranging from 10 mg/mL to 0.5 mg/mL in nuclease-free water. A 5 μL aliquot of each standard was diluted to 25 μL in water, and combined with 200 μL of the Working Reagent in a 96-well plate. The plate was covered, vortexed, and allowed to incubate at room temperature for 30 minutes before measurement with a spectrophotometer. All standards were prepared in triplicate. Measurement was completed at an absorbance of 562 nm.
Table 3.1: Patient Information for Analysis of Protein Containing BMAA.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Race</th>
<th>Gender</th>
<th>Neuropathological Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>81</td>
<td>Caucasian</td>
<td>M</td>
<td>Alzheimer’s Disease</td>
</tr>
<tr>
<td>ALS</td>
<td>70</td>
<td>Caucasian</td>
<td>M</td>
<td>Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>Control</td>
<td>78</td>
<td>Caucasian</td>
<td>F</td>
<td>Non-Neurologic Control</td>
</tr>
</tbody>
</table>
3.2.5 Protein Denaturation

A 100 μL aliquot of supernatant from each subcellular protein fraction was transferred to a clean microcentrifuge tube and then diluted with 100 μL of 50 mM ammonium bicarbonate. A 20 μL aliquot of 10% deoxycholate (DOC) was added to each tube (for a final concentration of 1%) to denature protein. An aliquot of 22 μL of 50 mM TCEP was added to reduce disulphide bonds (final concentration of 5 mM). Samples were vortexed and incubated at 60° C to complete the process. Alkylation was completed with the addition of 24 μL of 100 mM iodoacetamide (IAA) to reach a final concentration of 10 mM, and incubated for 30 minutes at 37° C. The remaining IAA was quenched by adding 26 μL of 100 mM dithiothreitol (DTT) and allowing for incubation at 37° C for 30 minutes.

3.2.6 Protein Digestion

Promega trypsin was reconstituted to 40 ng/μL, and a 20 μL aliquot was added to each denatured subcellular protein fraction. After gently vortexing, the samples were allowed to incubate for 16 hours at 37° C to facilitate enzymatic digestion. After cooling to room temperature, 30 μL of 1% formic acid was added to each sample to precipitate DOC. After centrifugation (13,000 g x 10 minutes) to pellet the DOC, a volume equivalent to 100 μg of protein (as determined by the BCA assay) was mixed with 700 μL of 0.1% formic acid and vortexed prior to cleanup and removal of other detergents by solid phase extraction (SPE). SPE was completed using 10 mg Waters Oasis HLB cartridges. Each cartridge was washed with 1 mL of methanol and conditioned with 1 mL of water. Samples were then loaded onto the cartridges, and subsequently washed with an additional 1 mL of water. Elution was performed...
with 200 µL of 50/50 acetonitrile/0.1% formic acid. Samples were frozen at -80°C, lyophilized overnight, and stored at -80°C until analysis.

3.2.7 Proteomics Analysis

Each sample was reconstituted in 0.1% formic acid such that 1 µg of protein was loaded on the column with each injection. Separation was carried out using a Thermo Scientific Easy-nLC II configured with a 100 µm I.D., 30 mm trap column with 5 µm, 100Å C18 (Magic C18 AQ), and a 75 µm I.D. 150 mm analytical column packed with the same silica. Solvent A was 98:2 0.1% formic acid in water:acetonitrile, and B was 10:90 water:acetonitrile with 0.1% formic acid. The gradient used for separation was as follows: Initial: 95% A; 45 min., 55% A; 47 min., 0%A; 55 min., 0% A. Data acquisition was performed on a Thermo Scientific LTQ Orbitrap Velos, operated in ESI+, with a source voltage of 2.2 kV and capillary temperature of 250°C. A 500 ms survey scan from m/z 400-2000 (60,000 FWHM) was used, with the 15 highest concentration peaks with charge states of 2-4 selected for MS/MS with a normalized collision energy of 35%. All data was collected in centroid mode. A lock mass reference of 445.120024 (siloxane) was used for internal calibration. A dynamic exclusion list (500 items) was used to prevent re-analysis of peaks for 60 seconds within a 10 ppm window. Raw data was exported in MGF format.

3.2.8 Data Analysis

PEAKS 6.0 (Bioinformatics Solutions, Waterloo, ON) was used for de novo sequencing of the obtained peptides sequences. An error tolerance of 1.0 Da was set for precursor ions (monoisotopic mass), and 0.8 Da for product ions. Non-specific cleavage was allowed at one end of the peptide, with only cleavages resulting from trypsin allowed. The maximum number
of missed cleavages permitted was 3, and an average local confidence (ALC) score cutoff of 30% used in the data analysis. Allowed post-translational modifications (PTM) were carbamidomethylation, and a custom modification of +3.05 on asparagine used to simulate the presence of BMAA in a peptide.

An ANOVA was completed using R for the nuclear subcellular fraction proteins. Treatment groups were used as the independent variable, while the relative number of peptides identified containing BMAA was taken as the dependent variable in order to determine overall significance of the model, with $\alpha$ set to 0.05.

### 3.3 Results

#### 3.3.1 BMAA, AEG, and DAB measured in Human Brain Samples

The measured concentrations of BMAA, AEG and DAB in the three human patient samples are listed in Table 3.2, below.

<table>
<thead>
<tr>
<th>Patient</th>
<th>BMAA ± SE (μg/g)</th>
<th>AEG ± SE (μg/g)</th>
<th>DAB ± SE (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>2.12 ± 0.18</td>
<td>0.35 ± 0.02</td>
<td>ND</td>
</tr>
<tr>
<td>ALS</td>
<td>1.26 ± 0.21</td>
<td>0.16 ± 0.07</td>
<td>ND</td>
</tr>
<tr>
<td>Control</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

#### 3.3.2 De Novo Sequencing Output Files

Output files from *de novo* sequencing can be sorted by several parameters, including PTM’s. An example of the interface used to accomplish this is provided in Figure 3.1.
Figure 3.1: PEAKS user interface showing determined peptides sequences, MS/MS spectra, and sorting parameter options.
3.3.3 ALC Confidence Scores

ALC scores represent an averaged value for determined confidence levels (Average Local Confidence) at each amino acid in a sequenced peptide; peptides with higher ALC scores have a greater probability of being the determined peptide. Processed data were sorted by PTM type, then by ALC score. An example of a typical ALC score plot is given in Figure 3.2.

Figure 3.2: Typical ALC score diagram for samples obtained in this study; samples possessing an ALC lower than 30 were discarded.

Samples deemed to have an appropriate ALC score were sorted by PTM type to separate those identified as containing BMAA and those not. The data are presented in Table 3.3.
Table 3.3: Peptides Identified As Containing BMAA in Each Subsample.

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Patient Designation</th>
<th>Fraction Type</th>
<th>Total MS/MS Spectra</th>
<th>Total Number of Peptide IDs</th>
<th>Number of Peptides with BMAA</th>
<th>Peptides with BMAA/Total Peptides (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>ALS</td>
<td>Cytoskeletal</td>
<td>13777</td>
<td>3144</td>
<td>983</td>
<td>31</td>
</tr>
<tr>
<td>A2</td>
<td>ALS</td>
<td>Cytoskeletal</td>
<td>14736</td>
<td>3134</td>
<td>989</td>
<td>32</td>
</tr>
<tr>
<td>A3</td>
<td>ALS</td>
<td>Nuclear</td>
<td>11605</td>
<td>2644</td>
<td>808</td>
<td>31</td>
</tr>
<tr>
<td>A4</td>
<td>ALS</td>
<td>Membrane</td>
<td>6992</td>
<td>1782</td>
<td>561</td>
<td>31</td>
</tr>
<tr>
<td>A5</td>
<td>ALS</td>
<td>Chromatin-Bound</td>
<td>3432</td>
<td>862</td>
<td>280</td>
<td>32</td>
</tr>
<tr>
<td>B1</td>
<td>ALS</td>
<td>Cytoskeletal</td>
<td>12206</td>
<td>2877</td>
<td>837</td>
<td>29</td>
</tr>
<tr>
<td>B2</td>
<td>ALS</td>
<td>Cytoplasmic</td>
<td>13085</td>
<td>2202</td>
<td>853</td>
<td>39</td>
</tr>
<tr>
<td>B3</td>
<td>ALS</td>
<td>Nuclear</td>
<td>11988</td>
<td>2651</td>
<td>785</td>
<td>30</td>
</tr>
<tr>
<td>B4</td>
<td>ALS</td>
<td>Membrane</td>
<td>9142</td>
<td>2088</td>
<td>551</td>
<td>26</td>
</tr>
<tr>
<td>B5</td>
<td>ALS</td>
<td>Chromatin-Bound</td>
<td>2217</td>
<td>580</td>
<td>185</td>
<td>32</td>
</tr>
<tr>
<td>C2</td>
<td>ALS</td>
<td>Cytoplasmic</td>
<td>13055</td>
<td>2851</td>
<td>855</td>
<td>30</td>
</tr>
<tr>
<td>C3</td>
<td>ALS</td>
<td>Nuclear</td>
<td>11084</td>
<td>2460</td>
<td>707</td>
<td>29</td>
</tr>
<tr>
<td>C4</td>
<td>ALS</td>
<td>Membrane</td>
<td>8249</td>
<td>1997</td>
<td>529</td>
<td>26</td>
</tr>
<tr>
<td>C5</td>
<td>ALS</td>
<td>Chromatin-Bound</td>
<td>2157</td>
<td>549</td>
<td>163</td>
<td>30</td>
</tr>
<tr>
<td>D1</td>
<td>AD</td>
<td>Cytoskeletal</td>
<td>10635</td>
<td>2492</td>
<td>721</td>
<td>30</td>
</tr>
<tr>
<td>D2</td>
<td>AD</td>
<td>Cytoplasmic</td>
<td>12244</td>
<td>2688</td>
<td>805</td>
<td>30</td>
</tr>
<tr>
<td>D3</td>
<td>AD</td>
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3.3.4 Occurrence of BMAA in Peptides

Visual examination of three MS/MS spectra indicate that at least some peptides identified as containing BMAA possess low signal-to-noise ratios and poor ALC scores (<40%) (Figure 3.3), while others found to contain BMAA possess excellent signal-to-noise ratios (<70%) (Figure 3.4). An example of a peptide that does not contain BMAA, but does have a similar confidence score, is provided in Figure 3.5.
Figure 3.3: Low confidence score (ALC < 40%) MS/MS spectrum of a peptide identified as containing BMAA.
Figure 3.4: High confidence score (ALC > 70%) MS/MS spectrum of a peptide identified as containing BMAA.
Figure 3.5: High confidence score (ALC > 70%) MS/MS spectrum of peptide identified without BMAA.
3.3.5 Relative Number of Peptides Containing BMAA

The percentage of peptides found to contain BMAA in each subsample type was averaged and plotted in Figure 3.6. Error bars represent the standard error of the individual means. The ANOVA completed for the nuclear fraction revealed statistically significant differences between the three groups; the control patient was determined to be statistically different from the ALS and AD patient groups, with a p-value of 0.0318. An ANOVA containing all data supplied in Figure 3.6 revealed no statistical significance when all groups were considered.
Figure 3.6: Relative number of peptides identified in each subcellular fraction as containing BMAA. Error bars represent the standard error.
3.4 Discussion

3.4.1 Identification of a Non-Protein Amino Acid in Protein

A variety of studies have examined the presence of non-protein amino acids in protein. Selenocysteine, while not one of the twenty amino acids normally identified as a protein constituent, is found in approximately 25 human proteins, largely those involved in redox reactions (Nauser et al., 2012) and is now considered the 21st proteinogenic amino acid. Similarly, canavanine, an arginine analogue and non-protein amino acid, is produced in the seeds of certain plant species (Rosenthal et al., 1986). Pests of these plant species showed incorporation of canavanine into protein as well as much higher levels of protein degradation and turnover were observed in response to canavanine (Rosenthal et al., 1986). A follow-up study (Melangeli et al., 1997) demonstrated that a genetic basis for the resistance to canavanine does exist in the form of a gene that hydrolyzes canavanine into L-homoserine and hydroxyguanidine. Similar examples have been reviewed recently (Huang et al., 2011).

Previous analyses of non-protein amino acids in protein have been completed with radiolabeling or standard amino acid analysis; the application of bottom-up proteomics to identify non-protein amino acids in protein is novel; at the time of thesis submission, I could find no reports examining the occurrence of non-protein amino acids in peptides by this method.

As currently available proteomics software is not generally aimed at the identification of non-protein amino acids in peptides or whole proteins, the work presented here required atypical application of a sequencing software package. While normal sequencing algorithm parameters were used in this study, no commercial software package allows for the use of
additional amino acids. As such, the best way to include BMAA as a possibility in a system such as this was to use generate a post-translational modification that represents the mass of BMAA. Furthermore, this approach allows the inclusion of BMAA to be optional in a peptide; if a mass corresponding to BMAA allows for a better ‘fit’ with experimental MS/MS spectra, then it can be included at the appropriate position. If not, it is excluded, and the peptide is sequenced with proteinogenic amino acids only.

A good ‘fit’ is determined by a calculated score by all sequencing algorithms; if the predicted fragment of an amino acid matches with experimental results well, then it is assigned a high score. With PEAKS, all amino acids in a sequence are given a score, which is then averaged to generate an Average Local Confidence (ALC) score. Sequenced peptides with a higher ALC score are viewed as having a higher likelihood of being the correct sequence. Sequences with low ALC scores, on the other hand, are then removed and not used in further analysis. The user determines this cutoff value; an ALC cutoff of 30% is used in this experiment, as it was the recommended value by the software manufacturer. Future work could examine the effects of optimization of sequencing parameters, as this would lead to increased confidence in the results of the analysis.

3.4.2 Occurrence of BMAA in Peptides

I have developed a method for identifying BMAA incorporated into peptides. While there are no other reports examining this application, a recent publication examined the effect of single, large doses of BMAA given to rats and examined subsequent neuropathological changes (Karlsson et al., 2012). In this study, a combination of targeted protein quantification and global profiling by matrix-assisted laser desorption ionization (MALDI) was performed. The
authors of this study did not find differences in the total number of ions detected by two-dimensional MALDI imaging, therefore indicating a lack of detectable changes in the treatment and control groups. Targeted quantification of several proteins revealed changes: proteins involved in transmembrane signaling were reduced in BMAA-treated rats, while the number of detected histones increased significantly. That study, in contrast to the one performed here, did not attempt to detect BMAA incorporated into a protein.

My approach differs in that it provides an unbiased look at many proteins. Extracting and digesting a large number of proteins provides as complete a view of the proteome as possible, and may succeed in detecting proteins that would otherwise be missed with methods such as those used by Karlsson et al. (2012). Subcellular fractionation provides a more in-depth view of the proteome than digestion alone, as performing LC-MS/MS on a less complicated mixture of peptides will reduce ion suppression from higher-abundance peptides (Huber et al., 2003), and thus allow for more detectable peptides, and a greater chance of identifying those containing BMAA.

My results demonstrate that incorporation of BMAA into protein is possible in the human brain, as a large number of peptides were sequenced in all samples that were more likely to contain than not (Figure 3.6). In all subcellular fractions, the data suggests a trend towards a higher number of peptides peptides with BMAA from the ALS patient than either the Alzheimer’s patient or the control patient. Similarly, the Alzheimer’s patient was found to have a larger number of peptides containing BMAA than the control patient in all fractions except the cytoplasmic one. This trend provides an early indication that BMAA may play a role in neurological diseases. These results are in accordance with an earlier study in which the
incorporation of L-DOPA into protein in place of tyrosine was investigated (Rogers et al., 2012); the authors found that incorporation of L-DOPA into protein resulted in greatly increased rates of protein degradation. If incorporation of BMAA into protein was to cause the same effects as incorporation of other non-protein amino acids such as canavanine and L-DOPA, the presence of BMAA in peptides identified here supports the observed toxic activity of BMAA.

3.4.3 Potential Limitations of this Approach

Reports differ on the affect of freezing tissue samples prior to subcellular fractionation (Lim et al., 2011; Cox et al., 2006); the ice crystals formed by freezing may induce a loss in structural integrity of the cell, thus causing proteins or other small molecules to cross-contaminate one another. This may hamper the efforts of subcellular fractionation. Indeed, in this study, larger standard errors were observed for all fractions except nuclear protein. The nucleus is more resistant to damage by the freeze-thaw process (Cox et al., 2006), and the relatively large standard errors observed in these fractions may be the result of cross-contamination between fractions prior to or during sample preparation. The nuclear fractions indicate a clear difference between the neurodegenerative disease patients and the control patient, thus supporting the hypothesis that BMAA is incorporated into protein and its hypothesized role in neurodegenerative disease.

A second potential limitation to this approach is in its reliance on sequencing to identify peptides that do or do not contain BMAA; because this work is based entirely on computer algorithms, the data obtained are limited to a model-of-best-fit approach. It is, however, worth noting that the addition of post-translational modifications to sequencing parameters, such as the modification of +3.05 Da used here to simulate the presence of BMAA on arginine, is a fairly
standard practice in proteomics (He et al., 2013). This weakness can be seen in Figure 3.6: analysis of tissue from the control patient did not show any BMAA, AEG or DAB, but peptides were identified as likely to contain BMAA in the control patient as well as the disease patients. The relative number of peptides identified (between 25 and 30%) as containing BMAA in the control patient indicates a high rate of potential false discovery or a contaminating factor as yet unidentified. The difference between the control and disease patients is only 2-3% in all subcellular fractions. If all peptides found in the control are assumed to be false positives, this leaves only a small number of peptides that actually contain BMAA in the patient samples. Given the number of peptides that BMAA could be incorporated into, as well as the relatively low level at which it is present, it is unlikely that a large number of peptides will be present in these samples.
Chapter 4: Cell-Free Expression as a Model for the Study of Incorporation of BMAA into Protein

4.1 Synopsis

A specific mechanism by which BMAA is associated with proteins remains unidentified. Such studies are challenging because of the complexity of biological systems and samples. A cell-free in vitro protein synthesis system offers an excellent alternative for investigation of changing amino acid composition in protein. The objective of this was to determine whether BMAA is incorporated into protein through an error in translational synthesis. A template DNA sequence was used in a series of reactions in which BMAA replaced traditional proteinogenic amino acids. This method was then tested with DNA extracted from post-mortem human tissues to determine if a genetic basis may underlie susceptibility to incorporation of BMAA. These studies show that BMAA can replace deficient amino acids in protein structure.

4.2 Methods

4.2.1 In vitro Protein Synthesis

The PURExpress protein synthesis kit (P/N E6840S; New England Biolabs) was used for all protein synthesis reactions according to the instructions included with no variation. All kit components were kept on ice prior to experiments as per the instructions. In preliminary reactions, the template DNA (DHFR) supplied with the kit was used for both experimental groups as well as the positive and negative controls. The 20 classic protein amino acids (60 mM; Sigma, St. Louis, MO) were used as a positive control for all reactions. The final
concentration of each amino acid in the mixed solutions was 3 mM as per the kit instructions. The negative control contained only the enzymes and template DNA with no added amino acid solutions to provide a baseline protein concentration for the reaction. Once combined, all kit components were gently mixed and incubated for sixteen hours at 37°C to facilitate protein synthesis. After incubation, all sample tubes were placed on ice to halt the synthesis reaction, and 25 μL of ice cold nuclease-free water were added to each reaction. Each sample was then split into two 25 μL aliquots to measure the total amount of protein and the BMAA content of the protein product.

4.2.2 Incorporation of BMAA

The positive control contained all 20 standard protein amino acids, but not BMAA. In preliminary experiments, amino acids were eliminated from the reaction solution individually to determine which amino acids were limiting for protein synthesis. BMAA was added to the positive control (All AA+BMAA) or substituted for limiting amino acids alanine (-ala+BMAA), glutamate (-glu+BMAA), glutamine (-gln+BMAA), isoleucine (-ile+BMAA), cysteine (-cys+BMAA), phenylalanine (-phe+BMAA), proline (-pro+BMAA), serine (-ser+BMAA), and threonine (-thr+BMAA). To determine whether the reactions with BMAA were unique or could be induced by its isomers, 2,4-diaminobutyric acid (DAB) and N-(2-aminoethyl)glycine (AEG) were incorporated in the same manner.

4.2.3 Human DNA Templates

Genomic DNA was extracted from post-mortem brain tissues of 3 human patients (the same 3 used for Chapter 3 of this thesis) using the Invitrogen PureLink Genomic DNA Extraction kit (Invitrogen, Carlsbad, CA) as per manufacturer’s instructions. In brief, 25 mg of tissue were
weighed, in triplicate, into a sterile microcentrifuge tube, and 200 μL of a 90:10 mixture of the supplied digestion buffer and proteinase K solutions were added to each sample. After vortexing and incubation at 55°C, samples were centrifuged and the supernatant removed. A 20 μL aliquot of ribonuclease was added to each tube prior to incubation for 2 minutes at room temperature. A 400 μL mixture of 50:50 binding buffer:ethanol was added to each sample; the lysate was then loaded on a supplied spin column and centrifuged. Two 500 μL washes were performed, with elution completed using 100 μL of the supplied elution buffer. Quantitation and purity determination of extracted DNA was performed using a nanodrop spectrophotometer (NanoDrop ND-1000, Fisher Scientific, Mississauga, ON); the A$_{260/280}$ and A$_{260/230}$ values were between 1.8 and 2.1 in all cases, indicating all extracts were quite free of protein and salt contamination. For protein synthesis reactions with human genomic DNA, exactly 125 ng of DNA were used to match the recommended concentration of the template DNA. Protein synthesis reactions were carried out using identical conditions and procedures as initial experiments.

4.2.4 Protein Quantification

The total protein in each reaction mixture was quantified by BCA analysis (Pierce BCA Kit, Fisher Scientific, Mississauga, ON). Reagents A and B of the BCA assay kit were combined at a ratio of 8:1, respectively, and vortexed vigourously to create the Working Reagent as per the instructions. Total protein was quantified by comparison to a Bovine Serum Albumin (BSA) standard curve in triplicate at concentrations ranging from 10 – 0.5 mg/mL. For all analyses, 200 μL of the Working Reagent were mixed with 5 μL of each standard in a microcentrifuge tube. The solution was incubated at 30°C for 30 minutes, after which each sample was transferred to
a 96-well microplate (Corning Costar) and the absorbance was measured at 540 nm (BioTek Synergy HT microplate reader).

4.2.5 Analysis of BMAA

Protein was precipitated by addition of 25 μL of 0.1 N tricholoroacetic acid (TCA), vortexing and centrifuging at 16,000 g for 5 mins. The resulting protein-free supernatant was transferred to a clean microcentrifuge tube. To ensure complete separation of the protein, an additional 25 μL of 0.1 N TCA was added to the protein pellets, vortexed and centrifuged. The supernatants were pooled and analyzed separately from the protein pellets. Amino acids were analyzed using optimized methods discussed in Chapter 2.

Each protein pellet was dissolved in 500 μL of 6 N HCl in a 4 mL glass vial, and hydrolyzed overnight under nitrogen at 110°C. A 100 μL subsample of each protein hydrolysate was lyophilized overnight (Labconco Freezone). When dry, 20 μL of 20 mM HCl and 60 μL of 0.2 M borate buffer were added and samples were vortexed. Protein samples were derviatized with 20 μL of AccQ-Fluor and analyzed as discussed in Chapter 2 of this thesis.

4.2.6 Denaturation of Protein

To determine whether BMAA was carried by proteins or incorporated into proteins, the amount of BMAA released by protein denaturation was determined in subsamples of the protein pellet. This pellet was denatured with 1.5% SDS and 2 mM dithiothreitol (DTT), then re-precipitated and washed with 0.1 N TCA. The free fraction supernatant and the protein pellet were prepared as described above.
4.3 Results

4.3.1 Controls

The negative control samples contained all kit components in the absence of amino acids, and therefore provide a measure of the protein content if de novo synthesis did not occur (Figure 4.1). The positive controls contained the standard 20 amino acids and provided a measure of the expected protein synthesis for each reaction batch (Figure 4.1). Positive and negative controls were included with each batch of samples and the other treatments were randomized across the sample batches. Substitution of isoleucine, glutamine or cysteine with BMAA did not appear to result in de novo protein synthesis (Figure 4.1).

4.3.2 Amino Acid Substitution

When DAB, AEG or BMAA were added to the positive control including all 20 protein amino acids, the total amount of protein produced was significantly less than the protein produced by the solutions containing normal protein amino acids but significantly greater than the negative controls indicating that protein synthesis was partially but not completely inhibited by the non-protein amino acids (Figure 4.1). Substitution of serine, phenylalanine, threonine, proline, glutamate, and alanine with BMAA resulted in significant de novo protein synthesis (Figure 4.1). Specifically, substitution of alanine with BMAA did not significantly reduce the amount of de novo protein synthesis as compared to the positive control (Figure 4.1). When the human DNA templates were used and serine was limiting, de novo protein synthesis continued with BMAA substitution (Figure 4.1). Statistical significance was determined for the human patients as described in Chapter 3 of this thesis.
Figure 4.1: Total protein in each cell-free synthesis reaction. Negative controls represent the protein present if no de novo protein synthesis occurs. Positive controls indicate the de novo protein synthesis with 20 amino acids present. Limiting amino acids are indicated by “-”. Reactions containing the human DNA template are indicated by patterned bars. Error bars represent the standard error of the individual mean. Samples were randomized across multiple batches with individual replicates indicated as “n” for each bar.
4.3.3 Detection of BMAA in Synthetic Proteins

BMAA was not detected in proteins from the negative or positive control samples as a check of method accuracy and efficiency (Figure 4.2). Small amounts of BMAA were detected in the protein fractions of the cysteine, threonine, glutamine, and isoleucine limited treatments (Figure 4.2). When phenylalanine, proline, alanine, glutamate, and serine were limiting, between 10% and 20% of the BMAA added was found in the protein fraction (Figure 4.2). Interestingly, when the human DNA templates were used in place of the standard bacterial template and serine was limiting, the incorporation of BMAA was increased to about 35% - 70% with significant differences between control and AD patients (Figure 4.2).

4.3.4 Incorporation of BMAA by Human DNA

When human DNA was used as the template in place of the *E. coli* standard, a greater proportion of BMAA was incorporated into proteins. DNA from the patient diagnosed with AD demonstrated the highest rate of BMAA incorporation into protein, significantly greater than the matched control patient (Figure 4.1). The ANOVA completed for the patient groups revealed partial statistical significance; the control patient was significantly different from the Alzheimer’s patient (*p*=0.0023), but the ALS patient was not found to be statistically significant from either the control or ALS patients (Figure 4.2).
Figure 4.2: Percent of BMAA incorporated into de novo protein as measured by LC-MS/MS. Limiting amino acids are indicated by “-“.

Reactions containing the human DNA template are indicated by patterned bars. Error bars represent the standard error of each individual mean. Samples were randomized across multiple batches with individual replicates indicated as “n” for each bar.
4.3.5 Protein Associated vs. Free BMAA

To determine whether the protein associated BMAA was actually incorporated into the proteins during synthesis or attached to the proteins, denatured proteins were analyzed. Our results indicate that both mechanisms are involved in the protein association with about 50% of the BMAA released by denaturation (Figure 4.3).

Figure 4.3: BMAA recovered after denaturation of the protein and hydrolysis of the protein pellet after denaturation. Error bars represent the standard error of the individual means.
4.3.6 Isomers

The relative rate of association of other non-protein amino acids was determined in studies with BMAA isomers, DAB and AEG. Significantly less of these isomers were recovered in the protein fractions, with about 7% of AEG and about 3% of DAB associated with proteins (Figure 4.4).

![Figure 4.4: Incorporation of AEG (left) and DAB (right) into protein as measured by LC-MS/MS. Error bars represent the standard error of individual means.]

4.4 Discussion

The hypothesis that the non-protein amino acid BMAA could be “protein associated” has been controversial and efforts to understand the mechanisms of BMAA-protein association
were required. The in vitro PURExpress protein synthesis system offered an ideal experimental design for these studies. Our data show that BMAA is both incorporated directly into the protein backbone during de novo synthesis and attracted to the three-dimensional structure. The idea that non-protein amino acids can be misincorporated into proteins during synthesis is relatively new, but recent researchers have proposed that mischarging of the transfer RNA is one potential mechanism (Yadavalli et al., 2013). Our data demonstrate that incorporation of BMAA into the protein structure through peptide bonding occurs when structurally similar amino acids such as serine or alanine are deficient.

The data presented in Figure 4.3 indicates that association of BMAA to protein is not likely to result entirely from covalent incorporation or electrostatic interaction, but rather a combination of the two. Denaturation was able to release approximately half of all BMAA from protein, but was unable to release the rest, indicating the data presented in Figure 4.2 is likely a combination of these two factors. Future work could add depth to this analysis by examining the effect of denaturation of all samples used in this study instead of a limited number used to explore this hypothesis here, as information about the relative amounts of BMAA in these two categories could yield significant results in the effort to determine the mechanism by which BMAA acts on proteins.

The idea that non-protein amino acids can be misincorporated into proteins during synthesis is relatively new. As discussed in section 3.4.1, a historical body of evidence exists for the incorporation of non-protein amino acids into protein, and gives cause for the examination of the mechanism by which BMAA may act when consumed over long periods of time, such as
that demonstrated by the Chamorro people in Guam (Murch et al., 2004a). While work examining the toxicology of BMAA has typically been focused on BMAA itself rather than its isomers, this evidence demonstrates the importance of testing its isomers in such a situation as well. If either AEG or DAB were able to act by the same mechanism, it would be expected that they would produce the same results as when BMAA is excluded from the synthesis reaction. When AEG is added to a serine-deficient preparation, it was found that approximately one-quarter of the analyte in question was found to be incorporated, and one-half this again for DAB (Figure 4.4). Interestingly, the same amount of each of these analytes is found in protein when serine is excluded or included, suggesting that what is found to be ‘incorporated’ is likely bound through electrostatic interactions rather than covalent attachment. It should be noted that this is only a possibility, and needs to be validated through further experimentation.

The incorporation or association of BMAA with proteins, specifically human neuroprotein, is significant for several reasons. BMAA has been proposed as a potential cause of progressive neurodegeneration (Cox et al., 2003; Cox et al., 2005; Bradley et al., 2009; Bradley et al., 2013; Field et al., 2013). The incorporation of BMAA into proteins and re-release with protein turnover is one potential mechanism that would result in a continual low recirculating dose that could result in a long latency period or the “slow” toxin mechanism that has previously been proposed. Incorporation of non-protein amino acids such as BMAA into proteins is a random process but “intrinsically disordered proteins” such as α-synuclein or tau may be more prone to errors (Rodgers, 2014; Uversky et al., 2008). Neuronal cells may be less able to breakdown these malformed proteins (Rodgers, 2014) and therefore the
misincorporation of non-protein amino acids such as BMAA may be the environmental factor that leads to protein misfolding and aggregation in neurodegenerative diseases.
Chapter 5: Conclusion

This research was designed to determine whether BMAA is incorporated into the peptide backbone of proteins. I hypothesized that the non-protein amino acid is incorporated into protein via an error in synthesis.

Despite a relatively large body of literature published on the subject (Reviewed in Chapter 1), accurate quantification of BMAA remains difficult (Cohen, 2012). To address this need, I developed an accurate and precise method for the analysis of BMAA, AEG and DAB using chemical derivatization and LC-MS/MS that is both sensitive and rapid. The recovery and precision of the method were both evaluated and found to be well within acceptable limits. This method was applied to several natural health products containing cyanobacteria, and it was determined that BMAA, along with its isomers AEG and DAB, is present in many of these samples. Further research is needed to determine what doses of BMAA and lengths of exposure are toxic, but analyses such as these provide evidence for a potential source of human exposure to BMAA.

To examine whether peptides containing BMAA can be found in protein, I using bottom-up proteomics coupled with de novo sequencing to identify peptides that contain BMAA in post-mortem human brain tissue. The results indicate that BMAA was detected in a large number of peptides identified in these samples, indicating that BMAA can, and does, incorporate into protein. The incorporation of BMAA into protein is likely to cause protein misfolding and potentially bring about neurodegenerative disease symptoms (reviewed in Rodgers, 2012). A large number of peptides identified as containing BMAA were also found; as
BMAA was not detected in the brain tissue, it is likely these identifications are false positives. Future work may include investigations into methods for the reduction of false positives, be it through developing new algorithms for sequencing of MS/MS spectra or optimization of parameters with current ones.

A model system was developed for the examination of incorporation of BMAA during protein synthesis through the use of a cell-free synthesis system. These systems are ideal due to the lack of any other metabolism, thus allowing for a simplified look at the role of BMAA during protein synthesis. A series of reactions was carried out using a template DNA sequence in reactions in which one amino acid was excluded, but BMAA included. Several of these reactions were replicated using DNA extracted from post-mortem human brain tissue. The results indicate that when amino acids similar in structure to BMAA are excluded, but BMAA is included, it is more likely that BMAA will be incorporated into protein. These results are in agreement with previous studies in which non-protein amino acid analogues were incorporated into protein through errors in tRNA synthetase charging (reviewed in Rodgers, 2014). Furthermore, our results show that DNA from neurodegenerative disease patients allows for more incorporation of BMAA into protein, indicating that a genetic susceptibility may underlie the ability of BMAA to incorporate into protein. Future work with cell-free expression may include the use of $^3$H or $^{14}$C labeled amino acids in the synthesis reaction, followed by gel electrophoresis and imaging to isolate and identify specific peptides formed in this process as containing BMAA. LC-MS/MS sequencing, such as that outlined in Chapter 3, would be of value in efforts to improve the fidelity of de novo sequencing, as would the use of synthetic peptides containing BMAA, as these would provide positive controls in which to use in the optimization
of sequencing parameters. Overall, my results demonstrate that BMAA can be present in dietary supplements and incorporated into protein during synthesis. Further research is needed to determine the effect of BMAA on protein structure and function.
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