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Extraction of Non-Protein Amino Acids from Cyanobacteria for Liquid Chromatography-Tandem Mass Spectrometry Analysis

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Abstract

Non-protein amino acids (NPAAs) are a large class of amino acids (AAs) that are not genetically encoded for translation into proteins. The analysis of NPAAs can provide crucial information about cellular uptake and/or function, metabolic pathways, and potential toxicity. β-methylamino-L-alanine (BMAA) is a neurotoxic NPAA produced by various algae species and is associated with an increased risk for neurodegenerative diseases, which has led to significant research interest. There are numerous ways to extract AAs for analysis, with liquid chromatography-tandem mass spectrometry being the most common, requiring protein precipitation followed by acid hydrolysis of the protein pellet. Studies on the presence of BMAA in algal species provide contradictory results, with the use of unvalidated sample preparation/extraction and analysis a primary cause. Like most NPAAs, protein precipitation in 10% aqueous TCA and hydrolysis with fuming HCl is the most appropriate form of extraction for BMAA and its isomers aminoethylglycine (AEG) and 2,4-diaminobutyric acid (2,4-DAB). The present protocol describes the steps in a validated NPAA extraction method commonly used in research and teaching laboratories.

Introduction

Amino acids are chemical compounds that contain at least one amine and carboxylic functional group. Some amino acids also contain an imino group, a functional acid group other than carboxylic; other amino acids have amine groups that are not attached to the α-carbon group[1]. There are over 500 amino acids[2], 22 of which are known as protein amino acids used for genetic coding in ribosomal protein synthesis[3]. These 22 amino acids can be further subdivided into essential and non-essential. Essential amino acids are necessary for an organism to function properly and can only be acquired by external sources. Non-essential amino acids can be synthesized within the organism. The classification of the 22 amino acids into essential/non-essential is unique to individual species. All other amino acids are non-protein

amino acids (NPAAs) that are not encoded for protein synthesis. Amino acids, whether protein or non-protein, can also play a signaling role within an organism and act as metabolic intermediaries[4]. Due to their important and varying roles, amino acid levels can provide an insight into organism condition, functionality, and metabolic pathways, etc. There are two main mechanisms by which amino acids can be incorporated into a polypeptide chain: ribosomal protein synthesis, which utilizes the 22 amino acids in encoding, and non-ribosomal peptide synthesis, which, alongside protein amino acids, allows for the use of some NPAAs in synthesis. Certain NPAAs can mimic protein amino acids, potentially leading to their misincorporation into peptides and proteins. The misincorporation causes a misfolding of proteins, which, in turn, has detrimental effects[5], such as the misincorporation of the NPAA L-3,4 dihydroxyphenylalanine (L-DOPA) in place of tyrosine, negatively impacting cellular function and health[6,7]. An additional source of incorporated NPAAs is through the post-translational modification (PTM) of amino acid residues. Amino acid residues are modified for various reasons, including changes to peptide or protein conformation, stability, and functionality. Upon hydrolysis of PTM-containing protein or peptides, these modified amino acid residues are released into their free NPAA form[8,9].

The NPAA β-methylamino-L-alanine (BMAA) produced by cyanobacteria, diatoms, and dinoflagellates[10] is a suspected neurotoxin that is implicated as a contributing factor to various neurodegenerative diseases such as amyotrophic lateral sclerosis/parkinsonism-dementia complex (ALS-PDC)[11,12], amyotrophic lateral sclerosis, and Alzheimer's disease[13]. It is suggested that BMAA is mistakenly incorporated into the polypeptide chain of proteins in place of L-serine[14] and/or other protein amino acids. The misincorporation of BMAA can lead to the misfolding of proteins, resulting in the deposition of protein aggregates in neurons[14]. In the last decade, interest in BMAA has significantly increased. A wide range of cyanobacterial species from freshwater, marine, and brackish environments have been discovered to produce BMAA[15], leading to their widespread distribution into various ecosystems[16,17]. Additionally, BMAA has been shown to biomagnify through the food chain to the human food web[18,19]. Due to the potential health effects, lack of understanding, and incongruences of BMAA toxicity, it is imperative to continue further research until either the toxicity of BMAA is ultimately understood or BMAA is deemed safe[20,21].

The analysis of amino acids in biological samples can be divided into four main steps: sample preparation, amino acid derivatization, separation and detection, and identification and quantification. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is the preferred analysis method as it provides a targeted and reproducible separation and analysis of amino acids.

Sample preparation techniques for analyzing cyanobacterial and other algal samples predominantly involve an extraction method for amino acids in their free and protein-bound forms. Over the years, the extraction methods have remained relatively consistent with common elements, including the desolvation of the sample to separate the free amino acids from their bound form, followed by protein precipitation and the release of the bound amino acids through hydrolysis with hydrochloric acid (HCl) at elevated temperatures[22]. This extraction form has been optimized for protein amino acids and employed for NPAAs. However, the detection and quantification of BMAA and its isomers (**Figure 1**), aminoethylglycine (AEG) and 2,4-diaminobutyric acid (2,4-DAB), in the same species of cyanobacteria

have shown inconsistent results in the literature, with a possible explanation lying in differences in the growth conditions and/or the strain of algae producing varying or no amounts of BMAA[23]. It has been argued that a more likely explanation for the inconsistencies in the detection and quantification of BMAA and its isomers is due to unvalidated experimental protocols, the use of a wide range of analytical techniques, and insufficient experimental detail in the reported methods[16,24] leading to irreproducible inter-laboratory data. However, Glover et al.[25] and Banack[26] have recently developed and validated an analytical technique for the detection and quantification of BMAA and its isomers using ultra-performance liquid chromatography (UPLC)-MS/MS in accordance with the International Society of Analytical Chemists (AOAC), US Pharmacopeia, and FDA guidelines necessary for single-laboratory validation.

These validation experiments focused on the separation and detection of BMAA and its isomers and did not address the inconsistencies in sample preparation protocols. Lage et al.[27] compared the performance of three common extraction methods for quantifying BMAA and its isomers in cyanobacterial samples *via* LC-MS/MS: solid phase extraction (SPE) of free amino acids[28,29]; a protein precipitation method involving a methanol extraction and acetone precipitation[30]; and the most commonly used extraction method for BMAA, protein precipitation with trichloroacetic acid (TCA)[31]. They concluded that the TCA protein precipitation was the optimal protocol, yielding higher BMAA concentrations in the test samples compared to the other extraction methods. Their study validated TCA extraction with derivatization of BMAA using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) in a cyanobacteria matrix, providing an established guide to achieving reliable and reproducible BMAA data. TCA amino acid extraction is an accepted and common sample preparation technique that can also be applied to other matrices. However, the stability of the amino acid during hydrolysis needs to be considered to prevent degradation or oxidation, which can be overcome with the use of chemical modifiers and reducing agents[32]. TCA extraction is routinely used and taught to new research students, and although the protocol is widely reported, a visual aid in the application of this method is a valuable resource, ensuring proper and consistent execution.

Reverse-phase chromatography is commonly used to separate amino acids, requiring a derivatization step prior to analyses. The derivatization of amino acids such as BMAA allows for chromatographic retention and can increase resolution between isomers. It also increases the molecular mass and improves ionization in the mass spectrometer. Several derivatizing reagents have been used for the analysis of amino acids *via* LC-MS/MS, including propyl chloroformate (PCF)[33], 6-aminoquinolyl-N-hydrosysuccinimidyl carbamate (AQC)[27], 9-fluorenylmethyl chloroformate (FMOC)[34], and dansyl chloride (DC)[35]. However, the only validated techniques for analyzing BMAA used either PCF[36] or AQC[24,26,37] as their derivatizing reagent.

The scope of this protocol is focused on the TCA extraction of NPAAs from cyanobacterial matrices. It is a labor-intensive method that is routinely used and taught in academic and industry research laboratories based on manuscripts that may be short on detail; therefore, this protocol provides details of the procedure and techniques involved in preparing samples for the analysis of free and bound BMAA as the model amino acid.

Protocol

The cyanobacteria species *Merismopedia* was used for the present study[38].

1. Raw sample preparation

1. Collect the algal scum from the aquatic source of interest or from a cyanobacterial cultured flask and place it into 50 mL centrifuge tube[38].
   NOTE: The sample must be frozen at −20 °C for later extractions and thawed prior to the following step.
2. Centrifuge the tubes containing the sample at 3,500 x *g* for 10 min at 25 ˚C. Decant the supernatant into a waste container and discard it into biological waste.
   NOTE: The supernatant may be reserved for future analysis of the exosome.
3. Cover the tube containing the sample pellet securely with a sealing film (see **Table of Materials**) and pierce a few holes in the film using a sharp, long nose tweezer. Store the tube in an upright position at −80 °C for 30 min.
4. Turn on and equilibrate the freeze dryer at 0.1 mbar and −80 °C (~30 min).
   NOTE: The parameters (step 1.4) are optimized for the freeze dryer used for the present study (see **Table of Materials**). Follow standard operating procedures according to the freeze dryer model available in the laboratory or set the lowest possible temperature if the freeze dryer model does not go as low as −80 ˚C.
   1. Place the centrifuge tube(s) upright into a freeze dryer glass container and place it inside the −80 °C freezer for 5 min to cool the jar.
   2. Remove the glass container from the freezer and attach the rubber lid.
   3. Ensure the handle on the rubber valve outlet of the freeze dryer is vented to the atmosphere (pointing upward) and attach the glass container firmly.
   4. Turn the handle of the rubber valve outlet very slowly to the pointing down position to expose the jar to the vacuum and allow up to 24 h of freeze-drying to ensure the sublimation of all the liquid.
   5. To release the vacuum in the jar, turn the handle to the pointing upward position, detach the glass container, and remove the freeze-dried samples.
5. Use an analytical balance to weigh 15-50 mg of dried sample pellets into a 15 mL centrifuge tube.
   NOTE: The samples may be placed into −80 °C storage if not processed further at this stage.

2. Cell lysing and fractionation of free NPAAs

1. Add 100 µL of 100 ng/mL of D5-2,4-DAB (see **Table of Materials**) standard using a micropipette to the sample tube (optional).
2. Add 300-600 µL of 10% w/v aqueous trichloroacetic (TCA, see **Table of Materials**) or 300-600 µL of 11.7%-13.3% TCA, respectively, if adding the D5-2,4-DAB standard.
   NOTE: Choose a volume of aqueous TCA that fully covers the pellet.
3. Place the sample tubes in a container filled with crushed ice.
4. Use a probe sonicator (see **Table of Materials**) at medium-high power (70%) and lyse the sample for 1 min following the steps below.

CAUTION: Ensure proper PPE with the use of noise-canceling earmuffs.

1. Follow the appropriate safety protocols in preparation for the use of the probe sonicator by placing in-use signage on the door, performing sonication in the fume hood, and using noise-canceling earmuffs.
2. Switch on the sonicator and enter the following parameters: amplitude, 70%; time, 1 min.
3. Spray 70% ethanol onto a lint-free paper wipe (see **Table of Materials**) and wipe down the probe.
4. Fully immerse the end of the probe into the sample, and press **start**.
5. When the probe sonicator stops, place the centrifuge tube containing the sample on ice for 1 min.
6. To ensure the cells are lysed, repeat steps 2.4.3-2.4.5 once more.

5. Place the sample into a fridge at 4 °C for 12-24 h to allow protein precipitation.
6. Centrifuge the 10% aqueous TCA sample at 3,500 x *g* for 15 min at 8 °C.
7. Use a micropipette to transfer the supernatant to a 2 mL tube labeled "Free Fraction".
8. Micropipette 400 µL of 10% aqueous TCA into the centrifuge tube containing the remaining sample pellet and break up the pellet either by vortex agitation or with the micropipette tip.
9. Repeat steps 2.6-2.7, transferring the supernatant to the same 2 mL "Free Fraction" tube.
10. Micropipette 400 µL of 10% TCA/acetone into the centrifuge tube with the remaining pellet and break up the pellet with vortexing or the micropipette tip.
11. Centrifuge the 10% TCA/acetone sample at 3,500 x *g* for 15 min at 8 °C. Use a micropipette to transfer the supernatant to the 2 mL tube labeled "Free Fraction".
12. Place the "Free Fraction" tube with the lid open into a centrifugal evaporator (see **Table of Materials**) until all the volatile liquids are removed (at least 1 h).
13. Once the sample is free of volatile liquids, cover the tube securely with sealing film and pierce the film with a few holes using a sharp, long nose tweezer. Place the sample into a −80 °C freezer.
14. Freeze dry the "Free Fraction" sample by repeating all the steps in step 1.4.
15. Micropipette 200 µL of 20 mM hydrochloric acid (HCl) into the "Free Fraction" tube to reconstitute the freeze-dried sample and place it in −80 °C freezer storage.
    NOTE: The "Free Fraction" of the sample is ready for filtration in step 4. The remaining pellet will be further processed in the following steps for protein fractionation.

3. Fractionation of the protein-bound NPAAs

1. Use a glass engraver to label glass shell vials with the sample details for identification.
   NOTE: Strong acid may dissipate ink labeling; therefore, engraving the labels on glass is recommended.
2. Micropipette 100 µL of 100 ng/mL D5-2,4-DAB standard onto the sample pellet (optional).
3. Micropipette 400 µL of 100% acetone onto the sample pellet and break up the pellet using either vortex agitation or the micropipette tip.

4. Use a 1 mL micropipette set at 1,000 µL to transfer the washed and resuspended pellet to the corresponding glass shell vial.

   NOTE: Another 400 µL of 100% acetone can be added to the agitated pellet to assist in the complete transfer of the pellet into the glass shell vial. This can be repeated a third time if necessary.

5. Centrifuge at 8,000 x *g* for 5 min at 25 ˚C and decant the liquid into biological waste.

6. Place the remaining pellet into a centrifugal evaporator until all the liquid is removed and the pellet is dry (~1 h).

7. Prepare the vacuum hydrolysis vial by adding 1 mL of 6 M HCl into the bottom of the hydrolysis vial.

8. Use tweezers to carefully insert the labeled shell vials containing the dried samples into the hydrolysis vial, ensuring an upright, stable position.

   NOTE: Empty shell vials can be used to help keep the sample vials upright if the number of samples is less than the capacity of the hydrolysis vial.

9. Attach the lid to the hydrolysis vial and push the red knob on the lid to close the valve.

10. Turn the vacuum pump on, attach the vacuum tube to the head of the hydrolysis vial lid, and press the green knob on the lid to open the valve.

11. Allow the vacuum pump (see **Table of Materials**) to remove the air from the vial for 1 min.

12. Close the vial by depressing the red knob on the hydrolysis vial lid, turn the vacuum pump off, and remove the vacuum tube.

13. Attach a rubber tube to the nitrogen gas tap on the laboratory bench and open the tap slightly. Place the thumb at the tube's end, seal it, and count until the gas begins to escape as pressure builds up. This will be the timeframe for the next step. Adjust the gas flow to a suitable timeframe.

14. Attach the other end of the rubber tube to the head of the hydrolysis vial lid and then immediately push the green knob of the lid. Count to the time determined in step 3.13 and quickly push the red knob on the lid and remove the rubber tube.

15. Repeat steps 3.10-3.14 twice to ensure the glass hydrolysis vial is free of air and filled with nitrogen gas.

16. Place the hydrolysis vial into a preheated oven set at 110 °C for 16-18 h.

17. Use oven gloves to remove the glass hydrolysis vial from the oven and allow it to cool inside the fume hood for 10 min. Inside the fume hood, while facing it away from you, push the green knob to release pressure and gas.

18. Use tweezers to remove the shell vials from the hydrolysis vial.

19. Reconstitute the hydrolyzed sample pellets by micro pipetting 200 µL of 20 mM HCl into the shell vial. Ensure the pellet is resuspended by either vortexing or using a pipette tip.

20. Centrifuge the shell vials containing the reconstituted sample pellets for 2 min at 5,300 x *g* and 25 ˚C.

4. Sample filtering

1. Label 2 mL filter tubes (see **Table of Materials**) containing 0.2 µm pore membrane filters as "Free Fraction" and "Protein Fraction".

2. Transfer the reconstituted samples from step 2.15 (free fraction) and step 3.20 (protein fraction) into the corresponding filter tubes.

3. Place them in the centrifuge for 30 min at 5,000 x *g* and 25 ˚C.

4. Remove the filters from the filter tubes and cap them. The samples are now ready for amino acid derivatization in step 5.

   NOTE: The samples can be placed into the −80 °C freezer for storage for later derivatization and analysis.

5. Amino acid derivatization

1. Derivatize the samples using propyl chloroformate (PCF)[39] or 6-aminoquinolyl-N-hydrosysuccinimidyl carbamate (AQC)[40] according to the manufacturer's instructions (see **Table of Materials**).

Representative Results

An illustration of the extraction protocol is provided in **Figure 2** as a summarized reference guide. The results obtained by Violi et al.[38] were chosen to represent a positive result from this extraction protocol for the analysis of BMAA isomers from cyanobacteria. Nineteen single species of cyanobacteria were cultured from 11 eastern Australian freshwater sites. Using the same protocol, the BMAA isomers were extracted into free and protein fractions, derivatized with PCF, and analyzed using LC-MS/MS. Seventeen of the cyanobacterial isolates were positive for BMAA, and all 19 contained the 2,4-DAB isomer. A positive result is confirmed by using a validated LC-MS/MS method and observing at least three multiple reaction monitoring (MRM) transitions, one as quantifier ion and two as qualifier ions at the expected retention time. A representative MRM chromatogram of a standard containing BMAA and its isomers, indicated by the color-coded lines, is shown in **Figure 3**. The presence and concentration of BMAA and isomers in all 19 samples, sectioned to show the BMAA and isomer concentration in the free and bound fractions, is summarized in **Table 1**. A positive detection for all three isomers was observed in the free fraction of the *Merismopedia* species collected from Lake Liddell (NSW, Australia), where the free fraction contained a BMAA concentration of 68.38 µg/g dry weight (DW) ± 2.25 µg/g DW, 2,4-DAB with a concentration of 1,223.98 µg/g DW ± 20.7 µg/g DW, and AEG with a concentration of 125.27 µg/g DW ± 4.19 µg/g DW. The chromatographic MRMs are shown in **Figure 4**. To illustrate a negative result for BMAA and its isomers in a sample, the bound fraction of the *Microcystis flos-aquae* species collected from Walka Water Works (NSW, Australia) is chromatographically presented in **Figure 5**. While the bound fraction contained no BMAA, 2,4-DAB, and/or AEG, its free fraction contained all three isomers, with concentrations of 79.86 µg/g DW ± 1.59 µg/g DW, 1,156.15 µg/g DW ± 8.46 µg/g DW, and 433.83 µg/g DW ± 8.92 µg/g DW, respectively.

Thus, through the use of this protocol, Violi et al.[38] confirmed the presence of BMAA isomers in eastern Australian freshwater cyanobacteria and determined which cyanobacteria had toxin-producing capabilities.

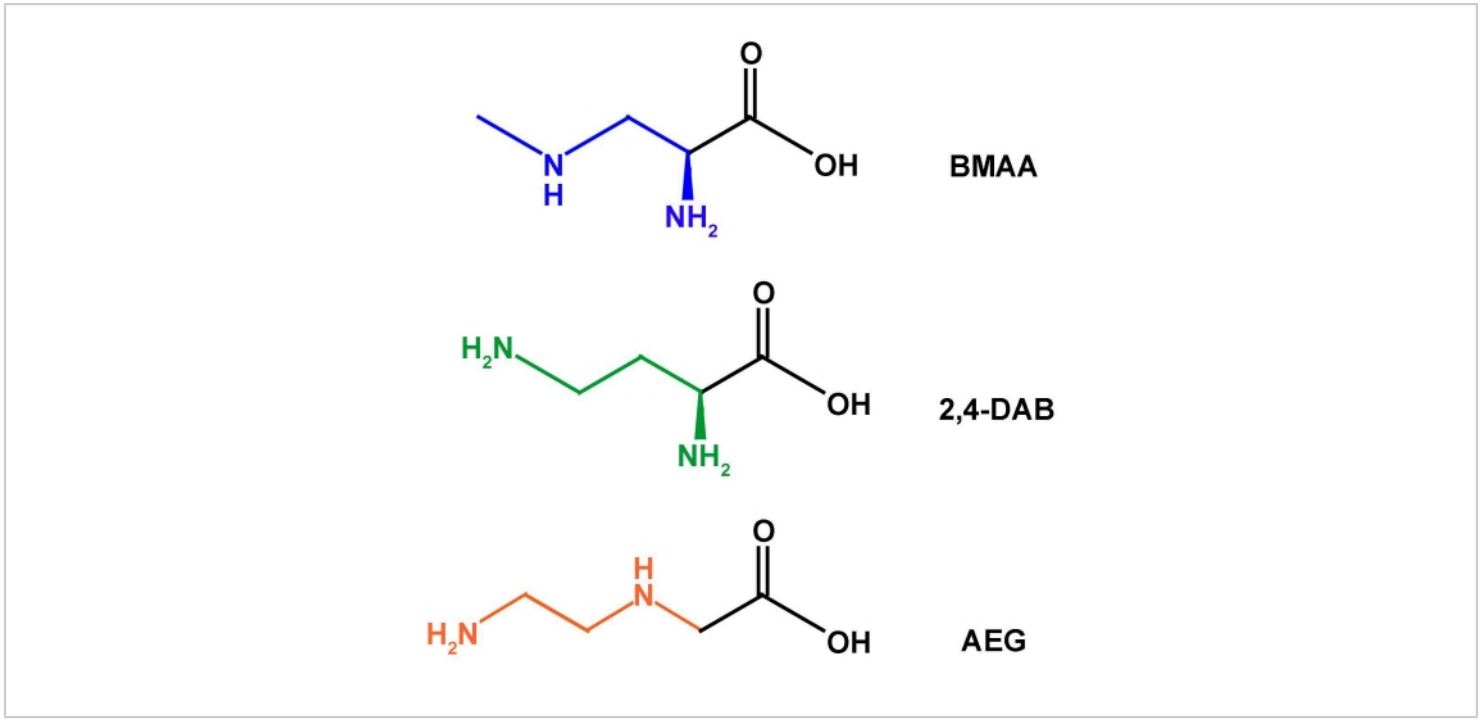

Figure 1: The chemical structure of BMAA and its isomers 2,4-DAB and AEG. Please click here to view a larger version of this figure.

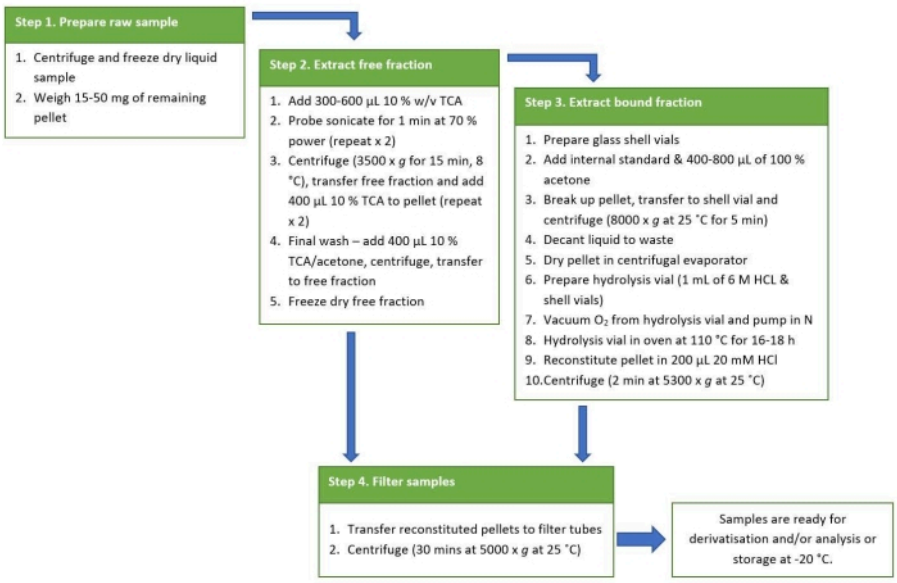

Figure 2: Summarized reference diagram of the extraction protocol. Please click here to view a larger version of this figure.

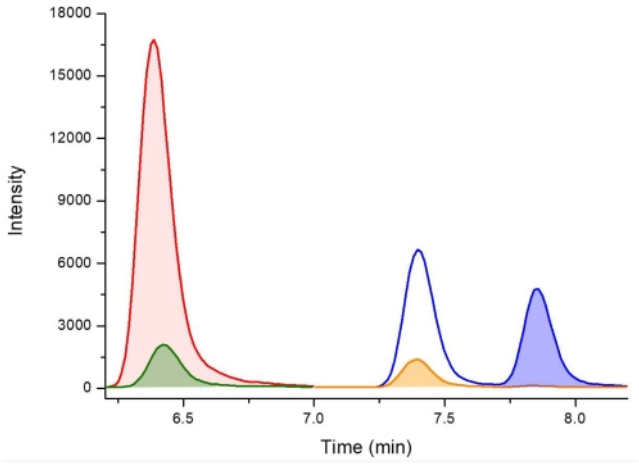

Figure 3: LC-MS/MS chromatogram of a calibration standard containing D5-2,4-DAB, 2,4-DAB, BMAA, and AEG for isomer identification based on retention, indicated by the highlighted peaks. Key: D5-2,4-DAB 338.01 m/z > 278.10 m/z at 6.4 min (red), 2,4-DAB 333.01 m/z > 273.10 m/z at 6.4 min (green), AEG 333.01 m/z > 88.00 m/z at 7.4 min (orange), and BMAA 333.01 m/z > 187.10 m/z at 7.8 min (blue). Please click here to view a larger version of this figure.

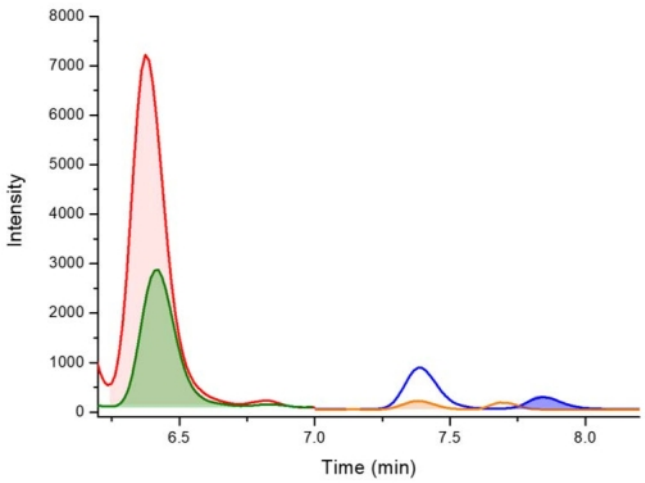

Figure 4: **LC-MS/MS chromatogram for detecting D5-2,4-DAB, 2,4-DAB, BMAA, and AEG in *Merismopedia* species collected from Lake Liddell (NSW, Australia), indicated by the highlighted peaks.** Key: D5-2,4-DAB 338.01 m/z > 278.10 m/z at 6.4 min (red), 2,4-DAB 333.01 m/z > 273.10 m/z at 6.4 min (green), AEG 333.01 m/z > 88.00 m/z at 7.4 min (orange), and BMAA 333.01 m/z > 187.10 m/z at 7.8 min (blue). Please click here to view a larger version of this figure.

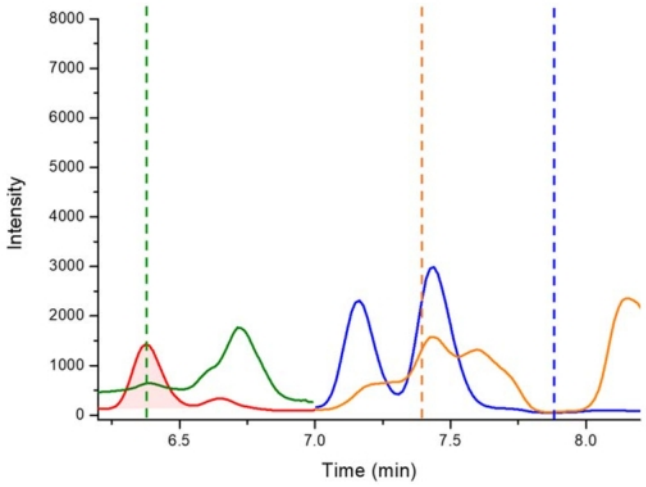

Figure 5: LC-MS/MS chromatogram of a negative control for 2,4-DAB, BMAA, and AEG in a bound fraction of *Microcystis flos-aquae* species collected from Walka Water Works (NSW, Australia). Key: D5-2,4-DAB 338.01 m/z > 278.10 m/z at 6.4 min (red - peak highlighted), 2,4-DAB 333.01 m/z > 273.10 m/z at 6.4 min (green), AEG 333.01 m/z > 88.00 m/z at 7.4 min (orange), and BMAA 333.01 m/z > 187.10 m/z at 7.8 min (blue). The dashed lines represent the retention times for missing 2,4-DAB, AEG, and BMAA. Please click here to view a larger version of this figure.

Table 1: BMAA, AEG, and 2,4-DAB concentrations in cyanobacterial isolates. Concentrations ± standard error of the mean (n = 3). ND denotes not detected. The highest concentration of each isomer detected is highlighted in green. The table is a modified version from Violi et al.[38]. Please click here to download this Table.

Discussion

The extraction protocol outlined here for the analysis of NPAAs applies to analyzing any amino acids in biological samples. For guides on isolating and culturing cyanobacterial strains, one can refer to the methods presented in the study by Violi et al.[38]. The first step in the protocol takes the sample to a point where normalization between samples against the dry weight can be achieved. The second step is cell lysis to release the analytes and can be performed using an array of techniques, including mechanical disruptions/lyses such as probe sonication as described in this protocol, freeze/thaw cycles, grinding and bead milling, and non-mechanical disruptions such as enzymatic, detergent and/or chemical lysis. Mechanical disruption is known to be advantageous over non-mechanical as it allows for a greater capacity of the sample to lyse while allowing the intracellular bonds and proteins to remain intact[41], although the sample matrix may dictate the optimal method for cell lysing.

Protein precipitation is the critical third step of this protocol when extracting amino acids for analysis. TCA is the most commonly used solvent; however, perchloric acid, acetone, methyl tert-butyl ether (MTBE), methanol, and/or acetonitrile[8,42] have also been used, where each extraction solvent is purposed to extract and precipitate different

substrates. The model described here extracts the NPAA BMAA and its isomers from a cyanobacterial matrix, and although a range of different solvents have been utilized, the two most common are 10% TCA in water (aqueous) and 10% TCA in acetone. In general, protein precipitation with TCA is commonly used to extract amino acids to fractionate free amino acids from the protein-bound amino acids. Additionally, TCA extraction allows for determining the total protein content, reducing contaminants for the free fraction, and reducing the activity of proteases with minimal protein degradation[43]. The TCA extraction of the free fraction works by initially rinsing out organic-soluble substances, leaving behind proteins and insoluble compounds such as cell wall remnants in the precipitate, which is then followed by thermal hydrolysis extraction of the protein-bound amino acids (bound fraction) using a strong acid.

The fractionation of the free fraction with 10% aqueous TCA plus sonication produces the most extensive protein precipitation compared to other organic solvents[44] and the best amino acid recoveries[42]. Some studies opt to use an acid combined with an organic solvent (i.e., 10%-20% TCA in acetone[43]) to precipitate more molecules, including smaller peptides/biomolecules, minimize protein degradation, and reduce contaminants such as salts[43]. Another advantage of 10% TCA in acetone is its faster drying rate in the preparation of the pellet for hydrolysis, minimizing moisture residue to prevent amino acid modification. However, 10% aqueous TCA plus sonication has better extraction efficiencies of free amino acids when compared to 10% TCA in acetone[44] alone. Additionally, 10% aqueous TCA precipitation has limitations such as a long drying time, not being able to precipitate all proteins or small peptides/biomolecules, and depending on the analyte of interest (i.e., proteins or amino acids), requiring additives and reducing agents to prevent oxidization and degradation[45,46].

This protocol uses a combination of 10% aqueous TCA and 10% TCA in acetone to increase protein precipitation, ensure smaller peptides/biomolecules are precipitated, allow for faster pellet drying times, and increase extraction efficiencies, taking advantage of both solvent extraction properties. However, the combination of 10% aqueous TCA and 10% TCA in acetone can precipitate small peptides/biomolecules in the free fraction after the 10% TCA in acetone supernatant is combined with the aqueous free fraction. In this case, the precipitates should be transferred to the bound fraction for hydrolysis.

The second half of this protocol involves the release of amino acids (i.e., BMAA) from the protein pellet *via* acid-vapor hydrolysis at elevated temperatures in an oxygen-free environment. The predominately limiting factor to the hydrolysis step is that it is time-consuming and laborious to prepare. The overnight incubation prevents fast and time-efficient sample preparation and analysis. In addition, the quantitative transfer of the pellet from a centrifuge tube to a shell vial (step 3.4) is an arduous process that requires diligence and patience to ensure an accurate point of normalization to the dry weight. Possible issues the user may face are the incomplete transfer of the pellet, wet precipitated proteins sticking to pipette tips and the original tube, and solid particles of the pellet blocking the pipette tip. A practical suggestion in aiding an easier transfer of the pellet is to remove about 0.3-0.5 mm off the end of the pipette tip with a pair of scissors, allowing larger pellet particles to be drawn and released into the shell vial. This transfer method is common practice for protein extraction for all amino acid analyses. However, modifications to improve the efficiency

and accuracy of quantitatively transferring the pellet into the shell vial should be investigated.

Two forms of hydrolysis techniques can be employed to release amino acids from their protein-bound state: liquid-phase hydrolysis and acid-vapor hydrolysis. Liquid-phase hydrolysis, which involves the addition of 6 M HCl onto the sample, which is then placed in a 110 °C oven overnight, is an alternative to the acid-vapor hydrolysis described in this protocol. Extraction techniques that employ liquid-phase hydrolysis may require further processing, such as desalination, especially if AQC derivatization is chosen, as it requires basic conditions for tagging[40]. Acid-vapor hydrolysis avoids desalination and allows the user the freedom to choose the derivatization technique upon reconstitution of the final pellet before filtration. Furthermore, independent of the chosen hydrolysis method, the samples may require further processing, such as SPE for matrix purification and concentration[29,47,48], depending on the choice of derivatization technique and/or analytical analysis (e.g., reverse phase LC-MS/MS vs. hydrophilic interaction liquid chromatography [HILIC] LC-MS/MS[37]). The reconstitution of the final pellet in 20 mM HCl in this protocol is appropriate for derivatization using PCF reagents[48] *via* a commercially available amino acid hydrolysis kit[39] (see **Table of Materials**). Both AQC and PCF will derivatize all the amino acids, protein, and non-protein in origin present in the sample, and the selectivity of the analytes is obtained through the use of LC-MS/MS and its combination of retention time matching and careful selection of the quantifier and qualifier MRMs[38].

Current hydrolysis protocols are not optimized for the release of BMAA, 2,4-DAB, and AEG but for the 22 protein amino acids based on existing methods for protein hydrolysis[32], where incubation for longer than 18 h results in the degradation and modification of certain amino acids, affecting LC-MS/MS analysis and, thus, the obtained concentrations[32]. Beach et al.[49] investigated hydrolysis over time (0.5-120 h) to optimize hydrolysis for analyzing BMAA and the proteinogenic amino acids. They found that although there was an early rapid release of BMAA during the first 0.5 h of the hydrolysis, the BMAA levels continued to increase as the hydrolysis time increased, without degradation, even after 5 days[49]. There are also still different views and questions about the true nature of bound BMAA and its isomers, with some studies suggesting that rather than BMAA bonding[50,51] or misincorporation into proteins[14], BMAA-protein association is superficial[52]. However, the current consensus in the analysis of "bound" BMAA requires the validated and widely accepted hydrolysis step that breaks apart peptides/proteins to release amino acids and, thus, BMAA and its isomers.

The recovery rates of 20 protein amino acids were determined in a study by Sedgwick et al.[42] using TCA extraction, resulting in approximately 100% recovery. In the case of BMAA and its isomers from algal matrices, studies comparing the efficiency of BMAA extraction using various extraction solvents have found 10% TCA to be the most efficient for free BMAA[27]. The recovery rates for protein-bound BMAA extracted using liquid-phase hydrolysis were established in studies by Glover et al.[25] and by Faassen et al.[53], where the accuracy was determined by spiked recovery methods, with an average BMAA recovery rate of 108.6% and 70%, respectively. Faassen et al. described procedures for spike-recovery experiments and illustrated how the recovery differs depending on the stage of the extraction process the spike was made[53]. Faassen et al. additionally determined the efficiency of the acid-vapor protocol presented here, with recovery rates of 83.6% for the BMAA isomers spiked

prior to extraction and 68.6% for those spiked prior to hydrolysis[24]. These recoveries, methods of extraction, and analyses were further validated by Banack[26], with recovery rates of 94%-106% for BMAA in a cyanobacterial matrix and a %Relative Standard Deviation (%RSD) between 5.6% and 20%, meeting the FDA criteria for accuracy[54]. It is recommended that each laboratory initially establish its recovery rates and accuracy before utilizing this protocol *via* spike recovery experiments. The recovery methods presented in Faassen et al.[53] and Glover et al.[25] may be followed as a guide.

Alternative forms of hydrolysis techniques can be utilized instead of overnight oven hydrolysis for faster protein-bound extraction of the analyte. For example, a microwave extractor could significantly reduce the hydrolysis time from 24 h to as little as 10 min[55]. Microwave hydrolysis for amino acids uses the same principles as the conventional thermal method, using a glove box to prepare and assemble the microwave vessel in an oxygen-free environment and adding 6 M HCl. Once air-tight, the vessel containing the sample undergoes microwave radiation. Microwave hydrolysis has been used to extract amino acids from various sample matrices[56,57,58]; however, microwave hydrolysis has yet to be developed and validated for the analysis of BMAA.

In conclusion, 10% aqueous TCA protein precipitation is the optimal method to extract free non-protein amino acids from cyanobacterial matrices[27], and thermal hydrolysis provides a reliable and reproducible extraction of the protein-bound fraction. This protocol to extract the NPAA BMAA and its isomers from cyanobacteria is validated and widely accepted. Future directions to further optimize the extraction of BMAA will focus on the hydrolysis step, which is performed according to the specifications of the 22 protein amino acids. However, the extraction protocol presented here should still be considered efficient and effective for analyzing BMAA and its isomers *via* LC-MS/MS.

Disclosures

The authors have nothing to disclose.

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References

1. Larsen, P. O. Physical and Chemical Properties of Amino Acids. In *Amino Acids and Derivatives.*, edited by Miflin, B. J., 225-269. Academic Press. Cambridge, MA (1980).
2. Wagner, I., Musso, H. New naturally occurring amino acids. *Angewandte Chemie International Edition in English.* **22** (11), 816-828 (1983).
3. Reeds, P. J. Dispensable and indispensable amino acids for humans. *The Journal of Nutrition.* **130** (7), 1835S-1840S (2000).
4. Bhagavan, N. V., Ha, C.-E. Amino Acids. In *Essentials of Medical Biochemistry (Second Edition).*, edited by Bhagavan, N. V., Ha, C.-E., 21-29. Academic Press. Cambridge, MA (2015).
5. Rubenstein, E. Misincorporation of the proline analog azetidine-2-carboxylic acid in the pathogenesis of multiple sclerosis: A hypothesis. *Journal of Neuropathology & Experimental Neurology.* **67** (11), 1035-1040 (2008).
6. Chan, S., Dunlop, R., Rowe, A., Double, K., Rodgers, K. l-DOPA is incorporated into brain proteins of

patients treated for Parkinson's disease, inducing toxicity in human neuroblastoma cells in vitro. *Experimental Neurology.* **238** (1), 29-37 (2012).

7. Rodgers, K. J., Hume, P. M., Morris, J. G., Dean, R. T. Evidence for L-dopa incorporation into cell proteins in patients treated with levodopa. *Journal of Neurochemistry.* **98** (4), 1061-1067 (2006).
8. Violi, J. P., Bishop, D. P., Padula, M. P., Steele, J. R., Rodgers, K. J. Considerations for amino acid analysis by liquid chromatography-tandem mass spectrometry: A tutorial review. *TrAC Trends in Analytical Chemistry.* **131**, 116018 (2020).
9. Hashiguchi, A., Komatsu, S. Posttranslational Modifications and Plant-Environment Interaction. In *Methods in Enzymology.*, Volume 586, edited by Shukla, A. K., 97-113. Academic Press. Cambridge, MA (2017).
10. Berntzon, L., Ronnevi, L. O., Bergman, B., Eriksson, J. Detection of BMAA in the human central nervous system. *Neuroscience.* **292**, 137-147 (2015).
11. Cox, P. A., Banack, S. A., Murch, S. J. Biomagnification of cyanobacterial neurotoxins and neurodegenerative disease among the Chamorro people of Guam. *Proceedings of the National Academy of Sciences of the United States of America.* **100** (23), 13380-13383 (2003).
12. Cox, P. A. et al. Cyanobacteria and BMAA exposure from desert dust: A possible link to sporadic ALS among Gulf War veterans. *Amyotrophic Lateral Sclerosis.* **10** (Suppl 2), 109-117 (2009).
13. Pablo, J. et al. Cyanobacterial neurotoxin BMAA in ALS and Alzheimer's disease. *Acta Neurologica Scandinavica.* **120** (4), 216-225 (2009).
14. Dunlop, R. A., Cox, P. A., Banack, S. A., Rodgers, K. J. The non-protein amino acid BMAA is misincorporated into human proteins in place of l-serine causing protein misfolding and aggregation. *PLoS One.* **8** (9), e75376 (2013).
15. Cox, P. A. et al. Diverse taxa of cyanobacteria produce β-N-methylamino-L-alanine, a neurotoxic amino acid. *Proceedings of the National Academy of Sciences of the United States of America.* **102** (14), 5074-5078 (2005).
16. Faassen, E. J. Presence of the neurotoxin BMAA in aquatic ecosystems: What do we really know? *Toxins.* **6** (3), 1109-1138 (2014).
17. Al-Sammak, M. A., Hoagland, K. D., Cassada, D., Snow, D. D. Co-occurrence of the cyanotoxins BMAA, DABA and anatoxin-a in Nebraska reservoirs, fish, and aquatic plants. *Toxins.* **6** (2), 488-508 (2014).
18. Wang, C. et al. Food web biomagnification of the neurotoxin β-N-methylamino-L-alanine in a diatom-dominated marine ecosystem in China. *Journal of Hazardous Materials.* **404** (Pt B), 124217 (2021).
19. Banack, S. A., Johnson, H. E., Cheng, R., Cox, P. A. Production of the neurotoxin BMAA by a marine cyanobacterium. *Marine Drugs.* **5** (4), 180-196 (2007).
20. Jiang, L., Johnston, E., Åberg, K. M., Nilsson, U., Ilag, L. L. Strategy for quantifying trace levels of BMAA in cyanobacteria by LC/MS/MS. *Analytical and Bioanalytical Chemistry.* **405** (4), 1283-1292 (2013).
21. Jonasson, S., Eriksson, J., Berntzon, L., Rasmussen, U., Bergman, B. A novel cyanobacterial toxin (BMAA) with potential neurodegenerative effects. *Plant Biotechnology.* **25** (3), 227-232 (2008).

22. Cohen, S. Analytical techniques for the detection of α-amino-β-methylaminopropionic acid. *Analyst.* **137** (9), 1991-2005 (2012).

23. Main, B. J., Rodgers, K. J. Assessing the combined toxicity of BMAA and its isomers 2,4-DAB and AEG in vitro using human neuroblastoma cells. *Neurotoxicity Research.* **33** (1), 33-42 (2018).

24. Faassen, E. J., Gillissen, F., Lürling, M. A comparative study on three analytical methods for the determination of the neurotoxin BMAA in cyanobacteria. *PLoS One.* **7** (5), e36667 (2012).

25. Glover, W. B., Baker, T. C., Murch, S. J., Brown, P. N. Determination of β-N-methylamino-L-alanine, N-(2-aminoethyl)glycine, and 2,4-diaminobutyric acid in food products containing cyanobacteria by ultra-performance liquid chromatography and tandem mass spectrometry: Single-laboratory validation. *Journal of AOAC International.* **98** (6), 1559-1565 (2015).

26. Banack, S. A. Second laboratory validation of β-N-methylamino-L-alanine, N-(2aminoethyl)glycine, and 2,4-diaminobuytric acid by ultra-performance liquid chromatography and tandem mass spectrometry. *Neurotoxicity Research.* **39** (1), 107-116 (2021).

27. Lage, S. et al. BMAA extraction of cyanobacteria samples: Which method to choose? *Environmental Science and Pollution Research.* **23** (1), 338-350 (2016).

28. Jonasson, S. et al. Transfer of a cyanobacterial neurotoxin within a temperate aquatic ecosystem suggests pathways for human exposure. *Proceedings of the National Academy of Sciences of the United States of America.* **107** (20), 9252-9257 (2010).

29. Spáčil, Z. et al. Analytical protocol for identification of BMAA and DAB in biological samples. *Analyst.* **135** (1), 127-132 (2010).

30. Jiang, L. et al. Diatoms: A novel source for the neurotoxin BMAA in aquatic environments. *PLOS One.* **9** (1), e84578 (2014).

31. Murch, S. J., Cox, P. A., Banack, S. A., Steele, J. C., Sacks, O. W. Occurrence of β-methylamino-l-alanine (BMAA) in ALS/PDC patients from Guam. *Acta Neurologica Scandinavica.* **110** (4), 267-269 (2004).

32. Davidson, I. Hydrolysis of Samples for Amino Acid Analysis. In *Protein Sequencing Protocols.*, edited by Smith, B. J., 111-122. Humana Press. Totowa, NJ (2003).

33. Esterhuizen-Londt, M., Downing, S., Downing, T. Improved sensitivity using liquid chromatography mass spectrometry (LC-MS) for detection of propyl chloroformate derivatised ²-N-methylamino-L-alanine (BMAA) in cyanobacteria. *Water SA.* **37** (2), 133-138 (2011).

34. Kisby, G. E., Roy, D. N., Spencer, P. S. Determination of β-N-methylamino-l-alanine (BMAA) in plant (Cycas circinalis L.) and animal tissue by precolumn derivatization with 9-fluorenylmethyl chloroformate (FMOC) and reversed-phase high-performance liquid chromatography. *Journal of Neuroscience Methods.* **26** (1), 45-54 (1988).

35. Lampinen Salomonsson, M., Hansson, A., Bondesson, U. Development and in-house validation of a method for quantification of BMAA in mussels using dansyl chloride derivatization and ultra performance liquid chromatography tandem mass spectrometry. *Analytical Methods.* **5** (18), 4865-4874 (2013).

36. Main, B. J. et al. Detection of the suspected neurotoxin β-methylamino-l-alanine (BMAA) in cyanobacterial blooms from multiple water bodies in Eastern Australia. *Harmful Algae.* **74**, 10-18 (2018).

37. Combes, A. et al. Validation of the analytical procedure for the determination of the neurotoxin β-N-methylamino-l-alanine in complex environmental samples. *Analytica Chimica Acta.* **771**, 42-49 (2013).

38. Violi, J. P., Mitrovic, S. M., Colville, A., Main, B. J., Rodgers, K. J. Prevalence of β-methylamino-L-alanine (BMAA) and its isomers in freshwater cyanobacteria isolated from eastern Australia. *Ecotoxicology and Environmental Safety.* **172**, 72-81 (2019).

39. Phenomenex. *EZ:faast For Amino Acid Analysis of Protein Hydroysates by LC-MS. User's Manual.* .http://phx.phenomenex.com/lib/3882_L6_LC_MS.pdf (2005).

40. Waters. *AccQ-Tag Ultra Derivatization Kit Care and Use.* .https://www.waters.com/waters/support.htm?lid=10008559 (2014).

41. D'Hondt, E. et al. Cell Disruption Technologies. In *Microalgae-Based Biofuels and Bioproducts.* edited by Gonzalez-Fernandez, C., Muñoz, R., 133-154. Woodhead Publishing. Sawston, Cambridge (2017).

42. Sedgwick, G. W., Fenton, T. W., Thompson, J. R. Effect of protein precipitating agents on the recovery of plasma free amino acids. *Canadian Journal of Animal Science.* **71** (3), 953-957 (1991).

43. Niu, L. et al. Modified TCA/acetone precipitation of plant proteins for proteomic analysis. *PLOS One.* **13** (12), e0202238 (2018).

44. Fic, E., Kedracka-Krok, S., Jankowska, U., Pirog, A., Dziedzicka-Wasylewska, M. Comparison of protein precipitation methods for various rat brain structures prior to proteomic analysis. *Electrophoresis.* **31** (21), 3573-3579 (2010).

45. Koontz, L. TCA precipitation. *Methods in Enzymology.* **541**, 3-10 (2014).

46. Novák, P., Havlíček, V. Protein Extraction and Preparation. In *Proteomic Profiling and Analytical Chemistry (Second Edition).*, edited by Ciborowski, P., Silberring, J., 51-62. Elsevier (2016).

47. Li, A. et al. Elucidation of matrix effects and performance of solid-phase extraction for LC-MS/MS analysis of beta-N-methylamino-L-alanine (BMAA) and 2,4-diaminobutyric acid (DAB) neurotoxins in cyanobacteria. *Analyst.* **137** (5), 1210-1219 (2012).

48. Baker, T. C., Tymm, F. J. M., Murch, S. J. Assessing environmental exposure to β-N-methylamino-l-alanine (BMAA) in complex sample matrices: A comparison of the three most popular LC-MS/MS methods. *Neurotoxicity Research.* **33** (1), 43-54 (2018).

49. Beach, D. G., Kerrin, E. S., Giddings, S. D., Quilliam, M. A., McCarron, P. Differential mobility-mass spectrometry double spike isotope dilution study of release of β-methylaminoalanine and proteinogenic amino acids during biological sample hydrolysis. *Scientific Reports.* **8**, 117 (2018).

50. Murch, S. J., Cox, P. A., Banack, S. A. A mechanism for slow release of biomagnified cyanobacterial neurotoxins and neurodegenerative disease in Guam. *Proceedings of the National Academy of Sciences of the United States of America.* **101** (33), 12228-12231 (2004).

51. Banack, S. A., Murch, S. J., Cox, P. A. Neurotoxic flying foxes as dietary items for the Chamorro people, Marianas

Islands. *Journal of Ethnopharmacology.* **106** (1), 97-104 (2006).

52. van Onselen, R., Cook, N. A., Phelan, R. R., Downing, T. G. Bacteria do not incorporate β-N-methylamino-l-alanine into their proteins. *Toxicon.* **102**, 55-61 (2015).
53. Faassen, E. J., Gillissen, F., Zweers, H. A. J., Lürling, M. Determination of the neurotoxins BMAA (β-N-methylamino-L-alanine) and DAB (α-,γ-diaminobutyric acid) by LC-MSMS in Dutch urban waters with cyanobacterial blooms. *Amyotrophic Lateral Sclerosis.* **10** (Suppl 2), 79-84 (2009).
54. Food and Drug Administration. *Bioanalytical Method Validation. Guidance for Industry.* U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Veterinary Medicine (2018).
55. Reichelt, M., Hummert, C., Luckas, B. Hydrolysis of microcystins and nodularin by microwave radiation. *Chromatographia.* **49** (11), 671-677 (1999).
56. Marconi, E., Panfili, G., Bruschi, L., Vivanti, V., Pizzoferrato, L. Comparative study on microwave and conventional methods for protein hydrolysis in food. *Amino Acids.* **8** (2), 201-208 (1995).
57. Chen, S.-T., Chiou, S.-H., Chu, Y.-H., Wang, K.-T. Rapid hydrolysis of proteins and peptides by means of microwave technology and its application to amino acid analysis. *International Journal of Peptide and Protein Research.* **30** (4), 572-576 (1987).
58. Aviram, L. Y., McCooeye, M., Mester, Z. Determination of underivatized amino acids in microsamples of a yeast nutritional supplement by LC-MS following microwave assisted acid hydrolysis. *Analytical Methods.* **8** (22), 4497-4503 (2016).