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LC/ESI-ITMS Detection of the Neurotoxic Amino Acids in Cultured Cyanobacterial Isolate *Nostoc* MAC PCC 8009

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Abstract

The non-protein amino acid β-methylamino-L-alanine (BMAA), is a neurotoxic agent that is produced by various strain of cyanobacteria. 2,4-diaminobutyric acid (2,4-DAB), and N-(2-aminoethyl) glycine (AEG) are the common isomers of BMAA. 2,4-DAB exhibit neurotoxic properties like BMAA. Various studies have shown that cyanobacteria produce BMAA and DAB, however, no studies have shown the detection of these amino acids using liquid chromatography-electrospray ionization ion trap mass spectrometry (LC/ESI-ITMS) method. This study aimed to assess the occurrence of BMAA and its isomer 2,4-diaminobutyric acid (DAB) in axenic laboratory culture of cyanobacterial strain *Nostoc* MAC PCC 8009. Axenic laboratory cultures were harvested after 12 weeks of growth and non-protein amino acids were extracted by trichloroacetic acid (TCA) and methanol extraction. Liquid chromatography-electrospray ionization ion trap mass spectrometry (LC/ESI-ITMS) was employed to analyse the presence of BMAA and 2,4-DAB. Both BMAA and 2,4-DAB were detected in the axenic cultures, which confirms the production of these neurotoxic amino acids by cyanobacteria. Multiple stage mass analysis by ESI-ITMS using $[M+H]^+$ was useful to distinguish between the two isomers.

Keywords: BMAA, Cyanobacteria, DAB, Derivatized, ESI-ITMS, *Nostoc*

1. Introduction

β-*N*-methylamino-L-alanine (BMAA) is a non-protein neurotoxic amino acid. Both animal and *In vitro* studies have suggested the neurotoxicity of BMAA [1-6]. Cyanobacteria produce BMAA but accumulate through trophic levels in different food webs and ecosystems [7-13]. The pioneer study on the production of BMAA by cyanobacteria was conducted in cyanobacterial strain *Nostoc sp*. which is a symbiont bacterium inhabiting *Cycas micronesica* roots [14]. This study instigated more research on the production of BMAA in other cyanobacterial strains and led to the suggestion that all known free-living and symbionts cyanobacterial strains could produce BMAA [7]. With some reports associating BMAA with neurodegenerative disease [15, 16], concern has been raised for human exposure to BMAA in food webs and ecosystems.

The complexity of analytical methods and sample matrices necessitate the development of a reliable protocol for the analysis of cyanobacterial blooms. The reactivity of BMAA with metal ions makes it difficult to analyse by mass spectrometry [17, 18]. LC-MS, HPLCUV, GCMS/MS, LC-MS/MS, GC-MS, HPLC-fluorescence and amino acid analyzers were among the different analytical methods used in the analysis of BMAA [19]. Some of the aforementioned analytical methods detected and even quantified BMAA directly from the complex sample matrices, but others involved derivatization. Presumably, extraction methods and sample preparation might cause the differences in results. Likewise, variations in results may as well be contributed by the chromatographic separations or detection parameters. To date, there has been very limited reports on the use of LC/ESI-ITMS method to assess the presence of BMAA and its isomer DAB in cyanobacteria. This study employed LC/ESI-ITMS analytical method to assess BMAA and DAB production in axenic laboratory culture of cyanobacteria *Nostoc* MAC PCC 8009.

2. Materials and Methods

2.1 Materials

BMAA and DAB standards were obtained from Sigma-Aldrich, and amino acid derivatizing reagent 6-aminoquinolyl-N-hydrosuccinimidyl carbamate (AQC) (Waters AccQ Tag Ultra kit) was purchased from Waters, USA. Trichloroacetic acid (TCA) from Fisher Scientific, formic acid from Sigma-Aldrich, methanol from BDH Analar, hydrochloric acid (HCl) from Fisher Scientific, ammonium hydroxide from Across Organic.

2.2. Methods

2.2.1. Sample preparation

A strain of cyanobacteria; *Nostoc* MAC PCC 8009 from Matica lagoon in Brazil was kindly provided by Nigel J. Robinson. The axenic culture was grown in BG11 media (Sigma BG11, freshwater). Media was prepared in 50 ml sterilized conical flasks, 600 µl/30 ml equivalent to 20 ml/L as specified by the manufacturer. Media preparation was carried out in

envair Bio2 (class 2 microbiological safety cabinet) to ensure the sterility of the media. 200 µl of the cryopreserved samples were added to the media. The cultures were incubated at 28℃ under 12-hour light/12-hour dark on a shaking table. The culture was grown for the period of eight (8) weeks after which the cells were harvested by centrifugation.

2.2.2. Amino acids extraction and solid phase extraction (SPE)

Methanol extraction of amino acids was carried out based on the previous work [20]. Solid phase extraction was performed based on method described by Li et al. [21].

2.2.3. Derivatization

The dried sample pellet from the previous SPE process was redissolved in 20 µl of 20 mM HCl followed by addition of 70 µl of borate buffer to the dissolved sample and then 30 µl of the ACQ tag was added. In case of the TCA extracts 20 µl of the samples were used. While for the derivatization of standard BMAA and DAB, 20 µl of standard solutions (10 µg of standards dissolved in 1 ml acetonitrile/water, 50:50 v/v) were used. The samples were vortexed and analyzed within 48 h.

2.2.4. Analytical HPLC-ESI-ITMS

Analytical HPLC-ESI-ITMS was accomplished as described previously [22]. LC separation was carried out with a Surveyor HPLC system (ThermoFinnigan Hemel Hempstead, UK) fitted with a Phenomenex (Macclesfield, UK) Gemini C18, 3µm HPLC column (150 × 2.0 mm I.D) and a security guard column of the same material. Separation was conducted at 300 µl/min flow rate, with the column oven temperature set at 30 ^{o}C. Two different solvents gradients were applied; solvent A: 0.1% formic acid in 93:7 water/acetonitrile and solvent B: 0.1% formic acid in acetonitrile. An aliquot of 15 µl sample was injected and for the standard solutions, 10 µg/ml in 50/50 acetonitrile/water was injected. LC-ESI-ITMS was performed using a ThermoFinnigan LCQ ion trap mass spectrometer equipped with an ESI source operated in positive ion mode. Settings were: capillary temperature 280 °C, spray voltage 4.0, sheath gas flow 30 and auxiliary gas 2 (arbitrary units). The instrument was tuned using solutions containing both BMAA and DAB and the target ion was m/z 459. Detection was achieved at an isolation width of m/z 3.0 and fragmentation with normalised collisional dissociation energy of 25%. The activation Q value (parameter determining the m/z range of the observed fragment ions) was set at 0.20 producing fragment ions in the range m/z 100-500. LC-ITMS was carried out in MS/MS scan mode with 1 scan event only: MS^2 of m/z 459.

3. Results and Discussion

The presence of BMAA and DAB in the samples was determined on the basis of retention time, mass-to-charge ratio, and ratio of the product ions; the three major criteria for determining the presence of BMAA and DAB in accordance with previous studies [23,24]. From the LC separation of the mixture of BMAA and DAB standard solution, BMAA exhibits a retention time of 8.17 min and DAB 8.24 min (Figure 3.1). From the LC analysis of the *Nostoc* MAC PCC 8009 sample, BMAA and DAB were identified by comparing the retention time of the sample with that of the standard and were found to be 8.16 min and 8.27 min for BMAA and DAB respectively (Figure 3.1). The LC-ESI-ITMS analysis of the BMAA standard reveal the characteristics product ions for BMAA as 119 m/z, 145.2 m/z, 171.1 m/z, 257.9 m/z, 288.9 m/z, 314.8 m/z and 458.9 m/z (Figure 3.2A) while the characteristic product ions for BMAA in *Nostoc* MAC PCC 8009 sample were found to be 145.5 m/z, 171.0 m/z, 258.0 m/z, 289.0 m/z, and 459 m/z (Figure 3.2A). The LC-ESI-ITMS analysis of the DAB standard reveal the characteristics product ions as 118.9 m/z, 145.1 m/z, 171.1 m/z, 187.6 m/z, 288.9 m/z, 314.9 m/z and 459.0 m/z (Figure 3.3A). For the *Nostoc* MAC PCC 8009 sample, the LC-ESI-ITMS analysis shows the characteristic product ions as 118.8 m/z, 145.1 m/z, 170.0 m/z, 186.9 m/z, 288.9 m/z, 314.7 m/z and 459.0 m/z (Figure 3.3B).

This study detected the presence of non-protein neurotoxic amino acids BMAA and DAB in the axenic culture of *Nostoc* MAC PCC 8009. Recently, BMAA was detected from cultures of *Nostoc* 29150 sample matrix using three different analytical methods [25]. BMAA and DAB standards exhibits identical molecular mass and to some degree a similar production spectrum that could lead to misidentification of these isomers. As previously reported, when the carbon-nitrogen bond in both BMAA and DAB is cleaved, the product ions 119 m/z, 145.1 m/z, 171.1 m/z, 289 m/z and 315 m/z of the precursor at 459 m/z are derived [24]. On the other hand, 145.1 m/z and 171.1 m/z are the $[M+H^+]$ of AQC tag that resulted from the carbonyl carbon-amide nitrogen bond cleavage [24], and this is not specific, hence, cannot distinguish BMAA from DAB. The product ions 315 m/z and 289 m/z are more relevant because they incorporate amino acid structures [24], however, they are still not efficient enough to distinguish between amino acids with the same molecular mass. The specific product ion fragment for BMAA standard which is not exhibited in DAB spectra is 258.1 m/z (Figure 3.2B), possibly this product ion fragment originated by the cleavage of the bond between the β-carbon and secondary amine nitrogen. The product ion fragment 188.1 m/z present in DAB spectra but absent in BMAA spectra originated from cleavage of the bond between the α-carbon and primary amine nitrogen of DAB [24].

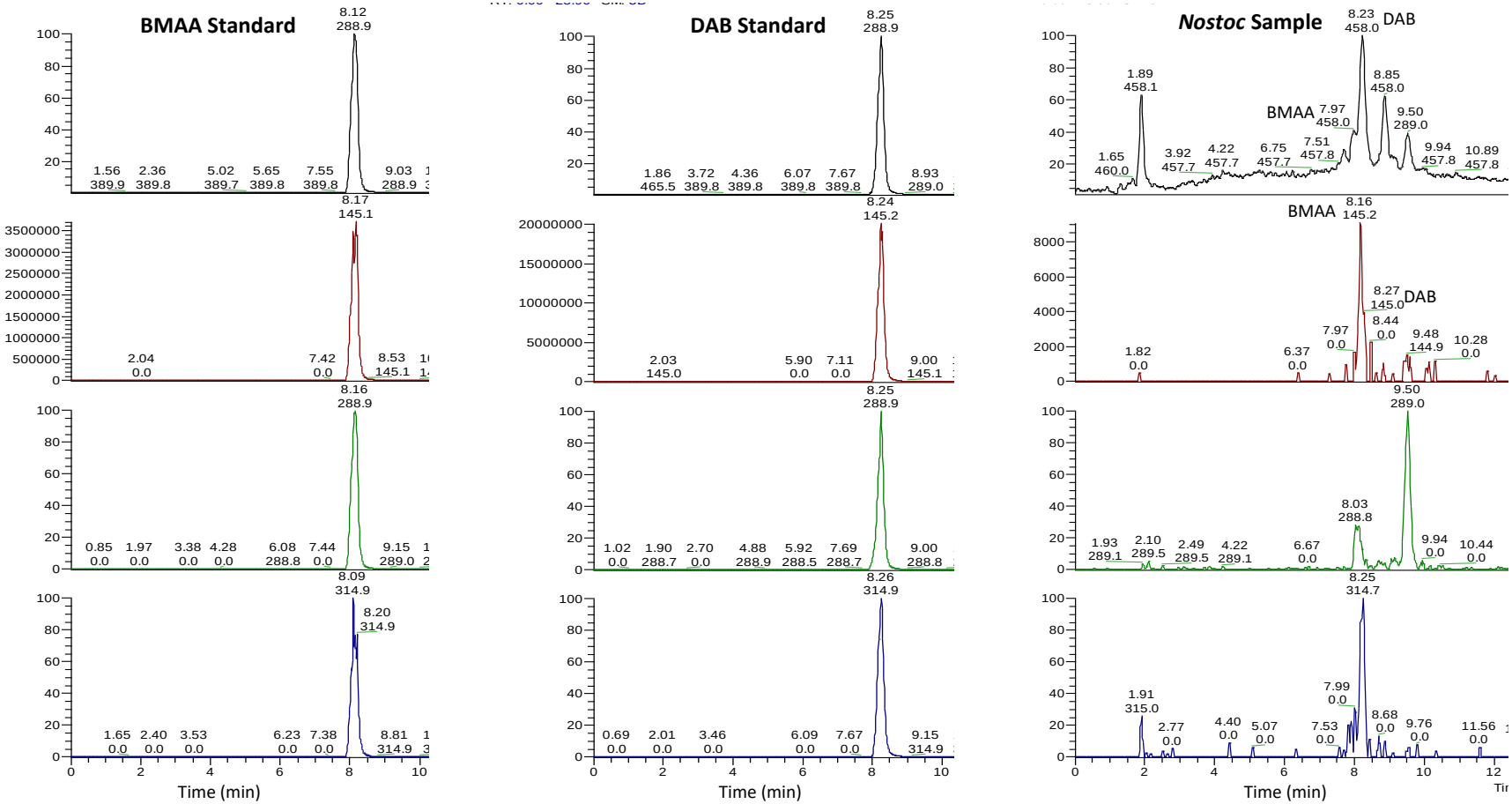

Figure 3.1: LC-ESI-ITMS Chromatograms of BMAA Standard, DAB Standard (10 µg/Ml) And *Nostoc* MAC PCC 8009 Sample (15 µl/Ml). ESI Full MS^2 Scan of The Precursor Ion 459 *M/Z* (Black). ESI Full MS^2 Scan of The Product Ion 144.5-145.5 *M/Z* (Red). ESI Full MS^2 Scan of The Product Ion 288.5-289.5 *M/Z* (Green). ESI Full MS^2 Scan of The Product Ion 315.5-314.5 *M/Z* (Blue).

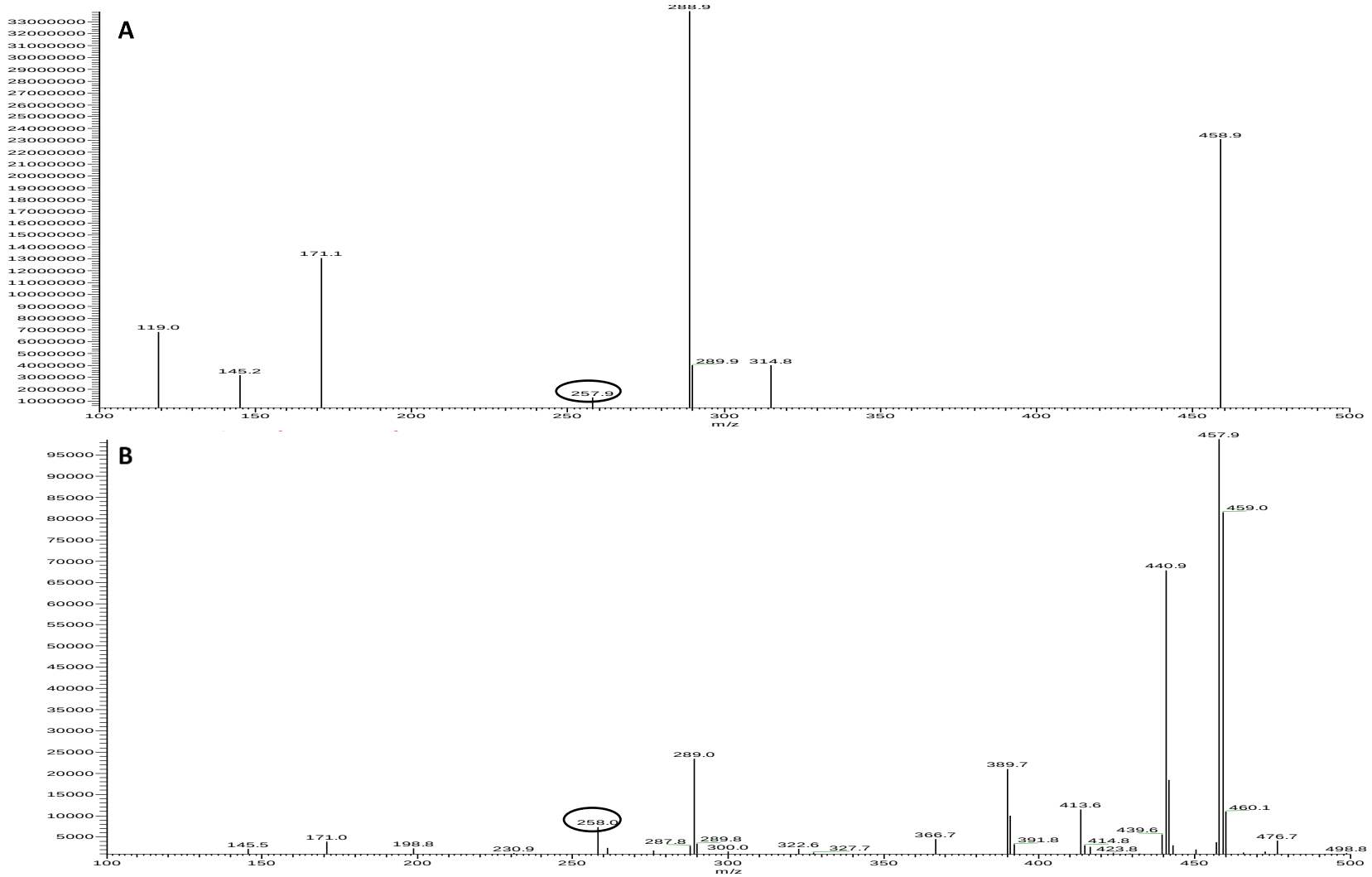

Figure 3.2: LC-ESI-ITMS Spectra of BMAA. **(A)** BMAA Standard **(B)** *Nostoc* MAC PCC 8009 Sample. Specific Product Ion Fragment Circled.

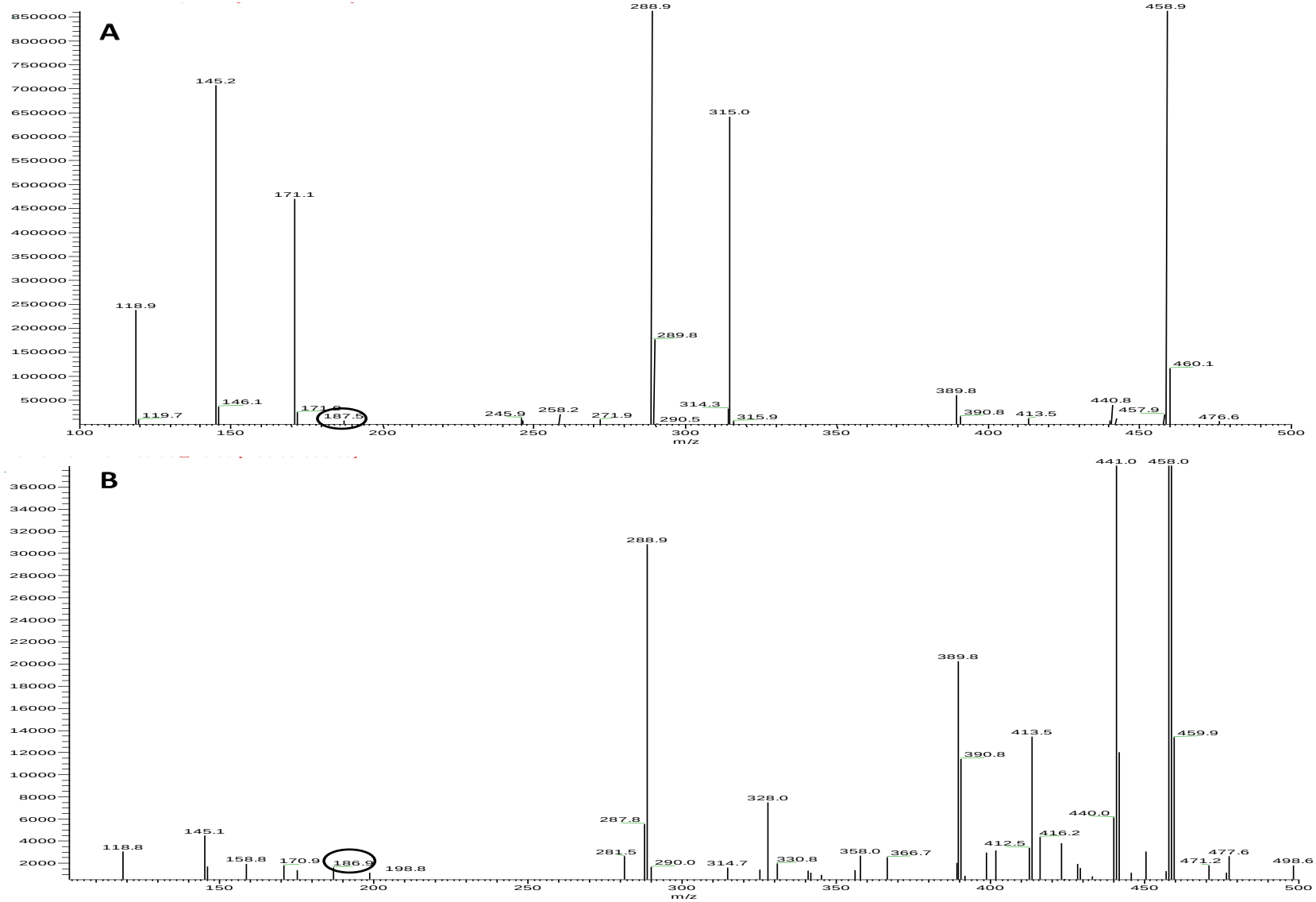

Figure 3.3: LC-ESI-ITMS Spectra for DAB. (A) DAB Standard (B) *Nostoc* MAC PCC 8009 Sample. Specific Product Fragment Ion Encircled.

In this study, the results obtained shows that the specific product ion fragment for BMAA standard and DAB standard were 257.9 and 187.6 respectively, whereas, the specific product ion fragment for BMAA and DAB from *Nostoc* samples were 258.0 and 186.9 respectively. This suggests that the present study detected the presence of BMAA and DAB in the axenic culture of *Nostoc* MAC PCC 8009 based on the criteria for detecting these amino acids as previously reported [24].

Cyanobacterial blooms in Nigeria are an increasing concern, more often these blooms are found at very near proximity to rural and urban localities. Rivers are the major sources of water for significant agricultural regions in Nigeria and, majority of these waters are used for irrigation. These waters also serve as the source of drinking water for the localities within the proximity of these rivers. The persistent occurrence of cyanobacterial blooms in waterbodies is therefore of great concern because previously it has been reported that plants take up and integrate BMAA into their proteins [26, 27]. BMAA uptake and its subsequent integration into plant proteins poses the risk of human exposure [28]. Several evidences have indicated that BMAA may bioaccumulate in animals that feed on plant tissues with integrated BMAA in their proteins [29, 30]. Previous reports on suckling rodent pups have suggested that BMAA can be transferred through milk [31, 32], which highlight the possibility that, in humans BMAA can be transferred from mother to child. In addition, it has been demonstrated recently that BMAA accumulate within eggs after ^{14}C BMAA was administered to laying quail hens and this shows that it is likely that humans can be exposed to BMAA through poultry products [33].

In support of previous studies that employed various different detection methods [8, 10, 12, 19, 22, 34-37] this study has detected the presence of BMAA and its structural isomer DAB in cyanobacterial samples by the application of LC-ESI-ITMS method. The detected BMAA and DAB were not quantified due to poor LC separation of the two isomers. Previously, Li and colleagues attempted to analyse BMAA production in cyanobacteria by ESI-ITMS method but was not able to detect BMAA although they used underivatized samples [21]. Our results suggest that it is possible to detect BMAA in cyanobacterial samples by the application of AQC-derivatized amino acids using LC-ESI-ITMS analytical method but the limitation of this method is the poor LC separation of the analytes of interest which hindered the quantification of the analytes. There is a need to further investigate the presence of BMAA in cyanobacteria by the use of advanced and more sensitive ESI-ITMS which will allow for both qualitative and quantitative determination of BMAA in cyanobacterial samples.

4. Conclusion

The non-protein neurotoxic amino acids BMAA and DAB were detected in axenic culture of cyanobacterial strain *Nostoc* MAC PCC 8009. The persistent occurrence of cyanobacteria that produces these toxins in waterbodies used as a source of drinking water and for irrigation presents a great risk to public health and food security in Nigeria. Regulating the prevalent occurrence of these emerging toxins in Nigerian waters is required to pave way for appropriate decisions to be made about the risks associated with their presence.

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