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ORIGINAL ARTICLE

Improving derivatization efficiency of BMAA utilizing AccQ-Tag® in a complex cyanobacterial matrix

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Abstract Two different assays have been developed and used in order to investigate the optimal conditions for derivatization and detection of acid *β*-*N*-methyl-amino-L-alanine (BMAA) in a cyanobacterial sample. BMAA was extracted from cyanobacterial cultures both from the cytosolic ("free") fraction and in the precipitated ("protein") fraction using a newly developed extraction scheme and the sample matrix was standardized according to protein concentration to ensure the highest possible derivative yield. A rapid and sensitive HPLC method for fluorescence detection of the non-protein amino acid BMAA in cyanobacteria, utilizing the Waters AccQ-Tag® chemistry and Chromolith® Performance RP-18e columns was developed. Using this new method and utilizing a different buffer system and column than that recommended by Waters, we decreased the time between injections by 75%. The limit of quantification was determined to be 12 nmol and limit of detection as 120 fmol. The linear range was in the range of 8.5 nmol–84 pmol. Accuracy and precision were well within FDA guidelines for bioanalysis.

Keywords *β*-*N*-methyl-amino-L-alanine (BMAA) · Cyanobacteria · Carbamate 6-aminoquinolyl-*N*-hydrosuccinimidyl (AQC) · Derivatization · High-performance liquid chromatography (HPLC)

Johan Eriksson and Sara Jonasson contributed equally to this work.

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Introduction

The non-protein amino acid *β*-*N*-methyl-amino-L-alanine (BMAA) is a neurotoxin produced by cyanobacteria (Cox et al. 2005). The neurotoxin was first discovered in 1967 in the seeds of *Cycas micronesica* (Hill 1994; Banack et al. 2006; Rao et al. 2006). At the time, the discovery was thought to be the key to the deadly neurodegenerative disease amyotrophic lateral sclerosis/parkinsonism dementia complex (ALS-PDC), which is common among the Chamorro people of Guam compared to the incidence of ALS-PDC elsewhere (Rao et al. 2006). It was hypothesized that the Chamorro people were exposed to the toxin through their diet, consumption of flying foxes, a prized food of the indigenous Chamorro people. The flying foxes were shown to be part of a biomagnification chain as they feed on the cycad seeds and accumulate high levels of BMAA that is transferred to man when eaten (Banack and Cox 2003; Banack et al. 2006). BMAA in the seed of cycads was shown to be produced by the symbiotic cyanobacteria *Nostoc*, located in the root of the plant (Cox et al. 2003). Moreover, the production of BMAA was shown to be a general trait among all cyanobacteria (Cox et al. 2005), which suggests that the BMAA toxin may have a global distribution to the ecosystem, via cyanobacteria.

BMAA has no chromophore and needs to be derivatized in order to be detectable with either fluorescence or by UV–VIS. Using pre-column derivatization, several reagents are frequently used, such as: phenylisothiocyanate (PITC), *o*-phtaldialdehyde (OPA), dimethylaminoaphtalensulphonyl chloride (Dansyl-Cl) and 9-fluorenylmethyl-chloroformate (FMOC), which yields derivates detectable by UV (Cohen and Michaud 1993; Sarwar and Botting 1993; Strydom and Cohen 1994; Liu et al. 1995). However, all of these reagents have shown drawbacks like long

derivatization time, unstable derivates, and the need for removing excess reagent prior to the chromatographic analysis (Bosch et al. 2006).

In our study, we have used the highly reactive carbamate 6-aminoquinolyl-*N*-hydrosuccinimidyl (AQC) that reacts both with primary and secondary amines and yields fluorescent derivates (λ excitation and emission at 250 and 395 nm, respectively) (Bosch et al. 2006). The advantage of the reagent is that it rapidly forms a stable derivative (up to 1 week) and has a minimum of reactive interference products due to the very short half-life of the underivatized reagent (Cohen and Michaud 1993). The derivative technique is also very reproducible and linear over a broad range of content ranging from 2.5 to 200 μM (Cohen and Michaud 1993).

A crucial hindrance in our work has been to determine the point at which the reagent and the sample have the correct proportions to ensure full derivatization of the analyte of interest, e.g., BMAA, as our starting material is crude cyanobacteria cultures. BMAA may occur in both free and protein-bound form and it has been shown that the ratio between the protein-bound and free BMAA is present in a ratio between 60:1 and 120:1 (Ince and Codd 2005). So far, the amino acid fraction ("free fraction") has not caused any problem as the amino acid content has been in the right range for our standard protocols, whereas the precipitated fraction (protein fraction) has had large variations in amino acid content due to amount of starting material and extraction efficiency of the sample.

The previously used TCA extraction procedure has been inefficient in some cases, e.g., it requires long evaporation time (Cox et al. 2003). Therefore, a methanol/acetone extraction method was used for a more time efficient extraction. Moreover, the methanol/acetone precipitation protocol also gave rise to chromatograms with less interfering peaks.

In this study, a rapid and efficient HPLC method using two coupled Chromolith® columns with 3 mm i.d. decreased the retention time for the BMAA peak from approximately 30 min on the column originally proposed from Waters, to 9 min. Moreover, two different assays to standardize the sample workup and correlate the efficiency of the derivatization yield of BMAA to the optimal protein concentration in the sample are described.

Materials and methods

Growth conditions

Axenic cultures of *Leptolyngbya* PCC 73110 were grown in batch cultures in BG11 media (Stanier et al. 1993). The cultures were grown in Nalgene polycarbonate flasks at a constant volume of 1 L and kept in a climate chamber at +20°C, with 80 μmol photons m^{-2} s^{-1} irradiance and a 16:8 h light:dark cycle. A slow moving magnetic stirrer bar kept the cultures homogenous and a continuous stream of air at the bottom of each flask ensured the saturated concentrations of dissolved inorganic carbon. After 6 days of growth, cells were harvested by centrifugation, and immediately frozen at −80°C for further amino acid extraction.

Extraction

The extraction of both the free and bound form of the amino acids was performed according to Ran et al. (2007) with some minor changes. Cells were dissolved in 450 μl of 70% methanol (70/30 methanol/water). Cell lysis was ensured by first freeze/thawing the suspension 3× in liquid nitrogen followed by sonication (BANDELIN SONOPLUS, Model HP 2070) in 4 cycles of 20 s at 70% intensity. Between each cycle, the sample was allowed to cool for at least 30 s on ice. During the sonication, the tube with the cell suspension was kept in an ice-water bath, to minimize protein degradation. Cell lysis was verified by light microscopy.

Ice-cold acetone (−20°C) was added at 4× the volume of the lyzed cell suspension and vortexed briefly. The samples were than allowed to precipitate at −20°C for a minimum of 3 h. The samples were then centrifuged at 12,000*g* for 10 min using a table centrifuge (Sigma 1K15) and the supernatant (free fraction) was transferred to a new tube. Both fractions were dried using a speed-vac (DNA Speedvac, Model DNA 100, Savant). The dried supernatant was saved for future analysis.

The pellet was re-dissolved in 200 μl of 90% methanol and protein concentration was measured using the RC/DC kit (BioRad, Sweden). Samples were then hydrolyzed in 6 M HCl for 24 h at 110°C. The hydrolysate was filtered using a Centricon® spin-filter (Millipore) device at 12,000*g* for 5 min. Excess HCl was evaporated to complete dryness at 55°C and thereafter, the pellet was reconstituted in 120 μl of 20 mM HCl (Murch et al. 2004).

All TCA extractions were performed according to Cox et al. (2003).

Sample work up

The original sample was first diluted according to Table 1. Then 4 μl of 0.1 mg/ml pure BMAA standard was added to each sample in the dilution series (Table 1, first assay). A sample with 4 μl of 0.1 mg/ml BMAA standard only was analyzed simultaneously with the cyanobacterial samples. In the additional assay, 120 μg of pure BMAA standard

Table 1 Sample dilution and addition of BMAA in the two assays

First assay: diluted and spiked samples		Second assay: spiked and diluted samples	
Dilution	BMAA added (μg)	Dilution	BMAA added (μg)
1:10	0.4	1:10	12
1:30	0.4	1:30	4
1:50	0.4	1:50	2.4
1:75	0.4	1:75	1.6
1:100	0.4	1:100	1.2
1:250	0.4	1:250	0.48
1:300	0.4	1:300	0.4
1:500	0.4	1:500	0.24
1:750	0.4	1:750	0.16
1:1,000	0.4	1:1,000	0.12

was added to the 1:1 sample prior to dilution (Table 1, second assay).

The BMAA peak was identified by its retention time compared with a commercial standard (Sigma–Aldrich, L-BMAA hydrochloride B-107).

Chromatographic conditions

The HPLC system (LaChrom, Hitachi) used in this study consisted of a fluorescence detector L-7485, column oven L-7300, interface D-7000, and binary gradient pump L-7100.

Separation was carried out using two serially coupled 3 mm i.d. Chromolith® Performance RP-18e (VWR, Sweden) columns joined by a column coupler. The pre-column was also supplied from VWR. The columns were thermostated at 37°C, the flow rate set to 2.0 ml/min and the injection volume was 10 μl. Detection was carried out by fluorescence (λ excitation 250 nm and emission 395 nm). Mobile phase A consisted of 20 mM sodium acetate set to pH 5.5 prepared with double distilled water (ddH_2O). Eluent B consisted of acetonitrile/ddH_2O (50/50, v/v). Gradient conditions were as described in Table 2. Between every third run, a cleaning step was performed using 80% acetonitrile at 2 ml/min for 10 min. Limit of quantification (LOQ) and limit of detection (LOD) was determined to be 12 nmol and 120 fmol, respectively, with 3:1 signal to noise ratio (S/N) using standards, and to approximately 40 nmol (LOD) in the cyanobacterial matrix. The linearity was $R^2 = 0.984$ in the range 8.5 nmol–84 pmol. Accuracy and precision were determined and were well within the FDA regulations for bioanalytical analysis (http://www.fda.gov/CDER/GUIDANCE/4252fnl.htm).

Table 2 Gradient conditions used for separation of BMAA

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	10	90
0.5	14	86
4.2	24	76
10	40	60
10.5	100	0
15	100	0
15.5	10	90
22	10	90

The presence of BMAA produced by *Leptolyngbya* PCC 73110 has previously been verified by liquid-chromatography–mass spectrometry (LC–MS) (Cox et al. 2005).

Results and discussion

In the present work, a method that enabled maximum derivative yield of the non-protein amino acid BMAA in a complex mixture of amine-containing compounds was developed using the cyanobacteria *Leptolyngbya* PCC 73110 as the model organism.

The highly reactive amine-derivatizing reagent AccQ-Tag® was used for detection of amino acids in hydrolyzed samples. In order to optimize the derivative yield of BMAA using 20 μl AccQ-Taq®, 0.03–5 μg/μl protein solutions was derivatized under similar conditions.

To avoid variations caused by competition between primary and secondary amines in the amino acid solution, as different amino acids have different reaction rates (Cohen and Michaud 1993), AccQ-Tag® had to be in the correct concentration range with respect to the analyte of interest, e.g., BMAA. In a complex sample of more or less unknown composition, the worst case scenario would be that the analyte of interest has a much lower affinity to the derivative reagent than the other amine-containing compounds and thereby, will be discriminated as other compounds will be derivatized to a higher degree.

Our work shows a correlation between sample protein concentration and efficiency of derivatization, where the BMAA peak area decreased as the protein concentration increased (Fig. 1). This is due to the competitive reaction between the different amine-containing compounds in the sample. As the protein concentration increases, the molar excess of AccQ-Tag® decreases, causing a decline in the area of the peak corresponding to BMAA.

Highest efficiency of the AccQ-Tag® was obtained when the protein concentration in the samples ranged between 0.06–0.15 μg/μl. Furthermore, the "area under peak" and the peak height for the peak corresponding to

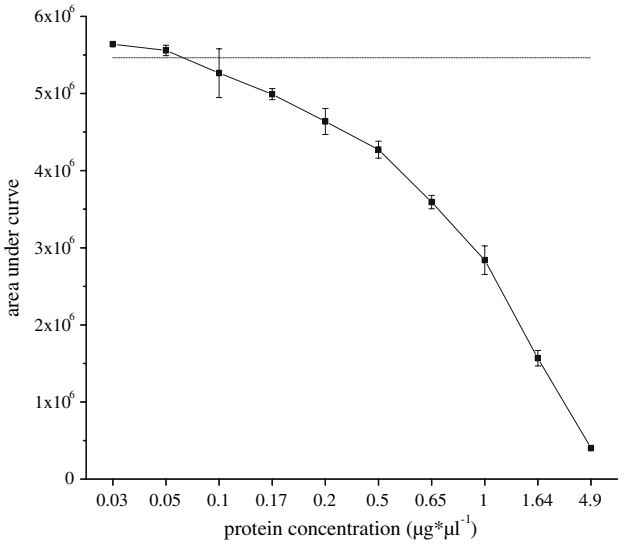

Fig. 1 The graph represents the decrease in BMAA peak area ($n = 10$) as the protein concentration increases in the sample. Also, the *horizontal line* represents the area of a pure BMAA sample of the same concentration as used in the dilution experiments. *Error bars* indicate standard deviation

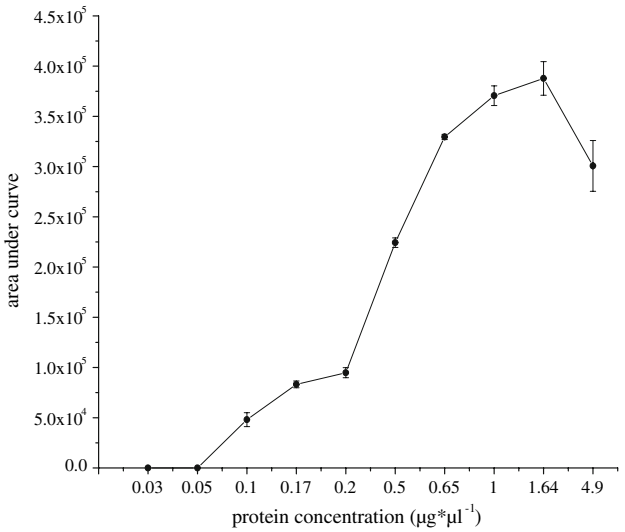

Fig. 2 BMAA peak area as an effect of dilution. The limit of detection (LOD) is clearly visible in the graph. *Error bars* indicate standard deviation

BMAA showed a similar trend (data not shown). When the protein concentration in the sample reached more than ~0.17 µg/µl, there was a decrease in the peak area and peak height corresponding to BMAA concentration.

The detection limit of derivatized BMAA in the complex matrix produced by the cyanobacteria is shown in Fig. 2. The undiluted sample was spiked with 120 µg BMAA, which is equivalent to the amount produced and which has already been reported for *Leptolyngbya* PCC

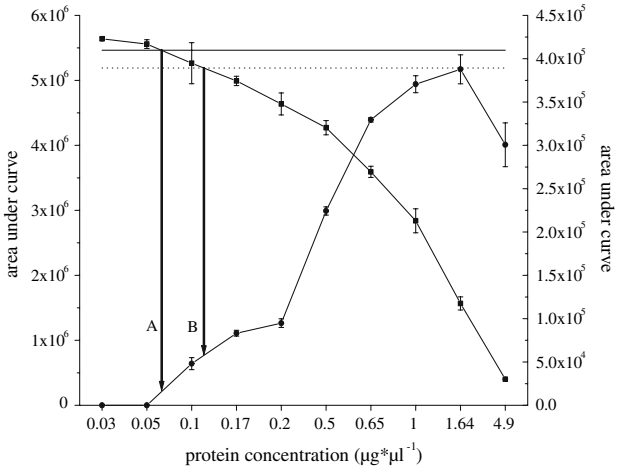

Fig. 3 The graph shows assay 1 combined with assay 2. *Arrow A* represents the protein concentration corresponding to the detection limit of the currently used HPLC system setup. *Arrow B* represents the protein concentration for optimal derivatization efficiency (approximately 95% derivatization efficiency) combined with sufficient detection of BMAA. *Squared symbols* represent assay 1, while *circled symbols* represent assay 2. The *horizontal* and the *dotted line* correspond to 100 and 96% derivatizing efficiency, respectively. *Error bars* indicate standard deviation

73110 in PNAS (Cox et al. 2005). The data points in Fig. 2 represent the same dilutions as the data points in Fig. 1. By combining the information in Figs. 1 and 2, conclusions can be drawn about the optimum dilution in order to achieve the optimal derivatization efficiency and detectable levels of BMAA in the cyanobacterial sample (Fig. 3).

Theoretically, the optimal BMAA derivative yield would be achieved in a very diluted sample (Fig. 1), but since the BMAA concentration is low in cyanobacteria (Cox et al. 2005), a highly diluted sample would eliminate any possibility of BMAA detection, as the concentration would be well below the detection limits of our method. As the combined graphs in Fig. 3 suggests; highest derivatization efficiency together with still not too diluted samples for BMAA detection is obtained when the protein concentration is ~0.12 µg/µl. At this concentration, the derivatization efficiency is 96%.

When comparing the methanol/acetone precipitation with the TCA precipitation, the methanol/acetone solution gave a chromatogram that was easier to interpret due to less interference peaks and was also quicker to evaporate. One of the major reasons for using methanol/acetone is that it is rapidly evaporated and thereby speeds up the time for analysis.

During the derivatization process, acidic cyanobacterial cell content in combination with residual acid, e.g., TCA, might cause low pH. This will cause inefficient buffering capacity by the borate buffer supplied in the kit with the AccQ-Tag®, which will lead to non-optimal derivatization

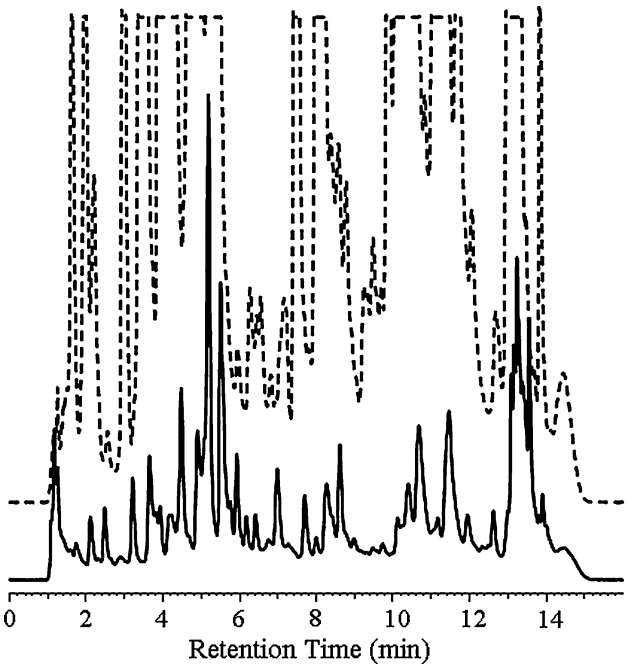

Fig. 4 The two chromatograms illustrate the difference in number and height of the peaks. The *dotted chromatogram* represents a 5 dilution whereas the *solid line* is a 2.5 dilution. The two chromatograms are from the same preparation and are only separated by the dilution factor

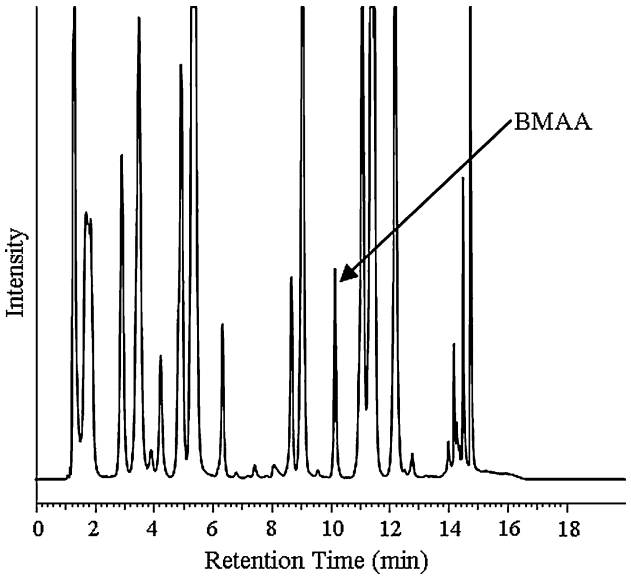

Fig. 5 A representative chromatogram showing the BMAA peak (R_t 10.1 min) in a 300× dilution according to assay 1

conditions. By standardizing the protein concentration before derivatization in borate buffer, we were able to overcome the inadequate derivatization of the analyte, and generate a reproducible and stable result.

Figure 4 shows the difference in peak yield at a 2× decrease in sample concentration. This shows the requirement of molar excess of AccQ-Tag® compared to reagent molecules. In our studies, we have seen that this effect is almost exponential, and as the sample concentration increases, the number of peaks is reduced at a certain breakpoint. Thereafter, the certainty of the analysis is highly compromised as the number and intensity of the peaks in the chromatogram is not representative of the actual sample. Figure 5 displays a chromatogram produced in a 300× dilution according to assay 1. The BMAA peak can be found at the retention time of 10.1 min.

Conclusion

We have developed a method to determine the optimal protein concentration in order to obtain maximum analyte derivatization efficiency. This was done by combining the results from two different assays. Put together, this gave us an optimum protein concentration range that led to highly reproducible results. In our study, the optimal protein amount was approximately 0.12 μg protein/μl.

We have also developed rapid and efficient chromatography for detection of BMAA utilizing two Chromolith® Performance RP-18e columns joined by a column coupler.

We have used methanol/acetone protein precipitation instead of the commonly used acidic protein precipitation, e.g., TCA. The methanol/acetone solution gave a chromatogram that was easier to interpret due to less interference peaks and was also quicker to evaporate.

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