UTS UNIVERSITY OF TECHNOLOGY SYDNEY

Analysis Of Protein And Non-Protein Amino Acids Via Liquid Chromatography-Tandem Mass Spectrometry

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Certificate of Original Authorship

I, Jake Patrick Violi declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the Faculty of Science at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise reference or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis. This document has not been submitted for qualifications at any other academic institution.

This research is supported by an Australian Government Research Training Program Scholarship.

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List of Publications

First author

Violi, J. P.; Bishop, D. P.; Padula, M. P.; Westerhausen, M. T.; Rodgers, K. J., Acetonitrile adduct analysis of underivatised amino acids offers improved sensitivity for hydrophilic interaction liquid chromatography tandem mass-spectrometry. Journal of Chromatography A 2021, 1655, 462530.

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Co-author

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Italiano, C. J.; Pu, L.; **Violi, J. P.**; Duggin, I. G.; Rodgers, K. J., Cysteine biosynthesis contributes to β-methylamino-l-alanine tolerance in *Escherichia coli*. Research in microbiology 2021, 172 (6), 103852.

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O'Rourke, M. B.; Town, S. E. L.; Dalla, P. V.; Bicknell, F.; Koh Belic, N.; **Violi, J. P**.; Steele, J. R.; Padula, M. P. 2019. What is Normalization? The Strategies Employed in Top-Down and Bottom-Up Proteome Analysis Workflows. Proteomes 7, (3).

Facey, J.A., Steele, J.R**., Violi, J.P**., Mitrovic, S.M., Cranfield, C. 2019. An examination of microcystin-LR accumulation and toxicity using tethered bilayer lipid membranes (tBLMs). Toxicon 158, 51-56.

Abstract

Amino acids are a class of small polar compounds, known mainly for their roles as the substrates in ribosomal protein synthesis. In addition to their use in protein synthesis, amino acids have many biological functions including, modulation of homeostasis, and in neurotransmission. The majority of amino acids found in nature are not used in ribosomal protein synthesis and are classified as non-protein amino acids. Some of these non-protein amino acids are produced by fungi, algae and bacteria and are of interest as they can negatively impact on human health and have been suggested to play a causal role in sporadic neurological diseases such as sporadic motor neuron disease. β-Methylamino-L-alanine, more commonly referred to as BMAA, is a non-protein amino acid produced by cyanobacteria and marine diatoms, that is implicated as a potential environmental factor that could play a role in sporadic motor neuron disease, however there is still much to be known regarding its mechanism of toxicity. The studies presented in this thesis aimed to firstly design new methods for amino acid and metabolite analysis, and secondly, investigate sources of BMAA and its effect on the human metabolome to provide insight into its neurotoxicity and the potential for human exposure.

Amino acids are small polar zwitterionic molecules, and their analysis is complicated due to their physicochemical properties. The most sensitive technique for amino acid analysis is LC-MS/MS. LC-MS/MS while allowing for sensitive analysis of native amino acids it is problematic due to low ionisation efficiencies, in source fragmentation, and difficulty in the correct application of chromatographic techniques. This has led most researchers to derivatising amino acids prior to analysis, this however, has the disadvantages of increasing the time and cost of analysis. Chapter 2 in the thesis is a published tutorial review that examines the ways amino acids are currently analysed via LC-MS/MS and discusses the advantages and disadvantages of different approaches. Chapter 3 discusses the development of a sensitive protein amino acid analysis method for native amino acids. A novel acetonitrile (ACN) adduct (M+H+ACN+) was discovered for each of the protein amino acids and was found to increase the detection sensitivity of 16 out of the 20 protein amino acids, with improvements to the signal-to-noise ratio ranging from 23% to 1762%.

Non-protein amino acids such as BMAA are readily taken up by human cells and in some cases can mimic protein amino acids in intracellular processes including in protein synthesis. In Chapter 4, we treat neuroblastoma cells with BMAA and examine changes in levels of protein amino acids over a 48 hour period using the analysis method developed and validated in Chapter 3. Levels of 16 of the 19 amino acids detected were significantly changed in at least one timepoint with 3 the amino acids, histidine, tyrosine and serine having consistent decreases in concentration at 3 timepoints. Serine was the most heavily affected amino acid, decreasing in concentration in 4 sequential timepoints, suggesting serine may be integral to BMAA's mechanism of toxicity.

The amino acid data presented in chapter 4, while important, is only reveals some of the changes in the metabolome that occur in response to BMAA. In chapter 5 a novel untargeted method was developed and used to analyse metabolic changes in BMAA treated neuroblastoma cells. Many metabolic pathways were found to be impacted by BMAA namely the one-carbon metabolism and alanine, aspartate and glutamate metabolism. In addition, a number of neurotransmitters and markers of oxidative stress were found to be altered.

BMAA has been shown to be produced by cyanobacteria and marine diatoms but cyanobacteria are the only known BMAA producers in freshwater systems. Chapter 6 is a published manuscript in which we investigated if freshwater diatoms had the capacity to produce BMAA and its isomers like their marine counterparts. Five axenic diatom cultures were established from multiple locations across eastern Australia. Intracellular amino acids were extracted and analysed for BMAA and its isomers using LC-MS/MS. Four out of the five diatoms were shown to have detectable BMAA. These results show that BMAA production by diatoms is not confined to marine genera and that the prevalence of these non-protein amino acids in Australian freshwater environments cannot be solely attributed to cyanobacteria

The extraction of BMAA from sample matrices is a time-consuming procedure, with an overnight hydrolysis step required to release bound BMAA from the protein fraction. This hydrolysis step is based on those used to cleave the polypeptide bonds in proteins and release the constituent amino acids. The nature of the association between BMAA and proteins is not known and might differ between sample matrices. Recent amino acid studies have found that by utilising microwave assisted hydrolysis this step can be reduced to 5 minutes. Chapter 7 investigates the use of microwave assisted hydrolysis to allow a quicker more efficient sample extraction of protein-bound BMAA and its isomers. A 5-fold increase in recovery was found for BMAA in the two sample matrices examined. This suggests that current hydrolysis methods are not optimal for BMAA recovery and that concentrations of this toxic NPAA in nature is currently being underestimated.

Future studies should be aimed at optimising conditions for the release of bound BMAA from a range of sample matrices using the microwave approach to allow its accurate quantification. This could also be applied to brain samples which have previously been difficult to analyse. It could be valuable to conduct lipid analysis or lipidomics on BMAA treated cells to provide further information on how BMAA could impact on lipid synthesis and metabolism.

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List of Abbreviations

%RSD	Percentage relative standard deviation
(S)-NIFE)	N-(4-Nitrophenoxycarbonyl)-L-phenylalanine 2-methoxyethyl ester
2,4-DAB	2,4-diaminobutyric acid
2D-LC	Two-dimensional liquid chromatography
AAA	Amino acid analysers
ACN	Acetonitrile
AEG	N-(2-aminoethyl) glycine
AlaRS	Alanyl tRNA synthetase
ALS	Amyotrophic lateral sclerosis
ALS-PDC	Amyotrophic lateral sclerosis-Parkinson's dementia complex
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANP	Aqueous normal phase
APCI	Atmospheric pressure chemical ionisation
AQC	6-aminoquinolyl-N-hydroxysuccinimidyl carbamate
AZE	Azetidine-2-carboxylic acid
BCA	Bicinchoninic acid
BMAA	β-Methylamino-L-alanine
BOAA	β-N-oxalyl-L-α,β-diaminopropionic acid
BSA	Bovine serum albumin
CCC	Chiral column chromatography
CCS	Collision cross-section
CDA	Chiral derivatisation agents
CE	Capillary electrophoresis
CI	Chemical ionisation
CID	Collision-induced dissociation
CNS	Central nervous system
CSP	Chiral Stationary phases
CV	Compensation voltage
D5-DAB	2,4-Diaminobutyric-2,3,3,4,4-d5
DA	Domoic acid
DBEMM	Dibenzyl ethyl ethoxymethylene malonate
DC	Direct current
DDA	Data-dependent acquisition
DEEMM	Dibenzyl ethoxymethylene malonate
DFDNB	1,5-difluoro-2,4-dinitrobenzene
DIA	Data-independent acquisition
DIMS	Differential mobility spectrometry
DL	Desolation line
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTIMS	Drift-time ion mobility spectrometry
DTT	Dithiothreitol
DW	Dry weight

EI	Electron ionisation
EMEM	Eagle's Minimum Essential Medium
ESI	Electrospray ionisation
Ethos X	ETHOS X microwave extractor
FA	Formic Acid
FAIMS	Field asymmetric-waveform ion-mobility spectrometry
FBS	Fetal bovine serum
FDAA	1-fluoro-2-4-dinitrophenyl-5-L-alanine amide
FDVA	1-fluoro-2-4-dinitrophenyl-5-L-valinamide
FLD	Fluorescence detection
FLEC	1-(9-fluorenyl)-ethyl chloroformate
FMOC	9-fluorenylmethyloxycarbonyl chloride
FT-ICR-MS	Fourier-transform ion cyclotron resonance mass spectrometer
GABA	Gamma-aminobutyric acid
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GITC	2,3,4,6- tetra-O-acetyl-b-d-glucopyranosyl isothiocyanate
HCl	Hydrochloric Acid
H-ESI	Heated electrospray ionization
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
IBLC	N-isobutyryl-L-cysteine
IC	Ion chromatography
IEM	Inborn errors of metabolism
IMS	Ion mobility spectrometry
IPC	Ion-pairing chromatography
ISTD	Internal standard
iTRAQ	Isobaric tags for relative and absolute quantitation
LAT1	L-type amino acid transporter 1
LC	Liquid chromatography
LC-MS	Liquid chromatography- mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
L-Dopa	L-3,4 dihydroxyphenylalanine, levodopa
LLOD	Lower limit of detection
LOD	Limit of detection
LOQ	Limit of quantification
LOWESS	Locally weighted scatterplot smoothing
m/z	Mass-to-charge ratio
MBNA	MassBank of North America
Methanol	MeOH
MIPI	Microwave-induced plasma ionisation
MND	Motor neuron disease
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry

MTBE	Methyl tert-butyl ether
MW	Microwave
NAA	N-Acetyl-L-aspartate
NAC	N-acetyl-L-cysteine
NMDA	N-methyl-D-aspartate
NMR	Nuclear Magnetic Resonance
NPAA	Non-protein amino acids
OPA	Ophthaldialdehyde
P5CS	Delta-1-pyrroline-5-carboxylate synthase
PBS	Phosphate buffered saline
PCA	Perchloric acid
PCF	Propyl chloroformate
PE	Phosphatidylethanolamines
PHGDH	Phosphoglycerate dehydrogenase
PI	Phosphoinositides
PKU	Phenylketonuria
PRM	Parallel reaction monitoring
PSAT	Phosphoserine aminotransferase
PSTs	Paralytic shellfish toxins
PTFE	Polytetrafluoroethylene
QC	Quality control
QOMS	Quadrupole orbitrap mass spectrometry methods
QTOF	Quadrupole time of flight
RF	Radiofrequency
RPLC	Reverse phase liquid chromatography
S/N	Signal-to-noise ratio
SAMe	S-Adenosyl-L-methionine
SerRS	Seryl tRNA synthetase
SFC	Supercritical fluid chromatography
SFE	Supercritical fluid extraction
SIL	Stable isotope labelled
SPE	Solid phase extraction
SRM	Selected reaction monitoring
TAHS	p-N,N,N-trimethylammonioanilyl N0-hydroxysuccinimidyl carbamate iodide
TCA	Trichloroacetic acid
TIC	Total ion chromatogram
TIMS	Trapped ion mobility spectrometry
TLC	Thin-layer chromatography
TMG	Trimethylglycine
TMT	Tandem mass tags
TQMS	Triple quadrupole mass spectrometer
tRNA	Transfer ribonucleic acid
TWIMS	Traveling wave ion mobility spectrometry
UHPLC	Ultra-high performance liquid chromatography
UTEX	University of Texas
UV	Ultraviolet

Vis	Visible light
w/v	Weight per volume