Department of Chemistry

Reaction Pathways in the Chlorination of Amino Acids

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This thesis is presented for the Degree of
Doctor of Philosophy
Of
Curtin University

June 2016

Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature: _____

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Date: 30/06/2016

Acknowledgments

I am very grateful to many individuals for the help and support that I've received over the past years. Especially to the following people:

My Principal Supervisor, A/Prof. Cynthia Joll, for her continual guidance and insights for the project; for her support and encouragement; and for her thorough review of this Thesis.

My Co-Supervisor, Dr. Kathryn Linge, for her guidance and encouragement throughout my PhD program; for her advice on experimental planning and design; and her insightful feedback on this Thesis.

My Co-Supervisor, Dr. Francesco Busetti, for his helpful insight and technical advice on LC-MS; for his contribution to experimental planning and design; and his helpful feedback on the Thesis.

Mr. Geoff Chidlow, for his invaluable technical assistance and advice on the operation of GC-MS instrumentation.

The staff and my fellow students in the Department of Chemistry, past and present, for their support, assistance and companionship over the years, particularly Ina Kristiana, Deborah Liew, Jace Tan, Mark Maric, Patrick Fritz, Krishnan Murugappan and Rhys Tilbury.

The staff of the Water Corporation of Western Australia, for assistance in the provision of samples and operational information for my project.

Ms. Carolyn Bellamy and Mr. Gareth Roeszler from Water Research Australia for their wonderful support and encouragement throughout the course of my project.

My Water Research Australia mentor, Ms. Mary Drikas, for her invaluable advice and insight for my project.

I would also like to acknowledge the following institutions for financial support: Curtin University (Curtin International Postgraduate Research Scholarship) and Water Research Australia (WaterRA PhD Top-Up Scholarship). I also thank the Australian Research Council (LP110100548 and LP130100602) and the Water Corporation of Western Australia for their support of this project.

Last, but not least, I would like to thank my family and friends for their support, understanding and encouragement throughout the years. Special thanks to Michelle Wong for encouraging me to apply for the PhD program, as all this would not be possible without taking that first step.

Abstract

Water disinfection is a crucial step in the production of safe drinking water. In water disinfection, pathogenic microorganisms are removed or deactivated by means of physical or chemical methods. Chlorination and chloramination are the most widely used disinfection techniques as they are effective, inexpensive, and provide disinfectant residual within the distribution system for a considerable length of time. When chlorination and chloramination are used, the disinfectant is measured as free or combined chlorine. Although combined chlorine is used as the measurement for inorganic monochloramine, combined chlorine in fact includes all species of inorganic and organic chloramines. Organic chloramines are compounds that contain at least one chlorine atom directly bonded to an amine nitrogen atom in an organic molecule. In water chemistry, organic chloramines can also be used as a collective term for species like *N*-chloramines, *N*-chloramino acids, *N*-chloraldimines and *N*-chloramides, due to the fact that these species are included in the measurement of combined chlorine. Organic chloramines are known to form when chlorine-based disinfectants react with dissolved organic nitrogen or dissolved organic carbon, however minimal information on the occurrence of organic chloramines in drinking water is available, resulting in unknown health risks. In vitro studies and structural assessment of the toxicity of organic chloramines suggest that organic chloramines are potential carcinogens and mutagens. The formation of organic chloramines can also cause adverse effects in water treatment. The formation of organic chloramines has been found to increase the chlorine demand of treated water, and reduce germicidal efficiency of both chlorine and inorganic monochloramine. Most importantly, organic chloramines interfere in all analytical methods used to determine combined chlorine residuals, which can result in the overestimation of inorganic monochloramine concentrations in disinfected waters. In addition, *N*-chloramino acids are known to degrade into odorous aldehydes and *N*-chloraldimines, which could potentially result in odour issues in drinking water.

This Thesis focuses on better understanding the reaction of amino acids with chlorine, the reaction thought to be the main contributor to the formation of organic chloramines. Chapter 2 presents a critical review of previously published information on the

formation of organic chloramines and their impact on water treatment processes. The development of analytical methods for amino acids and organic chloramines is presented in Chapter 3. In Chapter 4, studies of the formation and stability of organic chloramines are reported, while the speciation and degradation of organic chloramines, specifically *N*-chloramino acids, are described in Chapter 5. Finally, the occurrence of free amino acids and the odorous degradation products of *N*-chloramino acids in both drinking waters and wastewaters is presented in Chapter 6. The conclusions and recommendations of this Thesis are summarised in Chapter 7.

The critical review on organic chloramines presented in Chapter 2 highlighted that one of the biggest challenges for the study of organic chloramines in water systems was the absence of analytical standards, resulting in difficulties in the identification and quantification of organic chloramines in water systems. In addition, many of the organic chloramines formed during disinfection were found to be unstable, which resulted in difficulties in sampling and detection using mass spectrometric methods. The review also indicated that the formation of organic monochloramines and dichloramines should be considered during water disinfection, as it is a common strategy to achieve breakpoint chlorination during disinfection. The practice of breakpoint chlorination involves a high chlorine to precursor molar ratio, making it more likely that organic dichloramines would be formed. Finally, the review found that organic chloramines can be formed from many different precursors and pathways. Therefore, by understanding the occurrence and concentrations of the precursors in water systems, better prediction of the formation of organic chloramines can be achieved.

Analytical methods developed for the analysis of amino acids and to monitor the degradation of organic chloramines are presented in Chapter 3. A novel analytical method for the analysis of 18 amino acids in natural waters, which utilised solid-phase extraction (SPE) for sample clean-up and extraction, followed by liquid chromatography-electrospray tandem mass spectrometry (LC-MS/MS) operated in multiple reaction monitoring mode, was developed and validated. The method limits of quantification (MLQ) for the SPE LC-MS/MS method in ultrapure water ranged from 0.1 to 100 µg L^{-1} as N for the different amino acids. The developed method was successfully applied for the analysis of free amino acids in three surface waters and

was used in Chapter 4 for the analysis of residual amino acids after chlorination and for the survey of free amino acids in drinking waters and wastewaters in Chapter 6. To study the formation and degradation of organic chloramines, a direct UV method and a modified standard iodometric method for colorimetric measurement were used. The modified standard iodometric method was only used for organic chloramines not suitable for direct UV detection due to interference peaks in the 250 to 280 nm range from either the precursors of the organic chloramines or from the degradation products of the organic chloramines. The combination of direct UV detection and the modified standard iodometric method allowed the stability of more organic chloramines to be investigated than in previous studies. This research is presented in Chapter 4.

The formation and stability of organic chloramines, from chlorination of three amines and 21 amino acids, and organic chloramides from two amides, and the competition between 20 of the amino acids (the proteinogenic amino acids) during chlorination, were studied and the results presented in Chapter 4. After chlorination at a chlorine to precursor (amine, amino acid or amide) molar ratio of 0.2 to 2, all three amines and 18 out of the 21 amino acids formed organic chloramines, while the amides and three amino acids (cysteine, methionine and tryptophan) did not form organic chloramides/chloramines. These three amino acids did not form organic chloramines at these low molar ratios because they have a second functional group which is more reactive to chlorine than the amine group. Among the 20 proteinogenic amino acids tested for their competitive reactivity towards chlorination, cysteine, methionine and tryptophan were the most reactive amino acids towards chlorination, however they did not form organic chloramines at the chlorine to precursor ratios used. Isoleucine, threonine and valine were found to be the least reactive amino acids towards chlorination. After formation, most of the organic chloramines from the amino acids were found to be unstable, with only six out of the 21 organic chloramines (18 from amino acids and three from amines) formed having a half-life of more than 3 hours, although this group included all the organic chloramines formed from amines. The organic chloramine from taurine was found to be more stable than the organic chloramines from the 17 proteinogenic amino acids that formed organic chloramines. The proteinogenic amino acids are all α-amino carboxylic acids, whereas taurine is a β-amino sulfonic acid. Degradation of these organic chloramines occurs *via* dehydrohalogenation of the chlorine attached to the nitrogen and a hydrogen attached

to the carbon bonded to the nitrogen. In the α-amino acids, these hydrogens are more acidic, and thus reactive to dehydrohalogenation, due to their location α to the carboxylic acid group. In taurine, these hydrogens are less acidic because they are located β to the sulfonic acid group, resulting in a more stable organic chloramine. Finally, a risk assessment relating the reactivity of the organic chloramine precursors and the stability of the organic chloramines, as determined in this study, to published toxicity and precursor abundance data indicated that only *N*-chloroglycine is likely to be of concern, due to its stability, toxicity and precursor abundance in drinking water source waters.

As most of the organic chloramines (*N*-chloramino acids) formed from the chlorination of amino acids were not stable, it is important to understand their speciation and degradation. The speciation of *N*-chloramino acids formed from glycine, isoleucine, leucine, lysine, taurine, tyrosine and valine, and the reaction pathways and rate constants of the chlorination of valine as a model amino acid, were investigated (Chapter 5). All amino acids tested demonstrated similar speciation of initial products, with formation of *N*-monochloramino acids at Cl:N $\leq$ 1 and formation of *N*,*N*-dichloramino acids at Cl:N $\geq$ 2. At Cl:N $\geq$ 2, tyrosine formed a mixture of the *N*,*N*-dichloramino acid and a dichloro derivative with one chlorine attached to the amino nitrogen and the other chlorine attached *ortho* to the hydroxy group on the phenol ring. When the Cl:N ratio was increased to 12, tyrosine degraded into other by-products as the phenolic functional group was much more reactive towards chlorination than the chloramine functional group and further chlorination and oxidation of the phenolic ring presumably led to ring-opening. This result demonstrated the importance of considering all reactive functional groups in a molecule to understand and predict the possible by-products formed from chlorination. The rate constant for each reaction in the pathway for the chlorination of valine was determined by modelling and/or from experimental data. At molar ratio Cl:AA = 2.8, the chlorine was found to first react quickly with valine to form *N*-monochlorovaline (k_1 = 5.4x10^4 M^{-1}s^{-1}) with a slower subsequent reaction with *N*-monochlorovaline to form *N*,*N*-dichlorovaline (k_2 = 4.85x10^2 M^{-1}s^{-1}), while some of the *N*-monochlorovaline degraded into isobutyraldehyde (k_3 = 1.00x10^{-4} s^{-1}). The *N*,*N*-dichlorovaline then competitively degraded into both isobutyronitrile (k_4 = 1.25x10^{-4} s^{-1}) and *N*-chloroisobutyraldimine (k_5 = 1.15x10^{-4} s^{-1}). The conversion (reaction yield) of valine to isobutyraldehyde,

isobutyronitrile and *N*-chloroisobutyraldimine was 5%, 35% and 30%, respectively. Modelling of the reaction pathways also predicted that isobutyraldehyde and isobutyronitrile would hydrolyse slowly into isobutyric acid. Since similar speciation of glycine, leucine, isoleucine and valine after chlorination was observed, it was expected that the chlorination reactions of these other amino acids would have similar trends to the reactions between valine and chlorine. Therefore, at a practical water treatment plant chlorine dose, where approximately 0.5 mg L^{-1} free chlorine residual is required, 30% of amino acids may be converted to the odorous and stable *N*-chloraldimines, which might result in chlorinous off-flavours in the drinking water. However, this process depends on the presence of amino acids at sufficient concentrations to give the products above their odour threshold concentrations at the consumer's tap.

It is therefore important to understand the concentration of free amino acids and their odorous chlorination degradation products in drinking waters. Hence, the occurrence of 18 free amino acids in four drinking water treatment plants, as well as four wastewater treatment plants, were studied (Chapter 6). In general, most drinking water treatment plants and wastewater treatment plants were effective in the removal of dissolved organic carbon (DOC), with efficiency up to 90%, while a general decrease in the concentrations of free amino acids was observed after treatment for both drinking water and wastewater treatment plants. The concentrations of free amino acids in raw drinking waters were 0.7 to 2.8 µg L^{-1} as N, contributing 0.06% to 2% of the total dissolved nitrogen. Among the 18 amino acids analysed, alanine, glycine, serine and valine had the highest contribution to the concentration of total free amino acids in drinking waters. The concentrations of individual free amino acids in the wastewater influents were found to be 0.4 to 59 µg L^{-1} as N, contributing 0.06% to 19% of the total dissolved organic nitrogen. Isoleucine, leucine and phenylalanine were the main contributors to the concentration of free amino acids in the wastewater influents. Isoleucine, leucine and valine had the highest contribution to the concentration of total free amino acids in wastewater effluents.

When chlorinated, free amino acids were shown to react to form odorous aldehydes and *N*-chloraldimines, however no aldehydes or *N*-chloraldimines were detected in the treated drinking waters. This was due to the fact that the concentrations of individual

free amino acids were very low (maximum concentration of 2 µg L^{-1} as N) in the treated waters. As a consequence, the maximum possible concentrations of the respective aldehydes and *N*-chloraldimines that could be formed were near or below the limits of detection of the analytical methods used (determined in formation potential (FP) experiments for the aldehydes and *N*-chloraldimines using raw drinking water). FP experiments for the aldehydes and *N*-chloraldimines on the secondary effluents from the wastewater treatment plants showed that, if chlorination was used for the disinfection of the secondary effluents, 0.002 to 0.02 µM of aldehydes would be produced, while 0.001 to 0.05 µM of *N*-chloraldimines would be produced. The concentrations of aldehydes produced would be generally lower than their odour threshold concentrations (odour threshold concentrations = 0.002 to 0.1 µM), while the concentrations of *N*-chloraldimines produced would be mostly above their odour threshold concentrations (odour threshold concentrations = 0.003 µM). In addition, FP experiments on model amino acids in the presence of ammonia found that the presence of ammonia in the water can change the initial rate of formation of aldehydes and *N*-chloraldimines from amino acids. However, no effect on the final concentrations of aldehydes and *N*-chloraldimines formed after 7 days was observed, where the concentrations of aldehydes and *N*-chloraldimines were found to be similar in water with and without ammonia. From the FP experiments, it was observed that aldehydes, nitriles and *N*-chloraldimines slowly degraded after formation. Based on the prediction in Chapter 5 that isobutyraldehyde and isobutyronitrile would hydrolyse into isobutyric acid, a FP experiment on the formation of isobutyric acid from the chlorination of valine was conducted in which isobutyric acid was detected. The detection of isobutyric acid in the FP experiment indicated that the respective carboxylic acid can be formed from the chlorination of amino acids. This is the first report of the formation of the respective carboxylic acid from chlorination of an amino acid in an aqueous system and also indicated that these carboxylic acids could potentially be present in distributed waters.

This Thesis presents an in-depth study of the reaction pathways and rates of reaction between amino acids and chlorine. The stability of *N*-chloramino acids is dependent on the reactivity of hydrogens attached to the carbon bonded to the amine nitrogen and most of the *N*-chloramino acids formed from the chlorination of α-amino acids were not stable due to the relatively high reactivity of these hydrogens. *N*-Chloroglycine is

the only *N*-chloramino acid likely to be of concern in drinking water due to its stability, toxicity and precursor abundance in drinking water source waters. After chlorination, amino acids without a secondary reactive functional group follow similar competitive degradation reaction pathways, where aldehydes, nitriles and *N*-chloraldimines are formed. Aldehydes and nitriles can then be converted into carboxylic acids through subsequent reactions. In field studies, it was found that, although both drinking water and wastewater treatment plants were generally efficient in the removal of DOC, poor removals of free amino acids were observed, resulting in the potential to form odorous aldehydes and *N*-chloraldimines in both water types upon disinfection. Organic chloramines formed from amino acids have little or no health risk to the public due to their low stability; however, their degradation products can result in chlorinous off-flavours in drinking waters and pose an unknown public health risk. Future work is recommended to understand the occurrence and potential health impacts of the carboxylic acids in distributed waters, as the carboxylic acids are likely to be key end products of the chlorination of amino acids. Consideration of removal of amino acids in the treatment of drinking water may assist in the reduction of formation of *N*-chloramino acids and odorous degradation products in the distribution system. In conclusion, this project has advanced the understanding of the formation of organic chloramines, particularly through the speciation, reaction pathways and kinetics of the reactions between chlorine and amino acids.

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List of Abbreviations

AA	Amino Acid
APCI	Atmospheric Pressure Chemical Ionisation
CAR	Carboxen
CI	Disinfection By-Product
Cl	Chemical Ionisation
$DANSO_2H$	5-(Dimethylamino)naphthalene-1-sulfinic acid
DBP	Disinfection By-Product
DOC	Dissolved Organic Carbon
DON	Dissolved Organic Nitrogen
DPD	*N*,*N*-Diethyl phenylene-1,4-diamine
DWTP	Drinking Water Treatment Plant
EI	Electron Ionisation
ESI	Electrospray Ionisation
GC	Gas Chromatography
HS	Headspace
IS	Internal Standard
LC	Liquid Chromatography
MCA	Inorganic Monochloramine
MeOH	Methanol
MRM	Multiple Reaction Monitoring
MS	Mass spectrometry
MS/MS	Triple Quadrupole Mass Spectrometry
N-DBP	Nitrogen-containing Disinfection By-Product
NMR	Nuclear Magnetic Resonance
NOM	Natural Organic Matter
PDMS	Polydimethylsiloxane
pI	Isoelectric Point
QA	Quality Assurance
QC	Quality Control
RP	Reversed Phase
RSD	Relative Standard Deviation

S/N	Signal to Noise
SD	Standard Deviation
SIM	Selected Ion Monitoring
SPE	Solid-Phase Extraction
SPME	Solid-Phase Microextraction
SRM	Single Reaction Monitoring
SS	Surrogate Standard
TDN	Total Dissolved Nitrogen
UV	Ultraviolet
WWTP	Wastewater Treatment Plant

List of Publications and Presentations Arising from this Thesis

Refereed Journal Articles

Z.T. How, K.L. Linge, F. Busetti, C.A. Joll. (2016). Organic chloramines in drinking water: An assessment of formation, stability, reactivity and risk. Water Research, 93, 65-73

Z.T. How, F. Busetti, K.L. Linge, I. Kristiana, C.A. Joll, J.W.A. Charrois. (2014). Analysis of free amino acids in natural waters by liquid chromatography tandem mass spectrometry. Journal of Chromatography A, 1370, 135-146

Book Chapters

Z.T. How, K.L. Linge, F. Busetti, C.A. Joll. (2016). Detection Methods to Monitor the Degradation of Organic Chloramines. In Disinfection by-products in drinking water. Thompson, C., Gillespie, S. and Goslan, E. (eds), pp. 267-276, Royal Society of Chemistry, Cambridge, UK.

Oral Presentations

Z.T. How, K.L. Linge, F. Busetti, C.A. Joll. "Organic chloramines: Identification of degradation by-products" at Micropol & Ecohazard Conference 2015, 9th IWA Specialist Conference on Assessment and Control of Micropollutants and Hazardous Substances in Water, 22-25 November 2015, Singapore.

Z.T. How, K.L. Linge, F. Busetti, C.A. Joll. "Occurrence of free amino acids and related disinfection by-products in drinking water treatment systems" at NOM6 – 6th IWA Specialist Conference on Natural Organic Matter Research, 7-10 September 2015, Malmö, Sweden.

Z.T. How, K.L. Linge, F. Busetti, I. Kristiana, C.A. Joll, J.W.A. Charrois. "Understanding the role of amino acids in nitrogenous disinfection by-products formation" at NOM5 Down Under - 5th IWA Specialist Conference on Natural Organic Matter Research, 1-4 October 2013, Perth, Australia.

Poster Presentations

Z.T. How, K.L. Linge, F. Busetti, C.A. Joll. "Detection and confirmation of selected organic chloramines and their degradation by-products" at NOM6 - 6th IWA Specialist Conference on Natural Organic Matter Research, 7-10 September 2015, Malmö, Sweden.

Z.T. How, K.L. Linge, F. Busetti, C.A. Joll. "Speciation of organic chloramines and the rates and pathways of their degradation" at Drinking Water Disinfection By-Products - Gordon Research Conference, 9-14 August 2015, South Hadley, Massachusetts, USA.

Z.T. How, K.L. Linge, F. Busetti, C.A. Joll. "Organic chloramines stability, kinetics and their risk in drinking water" at DBP 2014: Disinfection By-products in Drinking Water, 27-29 October 2014, Mülheim an der Ruhr, Germany.

Z.T. How, F. Busetti, K. Linge, I. Kristiana, C.A. Joll, J.W.A. Charrois. "Application of LC-MS/MS for the analysis of free amino acids in natural waters" at Singapore International Water Week Water Convention 2014, 1-5 June 2014, Singapore.

Chapter 1. Introduction

1.1 Background and Objectives

Amino acids are reported to be the major constituents of dissolved organic nitrogen in natural waters (Westerhoff and Mash 2002) and are precursors for several classes of nitrogenous disinfection by-products (DBPs), as well as some odorous DBPs. Amino acids have been found to react with free chlorine to form various DBPs through an *N*-chloramino acid intermediate (Freuze et al. 2005, Hirose et al. 1989, Yang et al. 2010). Depending on the chlorine to amino acid ratio, *N*-monochloramino acid or *N*,*N*-dichloramino acid can be formed (Conyers and Scully 1993). After formation, *N*-monochloramino acid can degrade into the corresponding aldehyde, while the *N*,*N*-dichloramino acid can degrade into either the corresponding nitrile or *N*-chloraldimine (Conyers and Scully 1993). Among the DBPs formed from the chlorination of amino acids, little information is available on *N*-chloramino acids and *N*-chloraldimines, which are both sub-classes of organic chloramines. Organic chloramines are formed when chlorine based disinfectants react with organic carbon and/or organic nitrogen in the water. Within the sub-classes of organic chloramines, *N*-chloramino acids are considered to be the main contributor to organic chloramines (Ellis and Soper 1954, Yoon and Jensen 1993). Organic chloramines have been found to be potentially harmful to human health (Bull et al. 2011, Laingam et al. 2012), and to reduce germicidal efficiency of chlorine and inorganic monochloramine (White 2010). Organic chloramines also to interfere with all measurements of combined chlorine (White 2010), potentially leading to an overestimation of inorganic monochloramine concentration in water treatment plants (Scully et al. 1996). Despite the fact that organic chloramines are known to form during disinfection and to have an adverse impact on water treatment, little information is available on organic chloramines in water systems, resulting in an unknown health risk to the public.

In this Thesis, an in depth investigation of the reaction pathways and kinetics of the reaction of chlorine with amino acids was conducted, to better understand the possible impact of the chlorination of amino acids on water quality.

The first objective of this Thesis was to develop analytical methods for the detection of free amino acids in natural waters and engineered water systems, and for the detection and monitoring of organic chloramines in synthetic solutions for kinetic studies. The second objective was to investigate the stability of organic chloramines

and to prioritise organic chloramines of highest concern based on their stability, toxicity and likelihood of formation. A third objective was to study the speciation, reaction pathways and kinetics of the reactions between chlorine and amino acids to understand which DBPs might be found in the distribution system from the chlorination of amino acids.

A survey of free amino acids in different drinking water and wastewater treatment plants to understand the occurrence and removal of free amino acids in these engineered systems was the final objective of this Thesis. A survey of odorous DBPs in drinking water treatment plants, and formation potential experiments on odorous DBPs using drinking water source waters and secondary-treated wastewater effluents, were also conducted to understand the impact of the presence of free amino acids on the production of odorous DBPs.

1.2 Thesis Overview

This Thesis consists of five journal articles and one book chapter which are presented in Chapters 2 to 6. Therefore some unavoidable repetition of background and method information occurs. Journal articles and book chapters that are already published have been reformatted to the style of this Thesis with few other modifications. Supporting information for all of the journal articles are presented sequentially in Appendices 1 to 4. Chapter 2 presents a critical review of the formation of organic chloramines and their impact on water quality, the formation of different sub-classes of organic chloramines, analytical methods available for the detection of organic chloramines and also the toxicity of organic chloramines. This critical review will be submitted to the journal *Water Research*.

Chapter 3 is divided into two sections. The first section describes the development of a novel analytical method for the analysis of free amino acids in natural waters and engineered water systems using solid-phase extraction followed by liquid chromatography-tandem mass spectrometry, published in the paper 'Analysis of free amino acids in natural waters by liquid chromatography-tandem mass spectrometry', *Journal of Chromatography A*, 2014, **1370**, 135-146. The second section describes the development of a direct UV detection method and a triiodide colorimetric method to monitor the degradation of organic monochloramines. These monitoring methods were

published in the book chapter 'Detection methods to monitor the degradation of organic chloramines', in *Disinfection By-products in Drinking Water*, 2015, Chapter 31, 267-276, The Royal Society of Chemistry.

Chapter 4 is the publication 'Organic chloramines in drinking water: An assessment of formation, stability, reactivity and risk', published in *Water Research*, 2016, **93**, 65-73. In this study, the formation and stability of organic chloramines from amines and amino acids, organic chloramides from amides, the competitive reactions between amino acids and chlorine during chlorination, and also a risk assessment of the organic monochloramines, based on their toxicity, stability and precursor abundance, were investigated.

Chapter 5 presents the speciation of organic chloramines from different amino acids and also the reaction pathways and kinetics for the chlorination of amino acids. The journal article on this study has been submitted to the journal *Environmental Science and Technology*.

The occurrence study of free amino acids and odorous DBPs in drinking water and wastewater treatment plants, along with the formation potential of odorous DBPs from drinking water source waters and secondary effluents from wastewater treatment plants, is presented in Chapter 6. The journal article on the research presented in Chapter 6 is in preparation and will be submitted to a relevant journal.

Chapter 7 presents the conclusions derived from the outcomes of all of the research conducted in this Thesis, and implications for the water industry. Recommendations for further studies are also suggested.

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Chapter 2. Organic Chloramines in Chlorine-Based Disinfected Water Systems: A Critical Review

2.1 Introduction

Water disinfection is a crucial step in the production of safe drinking water, whereby pathogenic microorganisms are removed or deactivated by either physical or chemical means. Some disinfection processes also provide a disinfectant residual to prevent microbial regrowth during water distribution, where the presence of a disinfectant residual is more important for large distribution systems with long retention times or when the replacement of distribution system pipes is infrequent (more than 50 years) (Rosario-Ortiz et al. 2016). Chlorination and chloramination are the most widely used disinfection practices in the world because they are effective, inexpensive, and provide disinfectant residual within the distribution system. However, while chlorine and chloramine are effective in deactivating pathogens, they also react readily with naturally occurring inorganic and organic compounds present in the water to form unintended disinfection by-products (DBPs).

Since the discovery of DBPs in chlorinated drinking water in the early 1970s, extensive research has been undertaken to understand the formation of DBPs and their management (Richardson 2003). While more than 600 DBPs have now been identified, minimal information on occurrence and toxicology is available for most DBPs and only a small fraction (less than 40%) of DBPs have been quantified in drinking water (Krasner et al. 2006). One group of DBPs that have not been extensively studied are nitrogenous disinfection by-products (N-DBPs). However, interest in N-DBPs has grown recently with studies showing that some N-DBPs are more genotoxic and cytotoxic than the currently regulated DBPs by several orders of magnitude (Muellner et al. 2007, Plewa et al. 2004, Plewa et al. 2008). In particular, haloacetamides, halonitriles, heterocyclic amines and organic halamines were identified to be of highest interest from a potential toxicity perspective (Bull et al. 2011). Within classes of DBPs, the toxicity has been reported to increase from the chlorine analogue to the bromine analogue and then to the iodine analogue, the iodine analogue being the most toxic (Plewa et al. 2010).

Organic chloramines (more fully referred to as organic *N*-chloramines) are compounds that contain at least one chlorine atom directly bonded to an amine nitrogen atom in an organic molecule. In the water industry, the term ‘organic chloramines’ is generally an operational term that refers to any organic halogen compounds that are detected by

the combined chlorine measurement (the difference between the free and total chlorine concentration), where the combined chlorine concentration represent both inorganic and organic chloramines (Figure 2-1). However this fraction can include organic chloramines, as well as *N*-chloraldimines and *N*-chloramides. In this review, we refer to organic chloramines formed from amines or from amino acids as *N*-chloramines and *N*-chloramino acids, respectively, and we use 'organic chloramine' as a collective term for *N*-chloramines, *N*-chloramino acids, *N*-chloraldimines and *N*-chloramides. The structures and precursors of these four classes are presented in Table 2-1.

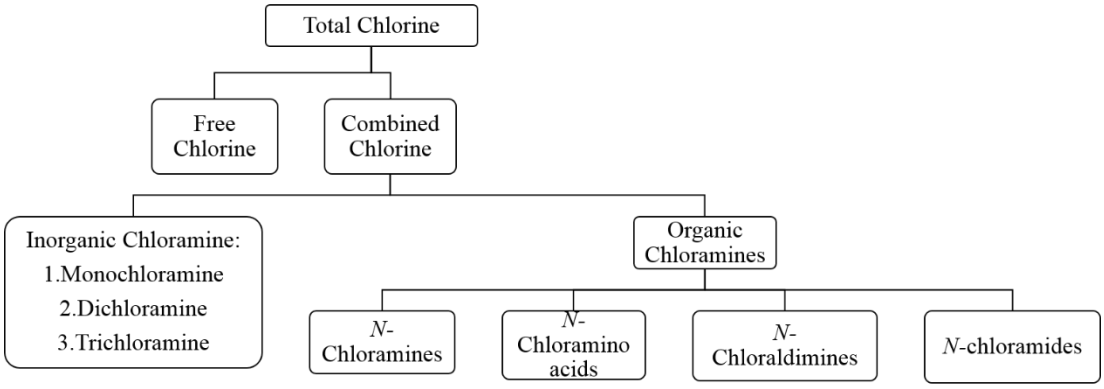
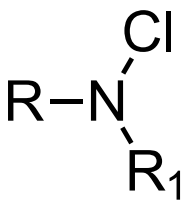
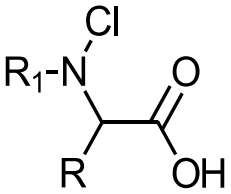
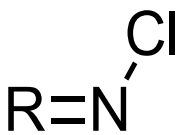
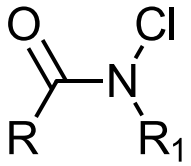


Figure 2-1: Chlorine species measured in water after chlorination or chloramination.

Table 2-1: Structure of different organic chloramines species

	***N*-Chloramine** R,R_1: Alkyl, halogen or hydrogen Precursors: Amines		***N*-Chloramino acid** R: Alkyl, halogen or hydrogen R^1: Alkyl, halogen or hydrogen Precursors: Amino carboxylic acids and amino sulfonic acids
	***N*-chloraldimine** R: Alkyl or hydrogen Precursors: Imine and amino acids		***N*-chloramide** R,R^1: Alkyl, halogen or hydrogen Precursors: Amides and imides

Since organic chloramines are known to form during chlorination and chloramination, it is important to understand the formation, reactivity and toxicity of organic chloramines, and their impacts on water treatment and quality. This paper presents a critical review of current knowledge of organic chloramines in water systems;

including the formation, stability and toxicity of organic chloramines, analytical methods for organic chloramines and the impact of organic chloramines on water treatment and quality.

2.2 Formation and Degradation of Organic Chloramines

Organic chloramines can form when dissolved organic nitrogen (DON) or dissolved organic carbon (DOC), naturally present in water systems, react with either free chlorine (Hunter and Faust 1967) or inorganic chloramines (Isaac and Morris 1983, Snyder 1982). The reaction is much quicker for chlorination than chloramination, for example organic chloramine formation from natural organic matter (NOM) isolates reached its maximum concentration within 10 minutes of chlorination, but took up to 120 hours to reach its maximum concentration after chloramination (Lee and Westerhoff 2009). The formation of organic chloramines increased as the DON/DOC ratio of the NOM isolates increased for both chlorination and chloramination (Lee and Westerhoff 2009)

The formation of organic chloramines is favoured at high pH (Saunier 1979), while the reaction kinetics vary between nitrogen species (Table 2-2). For example, the reaction rates of free chlorine with amino acids and organic amines are between 2 and 80 times faster than the reaction rate of free chlorine with ammonia (Hunter and Faust 1967, Yoon and Jensen 1993). However, amides react very slowly with chlorine to form organic chloramine related compounds (Hureiki et al. 1994), typically nine magnitudes slower than the reaction with ammonia (Table 2-2). Once formed, organic chloramines are relatively less reactive to undergo hydrolysis ($k < 10^{-5}\ s^{-1}$) (Yoon and Jensen 1993) than either chlorine gas ($k = 22.3\ s^{-1}$) (Wang and Margerum 1994) or inorganic chloramines ($k = 2.1 \times 10^{-5}\ s^{-1}$) (Morris and Isaac 1983). These results suggest that organic chloramines are more stable than free chlorine and inorganic chloramines in water.

After formation, organic chloramines can degrade into numerous disinfection by-products including aldehydes and nitriles (Nweke and Scully 1989). Many factors affect the stability of organic chloramines, such as basicity of the amine nitrogen in the organic chloramines (Pitman et al. 1969). The presence of an α-hydrogen promotes the degradation of organic chloramines through dehydrohalogenation (Hui and

Debiemme-Chouvy 2013). Details of the formation and degradation of specific classes of organic chloramines are discussed in the following sections.

Table 2-2: Reported reaction rates of chlorination of selected organic and inorganic nitrogen compounds

Compounds	pKa	k_{HOCl} $(M^{-1}S^{-1})(25°C)$	References
Inorganic nitrogen			
Ammonia	9.25	3.07×10^6	Qiang and Adams (2004)
		4.2×10^6	Morris and Isaac (1983)
		2.9×10^6	Margerum et al. (1979)
Monochloramine		1.5×10^2	Margerum et al. (1979)
		3.5×10^2	Morris and Isaac (1983)
Organic nitrogen			
Primary amine			
Methylamine	10.66	1.9×10^8	Margerum et al. (1979)
		3.6×10^8	Deborde and von Gunten (2008)
Ethylamine	10.81	1.98×10^8	Abia et al. (1998)
Secondary amine			
Dimethylamine	10.72	6.05×10^7	Abia et al. (1998)
		3.3×10^8	Deborde and von Gunten (2008)
		5×10^7	Deborde and von Gunten (2008)
Diethylamine	11.02	3.71×10^7	Abia et al. (1998)
		1.4×10^7	Deborde and von Gunten (2008)
		1.4×10^8	Deborde and von Gunten (2008)
Tertiary amine			
Trimethylamine	9.75	5×10^4	Abia et al. (1998)
(*N*-Me)-piperidine	10.08	8×10^4	Abia et al. (1998)
Amide			
N-Acetylalanine		1.58×10^{-3}	Jensen et al. (1999)
N-Methylformamide		1.70×10^{-3}	Thomm and Wayman (1969)
N-Methylacetamide		1.70×10^{-3}	Thomm and Wayman (1969) Deborde and von Gunten (2008)
		1.40×10^{-3}	
Urea		0.075	Deborde and von Gunten (2008)
Amino acids			
Glycine	9.78	1.13×10^8	Armesto et al. (1993)
		5×10^7	Armesto et al. (1994)
Alanine	9.87	3.4×10^7	Armesto et al. (1993)
		5.4×10^7	Armesto et al. (1994)
		5.4×10^7	Margerum et al. (1979)
β-alanine	10.06	8.9×10^7	Margerum et al. (1979)
Sulfur-containing amino acids			
Cysteine	8.15 and 10.29	1.2×10^9	Armesto et al. (2000)
		3.3×10^8	Pattison and Davies (2001)
Methionine	9.05	9×10^8	Armesto et al. (2000)

2.2.1 *N*-Chloramines

Primary and secondary amines react rapidly with free chlorine to form *N*-chloramines (Abia et al. 1998). The rate constants for the reaction with chlorine range between 10^7 and 10^8 M^{-1} s^{-1} for primary and secondary amines (Table 2-2). Tertiary amines react with free chlorine to form *N*-chlorinated quaternary ammonium salts, rather than *N*-chloramines, with much lower rate constants (10^3 - 10^4 M^{-1} s^{-1}) (Abia et al. 1998). Common primary and secondary amines, like methylamine, dimethylamine, diethylamine, ethylamine and some heterocyclic amines (piperidine and pyrrolidine) have been found in drinking waters (Scully and Bempong 1982, Wang et al. 2011), while primary and secondary amines, histamine, ethanolamine, propylamine and pyrrolidine, have been previously identified in human urine (Perry et al. 1962) and may be present in wastewaters. When the drinking water or wastewater is chlorinated, these amines can form *N*-chloramines. While alkanolamines such as ethanolamine have both hydroxyl and amine functional groups, the hydroxyl functional group has negligible reactivity with chlorine (Prütz 1996) and thus they can be considered to react to form *N*-chloralkanolamines.

After formation, *N*-chloramines have been found to be more stable than most *N*-chloramino acids (How et al. 2016, Scully and Bempong 1982) (Table 2-3). For example, the half-lives of *N*-chloropiperidine and *N*-chlorodiethylamine were both found to be more than 2 days (Scully and Bempong 1982), while most *N*-chloramino acids like *N*-chloroleucine, *N*-chlorophenylalaine and *N*-chlorovaline have half-lives of more than 3 hours (How et al. 2016). *N*-Chloramines degrade more quickly when the pH is greater than 10 or less than 7 (Antelo et al. 1996).

The mechanism of *N*-chloramine degradation involves β-elimination of HCl to form an imine that hydrolyses rapidly to an aldehyde or ketone (Figure 2-2) (Antelo et al. 1996). The degradation mechanism for *N*-chloralcoholamines is an E1 reaction which involves the formation of a nitrenium ion and subsequent loss of the α-carbon substituent, but ultimately results in the same by-products as an *N*-chloramine (Figure 2-2) (Antelo et al. 1996).

Table 2-3: Rate of decomposition of various organic monochloramines k ($\times 10^{-4}$)(s^{-1})

Precursors	Rate*	References
Amino acids		
Alanine	2.8	Armesto et al. (1996)
	1.9 (>385)	Coker et al. (2008)
	1.8	How et al. (2016)
Asparagine	11 (>385)	Coker et al. (2008)
	6.7	How et al. (2016)
Aspartic acid	8.1 (>385)	Coker et al. (2008)
	15	How et al. (2016)
Glutamine	3.2 (39)	Coker et al. (2008)
	14	How et al. (2016)
Glutamic acid	3.1 (>385)	Coker et al. (2008)
	3.1	How et al. (2016)
Glycine	0.04	Hand et al. (1983)
	<1.0 (8.9)	Coker et al. (2008)
	0.02	How et al. (2016)
Histidine	2.0	How et al. (2016)
Isoleucine	1.97	Armesto et al. (1996)
	1.3	How et al. (2016)
Leucine	3.2	Armesto et al. (1996)
	2.5	How et al. (2016)
Lysine	0.42	How et al. (2016)
Phenylalanine	1.6	How et al. (2016)
Proline	8.8	Hand et al. (1983)
	56	How et al. (2016)
Serine	2.1	How et al. (2016)
Taurine	<1.0 (<1.0)	Coker et al. (2008)
	0.02	How et al. (2016)
Threonine	2	Hand et al. (1983)
	1.2	How et al. (2016)
Valine	2	Armesto et al. (1996)
	1.0	How et al. (2016)
Amines		
Diethylamine	0.04	Scully and Bempong (1982)
	0.60	How et al. (2016)
Dimethylamine	0.04	Scully and Bempong (1982)
	0.39	How et al. (2016)
Ethanolamine	0.05	How et al. (2016)
Piperidine	0.02	Scully and Bempong (1982)
N-chloraldimines		
N-chloroisobutyraldimine	0.06	McCormick et al. (1993)
N-chlorophenylacetaldimine	0.06	Conyers and Scully (1993)
	0.04	Freuze et al. (2004)

*Value in brackets were the degradation constant for the *N,N*-dichloramine species

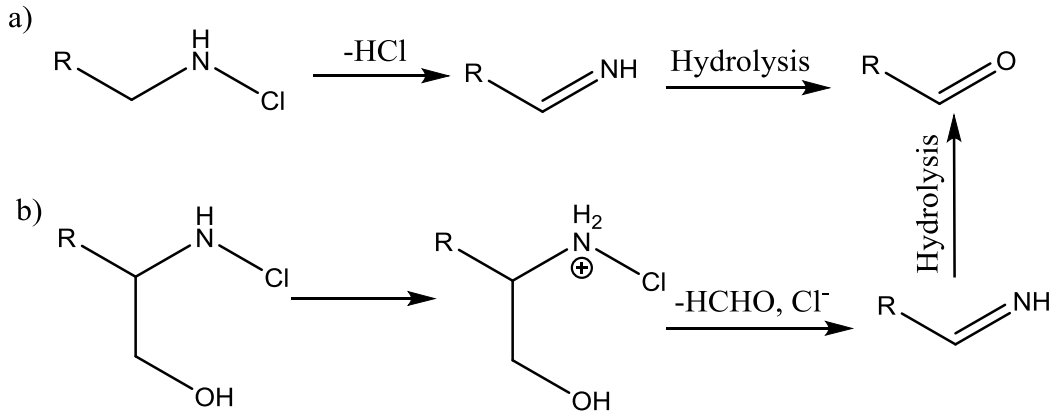


Figure 2-2: a) Degradation mechanism of *N*-chloramines, b) Degradation mechanism of *N*-chloralcoholamines, adapted from Antelo et al. (1996).

2.2.2 *N*-Chloramino acids

The *N*-chloramino acids are the most widely studied organic chloramines, because they form from the reaction of free chlorine and amino acids, and amino acids are considered the main constituent of DON (Ellis and Soper 1954, Yoon and Jensen 1993). Amino acids have been found to contribute up to 75% of DON (Westerhoff and Mash 2002), especially in waters impacted by algae or sewage effluent. Studies have also shown that free amino acids are poorly removed during biological filtration (Prevost 1998), and the concentration of free amino acids might even increase after sand filtration (LeCloirec et al. 1986), hence it is likely that amino acids will be present in waters during disinfection. The reported reaction pathways of amino acids with chlorine are presented in Figure 2-3.

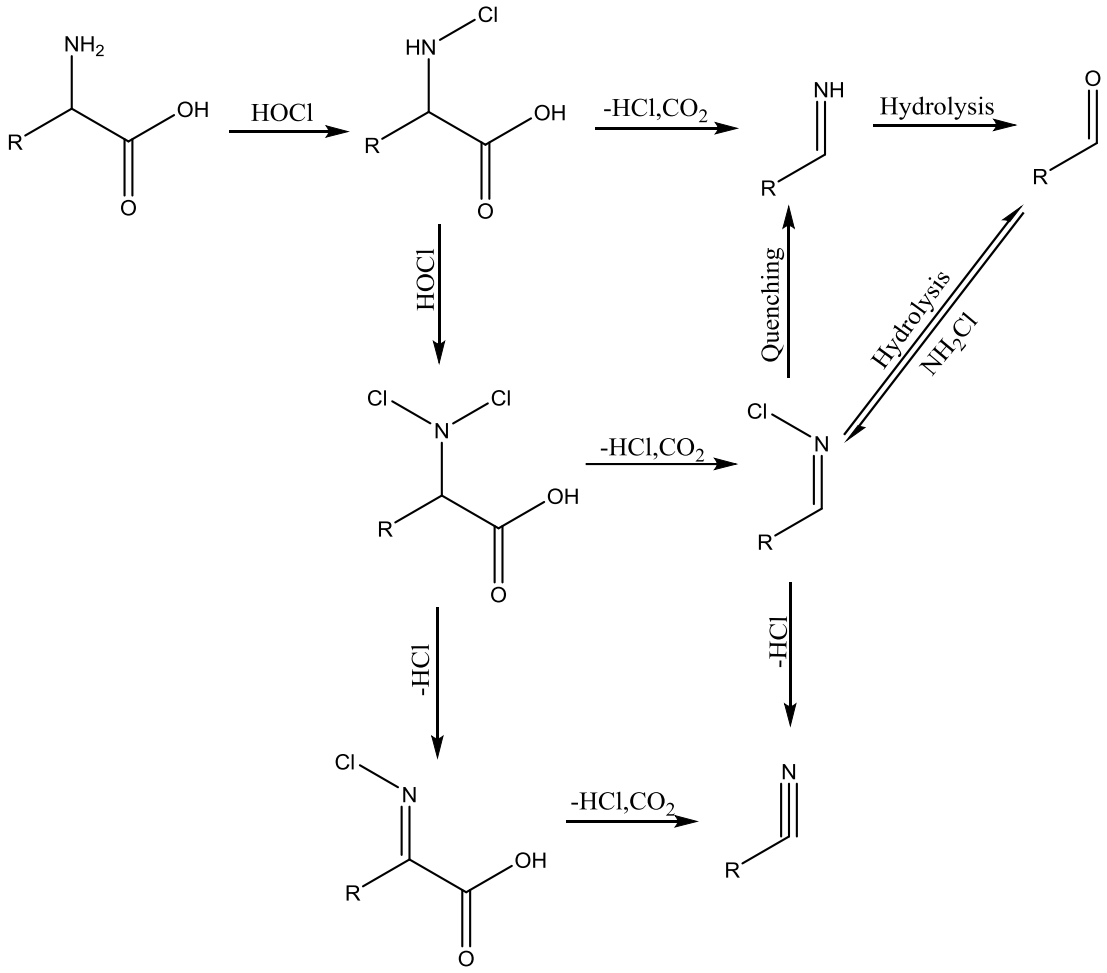


Figure 2-3: Reaction pathways for the reaction of amino acids and chlorine, adapted from Conyers and Scully (1993). *N*-Chloraldimines were suggested as intermediates for the formation of nitriles and aldehydes.

The half-lives of *N*-monochloramino acids have been found to vary from 13 min to greater than 3 days (Armesto et al. 1996, Hand et al. 1983, How et al. 2016, Li et al. 2011). As illustrated by the rate constants in Table 2-3, most *N*-monochloramino acids are less stable than *N*-monochloramines, with the *N*-monochloramino acids exhibiting half-lives of less than 90 min ($k < 1.3 \times 10^{-4}$ s^{-1}) (How et al. 2016). As Pitman et al. (1969) suggested that, in general, a more basic amine would form a more stable organic chloramine, amino acids with a more basic amine nitrogen would be expected to be more stable, however no trend between the pK_a of the amine nitrogen and the stability of the *N*-monochloramino acids was observed (Table 2-3). The stability of *N*-chloramino acids (e.g. of alanine) is highly influenced by the presence and reactivity of the α-hydrogen (α to the acid group), where more reactive α-hydrogens result in less stable *N*-chloramino acids (Hui and Debiemme-Chouvy 2013). *N*-Chloramino acids (e.g. α-aminoisobutyric acid) with two substituents at the α position to the acid

functional group have been reported to be the least stable (Hand et al. 1983). Among the *N*-chloramino acids with the same number of substituents, a larger substituent is also reported to reduce the stability of the *N*-chloramino acids (Hand et al. 1983), e.g. α-aminoisobutyric acid is less stable than proline. The type of acid group in an amino acid was also found to have an impact on the stability of the *N*-chloramino acids, where amino sulfonic acids reportedly form more stable *N*-chloramino acids as compared to amino carboxylic acids (Gottardi and Nagl 2010). In contrast, pH appears to have little impact on the stability of *N*-chloramino acids, since no significant change in the rate of decomposition of *N*-chloramino acids was observed with changing pH (Hui and Debiemme-Chouvy 2013).

N,N-Dichloramino acids are less stable than their analogous *N*-monochloramino acids (Coker et al. 2008), possibly due to the increased likelihood of dehalogenation. The stability of the *N,N*-dichloramino acids is also reported to be influenced by the substituent/s on the α-carbon, similar to the *N*-monochloramino acids (Coker et al. 2008).

2.2.3 *N*-Chloramides

N-Chloramides can form from the chlorination or chloramination of amides, or as a minor product from the chloramination of aldehydes. The rates of formation of *N*-chloramides from chlorination and chloramination of amines are reported to be very low (Deborde and von Gunten 2008, Jensen et al. 1999, Thomm and Wayman 1969), with formation rate constants ranging from 1.70×10^{-3} to 0.075 M^{-1} s^{-1} at pH 7 - 8 and temperature 20-25 ºC, conditions expected during water disinfection (Table 2-2). Alternatively, *N*-chloramides can be formed from the chloramination of aldehydes (Kimura et al. 2013). As illustrated in Figure 2-4, the aldehyde can react with inorganic monochloramine to form a *N*-chloramino alcohol, which can either react with inorganic monochloramine to form an *N*-chloramide or undergo dehydration to form a *N*-chloraldimine. This alternative pathway for the formation of *N*-chloramide is much faster than chlorination of most amides, with the rate constant for chloramination of acetaldehyde being 24.3 M^{-1} s^{-1} at 25 ºC. However, in the competitive reactions from the aldehydes, the formation of the *N*-chloraldimine is reported to be preferred (Kimura et al. 2015), as the dehydration of the *N*-chloramino alcohol is more

favourable than the oxidation of the *N*-chloramino alcohol by inorganic monochloramine.

Although no experimental data on the stability of *N*-chloramides is available, they are predicted to be less stable than *N*-chloramines based on their hydrolysis constants, where the hydrolysis constant of *N*-chloramide is 10^{-9} M^{-1} s^{-1} while *N*-chloramine is 10^{-12} M^{-1} s^{-1} (Qian and Sun 2003), but the *N*-chloramide is more stable than the *N*-chloramino acid, where the hydrolysis constant is 10^{-5} M^{-1} s^{-1} (Yoon and Jensen 1993). However, despite their relative stability, the formation of *N*-chloramides is either very slow from the chlorination of the corresponding amide or as a minor product from the chloramination of the aldehyde. Therefore, it is unlikely that *N*-chloramides form in real water systems, or, if they are formed, their contribution to the total organic chloramine fraction would be very small.

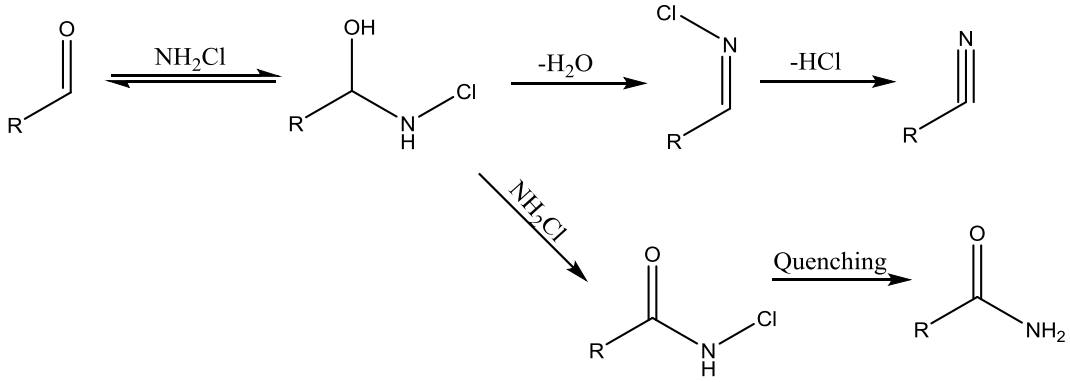


Figure 2-4: Reaction pathways for the reaction of aldehydes and inorganic monochloramine, adapted from Kimura et al. (2015). *N*-Chloraldimine was suggested as an intermediate for the formation of the nitrile.

2.2.4 *N*-Chloraldimines

N-Chloraldimines can be formed by either the decarboxylation and dehydrohalogenation of *N,N*-dichloramino acids (Figure 2-3) or by the chloramination of aldehydes (Figures 2-3 and 2-4) (Kimura et al. 2015, Nweke and Scully 1989, Pedersen et al. 1999), and therefore result from the presence of amino acids. *N*-Chloraldimines are often considered as intermediates in the reaction pathway for the formation of nitriles (Figures 2-3 and 2-4).

Formation of *N*-chloraldimines from aldehydes is reported to first involve a rapid, but reversible, reaction with inorganic monochloramine to form an *N*-chloramino alcohol,

which then undergoes slow dehydration to the *N*-chloraldimine (Pedersen et al. 1999). The rate of dehydration is the rate limiting step (Pedersen et al. 1999), and the rate constant for this step ranges from 1.24 to 277 M^{-1} s^{-1}, depending on the aldehyde species, temperature, and pH (Kimura et al. 2015, Pedersen et al. 1999). Overall, the formation of *N*-chloraldimines from chloramination of aldehydes is reported to be slow, and the rate constant for the formation of the *N*-chloraldimine from chloramination of acetaldehyde was found to be 9.8×10^{-3} M^{-1} s^{-1} at pH 6.5 by Scully et al. (1997) and 23.2 M^{-1} s^{-1} at pH 7.8 by Kimura et al. (2015). The rate constant of the reaction between inorganic monochloramine and acetaldehyde was also found to increase with increasing pH by Kimura et al. (2015) but decrease with increasing pH by Scully et al. (1997).

Despite being considered as intermediates, *N*-chloraldimines have been found to be very stable (half-lives of more than 60 minutes) in the absence of ammonia (Nweke and Scully 1989). In a study by Brosillon et al. (2009), *N*-chloroisobutyraldimine, *N*-chloro-3-methylbutan-1-imine and *N*-chloro-2-methylbutan-1-imine were found in a drinking water distribution network even with more than 20 hours of chlorine contact time. Interestingly, *N*-chloraldimines could contribute to off-flavours in drinking water. The odour threshold concentrations of *N*-chloroisobutyraldimine and *N*-chloro-3-methylbutan-1-imine were found to be 0.2 and 0.25 µg L^{-1}, respectively (Freuze et al. 2005). Thus, among the four classes of organic chloramines discussed, *N*-chloraldimines could be considered to be of the highest interest for drinking water, due to their stability, their odorous properties and the abundance of their amino acid precursors.

2.3 Analytical Methods for Detection of Organic Chloramines

A variety of analytical methods have been used for the detection of organic chloramines, either as a bulk parameter (all organic chloramines) or as individual compounds, in water (Table 2-4). Organic chloramines behave identically to inorganic chloramines (monochloramine, dichloramine, trichloramine) in most available analytical methods for determining concentrations of inorganic chloramines in aqueous solutions, including standard colorimetric methods (iodometric titration and the DPD (*N*,*N*-diethyl-*p*-phenylenediamine) method), UV-based methods, and chromatographic methods (Smallwood et al. 1994). Indeed, none of the standard

methods for the analysis of chloramines can distinguish between organic and inorganic chloramines (Donnermair and Blatchley 2003, Tao et al. 2008, White 2010). This may lead to an overestimation of the true concentration of inorganic chloramines in samples containing large amounts of nitrogen-containing organic matter (Scully et al. 1996).

The DPD method has been used for monitoring the formation and degradation of organic chloramines (Laingam et al. 2012, Li et al. 2011), through the measurement of combined chlorine in the samples, or used to determine if all chlorine has reacted (Li et al. 2011, Scully and Bempong 1982). Organic chloramines can also be measured as free residual chlorine using the standard colorimetric DPD method (Jensen and Johnson 1990), which leads to interferences in the measurement of free chlorine when the combined chlorine concentration is higher than 0.5 mg L^{-1} (APHA et al. 2005). In an earlier study, How et al. (2015), we showed that high concentrations of organic chloramines (> 4 mg L^{-1} as Cl) resulted in high variability in free chlorine measurement and the addition of thioacetamide immediately after the DPD reagent to stop the reaction between the DPD reagent and the oxidant did not reduce the variability of the free chlorine measurement. To date, the only method that has been able to accurately differentiate between free chlorine, individual inorganic chloramines and organic chloramines (as a bulk parameter) is membrane introduction mass spectrometry (Ferriol et al. 1991, Shang et al. 2000).

Direct UV detection has been used for the analysis of pure organic chloramines and bromamines (Antelo et al. 1999, Antelo et al. 1995, Olszanecki and Marcinkiewicz 2004). The maximum absorbance of organic chloramines was found to be around 250 nm with molar extinction coefficients of 350 to 380 M^{-1} cm^{-1} (Antelo et al. 1995), while the maximum absorbance of organic bromamines was around 290 nm with molar extinction coefficients of 430 to 470 M^{-1} cm^{-1} (Antelo et al. 1993).

Typically, *N*-chloramino acids and *N*-chloramines in ultrapure water have been analysed using liquid chromatography coupled with a mass spectrometer (LC-MS) (How et al. 2016, Li et al. 2011, Takats et al. 2001, Yang et al. 2010); derivatisation with UV or fluorescence detection (Furness-Green et al. 1998, Scully et al. 1984); and with post-column electrochemical detection (Bedner et al. 2002). Derivatisation of *N*-chloramines and *N*-chloramino acids with UV or florescence detection was suitable

for the screening of *N*-chloramines and *N*-chloramino acids, while mass spectrometry was more suitable for analysis of individual species (Li et al. 2011). Membrane introduction mass spectrometry has also being used for the detection of *N*-chloramines formed from 2-aminobutane and 1,3-diaminoproane (Kotiaho et al. 1991).

Generally, gas chromatography coupled with a mass spectrometer (GC-MS), using liquid injection or headspace techniques, has been used for the detection of *N*-chloraldimines, consistent with *N*-chloraldimines being more volatile than *N*-chloramino acids. *N*-Chloroisobutaldimine, *N*-chloro-3-methylbutan-1-imine, *N*-chloro-2-methylbutan-1-imine and *N*-chlorophenylacetaldimine were detected with varying degrees of confidence from formation potential experiments on the *N*-chloraldimines from chlorination of a wastewater sample by Conyers et al. (1993). In another study by Brosillon et al. (2009), *N*-chloroisobutaldimine, *N*-chloro-3-methylbutan-1-imine, *N*-chloro-2-methylbutan-1-imine and *N*-chlorophenylacetaldimine were reported to be detected in drinking water using purge and trap GC-MS.

The detection of *N*-chloroacetamide is not reported until a recent study by Kimura et al. (2015), where *N*-chloroacetamide was reported to be detected using a double focusing mass spectrometer by direct injection of samples.

Despite the existence of published methods for all of the classes of chloramines reviewed in this study, most methods have not been used for analysis of organic chloramines in real water samples. This is because further improvements in methods for the extraction and isolation of organic chloramines and sensitivity are required for the analysis of organic chloramines at the concentrations found in real water samples. One of the challenges, as seen from Table 2-3, is the instability of the organic chloramines, especially the *N*-chloramino acids, such that a rapid extraction method (no longer than 45 minutes) is required. The extraction methods used for the extraction of organic chloramines to date are listed in Table 2-5.

An additional challenge for the analysis of organic chloramines is the effect of oxidant quenching, a common sample preparation procedure for water samples that contain free chlorine or other oxidants. Quenching of the oxidant residual is typically

undertaken to prevent further formation of DBPs during the holding time between sample collection and analysis (Kristiana et al. 2014). Most of the commonly used quenching agents in water analysis (e.g. ascorbic acid, sodium sulphite and sodium thiosulfate) are reducing agents, and therefore 'quench' the disinfectant (the oxidant) through a redox reaction (Kristiana et al. 2014). However, some organic chloramines are also oxidants, and thus quenching transforms these organic chloramines into other by-products. For example, addition of a quenching agent (sodium thiosulfate) has been found to cause the reduction of *N*-chloroacetamide to acetamide (Kimura et al. 2015). The conversion of organic chloramines into other by-products via quenching therefore alters the DBP concentrations and distribution in the sample (Kimura et al. 2015) and results in the false negative detection of organic chloramines. Therefore, the use of quenching agents should be avoided. However, the counterbalancing issue is that, without quenching, oxidants such as residual chlorine or inorganic monochloramines, and even the organic chloramines themselves, may result in damage to the analytical instrument.

Finally, the lack of analytical standards limits the number of organic chloramines that can be quantified, or even identified. The relatively short half-lives of organic chloramines mean that standards have short shelf-life, and many standards must be made in-house and verified before use and used immediately. This means that verification of methods through traceability studies or by use of certified reference materials is generally not possible.

Table 2-4: Analytical methods for the detection of various organic chloramine species

Species	Analytical method	References
Bulk (all organic chloramines)	1. Subtraction of total chlorine and free total chlorine from DPD method (assumption of no inorganic chloramines)	APHA (2005)
	2. Subtraction of inorganic chloramines from combined chlorine: DPD method for combined chlorine and membrane introduction mass spectrometry for inorganic chloramines	Shang et al. (2000)
N-Chloramines	1. Pre-column derivatisation into dansyl derivatives using $DANSO_2H$. LC-RP separation with fluorescence detection	Scully et al. (1984) Takats et al. (2001)
	2. Infusion with APCI or ESI-MS/MS in positive mode	
	3. Membrane introduction mass spectrometry for inorganic chloramines	Kotiaho et al. (1991)
N-Chloramino acids	1. Derivatisation into dansyl derivatives using $DANSO_2H$ a. HPLC-RP separation with fluorescence detection b. GC/MS with CI	Scully et al. (1984) Scully (1990) Li et al. (2011)
	2. LC-RP-ESI-MS in both negative and positive mode	Furness-Green et al. (1998)
	3. LC-RP/HILIC with post-column reaction and detection using UV detection, organic chloramine reacted with iodine to form triiodide	Yang et al. (2010) How et al. (2016)
	4. LC-RP-ESI-MS in negative mode	
	5. LC-RP-ESI-HRMS in positive mode	
N-chloramides/ *N*-chlorimides	1. LC-RP-ESI-MS in positive mode	Li et al. (2011) Kimura et al. (2015)
	2. Double focus mass spectrometry	
N-chloraldimines	1. GC-MS in EI or CI	Conyers et al. (1993)
	2. GC-MS	Brosillon et al. (2009)

Table 2-5: Extraction methods developed for organic chloramines

Extraction method	Target	Reference
Reverse phase solid-phase extraction	*N*-Chloramines (after derivatisation), *N*-chloramino acids (after derivatisation), *N*-chlorimides, *N*-chloraldimines	Scully et al. (1984) Scully (1990) Li et al. (2011) Freuze et al. (2004)
Liquid-liquid extraction using trichloromethane	*N*-Chloraldimines	Conyers et al. (1993) Scully (1997)
Headspace 1. Direct headspace extraction 2. Purge and trap	*N*-Chloraldimines	 Conyers et al. 1993 Brosillon et al. 2009

2.4 Toxicology of Organic Chloramines

Although water-related toxicological studies are limited, several biomedical studies describing the potential adverse health effects of organic chloramines have been published. In the human body, inflammation is reported to cause generation of HOCl from activated phagocytes, and the HOCl can then react with nearby amino acids, peptides, or proteins (Hawkins and Davies 1999, Hawkins et al. 2003). The formation of organic chloramines from such reactions can result in damage to tissue (Hawkins and Davies 1999, Hawkins et al. 2003) or to RNA and DNA (Hawkins and Davies 2002), which can ultimately contribute to aging and cancers (Ames 1989, Hoeijmakers 2009). The principal mechanism for the toxicity of organic chloramines is not well understood. Grisham et al. (1984) hypothesised that organic chloramines convert to toxic forms when in contact with cells, while Cemeli et al. (2006) attributed measured cytotoxicity and genotoxicity to cellular oxidative stress. Studies have shown that organic chloramines produced from the reaction of HOCl and plasma proteins give rise to aminyl radicals (Hawkins and Davies 1999) which result in radical-induced damage to the DNA (Sortino et al. 1999). Oxidative damage was reported to be obvious in collagen treated with organic chloramines (Davies et al. 1993). It has previously been found that an electrophilic nitrogen from aromatic amines was responsible for alkylamination of DNA (Miller 1978) and this mechanism may also be relevant here as the nitrogen in organic chloramines is also electrophilic (Calvo et al. 2007, Scully and Bempong 1982).

Specific studies of the carcinogenicity of *N*-chloramino acids have indicated that *N*-chloramino acids can cause protein-DNA cross-links (Kulcharyk and Heinecke 2001), inhibit DNA repair (Pero et al. 1996), and affect the kinetics of the cell cycle, including cellular apoptosis (Englert and Shacter 2002, Hosako et al. 2004), all of which are commonly observed carcinogenic effects.

A number of in vitro studies have demonstrated that several organic chloramines are mutagenic towards bacteria (Nakamura et al. 1993, Scully and Bempong 1982, Süssmuth 1982, Thomas et al. 1987), as well as cytostatic or cytotoxic to Chinese hamster ovary cells (Bempong and Scully 1980). In a recent in vitro study by Laingam et al. (2012), significant cytotoxicity and genotoxicity were observed for WIL2-NS cells (human lymphoblastoid) that were treated with in situ-formed *N*-chloroethanolamine, *N*-chloroglycine, *N*-chlorohistamine and *N*-chlorolysine at the micromolar concentrations relevant for drinking water. All four of these compounds have also demonstrated mutagenic effects in bacterial assays (Nakamura et al. 1993, Süssmuth 1982, Thomas et al. 1987). Two studies of the effect of *N*-chloroacetamide on Chinese hamster ovary cells found that *N*-monochloroacetamide was cytotoxic but not genotoxic (Kimura et al. 2015), but that *N,N*-dichloroacetamide was both cytotoxic and genotoxic, and more potent than *N*-monochloroacetamide (Antelo et al. 1993).

To date, there have been no in vivo toxicity studies conducted for organic chloramines, and comparison of the toxicity of organic chloramines to other DBPs is difficult due to differences in methodology, including the cells tested and the length of exposure. However, organic bromamines would be expected to exert similar biological effects to organic chloramines (Olszanecki and Marcinkiewicz 2004) and, from the trends of other DBPs where the bromine analogue is more toxic than the chlorine analogue (Plewa et al. 2010), organic bromamines can be expected to be more toxic than organic chloramines.

2.5 Occurrence of Organic Chloramines and Impact on Water Treatment and Quality

Despite the fact that *N*-chloramines are relatively stable, there are currently no reports of their occurrence in either drinking water or wastewater systems, due to a lack of suitable analytical methods. In addition, there is little information available on the

occurrence and concentration of organic amines in water systems. Given that concentrations of DON in water systems are often less than 2.5 mg L^{-1} (Westerhoff and Mash 2002) and that amines are likely to be a minor contributor of DON, analytical methods for organic amines in water systems are likely to need detection limits in the µg L^{-1} range. Thus, this paucity of knowledge makes it difficult to assess the impact of *N*-chloramines on human health and the environment.

Although free amino acids readily form *N*-chloramino acids, free amino acids are only a very small fraction of total amino acids in natural waters, typically about 1% (Chinn and Barrett 2000, Dotson and Westerhoff 2009, Thurman 1985) and 7% to 29% in wastewater effluent (Confer et al. 1995), this low abundance contributing to the lack of occurrence data on *N*-chloramino acids in water systems. Most of the amino acids exist in the total amino acid fraction as combined amino acids in peptides and proteins and, when chlorinated, *N*-chloropeptides were found to be stable for a few days (Jensen et al. 1999); however, to date, no occurrence studies of *N*-chloropeptides have been reported. Another factor in the lack of occurrence data on *N*-chloramino acids in water systems is the instability of most *N*-chloramino acids, such that the *N*-chloramino acids would have degraded into other by-products, such as aldehydes or nitriles.

The presence of organic nitrogen in drinking water treatment and distribution increases the chlorine demand of the water (White 2010). Furthermore, as described in Section 3, none of the standard methods for the analysis of chloramines can distinguish between organic and inorganic chloramines (Donnermair and Blatchley 2003, Tao et al. 2008, White 2010), and combined chlorine concentrations higher than 0.5 mg L^{-1} can interfere in the measurement of free chlorine (APHA et al. 2005). Thus, the presence of organic chloramines can lead to overestimation of the concentration of the disinfectant during treatment and in the distribution system. This is of particular health significance because organic chloramines have been reported to have a much lower germicidal efficiency compared to both chlorine and inorganic monochloramine (Donnermair and Blatchley 2003, Scully et al. 1996, White 2010, Wolfe et al. 1985). For example, the maximum inactivation rate of *E.coli* was 0.09 L mg^{-1} min^{-1} for *N*-monochloramino acids, compared to 2.56 L mg^{-1} min^{-1} for free chlorine and 0.72 L mg^{-1} min^{-1} for inorganic monochloramine (Donnermair and Blatchley 2003). The formation of organic chloramines from free chlorine can continue over a long period

of time, resulting in a gradual decline in disinfection efficiency in the distribution system (White 2010).

In many distribution systems, a free chlorine residual is required to prevent bacterial regrowth. In vivo formation of organic chloramines is possible when free residual chlorine reacts with amines in the saliva and stomachs of consumers. A study by Scully (1990) identified *N*-chloroglycine, either *N*-chloroleucine or *N*-chloroisoleucine, and *N*-chlorophenylalanine in chlorinated rat stomach contents. The presence of organic chloramines in distribution systems can result in the in situ formation of other DBPs. For example, stable organic chloramines like *N*-chloroglycine (Hand et al. 1983) can also act as an intermediate for the formation of other DBPs, like aldehydes and ketones (Bull et al. 2006). The presence of organic chloramines can also artificially enhance the measured concentration of other DBPs, due to the conversion of organic chloramines into other DBPs when quenched (Kimura et al. 2015).

2.6 Conclusions and Recommendations

Organic chloramines are potentially cytotoxic and genotoxic to humans, while some species, like *N*-chloraldimines, may cause aesthetic issues in drinking water and treated wastewater. The formation of organic chloramines during water disinfection can also reduce the germicidal efficiency of the water, and lead to overestimation of inorganic monochloramine concentration. However, currently there is limited information on the occurrence of organic chloramines in water systems, due to the lack of analytical methods of sufficient sensitivity and the destruction of organic chloramines during current quenching procedures. Furthermore, a lack of traceable analytical standards has made both identification and quantification of many organic chloramines challenging. Many of the organic chloramines formed during disinfection are too unstable for current sample preparation methods. Future analytical method development should focus on the most stable organic chloramines formed during water disinfection, as they are more likely to be detected and more likely to reach the consumer.

To date, research has focussed on the formation of organic monochloramines rather than organic dichloramines. However, it is a common disinfection strategy to achieve

breakpoint chlorination during disinfection, and higher chlorine to precursor ratios are more likely to result in the formation of organic dichloramines.

The formation of organic chloramines can only occur in the presence of suitable N-containing precursors (e.g. amino acids) and therefore improved understanding of the occurrence and concentration of precursors in water systems would help to predict the formation of organic chloramines. Given the challenges identified in analysing organic chloramines, analysis of precursors may provide another avenue to access the health risk of organic chloramines in drinking waters and to assess the efficacy of current and future water treatment processes.

2.7 Acknowledgements

The authors thank the Australian Research Council (LP110100548 and LP130100602), Water Corporation of Western Australia, Water Research Australia and Curtin University for supporting this study. Curtin University (Curtin International Postgraduate Research Scholarship) and Water Research Australia (WaterRA PhD Scholarship) are also acknowledged for providing financial support for Z. How.

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Chapter 3.1 Analysis of Free Amino Acids in Natural Waters by Liquid Chromatography-Tandem Mass Spectrometry

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Journal of Chromatography A, 1370, pp.135-146, 2014
DOI: http://dx.doi.org/10.1016/j.chroma.2014.10.040

Statement of Contribution to Co-authored Published Paper

This Chapter includes the co-authored paper '*Analysis of free amino acids in natural waters by liquid chromatography–tandem mass spectrometry*', published in Journal of Chromatography A. The bibliographic details of the co-authored paper, including all authors are:

How, Z. T., Busetti, F., Linge, K. L., Kristiana, I., Joll, C. A. & Charrois, J. W. A. 2014. Analysis of free amino acids in natural waters by liquid chromatography tandem-mass spectrometry *Journal of Chromatography A,* 1370**,** 135-146.

I, Zuo Tong How, contributed to the paper by by conducting all the laboratory work and data analysis, being the primary writer, including creating figures and tables, and writing and editing the manuscript.

I, as a Co-Author, endorsed that this level of contribution by the candidate indicated above is appropriate.

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3.1.1 Abstract

This paper reports a new analytical method for the analysis of 18 amino acids in natural waters using solid-phase extraction (SPE) followed by liquid chromatography-

electrospray tandem mass spectrometry (LC-MS/MS) operated in multiple reaction monitoring mode. Two different preconcentration methods, solid-phase extraction and concentration under reduced pressure, were tested in development of this method. Although concentration under reduced pressure provided better recoveries and method limits of detection for amino acids in ultrapure water, SPE was a more suitable extraction method for real samples due to the lower matrix effects for this method. Even though the strong cation exchange resin used in SPE method introduced exogenous matrix interferences into the sample extracts (inorganic salt originating from the acid-base reaction during the elution step), the SPE method still incorporates a broad sample clean-up and minimised endogenous matrix effects by reducing interferences originating from real water samples. The method limits of quantification (MLQ) for the SPE LC-MS/MS method in ultrapure water ranged from 0.1 to 100 µg L^{-1} as N for the different amino acids. The MLQs of the early eluting amino acids were limited by the presence of matrix interfering species, such as inorganic salts in natural water samples. The SPE LC-MS/MS method was successfully applied to the analysis of amino acids in 3 different drinking water source waters: the average total free amino acid content in these waters was found to be 19 µg L^{-1} as N, while among the 18 amino acids analysed, the most abundant amino acids were found to be tyrosine, leucine and isoleucine.

3.1.2 Introduction

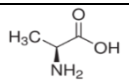
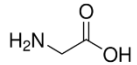
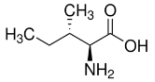
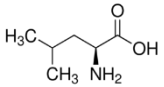
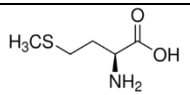
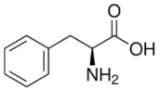
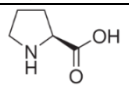
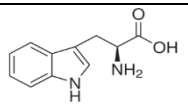
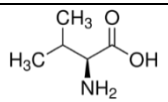
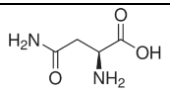
Total organic carbon (TOC) or dissolved organic carbon (DOC) is commonly used to measure the amount of organic material in drinking water source waters and to indicate the concentration of disinfection by-product (DBP) precursors. Dissolved organic nitrogen (DON) is a subset of DOC which includes any nitrogen-containing compounds present in water (Matilainen et al. 2011). The concentration of DON in surface waters, such as seawater, lakes, rivers, is reported to typically range from 0.1 to 10 mg L^{-1} as N, which is approximately 0.5 - 10% of the DOC content (Westerhoff and Mash 2002). The major sources of organic nitrogen in surface waters are algal breakdown products, agricultural runoff, urban runoff (Bronk et al. 1994, Westerhoff and Mash 2002) and wastewater input. Examples of nitrogen-containing functional groups within DON include amines, amides, nitriles and amino acids (Westerhoff and Mash 2002).

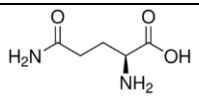
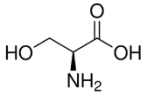
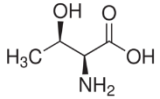
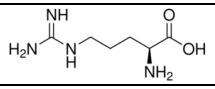
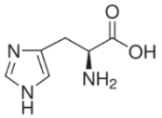
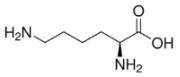
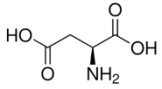
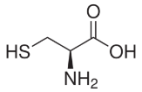
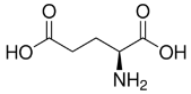
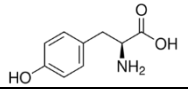
Amino acids are reported to be major constituents of DON, contributing up to 75 % of DON in surface waters (Hagedorn et al. 2000). The concentrations of total amino acids in seawaters, groundwaters and lakes were reported to range from 20 to 6000 µg L^{-1} (Thurman 1985), while the concentrations of free amino acids ranged from 1 to 80 µg L^{-1} as N (Chinn and Barrett 2000, Dotson and Westerhoff 2009, Thurman 1985). Glycine, alanine and serine have often been reported to be detected in the highest concentrations (Chinn and Barrett 2000, Dotson and Westerhoff 2009). The structure and physical properties (Blackman et al. 2007, Haynes 2013) of 20 of the 22 proteinogenic amino acids are presented in Table 3.1-1; selenocysteine and pyrrolysine were not included in this study as they were not commercially available. Amino acids have been reported to be precursors for several classes of N-DBPs, including halonitriles (Yang et al. 2010) and cyanogen halides (Hirose et al. 1989), as well as some odorous DBPs, such as *N*-chlorophenylacetaldimine (Freuze et al. 2005). The concentration of amino acids in natural water samples can be related to algal blooms and can also affect the level of other natural organic matter in the water (Ram and Morris 1980). Amino acids are also an important source of carbon for marine bacteria (Jørgensen et al. 1993). However, little information is reportedly available on amino acids in various natural waters partially due to the relatively low concentration of amino acids in the environment and difficulties with analytical methods (Chinn and Barrett 2000). In addition, when chlorinated, amino acids demonstrate a breakpoint curve phenomenon similar to ammonia, resulting in a higher chlorine demand for the distributed waters (Hureiki et al. 1994). If inorganic chloramine is used as the disinfectant, the presence of amino acids introduces a risk of overestimation of disinfection capabilities (Donnermair and Blatchley 2003). Therefore, knowledge of the occurrence of amino acids in source waters is important in understanding the formation and occurrence of N-DBPs, understanding the impact of algal blooms on water treatment and also to ensure that sufficient disinfectant is added during water treatment.

Given their polarity, reversed-phase liquid chromatography is commonly used for the separation of amino acids, followed by spectroscopic (UV-visible or fluorescence) detection with pre- or post-column derivatisation (Kaspar et al. 2009, Molnár-Perl 2003), as amino acids are generally neither chromophores nor fluorophores (Molnár Perl 2005). However, derivatisation may result in inconsistent results caused by varying

stability of the derivatives and incomplete derivatisation (Molnár Perl 2005). In addition, spectroscopic detection may lack the selectivity and sensitivity required for trace analysis, which is essential for the analysis of amino acids in natural waters. Mass spectrometry (MS) is more selective than spectroscopic techniques, as spectroscopic techniques can only differentiate between compounds by their retention time, while MS is also able to differentiate between compounds by their unique isotopic mass and fragmentation pattern. The separation and detection of 20 free amino acids in ultrapure water by liquid chromatography-electrospray ionisation-mass spectrometry (LC-ESI-MS) was first reported by Chaimbault et al. (1999). The same research group later also introduced a method using tandem mass spectrometry for the analysis of free amino acids in ultrapure water (Petritis et al. 2000). Since then, amino acids have been analysed by LC-MS in a range of applications involving a variety of matrices, including foods (Rebane 2010, Thiele et al. 2008) and biological samples (Armstrong et al. 2007, Swann et al. 2012). However, there are no reports to date on the application of LC-MS for the analysis of amino acids in natural waters, particularly waters containing natural organic matter.

Table 3.1-1: Selected amino acids, their structure, molecular weight, isoelectric point (pI) and acid-base constants.

Name	**Classification**	**Structure**[a]	**Molecular weight (Da)**	**pI**[b]	**pK_a** [b]	**pK_b** [b]	**pK_c** [b]
Alanine	Non-polar		89.1	6.00	2.33	9.71	
Glycine	Non-polar		75.1	5.97	2.34	9.58	
Isoleucine	Non-polar		131.2	6.02	2.26	9.60	
Leucine	Non-polar		131.2	5.98	2.32	9.58	
Methionine	Non-polar		149.2	5.74	2.16	9.08	
Phenylalanine	Non-polar (aromatic)		165.2	5.48	2.18	9.09	
Proline	Non-polar		115.1	6.30	1.95	10.47	
Tryptophan	Non-polar (aromatic)		204.2	5.89	2.38	9.34	
Valine	Non-polar		117.2	5.96	2.27	9.52	
Asparagine	Polar		132.1	5.41	2.16	8.73	

Glutamine	Polar		146.2	5.65	2.18	9.00	
Serine	Polar		105.1	5.68	2.13	9.05	
Thereonine	Polar		119.1	5.60	2.20	8.96	
Arginine	Basic		174.2	10.76	2.03	9.00	12.1
Histidine	Basic		155.2	7.59	1.70	9.09	6.04
Lysine	Basic		146.2	9.74	2.15	9.16	10.7
Aspartic acid	Acidic		133.2	2.77	1.95	9.66	3.71
Cysteine	Acidic		121.2	5.07	1.91	10.28	8.14
Glutamic acid	Acidic		147.1	3.22	2.16	9.58	4.15
Tyrosine	Acidic (Aromatic)		181.2	5.66	2.24	9.04	10.1

[a]Blackman et al., 2007, [b]Haynes, 2013

As the concentrations of amino acids in natural waters have been reported to be in the microgram per litre range (Chinn and Barrett 2000, Dotson and Westerhoff 2009, Thurman 1985), a preconcentration method is needed for analysis of amino acids in natural waters to concentrate the analytes and, if possible, to also remove matrix interferences before analysis. Concentration under reduced pressure is one of the most common techniques used for concentration of non-volatile analytes in an aqueous matrix, however it is a time consuming process due to the low volatility of water. Solid-phase extraction (SPE) is a preconcentration technique known to provide sufficient sample concentration for sub-nanogram per litre analysis in environmental samples (Gros et al. 2006b). In addition, SPE is also able to remove some matrix species (Gros et al. 2006b) and isolate the analytes from the sample. However, SPE is costly, can be time consuming and often suffers from low recoveries due to loss of analytes during the loading or the washing step (Gros et al. 2006a). In addition, the likely interferences in the water matrix are often similar to the analytes in terms of polarity and retention (Cech and Enke 2001) and so may not be separated during the SPE stage. Concentration under reduced pressure has been reported to be used for concentration of amino acids in natural waters (Chinn and Barrett 2000, Dotson and Westerhoff 2009). However, there have been no reports to date of the use of SPE for the extraction and isolation of amino acids from natural waters, even though SPE has previously been reported for the extraction of amino acids from various matrices, including plant roots (Persson and Näsholm 2001), tea leaves (Wang et al. 2010) and human plasma (Armstrong et al. 2007). Concentration under reduced pressure and SPE were chosen to be trialled for the preconcentration and isolation of amino acids from natural waters in the current study.

In this study, a novel method for the analysis of amino acids in natural waters was developed using liquid chromatography-electrospray ionisation-tandem mass spectrometry (LC-ESI-MS/MS) with solid-phase extraction (SPE) pre-treatment. The method was successfully developed and validated for the analysis of 18 out of 20 proteinogenic amino acids in natural waters. The suitability of SPE for analyte extraction of amino acids and matrix removal for natural waters was also compared to the more traditional approach of concentrating samples under reduced pressure.

3.1.3 Experimental

3.1.3.1 Sampling and Sample Pre-treatment

Grab samples were collected from a river in South Perth, Western Australia (River water), from a tap located in the Curtin Water Quality Research Centre laboratory (Tap water) and a groundwater sample from a local groundwater bore (Groundwater). Surface water samples (Surface water A-C) were collected from the raw water inlets of various drinking water treatment plants in Western Australia (basic characteristic presented in Table A1-1). All water samples were collected in amber glass bottles, previously annealed at 550 °C overnight and rinsed with the sample several times prior to sample collection. Samples were kept cold with ice packs during transport. On arrival at the laboratory, all natural water samples were filtered through 0.45 µm polyethersulfone membrane filters (Pall Life Science, Michigan, USA) and stored at 4 °C until extraction to prevent analyte degradation.

3.1.3.2 Analytical Standards and Chemicals

The amino acids, alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glycine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine, were purchased from Sigma Aldrich (New South Wales, Australia). The purity of all analytical standard compounds was ≥97%. The surrogate standards, [^{2}H3] alanine (alanine-d_3), [^{2}H3] leucine (leucine-d_3), [^{2}H3] glutamic were purchased from acid (glutamic-d_3 acid), were purchased from CDN Isotopes (Quebec, Canada, distributed by SciVac, Hornsby, Australia); [^{2}H2] glycine (glycine-d_2) and [^{2}H5] phenyl [^{2}H3] alanine (phenyl-d_5-alanine-d_3) were purchased from Sigma Aldrich (New South Wales, Australia). Methanol (MeOH) and acetonitrile (ACN) (ChromHR grade) were purchased from Mallinckrodt Baker (New Jersey, USA). Formic acid (purity 99%), concentrated hydrochloric acid (32%, HCl) and ammonium solution (28% ammonium) were purchased from Ajax FineChem (New South Wales, Australia). Ultrapure water (H_2O) was purified using an ion exchange system (IBIS Technology, Perth, Australia), followed by an Elga Purelab Ultra system with a 0.2 µm filter (Elga, High Wycombe, UK). Single standard stock solutions (1000 ng μL^{-1}) of the 20 amino acids and mixed working solutions of all 20 amino acids (100 ng μL^{-1} and 1 ng μL^{-1}) were prepared using 30:70 (*v:v*) MeOH:H_2O solvent. Individual surrogate standard stock solutions

(1000 ng μL^{-1}) and a mixed surrogate standard working solution (100 ng μL^{-1}) were prepared in 30:70 (*v:v*) MeOH:H_2O. All solutions were kept at -13 °C to avoid degradation.

3.1.3.3 Solid-phase Extraction Preconcentration and Isolation Procedure

Two types of reversed-phase stationary phases (Oasis HLB from Waters, Mildford, USA; Strata-E from Phenomenex, New South Wales, Australia) and one type of strong cation exchange stationary phase (Strata-X-C from Phenomenex) solid-phase extraction (SPE) cartridges were trialled for analytes preconcentration and isolation. All cartridges had 500 mg of resin and a 6 mL bed volume. Strata-X-C cartridges were selected for the preconcentration and isolation of amino acids in natural waters as they provided the highest recoveries and precision among the 3 types of SPE cartridge trialled. The pH of the water samples was adjusted to 1.3 using concentrated HCl solution before loading the samples onto the SPE cartridges. An automated Aspec XLi extractor (Gilson, Middleton, USA) was used for the conditioning, washing and elution of the cartridges, as described in Table 3.1-2. After cartridge conditioning, samples were loaded onto the SPE cartridges using two 8-channel off-line peristaltic pumps (Gilson, Middleton, USA) at a flow rate of 2 mL min^{-1}. The cartridges were dried under vacuum of 20 mmHg for 5 min to remove excess moisture. Analytes were then eluted into 12 mL glass test tubes in the Aspec XLi collection rack. Analytes were eluted with a delay of 1 min between each aliquot of solvent dispensed to ensure that the stationary phase was efficiently soaked with the eluent. The eluent (11.5 mL) from each sample was evaporated to dryness under a gentle stream of nitrogen using a dry block heater fitted with nitrogen blowdown (Ratek 30D, Boronia, Australia), set at 40 °C. The dried samples were re-dissolved in 500 µL of 30:70 (*v:v*) MeOH:H_2O. Sample extracts were then transferred via pipette into 2 mL screw cap amber glass vials (Agilent, USA) and stored in a freezer at -13 °C until analysis.

Table 3.1-2: Optimised SPE conditions adopted for the recovery and clean-up of amino acids in water

Step	Solvent and dispensed volumes
Conditioning	4.5 mL of MeOH 9 mL of 0.1 mol L^{-1} HCl solution (pH 1.3) in ultrapure water
Loading	500 mL of sample (pH 1.3) at 2 mL min^{-1}
Washing	5 mL of 4.5% MeOH in 0.1 mol L^{-1} HCl solution
Elution	11.5 mL of 5% ammonium hydroxide in MeOH

3.1.3.4 Concentration of Amino Acids under Reduced Pressure

The concentration under reduced pressure procedure was adapted from the method by Chinn and Barrett (Chinn and Barrett 2000) with some modifications. Each sample (500 mL) was placed into a round-bottom flask and concentrated to approximately 10 mL using rotary evaporation (Heidolph Instrument, Schwabach, Germany) at 60 °C under 9 mbar of vacuum. The reduced pressure allowed faster concentration time (~ 2 h per sample) and minimised the heating of the sample which could have resulted in some thermal degradation of amino acids (Sohn and Ho 1995). The concentrate (~10 mL) was then evaporated to dryness using a dry block heater fitted with nitrogen blowdown, set at 40 °C and under a gentle stream of nitrogen. The samples were then redissolved using 500 µL of 30:70 (*v:v*) MeOH:H_2O and transferred via pipette to a 2 mL screw cap amber glass vial and stored in a freezer at -13 °C until analysis.

3.1.3.5 Separation and Detection of Amino Acids by LC-MS/MS

Unless otherwise stated, all liquid chromatography and tandem mass spectrometry conditions adopted in this work were the same as we have previously reported in Swann *et al*. (Swann et al. 2012). Briefly, chromatographic separation was achieved using an Agilent 110 HPLC system (Palo Alto, CA, USA) and the amino acids were separated using a Gemini C18 column from Phenomenex® at a flow rate of 150 µL min^{-1}. The amino acids were detected using a Triple Quadrupole Mass Spectrometer (Micromass Quattro, Manchester, UK) fitted with an electrospray ionisation (ESI) interface operated in positive ion mode.

Retention time was the main parameter used to identify analytes, and multiple reaction monitoring (MRM) ratio was used as a second confirmation for analytes where two

stable transitions were monitored. In order to increase the sensitivity of the analytical assay, the MRM transitions were grouped into three separate windows based on their retention times. Moreover, given that 14 analytes and three surrogate standards eluted in the first 10 min of the chromatographic run, in order to optimise the sensitivity, as well as to increase the number of points collected across each chromatographic peak by the MS, only one transition (single reaction monitoring, SRM) was monitored for each analyte in the first window.

Analytes were quantified using the ratio of the analyte peak area to the surrogate standard (Table A1-3) peak area and using an external calibration curve obtained by diluting working standards with MeOH:H_2O (*v:v*) 30:70. Deuterated amino acids were used as surrogate standards and the corresponding surrogate standard with its analytes are listed in Table A1-2. Data processing was carried out using MassLynx NT4.0 software, while data quantification was performed using QuanLynx 4.0.

3.1.4 Results and Discussion

We have previously developed a method for the analysis of amino acids and amines in mammalian decomposition fluids by LC-MS/MS (Swann et al. 2012). In this method, direct injection of samples was used. However, since the concentrations of amino acids in natural waters were expected to be much lower than those in mammalian decomposition fluids, direct injection was not appropriate for natural water samples. Therefore, a preconcentration method was required. The matrix characteristics for natural waters, as compared to mammalian decomposition fluids, must also be studied. In addition, only 15 amino acids of interest were analysed in our earlier method, thus there was a need to include a more complete suite of amino acids in the current method.

3.1.4.1 Optimisation of Tandem Mass Spectrometry Conditions for Additional Analytes

Infusion experiments were conducted to determine the MRM transitions of the amino acids not analysed previously (Swann et al. 2012), i.e., alanine, aspartic acid, cysteine, glycine, glutamine and the deuterated surrogates, alanine-d_3, leucine-d_3, glutamic-d_3 acid, glycine-d_2 and phenyl-d_5-alanine-d_3, so that they could be incorporated into the present analytical method. The parent ion to product ion transition data for the remaining amino acids were as obtained previously (Swann et al. 2012). A significant

improvement on the previous method was the introduction of deuterated standards as surrogate standards to account for matrix effects and recovery, and also for quantification. One deuterated standard was assigned to multiple amino acid species as not all homologue deuterated standards for the amino acids were commercially available. The parent ion to product ion transitions used for SRM or MRM were selected based on their intensities in the MS/MS spectra (Table A1-2).

3.1.4.2 Instrumental Linearity, Detection Limits and Peak Identification Criteria

Instrument performance data is reported in Table A1-3. Instrumental linearity and instrumental detection limits were determined from analysis of 13 calibration standards ranging from 0.002 ng μL^{-1} to 20 ng μL^{-1}. Calibration curves showed good linearity (R^2>0.990) up to maximum concentrations that ranged between 5 to 20 ng μL^{-1} for all analytes. Instrumental detection limits, estimated at signal-to-noise (S/N) ratios of 3, ranged from 1 to 190 pg on column (0.2 - 38 pg μL^{-1}), which is consistent with our previous study (Swann et al. 2012). The variabilities of retention time and MRM ratio were calculated from repeat injections (n=10) of a solution containing 1 ng μL^{-1} of each amino acid. In general, the standard deviation (SD) of the retention time (t_R) was less than 20 s, indicating repeatable chromatography. However, leucine, isoleucine, tyrosine and phenylalanine showed higher SDs (ranging from 35s to 65 s). The reason for this variability of t_R is not known, but it was also observed in our previous work (Swann et al. 2012). The relative standard deviation (RSD%) of the MRM ratios (peak area ratio between the two MRM transitions) was generally less than 5 %, indicating repeatable fragmentation of parent ions in the collision cell.

3.1.4.3 Optimisation of the Solid-phase Extraction Procedure

3.1.4.3.1 Selection of the Type of Solid-phase Extraction Cartridge

Two types of reversed-phase, polymeric (Oasis HLB, Waters®) and octadecyl silica (Strata C18-E, Phenomenex®), and one type of strong cation exchange phase (Strata-X-C, Phenomenex®), solid-phase extraction (SPE) cartridges were trialled to determine the most suitable stationary phase for the extraction and concentration of amino acids from aqueous samples. As a preliminary comparison, one laboratory ultrapure water blank (1000 ng of deuterated surrogate standards in 500 mL of ultrapure water), two low concentration standards (250 ng amino acid standards + 1000 ng of deuterated standards in 500 mL of ultrapure water), and two high

concentration standards (1000 ng amino acid standards + 1000 ng of deuterated standards in 500 mL of ultrapure water) were separately loaded onto the three types of SPE cartridges without pH modification, as advised from the SPE manufacturer, and the analytes were extracted using the procedures outlined in the Appendix 1 (Table A1-4). Without pH adjustment, the pH of the sample was approximately 6.5, and thus most of the amino acids have no significant net charge. Neither the deuterated surrogate standards nor most of the amino acid standards (16 out of 20) were recovered from the reversed-phase cartridges at either the low or high concentrations tested (data not shown). Leucine-d_3 and phenyl-d_5-alanine-d_3 and 19 amino acids were recovered (average recovery = 23%) when using the Strata-X-C cartridge. These preliminary results suggested that strong cation exchange cartridges were more suitable than reversed phase cartridges for the extraction of amino acids, in agreement with the work previously reported by Spanik et al. (2007). The poor recoveries of analytes from the reversed-phase cartridges can be attributed to the fact that amino acids are polar at neutral pH, characteristics that reduce the retention of analytes under reversed-phase conditions, with the likely outcome that the analytes remained in the aqueous phase and were not retained on the SPE cartridges. The polar nature of the amino acids makes them more amenable to retention on the strong cation exchange cartridges. In addition, strong cation exchange packing material is polymeric and therefore designed to provide additional retention through reversed-phase mechanisms (e.g. π-π bonding, hydrogen bonding and hydrophobic interactions). The mixed-mode retention properties of the ion exchange packing material explained the improved retention of the amino acids when compared to the reversed-phase cartridges, where only π-π bonding, hydrogen bonding and hydrophobic interactions between the resin and the amino acids were possible. However, depending on the structure and isoelectric point (pI) of the amino acids, some of them (17 out of 20) are overall negatively charged at neutral pH, which results in the quite poor recoveries observed on the strong cation exchange resin.

It was noted that some of the amino acids tested were not recovered at all by the sample preconcentration methods (recovery data presented in Section 3.1.4.5). Arginine was not recovered by any of the cartridges trialled for the SPE method, while cysteine was not recovered by either the SPE or the concentration under reduced pressure method. As both arginine and cysteine are polar amino acids, they were not expected to be

retained on the reversed-phase SPE cartridge and therefore they were not expected to be recovered by this method. It was expected that arginine would be well-retained by the cation exchange phase because its pI value of 10.76 indicates that it will be positively charged at neutral pH, but, since the pH of the eluting solution was around pH 10, it is likely that the arginine was retained on the cartridge in a cationic form and was not eluted, resulting in minimal recovery. The fact that cysteine was not recovered by the concentration under reduced pressure method suggests that cysteine was not stable in the concentration step or that it has a limit of detection higher than the working concentration range. Higher concentrations of cysteine were not tested as the concentration of cysteine in natural waters is unlikely to be more than 2 mg L^{-1}.

The strong cation exchanger SPE cartridge provided the best recoveries among all the 3 types of SPE cartridges trialled and a lower pH will improve the recoveries of amino acids when using strong cation exchanger SPE by ensuring that all amino acids are in their cationic (positively charged) forms.

3.1.4.3.2 Optimisation of pH for solid-phase extraction on the strong cation exchange resin

Given the importance of charge for the retention of analytes on the strong cation exchange resin, a series of experiments were undertaken to investigate the effect of pH on the on the recovery of amino acids. In these experiments, the pH of the samples of amino acids in water was adjusted to pH 2 using concentrated HCl prior to application strong cation exchange SPE cartridge. The results showed that reducing the sample pH to 2 significantly improved the recovery of the amino acids (Table 3.1-3). For example, the recovery of proline increased from less than 1 % at neutral pH to 80 % at pH 2. This finding is in agreement with Spanik et al. (2007), and also expected since the lowest pI value of the amino acids studied was 2.77 (Table 3.1-1), and most of the amino acids have pI less than 6 (Table 3.1-1), such that at pH 2, all amino acids should be predominantly in their positively charged form. Lowering the pH has the effect of protonating the amino group while the carboxylic acid group undissociated, resulting in an overall positive charge on the amino acids. This promotes the interaction between the negatively charged resin and the positively charged amino acids, improving retention and recoveries.

Table 3.1-3: Accuracy (recovery %) and precision (RSD %) of recovery experiments of amino acids (2, 5 and 10 µg L^{-1}) conducted at acidic and neutral pH from buffered (30 mM phosphate) and unbuffered ultrapure water samples

Name	pH 2.5	pH 2	pH 6.5	pH 6.5
Lysine	10±15	80±20	<1	<1
Histidine	3±20	70±15	<1	<1
Arginine	<1	<1	<1	<1
Glycine-d_2	N.D.	25±50	N.D.	<1
Glycine	15±20	20±25	N.D.	<1
Serine	<1	10±25	N.D.	<1
Alanine-d_3	N.D.	30±10	N.D.	<1
Alanine	1±25	35±5	N.D.	<1
Asparagine	<1	10±1	N.D.	<1
Glutamine	1±30	5±10	N.D.	<1
Thereonine	1±25	15±10	1±30	<1
Glutamic – d_3 -acid	2±0	1±5	N.D.	<1
Aspartic acid	1±20	1±30	<1	<1
Cysteine	N.D.	N.D.	N.D.	N.D.
Glutamic acid	2±15	5±10	<1	<1
Proline	20±20	80±20	<1	<1
Valine	10±80	95±10	<1	<1
Methionine	10±40	10±5	N.D.	30±10
Isoleucine	30±60	80±10	<1	60±5
Leucine	50±45	40±1	<1	25±5
Leucine-d_3	45±20	50±5	N.D.	105±5
Tyrosine	60±10	165±10	<1	100±20
Phenyl-d_5-alanine-	60±15	60±10	1±15	60±2
Phenylalanine	55±10	85±10	2±10	105±0
Tryptophan	60±10	80±10	15±5	55±5

N.D. - not detected.

The use of buffer has been reported to increase the recoveries of amino acids where 30 mM phosphate buffer (pH 2.7-3.3) was found to be the most effective for the SPE extraction of amino acids (Rebane 2010). Therefore, the effect of a 30 mM phosphate buffer on the recoveries of the amino acids was investigated at both 2.5 and pH 6.5. The pH of the acidic buffer should be made near to the unbuffered acidic solution of pH 2 to minimise differences in recovery due to pH differences, however, an excessive volume of concentrated HCl solution would be required to lower the pH of the phosphate buffer to below pH 2.5. Amino acid recoveries were generally lower in buffered samples than in non-buffered samples (Table 3.1-3). The lower recoveries may be explained by competition between buffer cations and the amino acids on the

SPE cartridge or by the slightly higher pH of the buffered samples under acidic conditions. The concentration of potassium ions was calculated to be 1000 times higher than that of the amino acids in the solutions tested. The effect of this competition could be reduced by using a lower ionic strength buffer solution or an acidified non-buffered solution. An acidified non-buffered solution was chosen for the final method to reduce the chance of cation competition from the buffer solution.

While reducing pH had the effect of improving the recoveries, a loading pH of 2.5 (buffered solution) was not sufficiently low to protonate some of the amino acids. Some of the amino acids are overall negatively charged at pH 2.5, since they all have acidic pKa values below 2.5 (Table 3.1-1). Therefore the carboxylic acid groups in these amino acids would still be dissociated at pH 2.5, and the retention mechanism for these amino acids will be based on reversed-phase interaction rather than cation exchange. In order to further improve the recoveries of the amino acids, the loading pH was further reduced to 1.3, below the lowest acidic pKa of 1.70 for all of these amino acids (Table 3.1-1), using concentrated HCl solution. This pH guaranteed all amino acids to be positively charged, and thus better interaction between the amino acids and the cation exchange resin could be achieved. The reduction in pH from 2.5 to 1.3 significantly improved the recoveries and precision of analysis of the amino acids (Tables 3.1-3 and 3.1-4). The final procedure for the SPE extraction of amino acids to optimise their recoveries is shown in Table 3.1-2.

3.1.4.3.3 Optimisation of solid-phase extraction cartridge washing conditions

Despite modification to the pH of the sample in the loading step to optimise the SPE cation exchange process, recoveries of many of the amino acids were still very low (Table 3.1-3). In order to determine whether the analytes were not retained on the cartridges during loading, or whether they were being eluted in the washing step (3 mL of 0.1 mol L^{-1}HCl in H_2O followed by 3 mL of 0.1 mol L^{-1} HCl in MeOH), the eluent from the washing step was collected, concentrated to dryness, redissolved in MeOH:H_2O (*v:v*) (30:70) mixture, and analysed by LC-MS/MS. Serine, alanine, asparagine, glutamine, aspartic acid and glutamic acid were all detected in the extract from the washing step eluent (data not shown). According to the manufacturer (Phenomenex 2012), the eluent from the washing step should contain acidic and polar compounds previously retained on the resin, and this corresponds to the nature

of the amino acids detected. Detection of these acidic and polar amino acids in the washing step eluent suggested that retention of amino acids was by both reversed-phase and cationic interaction. Thus, the use of 50% methanol in the washing step eluted the compounds. In order to determine if the removal of the washing step would prevent the loss of analytes, an experiment to analyse the amino acids was conducted with the washing step removed. However, without the washing step, high ion suppression was observed.

In order to reduce the matrix effects but avoid loss of analytes, a series of experiments were conducted. A gentler washing step (10 mL of ultrapure water followed by 5 mL of 2.5% MeOH in ultrapure water) was tested and was found to reduce ion suppression. Four different organic washing solvent systems, 2.5 and 4.5% of isopropyl alcohol or MeOH in ultrapure water, were also trialled to further optimise the washing step. Cartridges were loaded with ultrapure water containing 2 µg L^{-1} of amino acids with the pH of the solution adjusted to 1.3 using concentrated HCl solution. The cartridges were then washed with 10 mL of ultrapure water and one of the different organic washing solvent systems. The eluent from each organic solvent wash was evaporated to dryness and redissolved using 500 µL of 30:70 (*v:v*) MeOH:H_2O. Each solution was then analysed for amino acids. No amino acids were detected in the four organic solvent systems investigated (Table A1-5), indicating that the amino acids were lost during the washing by the first 10 mL of ultrapure water, resulting in low recoveries for the amino acids. In the procedure for strong cation exchange, a basic solvent is used to elute the retained analytes and it is possible that, ultrapure water, with a pH of 7.3, may also have been able to elute the amino acids that were not strongly retained by the strong cation exchange resin. It was also noted that the less polar organic solvent (isopropyl alcohol) resulted in lower loss of analytes than the more polar organic solvent (MeOH) and a higher percentage of organic solvent in the washing solvent system also reduced the loss of analytes (Table A1-5). In order to reduce the loss of analytes due to the aqueous and/or the organic washing solution, an acidic wash of 5 mL of 0.1 mol L^{-1} HCl in 4.5% MeOH was tested. The results showed a general increase in recovery compared to washing the cartridges at neutral pH, especially in the first window where the losses of analytes were up to 100% for some of the amino acids when using a neutral washing step (Table A1-5). This indicated that the pH of

the washing step needed to be similar to that of the loading step to prevent the loss of analytes.

Although it was found that the washing step resulted in significant loss of some analytes, this step was required to reduce the matrix effect. A washing step using 5 mL 0.1 mol L^{-1} HCl in 4.5% MeOH was chosen as it resulted in the lowest loss of analytes.

3.1.4.4 Matrix Effects and Choice of Surrogate Standards

In LC-MS/MS, the signals for analytes can either be suppressed or enhanced by the matrix due to the competition between analytes and the matrix for the primary ions produced in the LC-MS/MS interface (Busetti et al. 2009, Cappiello et al. 2008). Ion suppression can result in the loss of sensitivity, accuracy and precision; while ion enhancement can result in the loss of accuracy and precision (Busetti et al. 2009, Cappiello et al. 2008). Many methods have been suggested to account for the matrix effect (Busetti et al. 2009, Gros et al. 2006a, Gros et al. 2006b), the use of deuterated standards being one of them. Deuterated standards usually co-elute with the homologue analytes and are subjected to almost identical matrix effects (Gros et al. 2006b), therefore deuterated standards represent the most effective way to account for matrix effects. However, not all homologue deuterated standards for the amino acids were commercially available and, to also minimise costs, a total of five deuterated standards were chosen for this analytical method to correct potential matrix effects, with one to three deuterated standards assigned to each monitoring window (Table A1-2).

3.1.4.4.1 Matrix Effects caused by Real Water Samples

In order to investigate the impact of matrix effects on both the SPE and concentration under reduced pressure preconcentration methods, peak areas of standards added into MeOH:H_2O water 30:70 (*v:v*) were compared to peak areas of standards added into a surface water, a tap water and a groundwater at 2 µg L^{-1} after filtration. The introduction of the real water sample matrices resulted in shifts in retention times of 30-60 s and caused ion suppression for most analytes for both SPE and concentration under reduced pressure preconcentration procedures. When concentration under reduced pressure was used as the preconcentration method, the ion suppression for most analytes was close to 100% for each of the three water samples (Figure 1).

However, when the final SPE procedure was used as the preconcentration method, ion suppression was lower than with the concentration under reduced pressure method (Figure 3.1-1). The suppression of signals for analytes in the river water, especially those analytes that eluted in the first 10 min, was expected since the river water was brackish, containing up to 30 parts per thousand of inorganic salts.

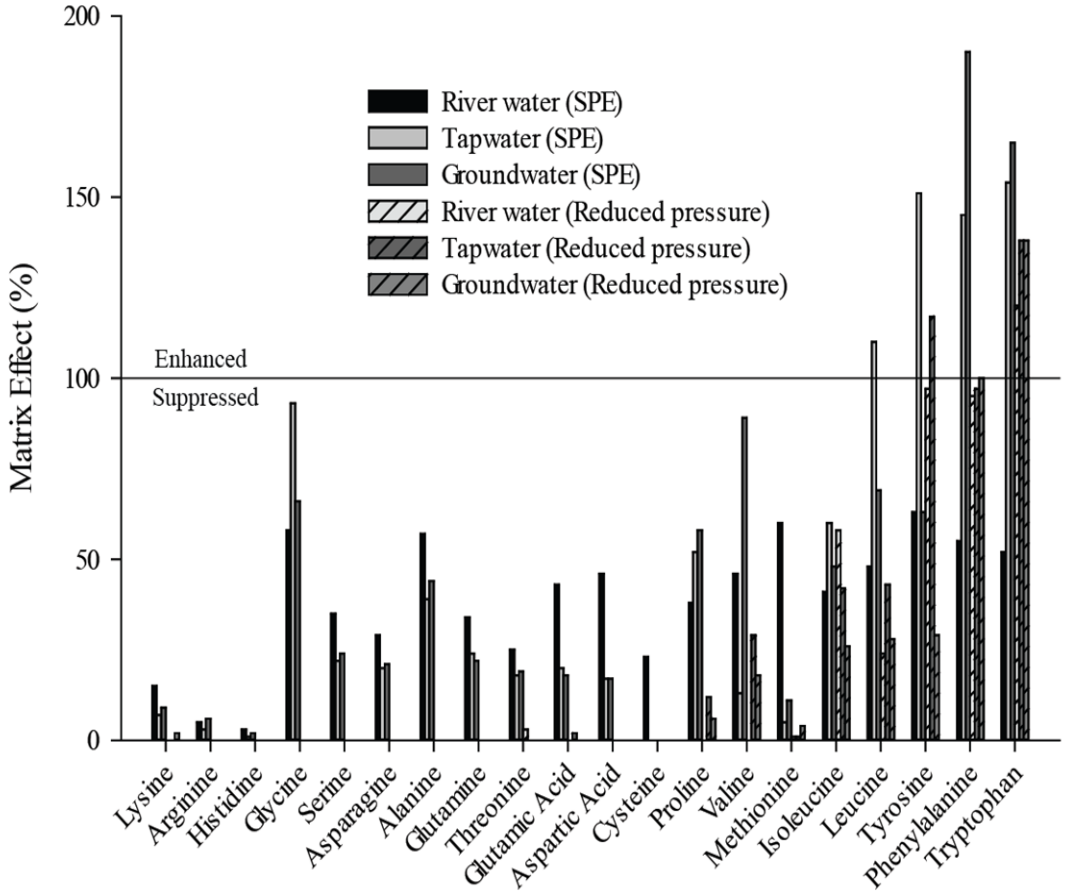


Figure 3.1-1: Matrix effects (%) from SPE and concentration under reduced pressure pre-concentrated samples (*n*=2) of river water, tap water and groundwater containing 2 ng μL^{-1} of amino acids. The matrix effects less than 100% represent ion suppression, while a percentage more than 100% represents ion enhancement. The suppression/enhancement effect of each matrix was determined by comparison to a standard solution of amino acids (2 ng μL^{-1}) dissolved in 70:30 MeOH: H_2O.

The high signal suppression experienced by samples pre-concentrated using the concentration under reduced pressure method results from the fact that this method only removes volatile compounds, with non-volatile compounds like inorganic salts being concentrated to the same extent as the analytes during the process. In addition, many of the non-volatile compounds are likely to be polar/ionic in nature and therefore elute in the first 10 min of the chromatogram, resulting in high interferences and high signal suppression in this part of the chromatogram. SPE is a preconcentration and

separation technique which provides a higher level of sample clean-up. Therefore, the SPE method was found to be more suitable as a preconcentration method for LC-MS/MS to reduce matrix effects.

3.1.4.4.2 Matrix effects caused by the SPE procedure

While SPE gave better clean-up of the sample matrix, it appeared to also introduce additional matrix that was not present in the samples from the concentration under reduced pressure method. Histidine, glycine and serine could only be detected, but not quantified, in SPE extracts, as these analytes co-eluted with an interference peak that could not be resolved from the analyte peaks. This co-elution was only observed in samples that were pre-concentrated using SPE, and not those pre-concentrated using the concentration under reduced pressure method, suggesting that the interferences were contributed by the SPE method, presumably from compounds leaching from the cation exchange resin.

The impact of such an interference peak can be reduced by improving the separation of the analytes from the interference peak or by increasing the selectivity of the detection method. As previously mentioned, the disadvantage of SPE is that the matrix that is not removed during clean-up is likely to have similar chromatographic properties to the analytes. Therefore, it is unlikely that changing the mobile phase and/or the elution gradient of the LC separation would significantly change the retention of the analytes and their separation from the interference peak. For example, using a mobile phase gradient with 90% water at the beginning of the HPLC analysis is designed in part to flush out inorganic salts. However, some amino acids are very polar and therefore have a similar retention time to the inorganic salts. A number of additional modifications were tested to improve the separation of the amino acids with the interference, including the use of a cation exchange column. However, no significant improvement in the separation of the amino acids and the interference was observed compared to our previously published LC method (Swann et al. 2012).

A comparison of ion suppression caused by SPE sample and solvent blank (cartridges subjected to the whole SPE procedure using ultrapure water as the sample) and SPE solvent blank (cartridges subjected to only conditioning and elution, without the sample loading step) showed that the SPE solvent blank could contribute up to 86% of ion

suppression and the SPE sample and solvent blank could contribute up to 96% ion suppression (Figure 3.1-2). The ion suppression might be caused by the ammonium chloride produced during the elution step where the basic solvent neutralised the acid from the sample and/or damage of the resins of the cartridges from the low pH during the conditioning and loading step. Even though the low pH had an adverse effect on the analysis, it was not possible to extract the amino acids at a higher pH, as the low pH was required to maintain the recovery and precision of the method (Section 3.1.4.3.2).

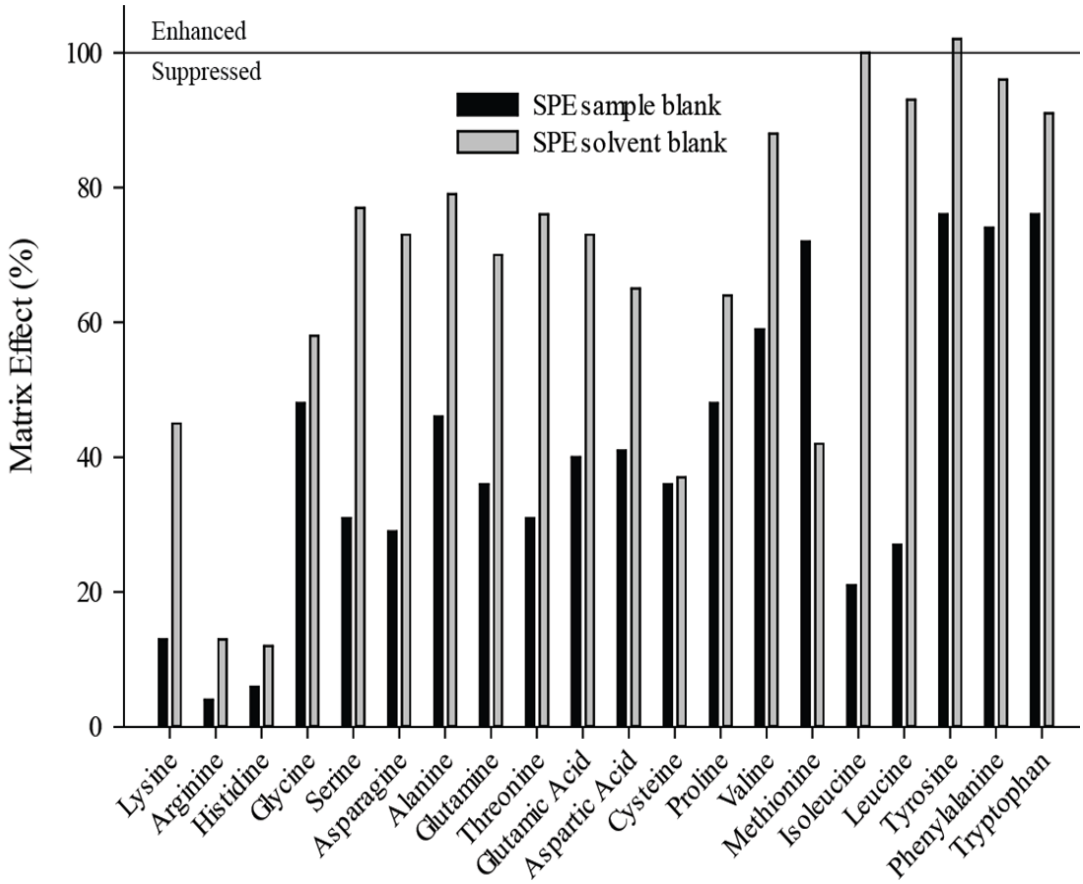


Figure 3.1-2: Matrix effects (%) by SPE sample and solvent blank with 2 ng μL^{-1} of amino acids. The matrix effects less than 100% represent ion suppression, while a percentage more than 100% represents ion enhancement. The suppression/enhancement effect of each matrix was determined by comparison to peak areas from a standard solution of amino acids (2 ng μL^{-1}) in 70:30 MeOH: H_2O.

As ammonium chloride (5 mg mL^{-1}) is likely to be produced during the SPE extraction of amino acids from water samples, an experiment was conducted to investigate the impact of ammonium chloride on the analysis of amino acids. Ammonium chloride (6 mg) was dissolved in 500 µL of MeOH:H_2O (*v:v*) 30:70 solvent, containing 2 ng μL^{-1} of amino acid standards and surrogate standards.

The peak areas of the analytes in the sample with added ammonium chloride were, on average, 30% lower in the first window and were, on average, 10% lower in the second and third windows as compared to a standard solution without ammonium chloride. When comparing the chromatograms (Figure 3.1-3), interference peaks similar to those found from samples that have gone through SPE were observed in the sample with added ammonium chloride. This suggested that ammonium chloride may have been formed during the eluting step in the SPE procedure and indicated that the matrix effect would be reduced if the ammonium chloride was removed or the formation was prevented.

Although an acidic washing step resulted in the formation of ammonium chloride, which was an interfering species for analytes eluting at the same time as the ammonium chloride (histidine, glycine and serine), an acidic wash in the SPE procedure was required to ensure higher recoveries and precision for analysis of the other amino acids. Therefore, the acidic washing step was utilised in the final SPE method.

The signal suppression/enhancement for the standards was similar to that of the surrogate standards (Table A1-6), indicating that the surrogate standards chosen were suitable for this application.

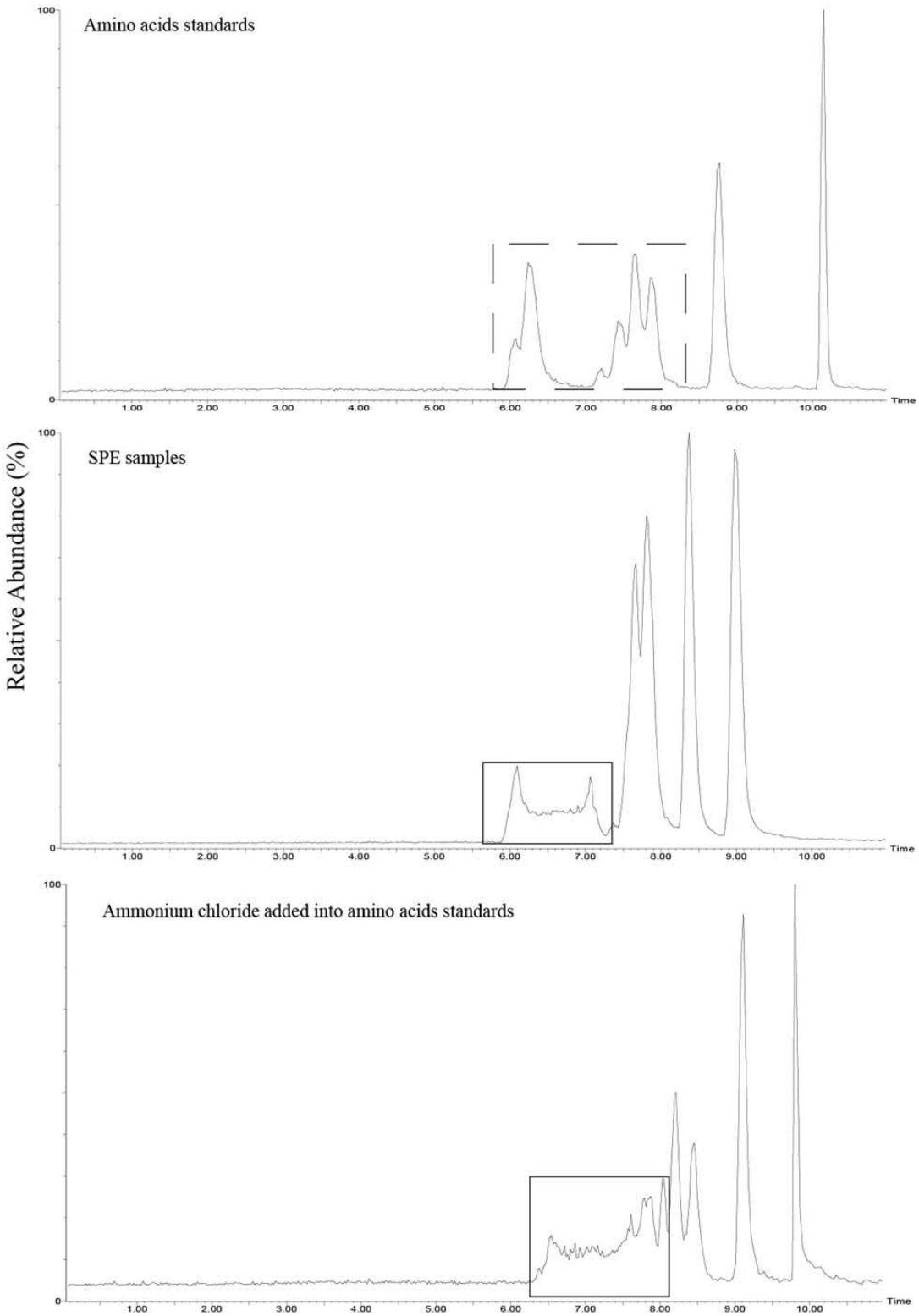


Figure 3.1-3: Impact of ammonium chloride on the chromatograms in the 10 min first window. The chromatogram from analysis of amino acids in ultrapure water with added ammonium chloride showed similar interference to the SPE sample and solvent blank, indicating that ammonium chloride was likely to be produced in the SPE elution step. Regions affected by matrix effects are highlighted by a solid line, as compared to the standard in a dashed line.

3.1.4.5 Method Validation

The recovery and precision of the two methods using SPE and concentration under reduced pressure preconcentration were determined using standard solutions of amino acids prepared in ultrapure water (Tables 3.1-4 and 3.1-5). The recovery was expressed as the percentage recovery relative to the surrogate standards, and the precision (repeatability) was expressed as the relative standard deviation (RSD) of the measured concentrations of amino acids after analysis. Results for recoveries and precision are presented as averages over three concentrations (2, 5 and 10 μg L^{-1}), each analysed in triplicate (n=9).

The relative recoveries of amino acids using the SPE preconcentration method had a median value of 105% and the precision varied from 5% to 25% (Table 3.1-4). The recoveries of amino acids using the concentration under reduced pressure preconcentration method had a median value of 95% and a precision comparable to the SPE method, varying from 5% to 35% (Table 3.1-5). Little information on the analytical recoveries of free amino acids from natural waters has been previously published, so comparison of the recoveries achieved in this method to previously published methods is not possible.

As the samples were loaded on the SPE cartridge at low pH, the amide functional groups of glutamine and asparagine would have been partially hydrolysed into the corresponding carboxylic acid groups, forming glutamic acid and aspartic acid, respectively, resulting in high relative recoveries of glutamic acid and aspartic acid in the SPE method (Table 3.1-4).

Cysteine was not detected in either preconcentration method and arginine was not detected in the SPE method due to poor recoveries (Section 3.1.4.3.1). Histidine, glycine and serine were not quantified due to interference from ammonium chloride formed during the SPE procedure (Section 3.1.4.4.2)

Table 3.1-4: Accuracy, precision and method limit of quantification (MLQ) achieved in analysis of amino acids (2, 5 and 10 µg L^{-1}) in ultrapure water using the strong cation exchange SPE preconcentration method. Recoveries are presented as average over the 3 concentrations in triplicate *(n*=9) of all analyses.

Name	Recovery (%)	Precision (RSD %)	MLQ (µg L^{-1} as N)
Lysine	90	10	80
Arginine	N.D.	N.D.	N.D.
Histidine	N.Q.	N.Q.	N.Q.
Glycine	N.Q.	N.Q.	N.Q.
Serine	N.Q.	N.Q.	N.Q.
Asparagine	80	20	65
Alanine	175	5	100
Glutamine	80	25	90
Thereonine	135	20	70
Glutamic Acid	245	5	30
Aspartic Acid	280	5	20
Cysteine	N.D.	N.D.	N.D.
Proline	90	10	5
Valine	130	20	5
Methionine	80	15	55
Isoleucine	100	10	0.5
Leucine	110	5	0.5
Tyrosine	120	10	0.5
Phenylalanine	105	5	0.1
Tryptophan	85	10	0.5
Median	**105**	**10**	**20**

N.Q. - not quantified; N.D. - not detected

The method validation was performed in ultrapure water; for analysis of amino acids in real water samples, QA/QC was assured by standard addition of amino acids to selected real water samples to ensure good recoveries and precision for each batch of samples processed.

Preconcentration of amino acids using the two methods, concentration under reduced pressure and SPE, gave comparable recoveries and precision, indicating that both methods could potentially be used for the preconcentration of amino acids in real water samples.

3.1.4.6 Method Limits of Quantification

For the two methods using SPE and concentration under reduced pressure, the method limits of quantification (MLQ) were calculated for triplicate (n=3) analysis of amino acids (2, 5 and 10 µg L^{-1}) in ultrapure water (Tables 3.1-4 and 3.1-5). Method limits of quantification were determined as the concentrations equivalent to signal to noise (S/N) = 10 by manual S/N calculation on unsmoothed chromatograms. The MLQ of the amino acids in the method using SPE as the preconcentration method were 0.1-100 µg L^{-1} as N (median: 20 µg L^{-1} as N) (Table 3.1-4), with the exception of arginine and cysteine which were not detected (discussed in Section 3.4.1) and histidine, glycine and serine which were not quantified (discussed in Section 3.4.2). The MLQ of most amino acids using the concentration under reduced pressure preconcentration method in ultrapure water was 0.1-40 µg L^{-1} as N (median: 1 µg L^{-1} as N) (Table 3.1-5), with the exception of cysteine which was not detected. The MLQ of amino acids using the concentration under reduced pressure preconcentration method was lower than the MLQ of amino acids using the SPE method due to the better absolute recovery of amino acids when using concentration under reduced pressure. Both analytical methods, therefore have the potential to be used for the analysis of amino acids in natural waters, since free amino acids have previously been found to be present in natural waters in the range of 1 - 80 µg L^{-1} as N (Chinn and Barrett 2000, Dotson and Westerhoff 2009, Thurman 1985). For real water samples, QA/QC, including the recoveries of amino acids, was assured by standard addition of amino acids to selected real water samples for each batch of samples processed.

Solid phase extraction was chosen for the preconcentration of amino acids in the rest of this study due to fact that SPE included a sample clean-up which reduced the ion suppression caused by real water samples and thus was more suitable for use as the preconcentration method for the detection of amino acids using mass spectrometry.

Table 3.1-5: Accuracy, precision and method limit of quantification (MLQ) achieved in analysis of amino acids (2, 5 and 10 µg L^{-1}) in ultrapure water using the concentration under reduced pressure preconcentration method. Recoveries are presented as average over the 3 concentrations in triplicate (*n*=9) of all analyses.

Name	**Recovery (%)**	**Precision (RSD %)**	**MLQ (µg L^{-1} as N)**
Lysine	20	35	30
Arginine	25	25	10
Histidine	20	35	40
Glycine	120	10	10
Serine	120	20	5
Asparagine	110	15	1
Alanine	90	10	5
Glutamine	95	20	1
Thereonine	115	20	1
Glutamic Acid	125	15	0.5
Aspartic Acid	85	10	5
Cysteine	N.D.	N.D.	N.D.
Proline	85	10	0.5
Valine	100	10	0.5
Methionine	90	40	1
Isoleucine	105	10	1
Leucine	100	10	1
Tyrosine	90	5	0.5
Phenylalanine	105	10	0.5
Tryptophan	70	10	0.1
Median	**95**	**10**	**1**

N.D. - not detected.

3.1.4.7 Application of the SPE LC-MS/MS Method to Drinking Water Source Waters

The free amino acid concentrations of three different surface waters (Surface waters A-C) were measured (Table 3.1-6) using the developed analytical method of SPE preconcentration followed by LC-MS/MS, with six amino acids present above their MLQs and the total free amino acid concentrations being 15, 16 and 26 µg L^{-1} as N for Surface Waters A, B and C, respectively. The developed method therefore shows promise for the detection and determination of amino acids in natural waters.

Table 3.1-6: Concentration (µg L^{-1} as N) of measured free amino acids for surface waters A, B and C. Arginine and cysteine were below their method limit of detection, while the other amino acids not listed were detected, but were present in concentrations below their respective method quantification limit

Name	Surface water A	Surface water B	Surface water C
Proline	<2	2	2
Isoleucine	3	3	5
Leucine	4	4	8
Tyrosine	4	4	7
Phenylalanine	3	3	4
Tryptophan	1	2	1
Total free	**15**	**16**	**26**

These concentrations are higher than total free amino acid concentrations measured in previous studies on surface waters in the USA analysed by spectroscopic detection with derivatisation, i.e., 7 µg L^{-1} as N (Chinn and Barrett 2000) and 0.69 µg L^{-1} as N (Dotson and Westerhoff 2009). The three amino acids present in highest concentrations in Surface Waters A-C were tyrosine, leucine and isoleucine, however the three amino acids present in highest concentrations in the previous studies (Chinn and Barrett 2000, Dotson and Westerhoff 2009) were alanine or histidine, serine and glycine.

Natural variation and/or analytical variation may account for the differences in measured amino acid concentrations in natural waters. According to Chinn and Barrett (Chinn and Barrett 2000), the concentrations of amino acids in water bodies change over time, and a single analysis may not capture all variations. The composition and concentration of natural organic matter, and thus naturally occurring amino acids, were reported to be very specific for each natural water source (Krasner et al. 1996) and strongly depended on biological activity (algae bloom) and season. In terms of

analytical variation, the use of derivatisation followed by UV detection in previous studies (Chinn and Barrett 2000, Dotson and Westerhoff 2009) may have resulted in lower concentrations of amino acids measured due to incomplete derivatisation, compared to the current mass spectrometric detection method without derivatisation. Another possible reason for the higher concentrations of amino acids detected in the current study may be due to the beneficial use of surrogate standards which allowed for correction from matrix effects and recoveries. In addition, 18 amino acids were analysed in the current study, as compared to only 16 amino acids analysed in previous studies (Chinn and Barrett 2000, Dotson and Westerhoff 2009), possibly resulting in differences in concentration and composition of total amino acids. Histidine, serine and glycine were the most abundant amino acids in natural waters in previous studies (Chinn and Barrett 2000, Dotson and Westerhoff 2009); however, these amino acids were not quantified in this study, resulting in differences in composition of total free amino acids in this study compared to previous studies.

3.1.5 Conclusions

A novel analytical method for the analysis of amino acids in natural waters, using SPE as the extraction and preconcentration method followed by separation and detection using LC-MS/MS, was developed and optimised. In the method, 18 out of the 20 amino acids tested could be successfully analysed, however, histidine, glycine and serine could only be semi-quantified due to exogenous matrix effects from the SPE cartridge. An alternative preconcentration method using concentration under reduced pressure was tested and it allowed for the analysis of 19 amino acids in ultrapure water. However, it is not suitable as a preconcentration method for natural waters as it does not incorporate a sample clean-up step, which could result in up to 100 % signal suppression for almost all amino acids. Although preconcentration using concentration under reduced pressure provided better recoveries, precision, and MLQs in ultrapure water, SPE was found to be a more suitable extraction and preconcentration method, as it incorporates a sample clean-up step, thus minimising matrix effects from real water samples. The developed analytical method using the SPE preconcentration step was successfully applied to the analysis of free amino acids in three surface water samples used as drinking water source waters. The average total free amino acid concentration in the natural water samples in this study was found to be 19 µg L^{-1} as N and the most abundant amino acids were found to be tyrosine, leucine and isoleucine.

Since the concentrations of amino acids vary in different source waters, it is necessary to characterise the amino acids in each water source to be able to optimise treatment methods to minimise the formation of DBPs from amino acids and prevent overestimation of disinfection capacity.

3.1.6 Acknowledgements

The authors would like to acknowledge the Australian Research Council LP110100548, Water Research Australia (project 1083) and the Water Corporation of Western Australia (WCWA) for funding this work, and Curtin University (Curtin International Postgraduate Research Scholarship) and Water Research Australia (WaterRA PhD Scholarship) for providing financial support for Z. How. The authors would also like to thank staff from WCWA for assistance in sample collection.

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Chapter 3.2 Detection Methods to Monitor the Degradation of Organic Chloramines

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Chapter 31 in Disinfection By-products in Drinking Water, 2015, Thompson, C., Gillespie, S. and Goslan, E. (eds), pp. 267-276, Royal Society of Chemistry, Cambridge, UK.
ISBN: 978-1-78262-088-4

Statement of Contribution to Co-authored Book Chapter

This Chapter includes the co-authored book chapter *'Detection methods to monitor the degradation of organic chloramines',* published in 'Disinfection By-products in Drinking Water'. The bibliographic details of the co-authored book chapter, including all authors are:

How, Z. T., Linge, K. L., Busetti, F. & Joll, C. A. 2015. Chapter 31: Detection methods to monitor the degradation of organic chloramines, in Disinfection By-products in Drinking Water, *The Royal Society of Chemistry,* 267-276.

I, Zuo Tong How, contributed to this book chapter by conducting all the laboratory work and data analysis, acting as the primary author, including writing and editing the manuscript and creating all figures and tables.

As Co-author, I endorse that the level of contribution by the candidate that is described above is accurate.

Kathryn Linge

Francesco Busetti

Cynthia Joll

3.2.1 Abstract

Organic chloramines have been found to form in drinking water systems and they have been reported to potentially cause cytotoxic and genotoxic effects even at micromolar concentration levels. However, little information on the occurrence and toxicity of organic chloramines in drinking water is currently available. In this study, a triiodide colorimetric method, which was a modified standard iodometric method for colorimetric measurement, was used to determine the formation of organic chloramines that were not suitable for direct UV detection due to interference peaks in the 250 to 280 nm range. The triiodide colorimetric and direct UV methods were successfully applied to determine the rate of degradation of *N*-chloroglycine, *N*-chloroserine, *N*-chloroasparagine and *N*-chlorophenylalanine. The stability of these organic chloramines was found to vary, with their half-life ranging from 15 to 5775 min. The stability of the organic chloramines appeared to increase with increasing basicity of the amine nitrogen. The half-life values found for some of the organic chloramines investigated in this study suggest that their presence and persistence may be relevant in drinking water systems. In order to better characterise the risk associated with the occurrence of organic chloramines in drinking water, the stabilities of a wider range of organic chloramines should be investigated.

3.2.2 Introduction

Organic chloramines have been predicted to be potentially cytotoxic and genotoxic and some organic chloramines, like *N*-chloroglycine, were found to be cytotoxic and genotoxic even at micromolar concentrations (Laingam et al. 2012). Recently, it was recommended (Bull et al. 2011) to give high priority to the characterisation of toxicological properties of organic chloramines with varying physical-chemical properties, as they are known to form in drinking water. However, little information is currently available on the occurrence or stability of organic chloramines in drinking water.

Organic chloramines may form when dissolved organic nitrogen-containing compounds, such as amino acids, amides and amines, present in water systems react with free chlorine (Hunter and Faust 1967) or inorganic chloramines (Isaac and Morris 1983, Snyder 1982) used for disinfection. However, the main pathway of formation of organic chloramines is considered to be involving reactions between amino acids and

free chlorine (Ellis and Soper 1954, Yoon and Jensen 1993). After formation, organic chloramines typically degrade to other disinfection by-products, including aldehydes and nitriles (Nweke and Scully 1989). Depending on the chemical structure, the half-life of an organic chloramine may vary from 13 minutes to more than 48 hours (Armesto et al. 1996, Hand et al. 1983, Scully and Bempong 1982). Organic chloramine stability is affected by many factors, although, in general, more basic organic chloramines have been found to be more stable (Pitman et al. 1969).

A number of analytical methods have been used to detect the presence of organic chloramines in water, including direct UV analysis, use of *N*,*N*-diethyl-*p*-phenylenediamine (DPD) to measure organic chloramines as part of combined chlorine, and iodometric methods to measure organic chloramines as part of total chlorine. When formed, organic chloramines have absorbance lambda maxima (λ_{max}) between 250 and 280 nm, and molar absorptivities of around 350 M^{-1} cm^{-1} (Hand et al. 1983). Many studies have utilised these spectroscopic properties to monitor the formation (Reckhow 2011) and degradation (Hand et al. 1983, Scully and Bempong 1982) of pure organic chloramines by direct UV analysis.

The DPD method measures the concentration of free chlorine and chloramines (commonly known as combined chlorine, and including both organic and inorganic chloramines) in a sample by the spontaneous reaction between free chlorine and DPD indicator to produce a red colour in the absence of iodide ion (APHA et al. 2005b). When iodide is subsequently added into samples containing chloramines, the iodide reacts with the chloramines releasing free chlorine to react with the DPD indicator to produce the red colour. A small amount of iodide is added to determine the monochloramines concentration, and excess iodide ion is added to determine the dichloramines concentration, as the excess iodide will release additional chlorine from the dichloramines which will react rapidly with the DPD indicator (APHA et al. 2005b). In fact, the measurement of free chlorine represents all species which can oxidise the DPD indicator reagent to the red coloured species, and is thus often referred to as free chlorine equivalents. Although the DPD method is not able to differentiate between inorganic and organic chloramines, it has previously been used to monitor the formation and degradation of organic chloramines (Laingam et al. 2012, Reckhow

2011) as well as confirm that all free chlorine has reacted (Reckhow 2011, Scully and Bempong 1982).

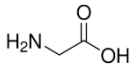
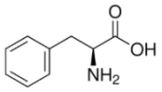
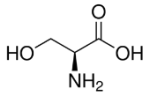
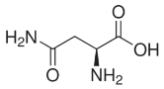
Iodometric titration is a commonly used analytical method to determine the concentration of oxidizing agents (Christian 2004). A strong oxidant, such as free chlorine, oxidises the iodide into triiodide producing a pale yellow solution in an acidic environment. When an excess of iodide is present in the solution, an equivalent amount of triiodide is produced to the amount of oxidant present (Equation 1). The triiodide is then titrated against sodium thiosulfate (Equation 2) until it become colourless; to ensure a better colour contrast, starch indicator is normally used during the titration to determine the end point. Although the iodometric titration cannot differentiate between different oxidants (e.g. between free chlorine and chloramines), previous studies have utilised Reaction (1) from the iodometric titration method to improve the sensitivity of organic chloramines for UV detection by converting the organic chloramines with low absorptivity (ε= 330-450 M^{-1} cm^{-1} at 250 nm) to a compound (triiodide) with higher absorptivity (ε= 25750 M^{-1} cm^{-1} at 353 nm) (Bedner et al. 2002, Reckhow 2011).

$$HOCl + 3I^- + H^+ \rightarrow I_3^- + H_2O + Cl^- \quad (1)$$

$$I_2 + 2S_2O_3^{2-} \rightarrow 2I^- + S_4O_6^{2-} \quad (2)$$

In the current study, a colorimetric measurement method was developed for the detection and measurement of the concentration of all organic chloramines which were not suitable for direct UV measurement due to interference in absorbance from the precursors or the by-products of organic chloramines at wavelengths between 250 and 280 nm. Both direct UV and the colorimetric measurement method were then used for the confirmation of the formation of organic chloramines and investigation of the degradation of selected organic chloramines. Four amino acids, asparagine, glycine, phenylalanine and serine, were selected for this study. This suite of amino acids included polar, non-polar and aromatic amino acids with a range of different amino group basicity (Table 3.2-1).

Table 3.2-1: Molecular weight, classification, pK_a and structures of the amino acids selected in this study

Name	Molecular Weight (g mol^{-1})	Classification	pKa of amino group	Structure
Glycine	75.07	Non-Polar	9.58	
Phenylalanine	165.19	Non-Polar (Aromatic)	9.09	
Serine	105.09	Polar	9.05	
Asparagine	132.12	Polar	8.73	

3.2.3 Experimental

3.2.3.1 Analytical Standards and Chemicals

Amino acids asparagine, glycine, phenylalanine and serine (purity ≥ 97%), aqueous sodium hypochlorite solution (10 – 15% chlorine), thioacetamide, and *N*,*N*-diethyl-*p*-phenylenediamine sulphate (purity ≥ 97%) were purchased from Sigma Aldrich (New South Wales, Australia). Potassium iodide, potassium dihydrogen phosphate and dipotassium hydrogen phosphate were purchased from Ajax FineChem (New South Wales, Australia); glacial acetic acid was purchased from Chem Supply (South Australia, Australia). Ethylenediaminetetraacetic acid was purchased from Asia Pacific Speciality Chemical (New South Wales, Australia), while sulfuric acid (purity 98%) was from Scharlab S.L. (Sentmenat, Spain). Monochlor F reagent was from Hach Pacific (Victoria, Australia). Ultrapure water was purified using an ion exchange system (IBIS Technology, Western Australia, Australia), followed by an Elga Purelab Ultra system with a 0.2 μm filter (Elga, High Wycombe, UK)

3.2.3.2 Formation of Organic Chloramines

Organic chloramines (0.06 or 0.6 mM) were prepared by adding sodium hypochlorite (HOCl) to solutions of the selected amino acids (Table 3.2-1) at a molar ratio of 1:5 (amino acids:HOCl). This molar ratio has been shown to minimise side reactions and ensure that the monochloramine is the major chloramine species formed.(Reckhow 2011) The formation of organic chloramines from glycine and serine was confirmed by performing UV absorbance scans between 195 and 400 nm using an Agilent

Technologies Cary 60 UV-Vis spectrometer (California, USA) with a deuterium arc lamp. As described in Section 3.1, a peak with λ_{max} between 250 and 280 nm is expected in the UV spectra if an organic chloramine is formed (Figure 3.2-1). For organic chloramines which have an interference peak at λ = 250 to 280 nm (e.g. phenylalanine), an alternative method to confirm organic chloramine formation was used. Such a method consists of first obtaining a UV spectrum of a chlorine solution followed by a UV spectrum after addition of the organic chloramine's precursor. The disappearance of the free chlorine peak at λ = 292 nm indicates that all chlorine has reacted (Figure 3.2-2), with the assumption that all chlorine reacted to form organic chloramines.

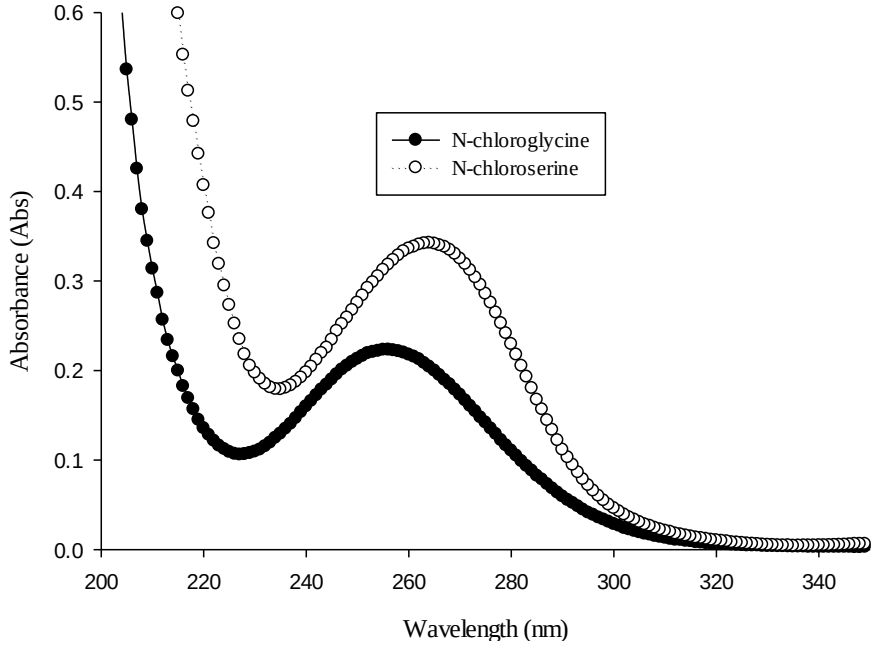


Figure 3.2-1. UV spectra of organic chloramines (0.6mM) formed from chlorination of glycine and serine; a peak with λ_{max} between 250-280 nm confirms the formation of organic chloramines.

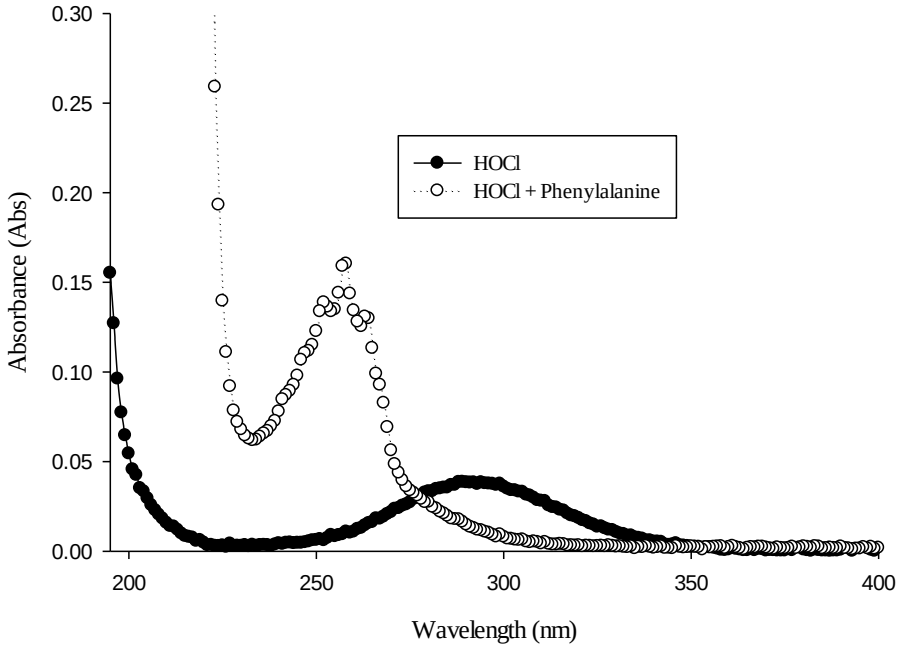


Figure 3.2-2: UV spectra to confirm the reaction of free chlorine and phenylalanine. The disappearance of the peak at $\lambda = 292$ nm indicates that all free chlorine had reacted. The peak at $\lambda = 254$ nm is attributed to the excess phenylalanine present in solution.

3.2.3.3 Measurement of Organic Chloramine Degradation Rates

The rates of degradation of organic chloramines were measured over 60 minutes at time intervals indicated in Table 3.2-2. Two measurement methods were used to determine the rate of formation and degradation of organic chloramines. The degradation of *N*-chloroglycine and *N*-chloroserine were monitored by direct UV measurement of the absorbance at $\lambda = 255$ nm. A colorimetric method, modified from a standard iodometric titration (APHA et al. 2005a) (referred to as the triiodide colorimetric method), was used to measure the degradation of *N*-chloroasparagine and *N*-chlorophenylalanine. For the triiodide colorimetric method, glacial acetic acid (125 µL) and potassium iodide (125 µL of a 15 g L^{-1} solution) were added to 2.5 mL of sample and the absorbance was measured at $\lambda = 353$ nm, assuming a molar absorptivity of triiodide of 25750 M^{-1} cm^{-1} (Plamer et al. 1984).

Table 3.2-2: Time interval and duration used for the measurement of the degradation rates of selected organic chloramines

Direct UV method	Time interval	1 min	5 min	10 min
	Duration	1-10 min	10-30 min	30-60 min
Triiodide method	Time interval	5 min		10 min
	Duration	1-30 min		30-60 min

3.2.4 Results and Discussion

3.2.4.1 Monitoring Formation and Degradation of Organic Chloramines using Direct UV Measurement

A peak at λ = 250 to 280 nm is expected if an organic chloramine is formed, and this was found for both *N*-chloroglycine and *N*-chloroserine (Figure 3.2-1). However, interferences in the UV spectra for both phenylalanine and chlorinated asparagine meant that the direct UV method could not be used to monitor the formation of organic chloramines from these amino acids. Comparison of the UV spectra for all tested amino acids (Figure 3.2-3) shows that phenylalanine has a peak at λ = 254 nm which means that chloramines could not be monitored between λ = 250 and 280 nm. While asparagine did not have an interference peak in the region of λ = 250 to 280 nm in the UV scan of the original amino acid, the degradation of *N*-chloroasparagine resulted in a by-product that had an absorbance at λ = 270 nm (Figure 3.2-4), indicating that direct UV was not a suitable method for the measurement of the degradation of *N*-chloroasparagine.

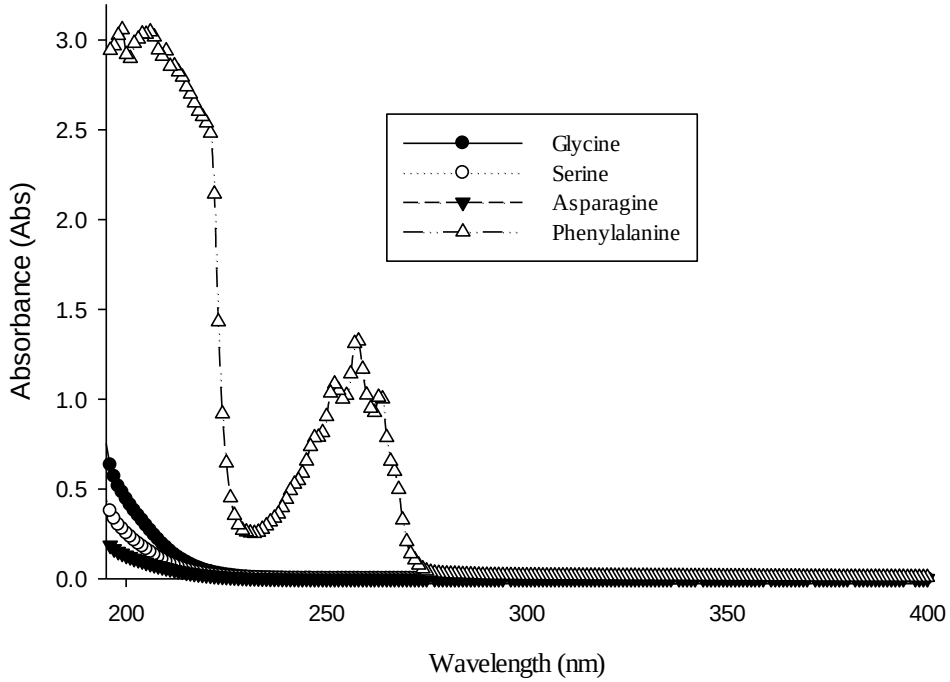


Figure 3.2-3: UV spectra of selected amino acids. Phenylalanine has a peak at 254 nm and therefore the direct UV method was not suitable to measure the degradation of *N*-chlorophenylalanine.

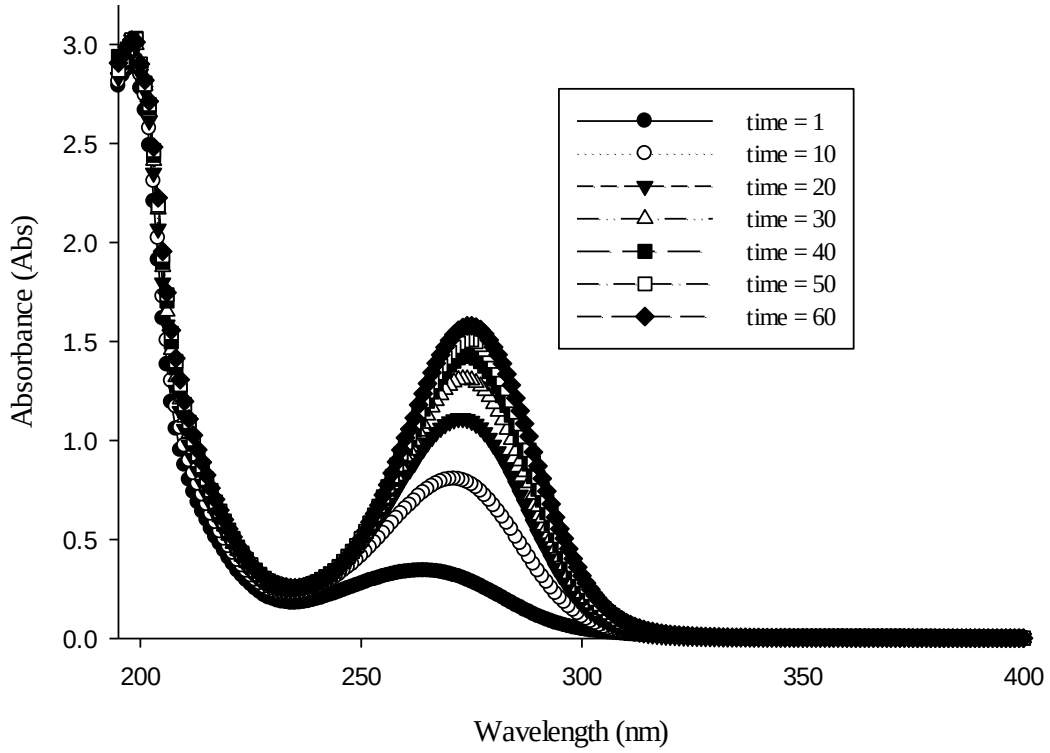


Figure 3.2-4: UV spectra of chlorinated asparagine between 1 to 60 min of chlorination. The increased absorbance and the shift of λ_{max} indicated that the degraded product of *N*-chloroasparagine had similar UV absorbance to the *N*-chloroasparagine. The direct UV method was therefore not suitable for the measurement of the degradation of *N*-chloroasparagine

3.2.4.2 Measurement Method for the Organic Chloramines not suitable for Direct UV Measurement

Direct UV detection is a suitable method for the detection and measurement of a number of organic chloramines. However, as discussed in Section 3.1, not all organic chloramines are suitable for direct UV measurement due to interference peaks between λ = 250 and 280 nm.

For those amino acids that cannot be monitored by direct UV measurement, chloramine rates of degradation can be estimated by measuring the changes in combined chlorine (inorganic and organic chloramines) over time using the standard DPD colorimetric method (4500-Cl G) (APHA et al. 2005c) with the assumption that no inorganic chloramine is present in solution. The DPD method has previously been used to monitor the formation and degradation of organic chloramines, (Laingam et al. 2012, Reckhow 2011) as well as confirm that all free chlorine has reacted (Reckhow 2011, Scully and Bempong 1982). However, in this study it was found that the free chlorine concentration measured by the DPD method was extremely variable,

increasing uncertainty in the combined chlorine measurements. It has previously been found that combined chlorine concentrations higher than 0.5 mg L^{-1}-Cl_2 can breakthrough into the free chlorine reading when using DPD methods (APHA et al. 2005b). The concentration of organic chloramines expected for this study was from 4 to 40 mg L^{-1}-Cl_2, therefore an interference in the free chlorine measurements was expected. While adding thioacetamide immediately after the DPD method is recommended to stop further reaction with combined chlorine (APHA et al. 2005b), this modification did not reduce the variability of the free chlorine concentration in this study. Therefore, the DPD method was not considered to be suitable for the measurement of the degradation of organic chloramines.

As an alternative, the standard iodometric titration method for total chlorine (free chlorine and combined chlorine) (4500-Cl B) (APHA et al. 2005a) was modified for colorimetric analysis (Bedner et al. 2002). Instead of determining the concentration of triiodide by titrating against sodium thiosulfate titrant, the concentration of triiodide was measured at λ = 353 nm. The total chlorine measurement was used as a proxy for organic chloramine concentration, but assuming that all free chlorine reacted with the excess amino acid, and that only organic chloramines formed. The assumption that all chlorine reacted with the amino acids was confirmed by the disappearance of the free chlorine peak at λ = 292 nm, as described in Section 3.2.3.2. It was confirmed that inorganic monochloramine did not form by using analysis with Monochlor F reagent. As di/tri inorganic chloramines could not be detected, it was assumed that they were not formed. Free chlorine was used as an oxidant to obtain the calibration for the response of triiodide to oxidant concentration, and a linear response (R^2=1) was achieved from 0 to 12 mg L^{-1}-Cl_2. Since the concentration of organic chloramines used for this experiment was between 4 to 10 mg L^{-1}-Cl_2, this indicated the degradation of organic chloramines (i.e. change in response) will be linear in the working concentration. Therefore, the triidode method would be a suitable and convenient method for the monitoring of the degradation of organic chloramines. Finally, the degradation of *N*-chloroglycine was measured by both the direct UV and the triiodide methods. The degradation rate constant for *N*-chloroglycine measured by the direct UV method ($k = 0.02 \times 10^{-4}$ s^{-1}) was found to be comparable to that obtained by the triiodide method ($k = 0.03 \times 10^{-4}$ s^{-1}).

3.2.4.3 Measured Organic Chloramine Degradation Rates

The degradation of organic chloramines was monitored by either the direct UV method or triiodide method described in Section 3.2.3.3. The measurements were then used to plot the first-order linear kinetic plot, ln (At/Ao) vs time in seconds, where At was the absorbance at a specific time and Ao was the initial absorbance (Figure 3.2-5). Figure 3.2-5 shows that the degradation of *N*-chloroglycine, *N*-chloroserine, *N*-chloroasparagine and *N*-chlorophenylalanine could all be modelled as pseudo first-order reactions, as the correlation coefficients of their linear regression curves were more than 0.97 for their first-order linear kinetic plots. The rate constants of the degradation of these organic chloramines were determined from the gradient of the linear regression curves of their first-order linear kinetic plots for the 4 organic chloramines, and their half-lifes were calculated using the half-life equation for first-order reactions (Equation 4). The half-life for the selected organic chloramines ranged from 15 to 5775 min (Table 3.2-3). Among the four organic chloramines, only the rate constant for the degradation of *N*-chloroglycine has been previously studied, and this study is the first to determine the rate constants for the degradation of *N*-chloroserine, *N*-chloroasparagine and *N*-chlorophenylalanine. The rate constant of 0.02×10^{-4} s^{-1} for the degradation of *N*-chloroglycine from this study agreed well with the rate constant of 0.04×10^{-4} s^{-1} previously reported by Hand *et. al.*.(Hand et al. 1983) Other studies have also found the degradation of organic chloramines to show first-order kinetics.(Armesto et al. 1996, Scully and Bempong 1982)

$$\text{Half-life} = \frac{\ln(2)}{k}, \text{ where } k \text{ is the rate constant} \qquad (3)$$

The stability of the organic chloramines (based on their half-life) varies greatly, with the non-polar amino acids (glycine and phenylalanine) producing more stable organic chloramines than those produced from the polar amino acids (serine and asparagine). In addition, the trend of the half-life of the selected organic chloramines is consistent with a previous report that more basic organic chloramines are more stable (Pitman et al. 1969), i.e. more basic precursors formed more stable organic chloramines (pK_a values in Table 3.2-1). However, further study with an extended number of amino acids is required to confirm the possible trend of stability with half-life.

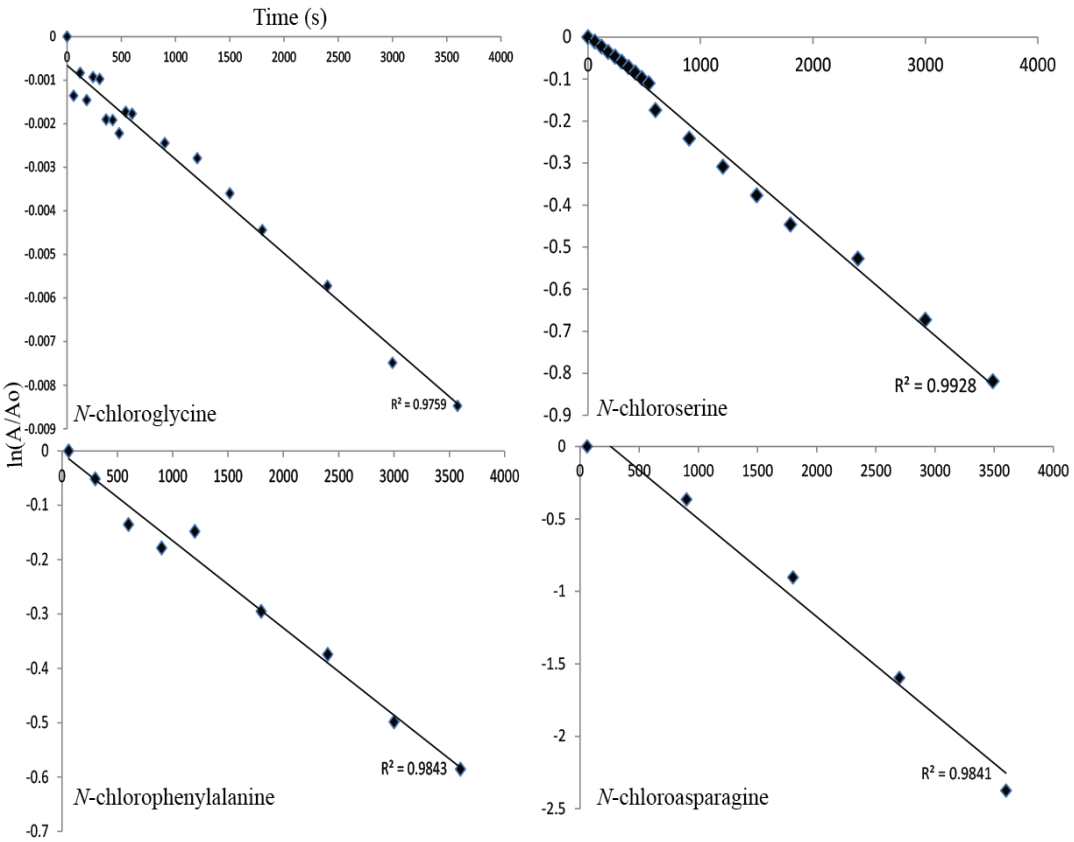


Figure 3.2-5: Pseudo first-order plots for the degradation of the selected organic chloramines

As *N*-chloroglycine was found to be stable (i.e. half-life > 5700 min) and since *N*-chloroglycine is known to be toxic at micromolar concentrations (Laingam et al. 2012), a need for a more in-depth investigation of the occurrence of this species in drinking water is required due to its potential risk to human health. In contrast, organic chloramines with shorter half-life are unlikely to be found in drinking water distribution systems. However, for these less stable organic chloramines, it is still necessary to consider the formation of additional by-products from these organic chloramines to understand their overall risk.

Table 3.2-3: Organic chloramines, and their rate constant of degradation and half-life.

Organic Chloramines	**k ± SD ($x10^{-4}$)(s^{-1}) (n=4)**	**Half-life (min)**
N-Chloroglycine	0.02 ± 0.01	5775
N-Chloroserine	2.1 ± 0.2	55
N-Chloroasparagine	6.7 ± 3	15
N-Chlorophenylalanine	1.6 ± 0.03	75

3.2.5 Conclusions

Two analytical methods, direct UV and an alternative triiodide colorimetric method, were successfully applied to the measurement of the degradation of organic chloramines.

The stability of organic chloramines was found to vary, with their half-life ranging from 15 to 5775 min, where more basic organic chloramines were found to be more stable. Although only *N*-chloroglycine was found to be the stable, more studies on the stability of different organic chloramines are required to better understand the risk associated with the presence of organic chloramines in drinking water.

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Chapter 4. Organic Chloramines in Drinking Water: An Assessment of Formation, Stability, Reactivity and Risk

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Water Research, 93, pp.65-73, 2016

DOI: http://dx.doi.org/10.1016/j.watres.2016.02.006

Statement of Contribution to Co-authored Published Paper

Chapter 4 is comprised of the co-authored paper *'Organic chloramines in drinking water: An assessment offormation, stability, reactivity and risk'*, published in Water Research. The bibliographic details of the co-authored paper, including all authors are:

How, Z. T., Linge, K. L., Busetti, F. & Joll, C. A. 2016. Organic chloramines in drinking water: An assessment of formation, stability, reactivity and risk, *Water Research*, 93, 65-73.

I, Zuo Tong How, contributed to the paper by conducting all the laboratory work and data analysis, acting as the primary author, including writing and editing the manuscript and creating all figures and tables.

As Co-author, I endorse that the level of contribution by the candidate that is describe above is accurate.

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4.1 Abstract

Although organic chloramines are known to form during the disinfection of drinking water with chlorine, little information is currently available on their occurrence or toxicity. In a recent *in vitro* study, some organic chloramines (e.g. *N*-chloroglycine) were found to be cytotoxic and genotoxic even at micromolar concentrations. In this paper, the formation and stability of 21 different organic chloramines, from chlorination of simple amines and amino acids, were studied, and the competition between 20 amino acids during chlorination was also investigated. For comparison, chlorination of two amides was also conducted. The formation and degradation of selected organic chloramines were measured using either direct UV spectroscopic or colorimetric detection. Although cysteine, methionine and tryptophan were the most reactive amino acids towards chlorination, they did not form organic chloramines at the chlorine to precursor molar ratios that were tested. Only 6 out of the 21 organic chloramines formed had a half-life of more than 3 hours, although this group included all organic chloramines formed from amines. A health risk assessment relating stability and reactivity data from this study to toxicity and precursor abundance data from the literature indicated that only *N*-chloroglycine is likely to be of concern due to its stability, toxicity and abundance in water. However, given the stability of organic chloramines formed from amines, more information about the toxicity and precursor abundance for these chloramines is desirable.

4.2 Introduction

Since the discovery in the early 1970s of disinfection by-products (DBPs) in chlorinated drinking water, extensive studies have been undertaken to understand the formation of DBPs and their management (Richardson 2003). Although more than 600 DBPs have now been identified, only minimal information on the occurrence or toxicity of many of these DBPs is available. A recent toxicological study identified several classes of nitrogen-containing DBPs, including haloacetamides, halonitriles and organic halamines, to be of highest interest with respect to potential toxicity (Bull et al. 2011). In addition, Bull et al. (2011) also suggested high priority be given to the characterisation of the toxicological properties of organic halamines that have varying chemical properties (e.g. stability and hydrophobicity). Since then, studies have been conducted on the haloacetamides, halonitriles and many other classes of nitrogen-containing DBPs (Bond et al. 2011, Liew et al. 2012), however there remains very

little published information regarding the occurrence or toxicity of organic halamines in drinking water.

Although water-related toxicological studies of organic chloramines are limited, several biomedical studies of the potential for adverse health effects from organic chloramines have been published. Organic chloramines have been found to cause protein-DNA cross-links (Kulcharyk and Heinecke 2001), inhibit DNA repair (Pero et al. 1996), and affect the rates of cellular apoptosis and the kinetics of the cell cycle (Englert and Shacter 2002, Hosako et al. 2004), which are all common characteristics of carcinogens. A significant *in vitro* cytotoxicity and genotoxicity has also been observed in WIL2-NS cells (human lymphoblastoid) that were treated with *N*-chloroglycine, *N*-chloroethanolamine, *N*-chlorohistamine, and *N*-chlorolysine formed *in situ* at low micromolar concentrations (Laingam et al. 2012).

Organic chloramines may be formed when dissolved organic nitrogen (DON), represented by functional groups such as amino acids, amides and amines within the dissolved organic carbon (DOC), present in water systems reacts with free chlorine (Hunter and Faust 1967) or inorganic chloramines (Isaac and Morris 1983, Snyder 1982). The general equations for the formation of organic (mono/di) chloramines from addition of hypochlorous acid are shown below:

$$R\text{-}NH_2 + HOCl \leftrightarrows R\text{-}NHCl + HOCl \leftrightarrows R\text{-}NCl_2 \quad (1)$$

$$R_2\text{-}NH + HOCl \leftrightarrows R_2\text{-}NCl \quad (2)$$

The reactions between amino acids and free chlorine are believed to be the main contributor to the formation of organic chloramines (Ellis and Soper 1954, Yoon and Jensen 1993). After formation, organic chloramines can degrade to different disinfection by-products, such as aldehydes and nitriles, depending on the chlorine to nitrogen ratio (Nweke and Scully 1989). The stability of different organic chloramines has been reported to be quite variable, ranging from a half-life of 13 minutes to more than 48 hours (Armesto et al. 1996, Hand et al. 1983, Scully and Bempong 1982). In general, more basic organic chloramines are more stable (Pitman et al. 1969). The

presence of an α-hydrogen can reduce organic chloramine stability, as it can promote dehydrohalogenation reactions (Hui and Debiemme-Chouvy 2013).

To accurately assess the risk associated with the presence of organic chloramines in drinking water, it is important to understand both the formation and stability of this class of compounds, in addition to their toxicity. In this study, the formation and degradation of a range of organic chloramines from 21 amino acids and three amines were investigated at pH 7-8. The chlorination of two amides was also studied. A health risk assessment of organic chloramines in drinking water was conducted based on the relationship between the stability and toxicity of the organic chloramines, and also their reactivity and precursor abundance.

4.3 Experimental

4.3.1 Analytical Standards and Chemicals

Amino acids (alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glycine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine) (purity ≥ 97%); amines (dimethylamine, diethylamine and ethanolamine) (purity ≥ 97%); amides (acetamide and acrylamide) (purity ≥ 97%); and aqueous sodium hypochlorite (10 – 15 % chlorine) were purchased from Sigma Aldrich (New South Wales, Australia). The amino acid taurine (purity 99%); was purchased from AK Scientific (California, USA); potassium iodide and formic acid (purity 99%) were purchased from Ajax FineChem (New South Wales, Australia); and glacial acetic acid was purchased from Chem Supply (South Australia, Australia). Monochlor F reagent was purchased from Hach Pacific (Victoria, Australia). The internal standards [^{2}H3] alanine (alanine-d_3), [^{2}H3] leucine (leucine-d_3) and [^{2}H3] glutamic acid (glutamic acid-d_3) were purchased from CDN Isotopes (Quebec, Canada; distributed by SciVac, Hornsby, Australia); [^{2}H2] glycine (glycine-d_2), [^{2}H5] phenyl [^{2}H3] alanine (phenyl-d_5-alanine-d_3) and *N*-acetylglycine were purchased from Sigma Aldrich. Methanol (ChromHR grade) was purchased from Mallinckrodt Baker (New Jersey, USA). Highly purified water (≥18.2 Ω.cm) was produced using an ion exchange system (IBIS Technology, Western Australia, Australia), followed by an Elga Purelab Ultra system with a 0.2 μm filter (Elga, High Wycombe, UK).

The concentration of sodium hypochlorite in the standard chlorination solution was confirmed by measuring the UV absorbance at 292 nm using a Cary 60 UV-Vis spectrometer from Agilent Technologies (California, USA) with a deuterium arc lamp. A molar absorptivity of sodium hypochlorite of 350 M^{-1} cm^{-1} (Morris 1966) was assumed.

4.3.2 Formation of Organic Chloramines

Organic chloramines were prepared by adding sodium hypochlorite to specific precursor aqueous solutions at a molar ratio of sodium hypochlorite to precursors (amino acids and amines) of 0.2. The same ratio was used for the chlorination of the amides. This molar ratio has been shown to minimise side reactions and to promote monochloramine formation (Li et al. 2011). The formation of organic chloramines (and chloramides) was assessed and confirmed by scanning the UV absorbance between λ= 195 and 400 nm, using the Cary 60 UV-Vis spectrophotometer. A peak at λ= 250-280 nm is expected if an organic chloramine (or chloramide) is formed. Some precursors (tyrosine, phenylalanine, tryptophan, dimethylamine, diethylamine, ethanolamine, acetamide and acrylamide) also have UV absorption in the region of λ= 250-280 nm. For these precursors, the formation of organic chloramines (or chloramides) was confirmed by obtaining a UV spectrum (λ= 195 to 400 nm) of the chlorine solution before and after addition of the N-containing precursor. It was assumed that disappearance of the free chlorine peak at 292 nm indicated that all chlorine had reacted, and that only organic chloramines (or chloramides) were formed. All UV measurements of chlorinated precursors were background subtracted using the UV absorbance from the solvent (highly purified water), cuvette and precursors used in the experiment.

4.3.3 Measurement of Degradation of Organic Chloramines

The rates of degradation of organic chloramines were measured over 60 minutes at time intervals as indicated in Table 4-1. The rate was measured four times for each individual organic chloramine. Two different measurement methods described in our previous work (How et al. 2015) were used to determine the rate of formation and degradation of organic chloramines. Briefly, a direct UV method measuring λ= 255 nm was used to measure the degradation of organic chloramines where there was no interference from its precursor or its by-products in the spectral region λ= 250-280 nm.

A triidodide colorimetric method was used for the measurement for organic chloramines with interferences from their precursor or their by-products in the spectral region λ= 250-280 nm. For the triiodide colorimetric method, glacial acetic acid (125 µL) and potassium iodide (125 µL of a 15 g L^{-1} solution) were added to 2.5 mL of sample. Organic chloramines will oxidise the iodide into triiodide, producing a pale yellow solution in an acidic environment. The absorbance of the sample was measured at λ= 353 nm and the concentration of organic chloramine was determined using an external calibration. The triiodide colorimetric method will measure the sum of all oxidants including inorganic monochloramine (MCA), inorganic di/tri-chloramines or organic di/tri-chloramines. Monochlor F was used to measure the concentration of inorganic monochloramine and it was assumed that inorganic di/tri-chloramines were not formed under the reaction conditions used. For both measurement methods, the degradation rate constant was determined from the slope of a least squares linear regression of ln (A_t/A_o) *vs* time where A_o was the absorbance at t=0 and A_t was the absorbance measured at a specific time (t).

Table 4-1: Time interval and duration used for the determination of the stability of selected organic chloramines.

Direct UV method	Time interval	1 min	5 min	10 min
	Duration	1-10 min	10-30 min	30-60 min
Triiodide method	Time interval	5 min		10 min
	Duration	1-30 min		30-60 min

4.3.4 Organic Chloramine Identification using Liquid Chromatography-Ultraviolet Absorbance-High Resolution Mass Spectrometry

In addition to the direct UV method, the formation, and the kinetics of degradation, of *N*-chloroisoleucine and *N*-chlorovaline were investigated using liquid chromatography coupled to both a photodiode array detector and a high resolution mass spectrometer (LC-UV-HRMS). *N*-Chloroisoleucine and *N*-chlorovaline were chosen because their stability (as discussed in Section 4.4.2) made them suitable for investigation by LC-UV-HRMS. Details of the LC-UV-HRMS system and its operation are presented in the Appendix 2. Instrumental conditions are reported in Table A2-1.

The organic chloramines for this experiment were formed using a molar ratio of chlorine to amino acids of 0.8 (as discussed in section 4.4.1) to maximise the formation of monochloramines and to reduce the chance that unreacted amino acids would

overload the LC column. The rate of degradation was determined by measuring the response (ratio of peak area of organic chloramines to peak area of internal standard) of the organic chloramines at 5, 50, 95, 140 and 185 min after chlorination. Because the chromatographic separation took 45 min, degradation was monitored for 185 min to ensure that sufficient data points were obtained. The relative abundance of *N*-chloroisoleucine and *N*-chlorovaline was the peak area ratio of the organic chloramine and the assigned internal standard, *N*-acetylglycine. *N*-Acetylglycine was chosen as an internal standard as it has been reported to be unreactive to free chlorine (Deborde and von Gunten 2008), therefore its concentration should not be affected by the presence of free chlorine. The mass spectra of highly purified water, HOCl, isoleucine and valine were also acquired to ensure that the suspected organic chloramine peak was not originally present in the reagents used. Therefore, any detection of a new peak was assumed to be due to the formation of organic chloramines as the expected degradation products: aldehyde, nitrile and *N*-chloraldimine (Nweke and Scully 1989) do not have an UV absorbance.

4.3.4 Competition between Amino Acids during Chlorination

Competitive chlorination experiments were undertaken in order to determine the relative reactivity of amino acids with chlorine, to provide insight into the likelihood of specific amino acids forming organic chloramines during chlorination of natural waters. The competitive chlorination experiments were conducted by reacting free chlorine with a mixture of 20 amino acids at equal concentration (30 µM) in 15 mL glass vials, with initial chlorine to total amino acid ratios ranging from 0.5 to 2. The mixture was allowed to react for 3 hours in the dark at room temperature, and then the sample was quenched with ascorbic acid solution to stop further reaction. The residual amino acid concentrations were determined using sub-samples (500 µL) of the reaction mixture using our previously reported LC-MS/MS method (How et al. 2014). Briefly, chromatographic separation was achieved using an Agilent 1100 HPLC system. Amino acids were separated using a Gemini C18 column (250 mm x 3 mm I.D., 3 µm particle size) from Phenomenex (New South Wales, Australia) at a flow rate of 150 µL min^{-1}. The amino acids were detected using a Micromass Quattro Ultima Triple Quadrupole Mass Spectrometer (Manchester, UK) fitted with an electrospray ionisation (ESI) interface operated in positive ion mode. Amino acids were quantified

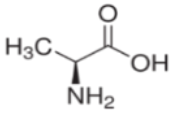
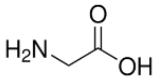
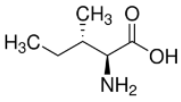
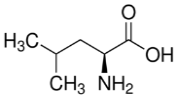
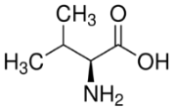
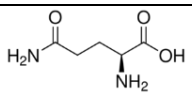
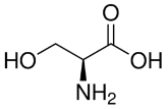
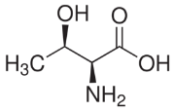
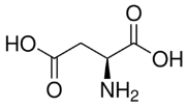
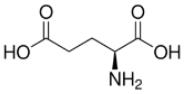
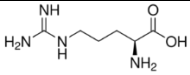
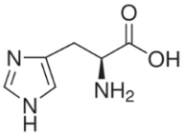
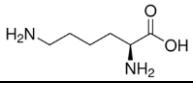
using the ratio of the analyte peak area to the assigned internal standard peak area and an external calibration curve.

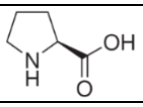
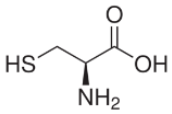
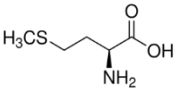
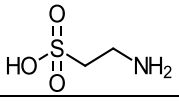
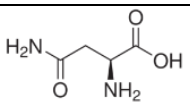
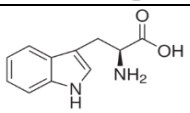
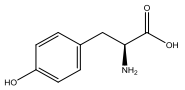
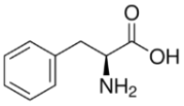
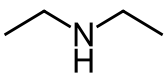
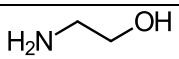
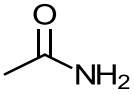
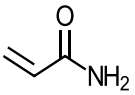
4.4 Results and Discussion

4.4.1 Formation of Organic Chloramines

All three amines and 18 out of the 21 amino acids reacted rapidly with hypochlorous acid (HOCl) to form organic chloramines (Table 4-2). Neither of the two amides tested formed organic chloramides, most likely due to the low reactivity of most amides with HOCl (Deborde and von Gunten 2008). This suggested that stable *N*-chloramides are unlikely to be formed from chlorination. Three amino acids (cysteine, methionine and tryptophan) did not form organic chloramines at the chlorine to precursor ratio used; for these amino acids, HOCl reacted with other functional groups in the molecules that are more reactive than the amino group (e.g. the thiol group for cysteine and methionine, and the indole group for tryptophan) to form other disinfection by-products like the corresponding sulfoxides from cysteine and methionine (Deborde and von Gunten 2008, Na and Olson 2007) and 7-chlorotryptophan from tryptophan (Flecks et al. 2008). It was found in a previous study by Shang et al. (2000) that organic chloramine can form from cysteine at a chlorine to precursor ratio of 4.4. The UV spectra of organic chloramines formed from precursors that do not have an interference at λ= 250-280 nm (Table 4-2) confirmed the formation of organic chloramines from the change in the UV spectrum before and after chlorination. All of these organic chloramines, except *N*-chlorolysine which has two primary amine functional groups that can form chloramines, had a UV spectrum similar to that of *N*-chloroglycine (Figure 4-1).

Table 4-2: Classification, structure, pKa of amine (or amide) nitrogen, and the rate constant of organic chloramine degradation from selected amino acids, amines and amides. Organic chloramines with interferences from their precursor or their by-products in the spectral region between λ= 250-280 nm were measured using a triiodide colorimetric method, rather than direct UV detection at λ= 255 nm and are indicated with an asterisk. Organic chloramines were formed from all amino acids except cysteine, methionine, tryptophan, acetamide and acrylamide.

Precursor	Classification	Structure	pKa of amine nitrogen	Organic chloramine degradation rate constant k ($x10^{-4}$)(s^{-1})	
				This work k ± SD (n=4)	Literature
Measured using direct UV method					
Alanine	Non polar		9.71	1.8 ± 0.1	2.7[a], 2.8[b], 3[c]
Glycine			9.58	0.02 ± 0.01	0.04[d]
Isoleucine			9.60	1.3 ± 0.1	1.97[c], 1.22[e]
Leucine			9.58	2.5 ± 0.01	3.2[c], 1.68[e]
Valine			9.52	1.0 ± 0.2	2[c], 1.22[e]
Glutamine	Polar		9.00	14 ± 1	Not reported
Serine			9.05	2.1 ± 0.2	Not reported
Threonine			8.96	1.2 ± 0.04	2[d]
Aspartic Acid	Acidic		9.66	15 ± 2	Not reported
Glutamic Acid			9.58	3.1 ± 0.3	Not reported
Arginine	Basic		9.00, 12.10	2.3 ± 0.2	Not reported
Histidine			9.09, 6.04	2.0 ± 0.2	Not reported
Lysine			9.16, 10.67	0.42 ± 0.03	Not reported

Proline	Secondary		10.47	56 ± 8	8.8 [d]
Cysteine	Sulfur-containing		10.28	Not Formed	Not reported
Methionine			9.08	Not Formed	Not reported
Taurine	Beta-amino sulfonic acid		9.06	0.02 ± 0.01	Not reported
Measured using triiodide colorimetric method					
Asparagine	Polar		8.73	6.7 ± 3	Not available
Tryptophan			9.34	Not Formed	Not available
Tyrosine	Aromatic		9.04	0.53 ± 0.2	Not available
Phenylalanine			9.09	1.6 ± 0.03	Not available
Dimethylamine	Secondary amine		10.73	0.39 ± 0.06	0.04 [f]
Diethylamine			10.84	0.60 ± 0.09	0.04 [f]
Ethanolamine	Primary amine		9.5	0.05 ± 0.07	Not available
Acetamide	Primary amide		15.1	Not Formed	Not available
Acrylamide			7.9	Not Formed	Not available

SD: standard deviation; [a]Armesto et al. 1996; [b]Stanbro and Smith 1979; [c]Armesto et al. 1993; [d]Hand et al. 1983; [e]Driessen Submitted; [f]Scully and Bempong 1982.

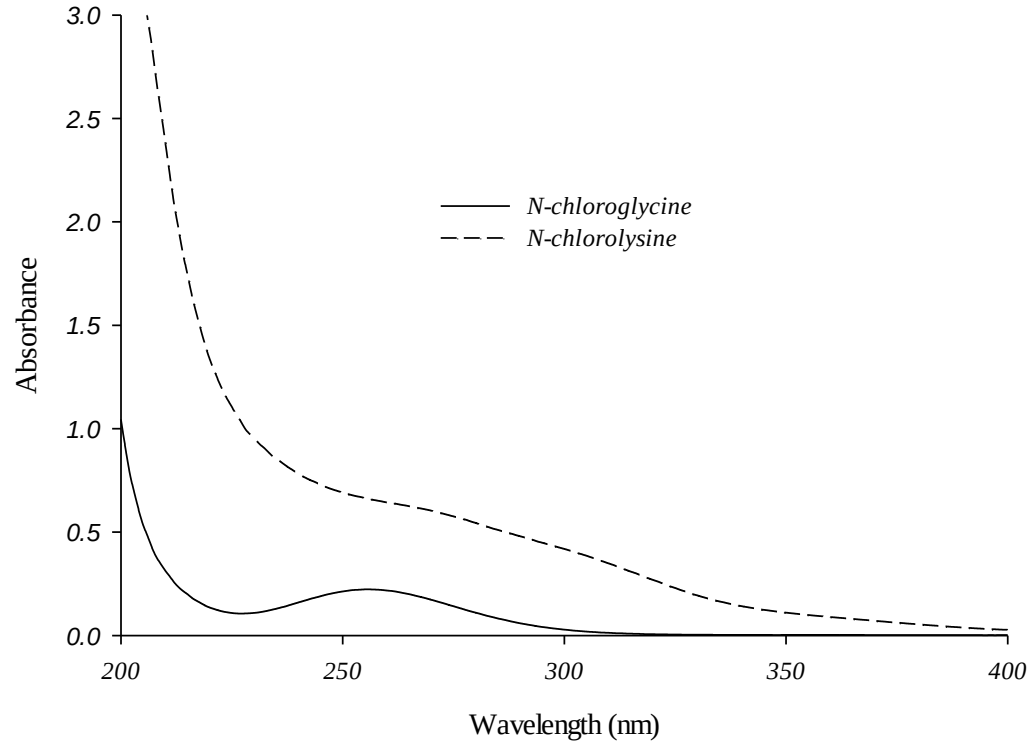


Figure 4-1: UV spectra of *N*-chloroglycine and *N*-chorolysine (0.6 mM). Organic chloramines formed from non-aromatic amino acids have similar UV spectra to *N*-chloroglycine

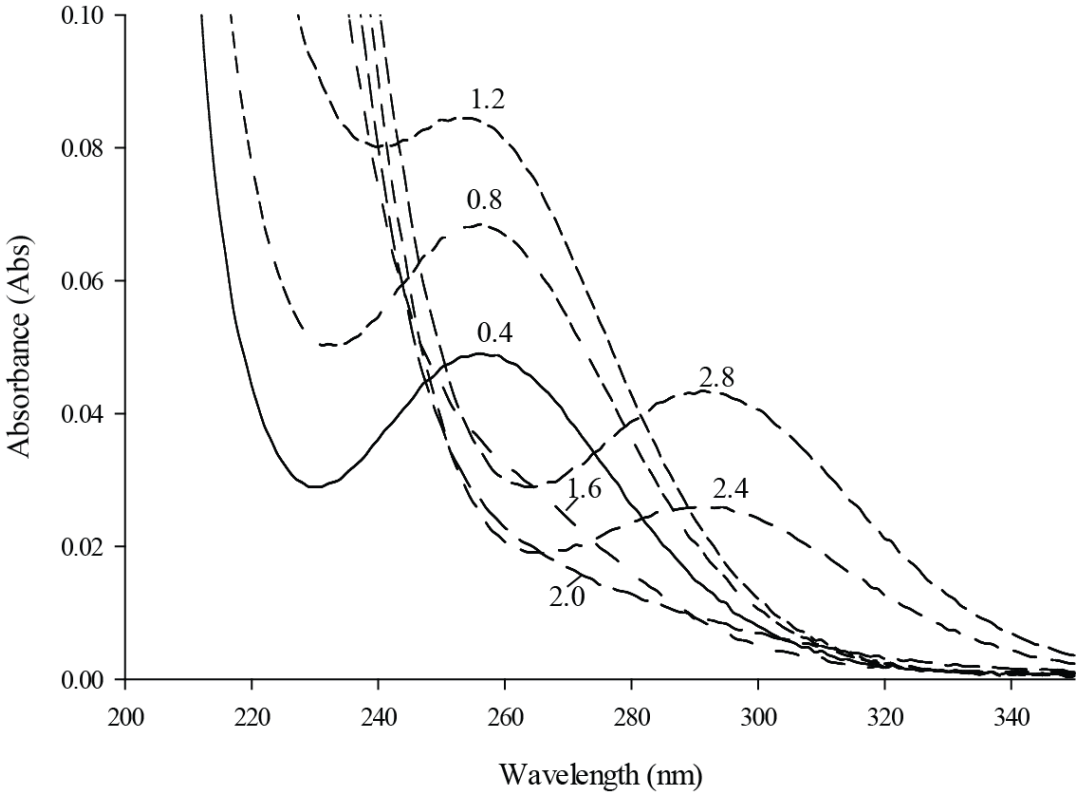


Figure 4-2: UV spectra from chlorination of isoleucine (0.6 mM) at different chlorine to isoleucine molar ratios (0.4-2.8)

In order to investigate the optimal chlorine to amino acids ratio to produce monochloramines, isoleucine was chlorinated at different chlorine to amino acid ratios (0.4 to 2.8) and allowed to react for 30 minutes before direct UV measurement. Figure 4-2 shows that, after background subtraction, the intensity of the peak at $\lambda = 256$ nm, attributed to *N*-chloroisoleucine increased when the molar ratio of chlorine to isoleucine was increased up to 1.2, but then decreased, completely disappearing at a molar ratio of 2. The increased intensity of the peak at $\lambda = 292$ nm above a molar ratio of 2 was attributed to an increased concentration of residual free chlorine, however no additional peaks were observed that could be identified as organic dichloramines in this study. While organic dichloramines may possess similar UV features as inorganic dichloramine ($\lambda = 295$ nm), this finding is in agreement with previous studies which have no identified organic dichloramines by UV-Vis (Conyers and Scully 1993, Freuze at el. 2004, Nweke and Scully 1989).

The increase in absorbance at $\lambda = 256$ nm when the molar ratio was 0.4 to 1.2 and the appearance of free chlorine when the molar ratio was above 2 is in agreement with the study of chlorination of isoleucine at different ratios by Nweke and Scully (1989), where they found that the highest concentration of monochloramine was formed at a chlorine to amino acid molar ratio of 1 and a molar ratio above 1 resulted in the formation of the organic dichloramine. Shang et al. (2000) also demonstrated that organic dichloramine was formed above molar ratio of 1.0 chlorine to amino acids, and that free chlorine was observed above molar ratio of 2.8 or higher depending on the type of amino acids. Therefore, a chlorine to amino acid ratio of 0.8 instead of 1.2 was used to maximise the formation of organic monochloramines, while preventing the formation of organic dichloramines (Section 4.4.3).

4.4.2 Kinetic Study of the Degradation of Organic Chloramines

The degradation of organic chloramines formed at chlorine to precursors' ratio of 0.2 was monitored by either the direct UV method or the triiodide colorimetric method over one hour and empirical rate constants were determined as described in Section 2.3 (Table 4-2). All organic chloramines, except *N*-chloroglutamine, *N*-chloroaspartic acid, *N*-chlorotaurine and *N*-chloroethanolamine, followed a first-order degradation reaction, indicating that their degradation rate was independent of the concentration of the precursors. The coefficients of determination for the first-order degradation of

organic chloramines are listed in Table A2-2. The half-life of each organic chloramine that followed a first-order degradation reaction was determined by calculations using the half-life equation for first-order reactions (Equation 3; Table 4-3). Whilst relatively rapid, the degradation of *N*-chloroglutamine, *N*-chloroaspartic acid and *N*-chloroethanolamine (Figure A2-1) was not linear, indicating that the degradation of these organic chloramines may be dependent upon other factors, such as the concentrations of precursor, by-products or the chloramine concentration, pH or temperature. These factors were not further investigated. The half-life of *N*-chloroglutamine, *N*-chloroaspartic acid and *N*-chloroethanolamine was determined as the time required for the initial concentration in the degradation experiments to be halved (Table 4-3). As *N*-chlorotaurine did not appear to degrade in the allocated monitoring period of one hour, degradation experiments for *N*-chlorotaurine were repeated over an extended period of 3 days, but the concentration of *N*-chlorotaurine was still greater than 50% after 3 days. While the degradation of *N*-chlorotaurine was not studied over an even longer period, it was apparent that *N*-chlorotaurine is very stable, with a half-life > 3 days.

$$\text{Half-life} = \frac{\ln(2)}{k}, \text{ where } k \text{ is the rate constant} \qquad (3)$$

Most of the degradation rate constants determined in this work were in agreement with previously reported values (Armesto et al. 1996, Hand et al. 1983), with the exception of *N*-chloroproline (Hand et al. 1983), *N*-chlorodimethylamine and *N*-chlorodiethylamine (Scully and Bempong 1982), where the rate constants were about one order of magnitude higher in the current study, compared to the respective previous studies. We note that the relative standard deviation of rate constants that we determined for *N*-chloroproline, *N*-chlorodimethylamine and *N*-chlorodiethylamine, being less than 15% over 4 replicate experiments (Table 4-2), indicated good experimental repeatability. One possible reason for the difference between the rate constants in the current study and the previous studies may be differences in the pH. In this study, the pH was about 8 and unbuffered, which is higher than the pH used in previous studies. The degradation rate constant for *N*-chloroproline was previously determined at pH 6.85 using 0.01 M phosphate buffer (Hand et al. 1983), while the degradation rates constants for *N*-chlorodimethylamine and *N*-chlorodiethylamine

were previously determined at pH 7 (Scully and Bempong 1982). The results suggested that higher pH destabilised the chlorine to nitrogen bond in organic monochloramines formed from secondary amines resulting in faster degradation of organic monochloramines. In contrast, Armesto et al. (1993) reported that pH had little impact on the stability of organic monochloramines formed from amino acids. However, the in work by Armesto et al. (1993), only amino acids with primary amines were studied. Further study is therefore required to confirm the impact of pH on the degradation of organic monochloramines formed from secondary amines.

Eleven of the 18 organic chloramines formed from amino acids had a half-life of less than 90 min (Table 4-3). In contrast, all three organic chloramines formed from amines had a half-life of greater than 3 hours (Table 4-3). This suggested that the residence time of drinking water in the distribution system will determine whether these organic chloramines can be detected in drinking water. For example, in many drinking water treatment plants with storage reservoirs, the water may have a residence time of 2-3 days before distribution. This suggests that, if formed, only organic chloramines that are stable for more than 2-3 days, for example *N*-chloroglycine and *N*-chlorotaurine, will enter the distribution system and have the possibility to be detected in distributed drinking water.

Table 4-3: Significance of organic chloramines for drinking water systems. Ranking is based on organic chloramines stability, toxicity, abundance of precursor and the relative reactivity of the precursor with chlorine expressed as the chlorine to amino acid ratio required to react with more than 50% of the individual amino acid after 3 hours.

Precursor	Half-life (min)	Toxicity at µM concentration [a]	Abundance in natural waters	Relative reactivity
Toxic & stable*				
Glycine	5775	Yes	High [b,c,d,e]	1.5
Ethanolamine	2310	Yes	N.A.	N.A.
Lysine	275	Yes	Low [b,c,d,e,f]	1.0
Stable* & High/unknown abundance of precursors				
Taurine	Not determined[#]	No	N.A.	N.A.
Dimethylamine	295	N.A.	N.A.	N.A.
Tyrosine	217	N.A.	High [f]	1.5
Diethylamine	190	N.A.	N.A.	N.A.
Valine	110	N.A.	High [c]	2.0
Threonine	100	N.A.	High [c]	2.0
Stable but low abundance of precursors; Moderately stable* but high abundance of precursors				
Isoleucine	90	No	Low [b,c,d,e,f]	2.0
Phenylalanine	75	No	High [f]	1.5
Alanine	65	N.A.	High [b,c,d,e]	1.5
Serine	55	N.A.	High [b,c,d,e]	1.5
Leucine	45	No	High [f]	1.5
Glutamic Acid	35	No	High [b,c]	1.5
Moderately stable* & low abundance of precursors; very unstable*				
Histidine	60	No	Low [b,c,d,e,f]	1.0
Arginine	50	No	Low [b,c,d,e,f]	1.0
Asparagine	15	N.A.	Low [b,c,d,e,f]	1.5
Glutamine	10	N.A.	Low [b,c,d,e,f]	1.5
Aspartic Acid	10	N.A.	High [b,c]	1.5
Proline	2	N.A.	High [d]	1.5

[a]Laingam et al. 2012, [b]Thurman 1985, [c]Berne et al. 1994, [d]Chinn and Barrett Sylvia 2000, [e]Dotson and Westerhoff 2009, [f]How et al. 2014

*Stable=half-life ≥90 min, moderately stable=half-life 16-89 min and unstable=half-life ≤15 min

[#]Half-life of *N*-chlorotaurine was not determined as no significant decrease in concentration after 3 days

N.A. = No available data

High abundance = Reported as top 5 contributors to total amino acids in water; low abundance = Amino acids that were not reported as main contributors to total amino acids.

Overall, the results showed that more basic organic chloramines (based on the pKa values of the amine nitrogen of the non-chlorinated amines; actual pKa values of chlorinated amine nitrogen are unknown but are assumed to follow a similar trend) were more stable, in agreement with previous research by Pitman et al. (1969), where more basic amines were reported to form more stable organic chloramines. For example, in the current study, basic amino acids like lysine, histidine and arginine formed more stable organic chloramines (half-lives of 275, 60 and 50 min, respectively) compared to acidic amino acids like glutamic acid and aspartic acid (half-lives of 35 and 10 min, respectively). Taurine, the only β-amino acid and also the only sulfonic acid tested, formed a more stable organic chloramine than any of the α-amino acids tested. This result is consistent with the finding of Gottardi et al. (2013) that β-amino acids form more stable organic chloramines compared to α-amino acids. The result also suggested that dehydrohalogenation may be an important mechanism in organic chloramine degradation, since, for example, *N*-chlorotaurine (a β-amino acid) was found to be the most stable organic chloramine, which is consistent with the lower reactivity of the hydrogen involved in the dehydrohalogenation reaction of β-amino acids (in the less reactive β- position to the carboxylic acid group) compared to the α-amino acids (more reactive α- position to the carboxylic acid group). In contrast, dehydrohalogenation becomes increasingly more likely for α-amino acids like *N*-chloroglycine and *N*-chloroleucine, and this is reflected in their decreased half-lives (5775 and 90 minutes, respectively). A similar trend was also reported in the work by Hui and Debiemme-Chouvy (2013) who found that β-amino acids formed more stable organic chloramines compared to α-amino acids. The lower stability of *N*-chloroleucine, compared to *N*-chloroglycine, is attributed to the additional alkane group on the α-carbon, as previous research has shown that α-amino acids with more substituents at the α-carbon are less stable (Hand et al. 1983).

4.4.3 Identification and Confirmation of Degradation Rates of Organic Chloramines using Liquid Chromatography-Ultraviolet Absorbance-High Resolution Mass Spectrometry

Identification of organic chloramines by LC-UV-HRMS required organic chloramines that would be relatively stable during the chromatographic separation (45 minutes) and detectable by HRMS. While very stable, the organic chloramines formed from the amines selected in this study resulted not detectable by LC-HRMS possibly due to

their poor ionisation/degradation in the ion source. Therefore, *N*-chloroisoleucine and *N*-chlorovaline, with half-lives of 90 and 110 min, respectively, were chosen for analysis by the LC-UV-HRMS technique, as they were relatively stable, but had half-lives short enough to allow analysis of degradation rates without more than 24 hours of LC-UV-HRMS instrument time. In addition, LC-HRMS showed good analytical sensitivity for *N*-chloroisoleucine and *N*-chlorovaline by LC-HRMS.

The organic chloramines, *N*-chloroisoleucine and *N*-chlorovaline, were formed using a molar ratio of chlorine to amino acid of 0.8 to maximise the formation of the monochloramine. The rate of degradation was determined by measuring the response (ratio of peak areas of *N*-chloroisoleucine and *N*-chlorovaline to *N*-acetylglycine) of the organic chloramines at 5, 50, 95, 140 and 185 minutes after chlorination. The formation of *N*-chloroisoleucine (Figure A2-2) and *N*-chlorovaline (Figure A2-3) was confirmed by LC-UV-HRMS. Retention times (t_R) determined by measuring λ= 250 nm on the UV detector (*N*-chloroisoleucine ~30 min and *N*-chlorovaline ~28 min) were similar to t_R determined by monitoring the target mass by HRMS (*N*-chloroisoleucine: *m/z*=166.0628 at ~31 min; *N*-chlorovaline: *m/z*=152.0466 at ~29 min). The mass accuracies of the ions detected were also within the limit of <5 ppm relative error (0.6212 ppm for *N*-chloroisoleucine and 4.754 ppm for *N*-chlorovaline). Identities of the suspected *N*-chloroisoleucine and *N*-chlorovaline were further confirmed by the $HRMS^2$ fragments (structures presented in Table A2-3) and comparison of measured isotopic patterns to isotopic patterns simulated by the X-calibur software (Figures A2-4 and A2-5).

The rate constants for the degradation of *N*-chloroisoleucine and *N*-chlorovaline derived by LC-UV-HRMS were 2.2×10^{-4} and 1.9×10^{-4} s^{-1}, respectively, which was in good agreement with the rate constants obtained from the direct UV measurement (1.3×10^{-4} and 1.0×10^{-4} s^{-1}, respectively) and with those reported previously (Table 4-2), with slight differences potentially attributable to the longer duration of monitoring for LC-UV-HRMS (3 hours, compared to 1 hour for the UV measurement).

4.4.4 Competition of Amino Acids during Chlorination

Experiments were conducted to determine the relative reactivity of 20 amino acids with chlorine to provide insight into the likelihood of specific amino acids to form

organic chloramines during chlorination of natural waters. The competitive chlorination experiments were conducted by addition of varying free chlorine concentrations to a mixture of 20 amino acids at equal concentration (30 μM each). The reaction mixtures were allowed to react for 3 hours in the dark at room temperature. The samples were analysed for their residual amino acids after quenching with ascorbic acid solution to stop further reaction with the free chlorine or combined chlorine. When the chlorine to total amino acid molar ratio was 0.5, the final concentration of most amino acids was greater than 90% of the initial concentration (Table 4-4). However, the concentrations of cysteine, methionine and tryptophan were below their limits of detection, indicating that these three amino acids were the most reactive and had reacted completely, although not reacting to form organic chloramines (Section 4.4.1). This high reactivity of cysteine and methionine is in agreement with studies by Shang et al. (2000) and Na and Olson (2007) where sulphur-containing amino acids were found to be the most reactive to chlorination. When equimolar concentrations of chlorine and total amino acids were present (i.e. a molar ratio of 1), six amino acids (isoleucine, leucine, valine, serine, threonine and proline) were still present at concentrations greater than 80% of the initial concentration. This suggested that free chlorine preferentially reacted with the other, more reactive amino acids, or their formed chloramines, rather than form organic chloramines from these six, less reactive amino acids. At a molar ratio of 1.5, most amino acids were found to have reacted with chlorine with the exception of phenylalanine, isoleucine, valine and threonine, where, even at a molar ratio of 2, they were still present at concentrations greater than 20-40% of the initial concentration, indicating that they were the least reactive amino acids with chlorine.

Table 4-4: Percentage of amino acids remaining 3 hours after chlorination at different ratios of chlorine to total amino acids.

Amino acids	Percentage (%) amino acid remaining at different ratios of chlorine to amino acid				Number of reactive sites	Second reactive functional group	Chlorine demand (Hureiki et al. 1994)
	0.5	1.0	1.5	2.0			
Non polar							
Alanine	90±20	60±7	30±4	9±1	1	-	2.8
Glycine	100±9	70±7	40±10	10±6	1	-	5.6
Isoleucine	100±6	80±10	50±6	30±0.5	1	-	2.6
Leucine	100±8	80±8	30±9	9±2	1	-	2.6
Valine	100±10	80±10	60±10	40±0.6	1	-	2.7
Polar							
Asparagine	100±10	50±10	10±3	8±0.7	1	-	5.6
Glutamine	100±10	60±10	20±4	9±0.4	1	-	3.8
Serine	100±20	90±10	30±9	1±1	1	-	5.3
Threonine	100±8	80±9	50±10	40±2	1	-	5.6
Basic							
Arginine	70±10	30±4	10±2	3±0.3	2	guanidinium	8.2
Histidine	50±5	9±3	0.8±0.2	0.4±0.2	2	imidazole	12
Lysine	70±10	30±5	8±2	2±0.8	2	amine	3.8
Acidic							
Aspartic acid	100±7	50±9	10±4	2±0	1	-	5.5
Glutamic acid	100±20	60±8	20±3	4±1	1	-	2.4
Secondary							
Proline	100±10	80±9	40±10	2±2	1	-	5.4
Aromatic							
Phenylalanine	80±8	50±10	30±7	20±0.6	2	benzyl	2.7
Tryptophan	4±6	0.1±0.1	0.2±0.1	0±0	2	phenol	16
Tyrosine	100±9	70±9	30±7	10±2	2	indole	13.4
Sulfur-containing							
Cysteine	0.7±0.1	0.7±0.4	0.3±0.02	0±0	2	thiol	6.2
Methionine	0.3±0.1	0.4±0.1	0.5±0.3	0.1±0.5	2	thiol	6.0

Given it is the practice for water utilities in many countries to maintain residual chlorine in the distributed water (chlorine to amino acids ratio much higher than 2), it could be assumed that the chlorine exposure would normally be sufficient to ensure that all amino acids would react with chlorine and is possible that most amino acids would form organic chloramine in distributed water. These results also confirm that the presence of amino acids and amines will increase chlorine demand in drinking water, in agreement with previous research on amino acids which has shown that between 2.6 and 16 mole of chlorine is required to fully react with each mole of amino acid (Hureiki et al. 1994, White 2010). The commonly reported (Berne et al. 1994, Chinn and Barrett 2000, Dotson and Westerhoff 2009, How et al. 2014, Thurman 1985) free amino acids in natural waters are glycine, tyrosine, valine, threonine, phenylalanine, alanine, serine, leucine, glutamic acid, aspartic acid and proline. Cysteine, methionine and tryptophan, which were found to be more reactive to chlorination, are not commonly detected in natural waters (Berne et al. 1994, Chinn and Barrett 2000, Dotson and Westerhoff 2009, How et al. 2014, Thurman 1985).

4.4.5 Assessing the Significance of Organic Monochloramines in Drinking Water Systems

The significance of organic monochloramine formation from amino acids and amines in drinking water systems was assessed by considering the reactivity of precursors, and formation and stability data collected in this study, to reported data for organic chloramine toxicity (Laingam et al. 2012) and precursor occurrence (Berne et al. 1994, Chinn and Barrett 2000, Dotson and Westerhoff 2009, How et al. 2014, Thurman 1985). The organic monochloramines were ranked by their 1) toxicity, 2) stability (half-life), 3) abundance in natural waters and 4) relative reactivity to chlorination, as described in Table 4-4. From this assessment, *N*-chloroglycine, *N*-chlorolysine and *N*-chloroethanolamine were of highest significance due to their stability and reported toxicity at micromolar concentrations (Laingam et al. 2012). However, lysine has been reported to be of low abundance in natural waters (Berne et al. 1994, Chinn and Barrett 2000, Dotson and Westerhoff 2009, How et al. 2014, Thurman 1985) and no data is available for the occurrence of ethanolamine in water systems, suggesting that *N*-chloroglycine is most likely to be detected in drinking water systems. More research to understand the occurrence and toxicity of the most stable organic chloramines, *N*-chloroglycine, *N*-chloroethanolamine, *N*-chlorotaurine, *N*-chlorotyrosine and *N*-

chlorodimethylamine, in drinking waters is recommended. Given their stability, organic chloramines formed from amines are of particular interest, however, little information is available on their toxicity and abundance of amine precursors in natural waters. Further study of the occurrence of organic chloramines and their precursors, particularly amines, would be beneficial to refine this risk assessment of organic chloramines in drinking water.

4.5 Conclusions

The results from this study suggest that most amines and amino acids form organic chloramines when treated with chlorine. Amino acids containing more reactive side groups (e.g. thiols and indole rings) did not form organic chloramines at the chlorine to amino acid molar ratios tested (cysteine, methionine and tryptophan), but potentially could form at the higher chlorine to amino acid molar ratios found in drinking water. A LC-UV-HRMS analytical method was successfully applied to confirm organic chloramine formation and also to determine the degradation rate of *N*-chloroisoleucine and *N*-chlorovaline. While the organic chloramines produced from amines exhibited half-lives of more than three hours, most of the organic chloramines produced from amino acids were found to have a half-life of less than 90 minutes. In general, the formation of organic chloramines will be most significant for water treatment plants with high dissolved organic nitrogen and direct distribution after chlorination, where minimal time exists for degradation of the organic chloramines between chlorination and distribution. However, the presence of amino acids and amines will increase chlorine demand in all drinking water systems.

Based on a screening health risk assessment of the organic chloramines, *N*-chloroglycine is proposed to be the organic chloramine studied of highest risk due to its stability, toxicity and precursor abundance. More information on the toxicity and precursor abundance for other organic chloramines that are likely to be stable in water distribution systems (e.g. *N*-chloramines and *N*-chlorotaurine) is required to provide a more comprehensive health risk assessment. The degradation products of less stable organic chloramines must also be considered, particularly those from amino acids found to be very reactive with chlorine to form organic chloramines.

4.6 Acknowledgements

The authors would like to acknowledge the Australian Research Council (LP110100548 and LP130100602), Water Corporation of Western Australia and Water Research Australia. Curtin University (Curtin International Postgraduate Research Scholarship) and Water Research Australia (WaterRA PhD Top-Up Scholarship) are also acknowledged for financial support for Z. How. The authors would also like to thank the anonymous reviewers for their suggestions and recommendations.

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Chapter 5. Formation and Degradation Pathways of Organic Chloramines: Speciation and Reaction Rates

5.1 Abstract

Chlorination of amino acids can result in the formation of organic monochloramines or organic dichloramines, depending on the chlorine to amino acid ratio (Cl:AA). After formation, organic chloramines degrade into aldehydes, nitriles and *N*-chloraldimines. In this paper, the speciation of organic chloramines formed from glycine, isoleucine, leucine, lysine, taurine, tyrosine and valine were investigated. With the exception of tyrosine, all amino acids demonstrated similar speciation of formation of the monochloramine at Cl:AA = 0.8 and dichloramine formation at Cl:AA = 2.8, although lysine only formed its dichloramine at Cl:AA $\geq$ 4. Tyrosine formed its monochloramine at Cl:AA = 0.8, but did not form a dichloramine at higher Cl:AA ratios; instead, it degraded into other by-products at Cl:AA = 12 as the phenolic functional group was much more reactive towards chlorination than the chloramine functional group. The rate constant for each reaction in the pathway for the chlorination of valine was determined by modelling and/or from experimental results. At Cl:AA = 2.8, the chlorine was found to first react quickly with valine to form *N*-monochlorovaline (5.4×10^4 $M^{-1}s^{-1}$), with a slower subsequent reaction with *N*-monochlorovaline to form *N*,*N*-dichlorovaline (4.85×10^2 $M^{-1}s^{-1}$), while some of the *N*-monochlorovaline degraded into isobutyraldehyde (1.00×10^{-4} s^{-1}). The *N*,*N*-dichlorovaline then competitively degraded into both isobutyronitrile (1.25×10^{-4} s^{-1}) and *N*-chloroisobutyraldimine (1.15×10^{-4} s^{-1}). Under a practical chlorination for disinfection regime, where a minimum chlorine residual of 0.5 mg L^{-1} may be the goal for drinking water, 30% of valine may be converted to the odorous and stable *N*-chloroisobutyraldimine, which might result in an odour in the drinking water.

5.2 Introduction

Organic chloramines, a subclass of nitrogenous disinfection by-products (N-DBPs), can form in water when organic matter reacts with free chlorine (Hunter and Faust 1967) or inorganic chloramines (Isaac and Morris 1983, Snyder 1982). Organic chloramines were identified by Bull et al. (2011) as a N-DBP subclass of high interest based on their potential carcinogenic hazards, while an *in vitro* study by Laingam et al. (2012) found that *N*-chloroglycine, *N*-chlorolysine, *N*-chloroethanolamine and *N*-chlorohistamine were potentially genotoxic and cytotoxic to humans at parts-per-billion concentrations. Amino acids are believed to be the main contributors to the formation of organic chloramines from organic matter (Ellis and Soper 1954, Yoon

and Jensen 1993). However, organic chloramines formed from amino acids have been found to be unstable (Armesto et al. 1996, How et al. 2016). A study of 18 organic chloramines formed from amino acids found that 12 had a half-life of less than 90 minutes (How et al. 2016). Consequently, as the time delay between chlorination and distribution of treated drinking water increases, it is more likely for consumers to be exposed to the degradation products of organic chloramines, rather than the organic chloramines themselves. Therefore, a full assessment of the health impact of organic chloramine formation in drinking water would benefit from a better understanding of the speciation and degradation pathways of the organic chloramines.

Previous studies of the organic chloramines formed from isoleucine, phenylalanine and valine have shown that organic chloramine degradation by-products retain structural components from the original amino acid precursor (Conyers and Scully 1993, McCormick et al. 1993, Nweke and Scully 1989). The main degradation product identified from organic monochloramine has been reported to be the corresponding aldehyde, while aldehyde, nitrile and *N*-chloraldimine degradation products have all been detected from organic dichloramines (Conyers and Scully 1993). A generalised formation and degradation pathway has previously been proposed to fit these experimental results (Figure 5-1), however not all the species proposed have been confirmed experimentally, in part due to the lack of suitable analytical methods. In addition, rate constants for the reactions in the generalised formation and degradation pathway have not yet been determined.

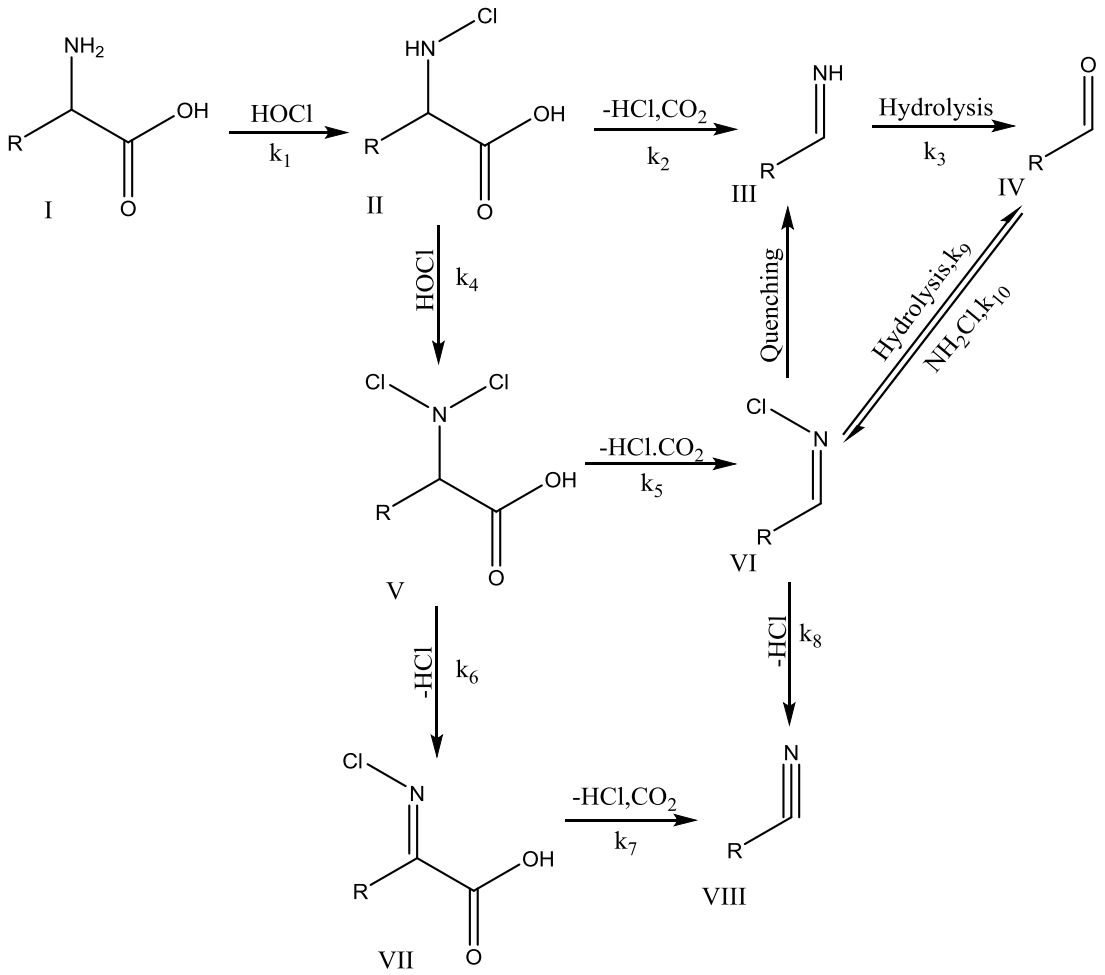

Figure 5-1: Formation and degradation pathway of organic chloramines formed from amino acids (Adapted from Conyers and Scully, 1993)

In this paper, we further investigated the formation and degradation pathways for organic chloramines. The effect of secondary functional groups (apart from the amino acid group) on the speciation of organic chloramines formed from the amino acids, glycine, lysine, tyrosine, isoleucine, leucine, phenylalanine and valine, was studied. To facilitate improved understanding of the organic chloramine degradation pathways, liquid chromatography- and gas chromatography-based mass spectrometric methods were developed for the identification of organic monochloramines, and aldehyde, nitrile and *N*-chloraldimine by-products. Finally, the rates of formation of the aldehyde, nitrile and *N*-chloraldimine were also investigated from chlorination of valine as a model amino acid, using both modelling and experimental results.

5.3 Methods and Materials

5.3.1 Formation of Organic Chloramines

Chemicals and reagents used are detailed in the Appendix 3 Text A3-1. The amino acids used in this study (Table A3-1) were chosen because of their known stability and toxicity (glycine), the presence of specific secondary functional groups (lysine and tyrosine), or because of data existing in previous studies (isoleucine, leucine, phenylalanine and valine). In addition, these amino acids were chosen as their organic chloramines were found to have half-lives of 45 min or longer which was the time required for each chromatographic separation (How et al. 2016). Organic chloramines (0.03 mM or 1.6 mM) were formed by chlorination of individual amino acids (0.04 mM or 2 mM) at chlorine to amino acid molar ratios (Cl:AA) of 0.8, 1.6 and 2.8. The low concentration organic chloramines (0.03 mM) were used for gas chromatography-mass spectrometric (GC-MS) experiments, while the high concentration organic chloramines (1.6 mM) were used for liquid chromatography-high resolution mass spectrometric (LC-HRMS) experiments. These ratios were chosen because previous studies have shown that only organic monochloramines form at Cl:AA = 0.8, a mixture of organic monochloramines and organic dichloramines form at Cl:AA = 1.6, and only organic dichloramines form at Cl:AA = 2.8 (How et al. 2016, Nweke and Scully 1989). The reaction mixtures were allowed to stand in the dark in air tight vials until analysis.

To measure the reaction rates of the formation and degradation of organic monochloramines, chlorinated amino acid solutions were injected into a liquid chromatograph coupled to an ultraviolet detector and a HRMS system (LC-UV-HRMS) 5 min after chlorination, without quenching. Both the amino acid and the organic chloramine were measured at 45 min intervals until 185 min after chlorination. To measure the rate of formation and degradation of by-products from organic chloramines, chlorinated amino acid solutions were also analysed by a headspace solid-phase microextraction GC-MS system (HS SPME-GC-MS), without quenching, at 5 min and 1, 2, 3, 24, 48 and 72 hours after chlorination.

All ^{1}H NMR spectra were obtained using a Bruker Avance III 400 MHz spectrometer (Bruker, Australia), by dissolution of amino acids or the *N*-chloramino acids in 90% water and 10% D_2O, with water signal suppression.

5.3.2 Liquid Chromatography coupled with Ultraviolet and High Resolution Mass Spectrometric (LC-UV-HRMS) Analysis

The chromatographic conditions were as described in our previous work (How et al. 2014). Amino acids and organic chloramines were detected first by UV (λ=255 nm) using an Accela photodiode array detector (Thermo Scientific, Waltham, USA), then by high resolution mass spectrometry (HRMS) using a LTQ Orbitrap XL (Thermo Scientific) fitted with electrospray ionisation operated in either positive or negative ionization mode, as previously described (How et al. 2016). HRMS screening used a full-MS scan in the range 70-300 *m/z* with a mass resolution of 15000 at 400 *m/z*. Organic chloramines were identified by comparing the measured mass and isotope pattern against the theoretical mass (< 5ppm, relative error) and the predicted isotope pattern, while amino acids were confirmed by comparing to their respective analytical standards. Data was processed using X-calibur QualBrowser 2.0.7 SP1 (Thermo Scientific). The HRMS instrumental conditions are provided in the Appendix 3 (Table A3-2).

5.3.3 Headspace Solid-phase Microextraction (HS SPME)-Gas Chromatography-Mass Spectrometric (GC-MS) Analysis

Volatile degradation by-products were extracted using an automated HS SPME method previously developed by our group (Driessen Submitted) using a 75 µm CAR/PDMS fibre. Sodium sulphate (3 g) and the internal standard solution (1,2-dibromopropane-d_6 in methanol, 100 µg L^{-1}, 2 µL) were added to each sample (10 mL in a 20 mL glass vial). Samples were agitated at 500 rpm at 60 °C for 10 minutes before the SPME fibre was inserted into the sample headspace for 15 minutes and then transferred to the GC injector port for thermal desorption at 300 °C for 3 minutes. GC-MS analysis used an Agilent 6890N gas chromatograph coupled with an Agilent 5975 mass selective detector using conditions reported in Table A3-3 available in the Appendix 3. The samples were analysed in both MS scan mode (20 – 320 *m/z*) to screen for the degradation products and single ion monitoring mode to quantify selected aldehydes, nitriles and *N*-chloraldimines (Table A3-4).

5.4 Results and Discussion

5.4.1 Detection and Identification of Organic Monochloramines

The formation of organic monochloramines and/or organic dichloramines (to be referred to as monochloramine and dichloramine) from chlorination of amino acids is controlled by the chlorine to precursor ratio and by the presence of secondary functional groups that may also react with chlorine (How et al. 2016). Formation experiments using a chlorine to amino acid (Cl:AA) ratio of 0.8 were used in this study to produce monochloramines, while avoiding dichloramine formation. In our previous work, we used LC-UV-HRMS to identify both *N*-chloroisoleucine and *N*-chlorovaline formation after the chlorination of isoleucine and valine, respectively (How et al. 2016). The same technique was used to detect and identify monochloramines formed from the chlorination of the other amino acids tested in the current study: glycine, leucine, tyrosine and taurine. For all amino acids, monochloramine peaks were detected in both UV spectra and MS chromatograms, and the mass to charge ratios of these suspected monochloramine peaks were within 5 ppm error of their theoretical values (Table 5-1). In addition, comparison of LC-UV-HRMS chromatograms of unquenched samples and samples quenched with ascorbic acid showed that the suspected organic monochloramine peaks were absent after quenching. As organic monochloramines are oxidants, addition of the quenching reagent would quench the organic monochloramines, resulting in the disappearance of the organic monochloramine peaks. Figure A3-1 shows the disappearance of the suspected *N*-monochlorovaline peak in the quenched solution in formation experiments using valine. Similar results were observed for all other amino acids (data not shown).

Formed organic chloramines always had longer retention times than the corresponding amino acid precursors (Table A3-5). Retention time is correlated to analyte hydrophobicity, with a longer retention time indicating greater hydrophobicity in reverse phase chromatography. Therefore, the organic chloramines formed were more hydrophobic than the amino acid precursors. This increase in hydrophobicity was caused by the substitution of chlorine for hydrogen at the amino nitrogen.

Table 5-1: List of the measured mass of suspected organic monochloramines compared to the theoretical mass. The difference (ppm) in the measured mass and theoretical mass was always less than 5 ppm. Suspected organic chloramines were measured in positive ion mode except for those produced from taurine which were measured in negative ion mode.

Compounds	Measured mass	Theoretical mass	Difference (ppm)
Positive $[M+H]^+$			
N-chloroglycine	110.00002	110.00033	-2.842
N-chloroisoleucine	166.06283	166.06293	-0.620
N-chlorolysine	181.07369	181.07383	-0.784
N-chlorotyrosine	216.04179	216.04220	-1.886
N-chlorovaline	152.04660	152.04728	-4.491
Negative $[M-H]^-$			
N-chlorotaurine	157.96722	157.96732	-0.620

5.4.2 Detection and Identification of Organic Dichloramines

Formation experiments using Cl:AA = 2.8 were designed to preferentially form dichloramines from the amino acid precursors (How et al. 2016), except for lysine where Cl:AA = 4.8 was used, as discussed in more detail below. As dichloramines are, in general, not detectable by UV, suspected dichloramine peaks were confirmed by LC-HRMS, including the exact mass and isotopic ratio. In addition, proton nuclear magnetic resonance (^{1}H NMR) spectroscopy was used to determine the positions of substitution of the chlorine atoms. *N*,*N*-Dichloramine species were not detected by LC-HRMS for glycine, isoleucine, lysine or valine. This might be due to the reported instability of the *N*,*N*-dichloramine species formed from α-amino acids (Coker et al. 2008). However, dichloramine species were detected for lysine, taurine and tyrosine, which was unexpected. The exact mass to charge ratios of detected dichloramines were within 5 ppm of the theoretical values (-2.742 ppm error for lysine, 0.544 ppm error for taurine and -1.886 ppm error for tyrosine). While a dichloramine species was detected from the chlorination of lysine, lysine has two amine nitrogens in its structure and, when chlorinated, can produce a chloramine functional group at both of the nitrogens. At Cl:AA = 0.8, two monochloramine peaks were detected (Figure 5-2a), suggesting that two species of monochloramine were detected. As the Cl:AA ratio increased to 2.8, only one dichloramine peak was detected (Figure 5-2b), most likely the *N*,*N*'-dichlorolysine (rather than *N*,*N*-dichlorolysine or *N*',*N*'-dichlorolysine

(structures presented in Figure A3-2)), as *N*,*N*-dichloramines where the two chlorines are bonded to the same nitrogen have been found to be unstable (Coker et al. 2008) and the *N*,*N*-dichloramines were not detected in this study for glycine, isoleucine, lysine and valine. When the Cl:AA ratio was increased to 4.8, no peaks corresponding to any dichlorolysines were detected. While *N*,*N*,*N*'-trichlorolysine or *N*,*N*',*N*'-trichlorolysine may have been formed, they were not detected by the MS technique, presumably because they each contain a nitrogen bonded to two chlorine atoms, a functional group which is not stable and not sensitive to detection by MS. The formation of *N*,*N*'-dichlorolysine at a Cl:AA ratio of 2 and *N*,*N*',*N*'-trichlorolysine at a Cl:AA ratio of 4 was also observed by Conyers and Scully (1997) through the detection of the corresponding aldehyde, 5-dichloraminopentanal.

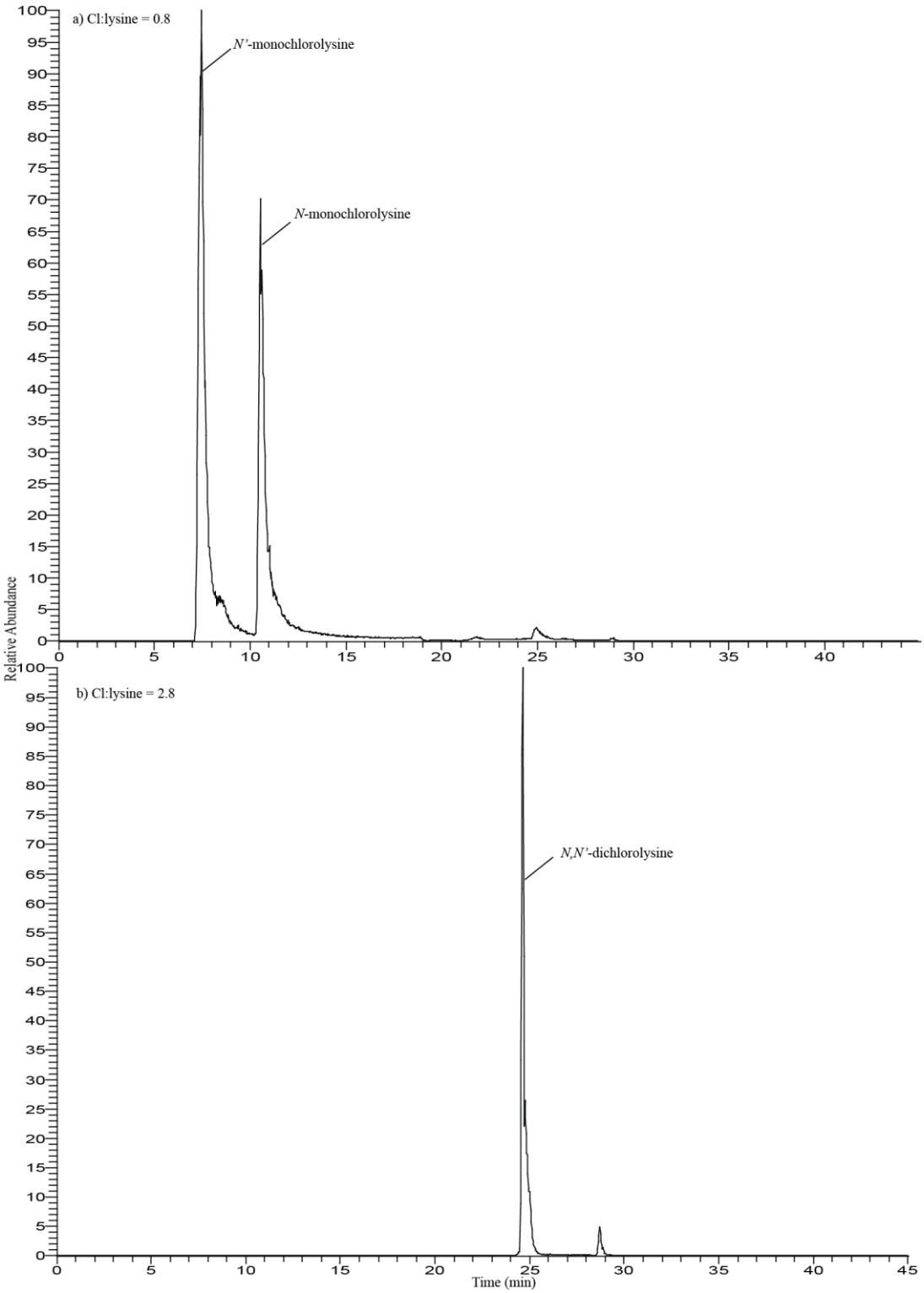

Figure 5-2: a) Two N-monochlorolysine peaks (m/z= 181.0737, at 5 ppm error) were detected in a sample of lysine (1.6 mM) treated with chlorine at Cl:AA = 0.8 by LC-MS using MS scan. b) N,N'-dichlorolysine peak (m/z= 215.0343, at 5 ppm error) was detected in a sample of lysine (1.6 mM) treated with chlorine at Cl:AA = 2.8 by LC-MS using MS scan.

Although mass spectrometry was able to provide the accurate chemical formula of the chlorinated products of tyrosine and taurine based on their accurate mass to charge ratio, MS could not definitively provide information on the position of the chlorine atoms, as the fragments yielded from the fragmentation of the chlorinated products were not diagnostic since chlorine was the first atom to be removed during the fragmentation. Therefore, the chlorinated products of tyrosine and taurine were analysed using ^{1}H NMR spectroscopy. For taurine, the ^{1}H NMR spectrum of the sample suspected to contain *N*,*N*-dichlorotaurine appeared to reflect an approximately 1:1 mixture of taurine starting material or *N*-monochlorotaurine (triplets at δ = 3.18 and 3.36 ppm; each 2H) and *N*,*N*-dichlorotaurine (triplets at δ = 3.16 and 3.34 ppm; each 2H) (methylene region of taurine and the chlorinated taurine sample (ratio Cl:AA = 2.8) shown in Figure 5-3). The peak at δ = 3.27 was an artefact created from the water signal suppression. The two triplets for the dichlorotaurine indicated that it was the *N*,*N*-dichlorotaurine isomer, since chlorine substitution onto a carbon would alter this pattern, while substitution of chlorine onto the sulfonic group is unlikely as the sulfonic group is not reactive to chlorination (Deborde and von Gunten 2008). The presence of remaining taurine starting material or *N*-monochlorotaurine in the ^{1}H NMR spectrum is in contrast to the LC-HRMS spectrum where only a peak for a dichlorotaurine was detected. This difference in progress of the chlorination reaction is presumably due to the different scales of these two experiments, where the concentration of the taurine for the NMR sample was 4 mM which was more than double the concentration (1.6 mM) used in the LC-MS sample of, resulting in some of the taurine remaining unreacted.

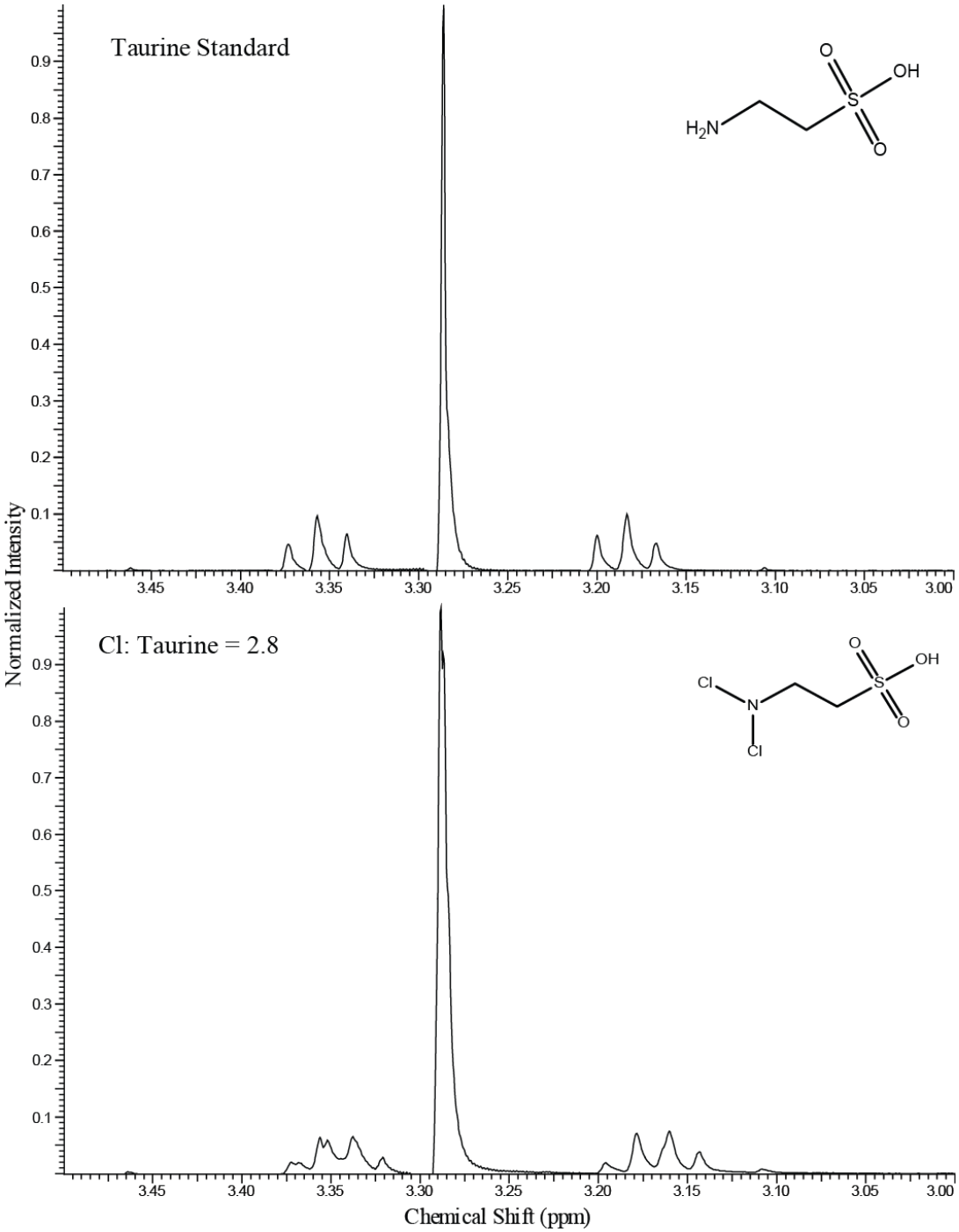

Figure 5-3: ^{1}H Nuclear magnetic resonance spectra (methylene region only) of taurine standard (4 mM) (top) and taurine (4 mM) treated with chlorine at Cl:AA = 2.8 (bottom). The peak at δ = 3.28 is an artefact resulting from the water signal suppression.

As tyrosine has two reactive functional groups (phenol and amino acid), ^{1}H NMR spectra for the samples suspected to contain *N*-monochlorotyrosine and *N,N*-dichlorotyrosine were obtained. The aromatic regions (δ = 6.00-7.50 ppm) of the spectra are presented in Figure 5-4. The aromatic region for tyrosine shows a characteristic AA'XX' pattern, with chemical shifts typical of aromatic protons in phenols (δ = 6.83 and 7.13 ppm), for the four aromatic hydrogens in the *para*-substituted aromatic ring of tyrosine (Figure 5-4a). In the aromatic region of the ^{1}H NMR spectrum of the tyrosine sample treated with chlorine at Cl:AA = 0.8, only the same AA'XX' signal (δ = 6.83 and 7.13 ppm) was observed (Figure 5-4b), indicating that no chlorine atoms were bonded to the aromatic ring and instead that the chlorine was bonded to the nitrogen for *N*-monochlorotyrosine. At Cl:AA = 2.8, a reduced intensity AA'XX' signal (δ = 6.83 and 7.13 ppm) was evident in the ^{1}H NMR spectrum (Figure 5-4c), with two new signals at higher chemical shift (δ = 7.30 and 7.45 ppm). Given the high Cl:AA ratio applied, the signals at δ = 6.83 and 7.13 ppm likely represent *N,N*-dichlorotyrosine. The higher chemical shift and integration ratio of 0.33:1.00 (δ 7.45:7.30 ppm) of the two new aromatic signals is consistent with chlorine substitution on the aromatic ring, where chlorination would occur *ortho* to the phenolic OH group, forming *N*-monochloro-3-chlorotyrosine (2-(chloramino)-3-(3-chloro-4-hydroxyphenyl)propanoic acid). The relative integration of the signals at δ 7.45: 7.30: 7.15: 6.85 ppm was 0.33:1.00:1.63:1.19, suggestive of a mixture of *N*-monochloro-3-chlorotyrosine (XI) and *N*-monochlorotyrosine (IX) or *N,N*-dichlorotyrosine (X) (Figure 5-4c) in a slightly lower than 1:1 ratio. Finally, the Cl:AA ratio was increased to 12. In this experiment, no signals were present in the aromatic region of the ^{1}H NMR spectrum (Figure 5-4d) or the ^{13}C NMR spectrum (Figure A3-3), indicating that further reaction with chlorine had resulted in degradation of the aromatic ring of tyrosine.

Depending on other functional groups in the amino acids, the formation of the *N,N*-dichloramine may occur at higher Cl:AA ratios or other dichloramine isomers may be present. Therefore, all amino acids tested demonstrated similar speciation, with formation of *N*-monochloramino acids at Cl:N ≤ 1 and formation of dichloramino acids at Cl:N ≥ 2, although the detection of *N,N*-dichloramines from α-amino acids using LC-HRMS was usually not possible.

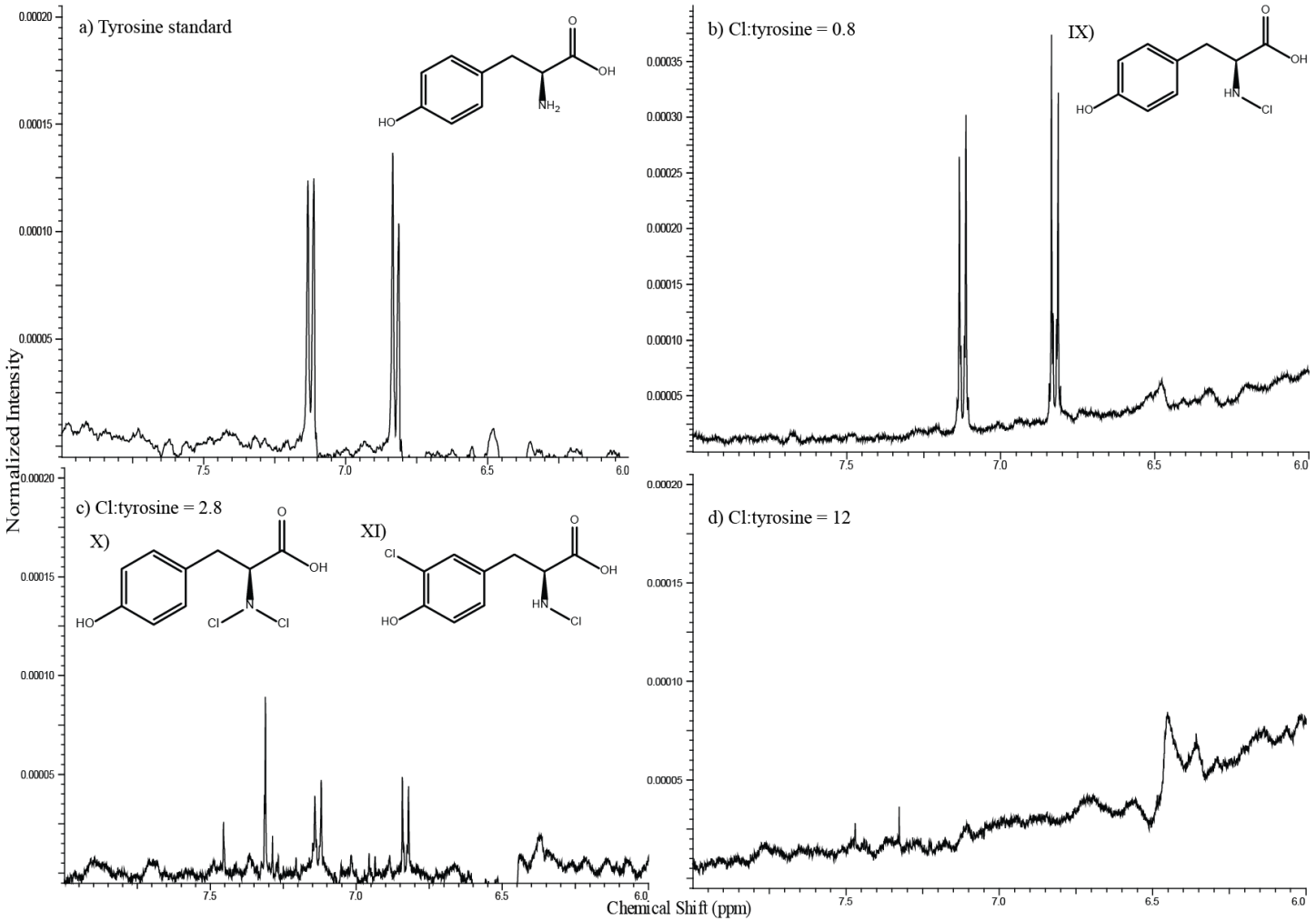

Figure 5-4: ^{1}H Nuclear magnetic resonance spectra (aromatic region only) of tyrosine standard (4 mM) (a) and chlorinated tyrosine (4 mM) at Cl:tyrosine ratio of 0.8 (b), 2.8 (c) and 12 (d).

5.4.3 Detection and Identification of By-products from the Degradation of Organic Chloramines Formed from Amino Acids

After formation from chlorination of amino acids, organic chloramines will degrade into other by-products (aldehydes, nitriles and *N*-chloraldimines) (Conyers and Scully 1993) as shown in Figure 5-1. In order to identify the by-products formed, and further understand the degradation pathways, chlorination studies were undertaken for the amino acids, glycine, isoleucine, leucine, lysine, taurine, tyrosine, phenylalanine and valine. The amino acids were chlorinated at a Cl:AA ratio = 2.8 to ensure that all three by-products (aldehyde, nitrile and *N*-chloraldimine) were formed (Conyers and Scully 1993, Freuze et al. 2004). After 24 hours in the dark in air tight vials, the reaction mixtures were analysed by HS SPME-GC-MS. Treatment of isoleucine, leucine, and valine with chlorine produced aldehydes, nitriles and *N*-chloraldimines (Table A3-6). Only the aldehyde and nitrile were detected from phenylalanine. Previous studies have shown that *N*-chlorophenylacetaldimine, the *N*-chloraldimine formed from phenylalanine, is not volatile enough for headspace analysis (Freuze et al. 2005) and therefore it is likely that *N*-chlorophenylacetaldimine was formed, but not detected. None of the three by-products were detected for chlorination of glycine, lysine, taurine and tyrosine. The fibre used for the HS SPME extraction in this study was suitable for the extraction of non-polar volatile compounds. As the predicted by-products from lysine, taurine and tyrosine are less volatile and more polar than the by-products produced from the chlorination of isoleucine, leucine, phenylalanine and valine, the by-products from lysine, taurine and tyrosine may have been formed but not detected. The by-products from glycine, on the other hand, will be very volatile and would likely elute with the solvent and not be detected in the analysis. Alternatively, organic chloramines like *N*-chloroglycine and *N*-chlorotaurine have been found to be stable (How et al. 2016) and therefore may not have degraded to produce significant concentrations of the three by-products.

The formation of stable aldehydes and *N*-chloraldimines from the chlorination of amino acids could be of significance in drinking water treatment processes as these by-products can result in odour problems in chlorinated water (Brosillon et al. 2009, Froese et al. 1999) (i.e., the odour threshold concentrations of the corresponding aldehydes and *N*-chloraldimines from isoleucine, leucine, phenylalanine and valine

range from 0.15 to 30 µg L^{-1} (Freuze et al. 2005, Froese et al. 1999) and from 0.20 to 3.00 µg L^{-1} (Freuze et al. 2005), respectively).

5.4.4 Investigation of the Reaction Pathways of Degradation of Organic Chloramines using Valine as a Model Compound

In order to better understand the generalised formation and degradation pathways for organic chloramines, the rate of reaction for each pathway was investigated using experimental and modelling methods. The amino acid valine was chosen for this investigation because it was one of the three amino acids for which the *N*-monochloroamino acid and all three degradation products were detected, the other amino acids being leucine and isoleucine. However, *N*-monochlorovaline is more stable than either *N*-monochloroleucine or *N*-monochloroisoleucine (How et al. 2016) and valine is reported to be one of the most abundant amino acids in surface waters (Berne et al. 1994, Brosillon et al. 2009). The reaction rates for each reaction step in the reaction of valine with chlorine were determined experimentally or predicted by modelling using the Kintecus software (Ianni 2015) (as described in the Appendix 3 test A3-2). The rates are presented in Table 5-2.

Table 5-2: Experimental and modelled reaction rates for the reaction pathways in the chlorination of valine.

Reaction	**Experimental**	**Modelled**
Valine + HOCl → *N*-monochlorovaline (k_1)($M^{-1}s^{-1}$)	$5.4x10^{4}$	$5.4x10^{4}$
N-Monochlorovaline → isobutyrimine (k_2) (s^{-1})	$1.0x10^{-4}$	$1.0x10^{-4}$
Isobutyrimine → isobuytraldehyde (k_3) (s^{-1})	Not Measured	$5.0x10^{4}$
N-Monochlorovaline + HOCl → *N*,*N*-dichlorovaline (k_4) ($M^{-1}s^{-1}$)	Not Measured	$4.9x10^{2}$
N,*N*-Dichlorovaline → *N*-chloroisobutyraldimine (k_5) (s^{-1})	$9.2x10^{-5}$	$1.2x10^{-4}$
N,*N*-Dichlorovaline → 2-(chlorimino)-3-methylbutanoic acid (k_6) (s^{-1})	$1.0x10^{-4}$	$1.3x10^{-4}$
2-(Chlorimino)-3-methylbutanoic acid → isobuytronitrile (k_7) (s^{-1})	Not Measured	$1.0x10^{2}$
N-Chlorobutyraldimine → isobuytraldehyde + isobuytronitrile (k_8 + k_{10}) (s^{-1})	$1.1x10^{-6}$	$1.0x10^{-6}$

When the Cl:AA ratio is less than 1, the aldehyde is reported to be formed from the monochloramine through an imine intermediate (structure III in Figure 5-1).(Conyers and Scully 1993) While detection and quantification of this imine would improve understanding of the degradation pathway, our attempts to detect imines using either LC-HRMS or GC-MS were unsuccessful. However, the rate of formation of *N*-monochlorovaline (k_1 = 5.4 x 10^4 $M^{-1}s^{-1}$ or 2.4 x10^2 s^{-1} based on the concentration of HOCl used at 4.48 mM as Cl) and the rate of formation of isobutyraldehyde (1.5 x10^{-4} s^{-1}) were determined experimentally. Comparison of these rates showed that the formation of *N*-monochlorovaline from the chlorination of valine is fast enough to not affect the rate of isobutyraldehyde formation. Therefore, the rate limiting step for the formation of isobutyraldehyde is either the degradation of *N*-chlorovaline into isobutyrimine (k_2) or the degradation of isobutyrimine into isobutyraldehyde (k_3). We previously found the rate of degradation of *N*-chlorovaline to isobutyrimine, k_2, to be 1.0 x 10^{-4} s^{-1} (How et al. 2016), which is similar to the rate of formation of isobutyraldehyde determined in the current study. This suggests that the rate of degradation of isobutyrimine into isobutyraldehyde, k_3, is faster than the degradation of *N*-monochlorovaline into isobutyrimine (k_2), and that formation of isobutyrimine is the rate determining step for formation of isobutyraldehyde from valine. Modelling using the Kintecus software indicated that k_3 = 5.00 x 10^4 s^{-1}, and this suggests that the imine was not detected experimentally due to its instability, with the imine very quickly undergoing hydrolysis to the aldehyde after formation.

It has previously been proposed that the formation of the aldehydes when the Cl:AA ratio is > 2 is through hydrolysis of the *N*-chloraldimine, a by-product of dichloramine degradation (Nweke and Scully 1989), rather than from degradation of the monochloramine (Figure 5-1). However, the same study (Nweke and Scully 1989) and subsequent studies (Conyers and Scully 1993, Freuze et al. 2004) suggested the *N*-chloraldimines are stable. To investigate the formation of the aldehydes from the hydrolysis of the *N*-chloraldimines over time, unquenched chlorinated valine samples (Cl:AA = 2.8) were analysed by HS SPME-GC-MS for isobutyraldehyde, isobutyronitrile and *N*-chloroisobutyraldimine at hourly intervals for the first 3 hours, and then at 24, 48 and 72 hours.

To assess the importance of *N*-chlorobutyraldimine in the formation of isobutyraldehyde, a model using the Kintecus software was used to predict isobutyraldehyde concentrations in the absence of monochloroamine degradation into isobutyraldehyde. The trend of the formation of isobuytraldehyde produced by the model was different from the experimental concentrations of isobutyraldehyde produced from chlorination of valine (Figure 5-5). Experimentally, isobutyraldehyde showed a sharp initial increase, followed by a sharp decrease, then a gradual decrease, whereas modelling without a contribution from monochloramine degradation showed a more gradual increase in isobutyraldehyde which plateaued to a lower concentration than in the experiment. This suggested that in the laboratory isobutyraldehyde was not formed solely from the hydrolysis of *N*-chlorobutyraldimine (VI→IV) but also from the degradation of the monochloramine (II→III→IV). This also indicated that at Cl:AA > 1, *N*-monochlorovaline would either react with HOCl to form *N*,*N*-dichlorovaline (II→V) or be degraded into isobutyraldehyde (II→III→IV). Figure 5-6 shows that, experimentally, the concentration of isobutyronitrile and *N*-chloroisobutyraldimine were much higher than isobutyraldehyde, indicating that most *N*-monochlorovaline must react with chlorine to form *N*,*N*-dichlorovaline (k_4) rather than degrading into isobutyraldehyde. Therefore, the reaction rate k_4 can be estimated to be faster than the degradation of *N*-monochlorovaline. When the final proposed reaction rates presented in Table 5-2 (i.e., modelled reaction rates in Table 5-2) were used, the simulated trends of formation of isobutyraldehyde, isobutyronitrile and *N*-chloroisobutyraldimine were similar to the experimental trends of formation (Figure 5-6), indicating that the proposed rate constants were accurate.

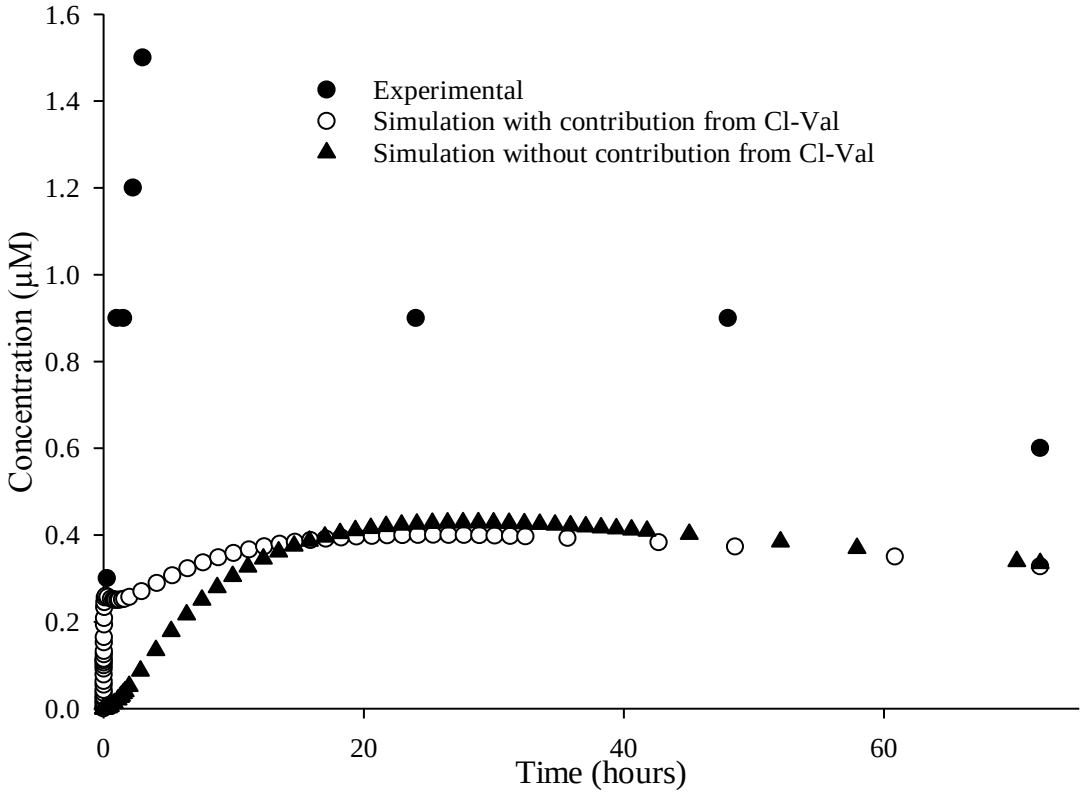

Figure 5-5: Concentrations of isobutyraldehyde from experimental chlorination of valine (30 µM), and from simulation of isobutyraldehyde formation with and without contribution from the degradation of N-monochlorovaline (Cl-Val).

Both isobutyronitrile and *N*-chlorobutyraldimine are degradation products of *N,N*-dichlorovaline (Figure 5-1). The rates of formation of isobutyronitrile and *N*-chlorobutyraldimine were experimentally determined from the increase in their concentration over the first three hours (Figure 5-6) and found to be very similar, at 1.0×10^{-4} s^{-1} and 9.2×10^{-5} s^{-1}, respectively, suggesting that both of these products can be formed from the *N,N*-dichlorovaline in their competitive pathways. While it is proposed that isobutyronitrile can also arise from the degradation of *N*-chlorobutyraldimine, the concentration of isobutyronitrile did not increase when the concentration of *N*-chlorobutyraldimine decreased (Figure 5-6). Previous work (Conyers and Scully 1993, Freuze et al. 2004) with other amino acids also showed that the formation of the respective nitriles and *N*-chloraldimines were both formed from the *N,N*-dichloramines, via the two competitive pathways.

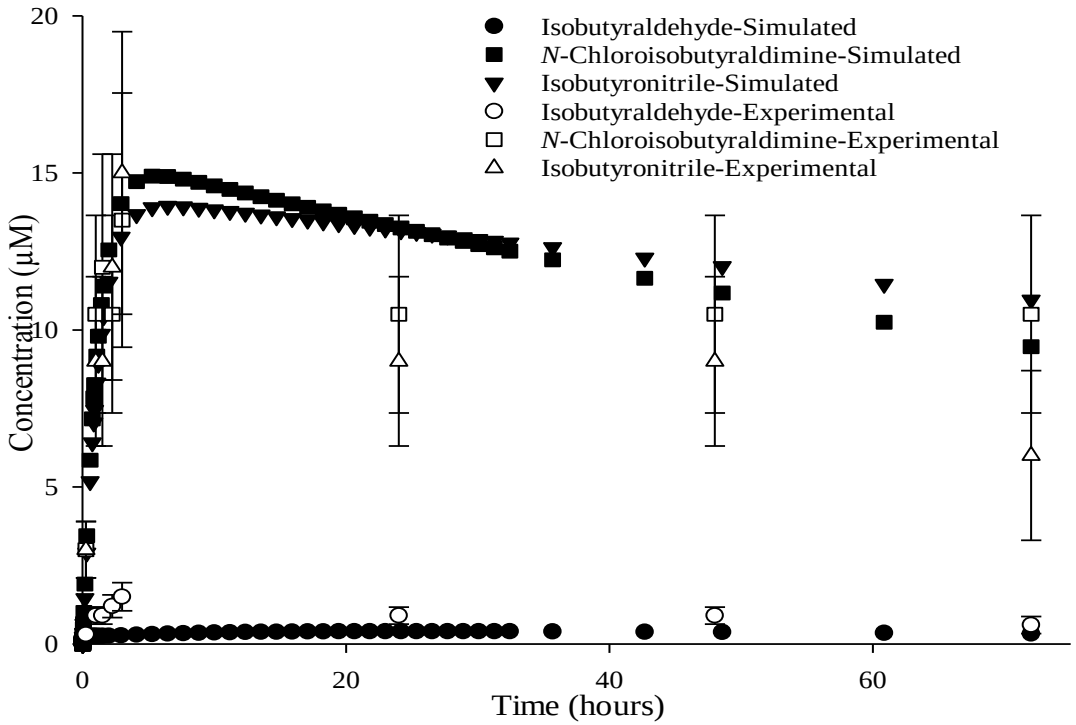

Figure 5-6: Concentrations of isobutyraldehyde, isobutyronitrile and N-chloroisobutyraldimine formed over 72 hours from both experimental and simulated chlorination of valine (30 µM). The experimental concentration of N-chloroisobutyraldimine was estimated from the difference in the isobutyraldehyde concentrations in quenched and unquenched samples.

Finally, *N*-chlorobutyraldimine degradation into isobutyraldehyde and isobutyronitrile was also investigated. In order to accurately measure the rate of degradation of *N*-chlorobutyraldimine into isobutyraldehyde and isobutyronitrile, pure *N*-chlorobutyraldimine solution is required. However, the synthesis of pure *N*-chlorobutyraldimine proved to be challenging. While *N*-chlorobutyraldimine can theoretically be formed through chloramination of isobutyraldehyde (Figure 5-1), there was no reaction observed between isobutyraldehye and inorganic monochloramine (MCA) in aqueous solution, even when MCA was present in 20 times molar excess. This result is consistent with previous reports that the formation of *N*-chloraldimines from the chloramination of aldehydes in aqueous solution is very slow (Kimura et al. 2015, Scully et al. 1997). Therefore *N*-chloroisobutyraldimine was produced in anhydrous conditions. Inorganic monochloramine was first produced in water and then extracted using diethyl ether. The concentration of MCA in the diethyl ether was assumed to be the difference in MCA concentration in the aqueous layer before and after the extraction. The isobutyraldehyde standard in methanol was then added to the MCA diethyl ether extract at a MCA:isobutyraldehyde molar ratio of 10.

The solution was then analysed by GC-MS for the formation of *N*-chloroisobutyraldimine every 60 minutes (time for each chromatographic separation) for 12 hours using liquid injection. The GC-MS program used was identical to that of the HS SPME-GC-MS methods (Table A3-4), except that the injector temperature was 250 ºC and initial holding time was 3 min instead of 2 min. Both *N*-chloroisobutyraldimine and isobutyronitrile were found in the reaction mixture, with the concentration of isobutyronitrile increasing over time (Figure A3-4). The formation of isobutyronitrile must be from the degradation of *N*-chloroisobutyraldimine as no other pathway to the isobutyronitrile was possible. This result confirmed that *N*-chloroisobutyraldimine degrades into isobutyronitrile. Any further experiments on this reaction mixture were not conducted because undesirable hydrochloric acid was also found in the GC-MS spectrum, being the result of thermal degradation of MCA during injection.

Both experimental and modelled results showed a decrease in the concentration of isobutyraldehyde and isobutyronitrile after formation. This is likely due to the degradation of isobutyraldehyde and isobutyronitrile into isobutyric acid.

From the rate of formation of *N*-chlorobutyraldimine and isobutyronitrile (Table 5-2), the half-life of *N*,*N*-dichlorovaline was found to be around 100 min. This suggests that *N*,*N*-dichlorovaline was not detected because of the absence of a suitable analytical technique for its detection, rather than *N*,*N*-dichlorovaline being too unstable.

The investigation of reaction rates for each reaction pathway in the chlorination of valine as a model amino acid (Table 5-2) showed that the *N*,*N*-dichloramine was formed from a stepwise reaction through the formation of the *N*-monochloramine, and the initial formation of the aldehyde at Cl:AA > 2 was from the degradation of the *N*-monochloramine. It also confirmed that the nitrile and the *N*-chloraldimine both formed from the *N*,*N*-dichloramine via competitive reaction pathways. After formation, the *N*-chloraldimine continued to degrade slowly to either the aldehyde or the nitrile. The nitrile and aldehyde also degraded slowly to presumably the corresponding carboxylic acid; in the case of valine, isobutyric acid.

5.5 Conclusions

This study of the formation and degradation pathways of the chlorination of valine indicates that, when Cl:AA > 2, chlorine will react quickly with valine to form *N*-monochlorovaline (5.4×10^4 $M^{-1}s^{-1}$), with a slower formation of *N,N*-dichlorovaline from *N*-monochlorovaline (4.85×10^2 $M^{-1}s^{-1}$). Some *N*-monochlorovaline will degrade to isobutyraldehye (1.00×10^{-4} s^{-1}), while *N,N*-dichlorovaline will competitively degrade to either isobutyronitrile (1.25×10^{-4} s^{-1}) or *N*-chloroisobutyraldimine (1.15×10^{-4} s^{-1}). As the reaction pathways of most amino acids with chlorine are similar, the speciation of degradation products (aldehydes, nitriles and *N*-chloraldimines) from other amino acids is expected be similar to that of valine, although the reaction rates of the various reactions would be dependent on each individual amino acid. Amino acids with a reactive side chain, like tyrosine and lysine, may not favour the formation of the *N,N*-dichloramine, depending on the reactivity of the side chain functional group, potentially producing different degradation products.

While organic chloramine formation is of interest, due to the potential toxicity of these compounds, this study has shown that the aldehyde, nitrile and *N*-chloraldimine by-products are more likely to be found in finished drinking water, with potential issues for odours in drinking water, and as yet unknown health implications. The formation of stable aldehydes and *N*-chloraldimines from the chlorination of amino acids could result in odour problems in chlorinated waters (Brosillon et al. 2009, Froese et al. 1999). For example, the odour threshold concentration of isobutyraldehyde is reported to range from 0.9 and 4 µg L^{-1} (Freuze et al. 2005, Froese et al. 1999), while the odour threshold concentration of *N*-chloroisobutyraldimine is reported to be 0.20 µg L^{-1} (Freuze et al. 2005). The maximum molar concentration of valine that has been found in a source water for drinking water was 63 nM (Brosillon et al. 2009, Chinn and Barrett 2000). If this raw water was chlorinated at a practical chlorination for disinfection regime, where a minimum chlorine residual of 0.5 mg L^{-1} (Cl:AA $\geq$ 2) was sought, without any treatment to remove the amino acids, 0.2 µg L^{-1} (3 nM) of isobutyraldehyde and 2.0 µg L^{-1} (19 nM) of *N*-chloroisobutyraldimine would be formed, based on the respective molar conversions of 5% and 30% found in the current study. The potential concentration of *N*-chloroisobutyraldimine of 2.0 µg L^{-1} is 10 times higher than its odour threshold concentration (0.2 µg L^{-1}), suggesting that formation of *N*-chloroisobutyraldimine during chlorination of waters containing valine

could result in an odour in the drinking water. Additionally, no toxicity information is available for aldehydes, nitriles and *N*-chloraldimines in drinking water, and therefore their presence would result in an unknown health risk. Therefore, improved knowledge of the occurrence of free amino acids and the odorous DBPs formed from the chlorination of amino acids would be beneficial for the water industry. In addition, if long retention times in the distribution system are expected, the degradation pathways of aldehydes and nitriles (e.g. to the corresponding carboxylic acids) should also be considered.

5.6 Acknowledgements

The authors would like to acknowledge the Australian Research Council (LP110100548 and LP130100602), Water Corporation of Western Australia, Water Research Australia and Curtin University for supporting this study. Curtin University (Curtin International Postgraduate Research Scholarship) and Water Research Australia (WaterRA PhD Scholarship) are also acknowledged for providing financial support for Z. How. The authors would also like to acknowledge Ching Goh for assistance in NMR analysis and Geoff Chidlow for assistance with GC-MS analysis.

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Chapter 6. Formation of Odorous Compounds from the Chlorination of Amino Acids in Drinking Waters and Wastewaters

6.1 Abstract

Aesthetic properties such as odour are commonly used by consumers to judge the quality of treated drinking water, and off-flavours often result in consumer complaints. In this study, the formation of odorous aldehydes and *N*-chloraldimines; and also nitriles that result from the chlorination of amino acids (AAs), in both drinking water and wastewater, were investigated. The effect of treatment on the removal of organic carbon and organic nitrogen, and the free AAs concentrations, was also studied in each system. Generally, good removal of DOC was observed in both drinking water and wastewater treatment, but the concentration of total free AAs did not significantly change. However, free AAs contributed less than 3% of total nitrogen in the drinking waters and less than 10% of organic nitrogen in the wastewaters, and there was limited formation of the three classes of disinfection by-products including the odorous aldehydes and *N*-chloraldimines in actual water systems. Formation potential experiments showed that carboxylic acids can form from the degradation of aldehydes and nitriles, and therefore should be considered as potential disinfection by-products in distribution systems with long retention times. Formation potential experiments also showed that the proportion of aldehydes, *N*-chloraldimines and nitriles that form changes in the presence of ammonia or at high chlorine to AA ratios (Cl:AA > 4). This suggests that results from DBP formation studies undertaken at controlled Cl:AA ratios cannot be directly applied to 'real' water systems, which typically have Cl:AA ratios that are orders of magnitude higher.

6.2 Introduction

Disinfection by-products (DBPs) are potentially harmful to human health (Nakamura et al. 1993, Richardson et al. 2007) and can also result in off-flavours in treated waters (Bruchet et al. 1992, Freuze et al. 2005, Hrudey et al. 1988). Off-flavours in treated waters are a major concern for water utilities, as consumers often use aesthetic properties to judge the quality and safety of their drinking water (McGuire 1995). Consideration of aesthetic properties, such as odours, is also important for wastewater treatment, particularly when the treatment plant is in a residential area and when the treated wastewater is intended for reuse or release into environmental waters.

Amino acids (AAs) are ubiquitous in most waters and are reported to be major constituents of dissolved organic nitrogen, contributing up to 75% in surface

waters (Hagedorn et al. 2000). Free AAs reportedly constitute a relatively small fraction of the total AAs, and have been found to range from 0.7 to 80 µg L^{-1} as N in seawaters, groundwaters and lakes (Chinn and Barrett 2000, Dotson and Westerhoff 2009, Thurman 1985), compared to the concentrations of total AAs (combined and free) which have been found to range from 20 to 6000 µg L^{-1} as N (Thurman 1985). However, studies have also shown that low molecular weight compounds, such as free AAs and short peptides, are poorly removed in a variety of water treatment processes. For example, only 50% of free AAs were reported to be removed during biological filtration in drinking water treatment (Prevost 1998) and only 25% of free AAs were reported to be removed in drinking water after chemical pre-treatment (either traditional oxidation or advanced oxidation), sedimentation and filtration (Dotson and Westerhoff 2009). Free AA concentrations have even been observed to increase after sand filtration (LeCloirec et al. 1986). Free AAs and short peptides can also pass through nanofiltration membranes (Escobar et al. 2000). Thus, even though the concentration of free AAs is typically much lower than the concentration of combined AAs, greater proportions of free AAs are likely to reach the disinfection step in a treatment process.

As shown in Figure 6-1, AAs produce a number of different DBPs after chlorination. Of particular interest are the aldehydes (IV in Figure 6-1) and *N*-chloraldimines (VI) that have been identified in chlorinated water (Freuze et al. 2005, Hrudey et al. 1988). In particular, aldehydes and *N*-chloraldimines formed from the AAs isoleucine, leucine, phenylalanine and valine have previously been detected in chlorinated drinking waters at concentrations greater than their odour threshold concentrations (Table 6-1).

Non-halogenated nitriles are also formed during the chlorination of AAs (VIII, Figure 6-1). However, they typically have a much higher odour threshold concentration, up to 1000 times higher than the odour threshold concentration of the corresponding aldehyde (Table 6-1). To date, only six different non-halogenated nitriles have been detected in drinking waters (Liew et al. 2012), including 3-methylbutyronitrile and benzyl cyanide. While non-halogenated nitriles are non-odorous, they have been found to be more toxic than their corresponding aldehydes, due to liberation of hydrogen cyanide during metabolisation (Tanii and Hashimoto 1984). Therefore, nitriles formed

from the chlorination of AAs are still potentially harmful to human health and the environment.

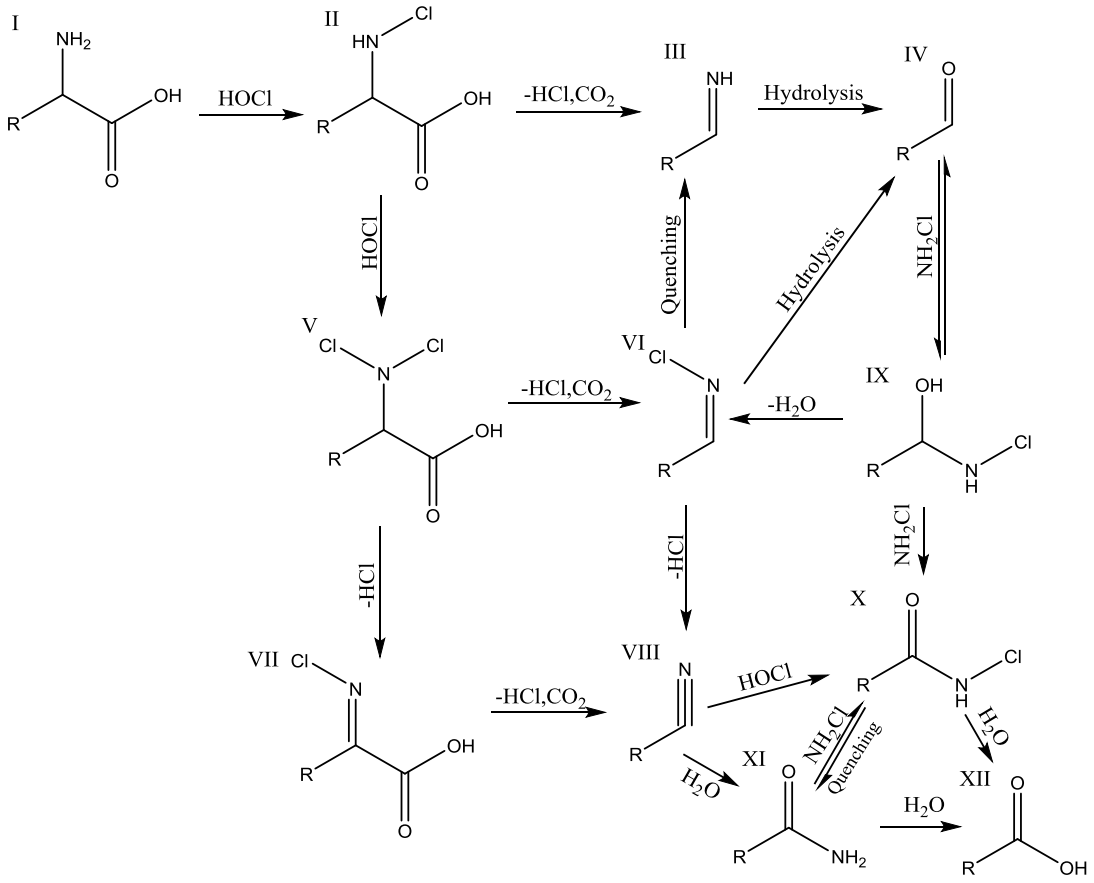

Figure 6-1: Reaction pathways for chlorination of amino acids (I) (Adapted from Conyers and Scully, 1993, Kimura et al., 2015, Yu and Reckhow, 2015). Key: I: Amino acid; II: *N*-Monochloramino acid; III: Imine; IV: Aldehyde; V: *N*,*N*-Dichloramino acid; VI: *N*-chloraldimine; VII: *N*-Chlorimino acid; VIII: Nitrile; IX: *N*-Chloramino alcohol; X: *N*-Chloramide; XI: Amide; XII: Carboxylic acid.

However, to date there is no study on how the treatment process prevent the formation of the odorous aldehydes and *N*-chloraldimines in drinking water and also these class of odorous DBPs have not being studied in wastewater treatment.

In this study, we investigate the formation of these three classes of DBPs that result from the chlorination of AAs in samples from four drinking water treatment plants (DWTPs) and four wastewater treatment plants (WWTPs). Aldehydes, *N*-chloraldimines and nitriles from the chlorination of four AAs that have been previously studied (isoleucine, leucine, phenylalanine and valine, Table 6-1) were measured, and

additional DBPs were identified. In order to understand how the presence of AAs influences the formation of these DBPs, the concentration of free AAs in all drinking water and wastewater treatment plant samples was also measured. Finally, the impact of the presence of ammonia, and of changes to the chlorine to AA ratio, were examined using formation potential experiments.

Table 6-1: Odour threshold and previously reported occurrence of aldehydes and *N*-chloraldimines produced from the chlorination of different amino acids.

Amino acid precursor	**Odorous by-product**	**Odour Threshold Concentration ($\mu g\ L^{-1}$)**	**Occurrence in drinking water ($\mu g\ L^{-1}$)**
Isoleucine	2-Methylbutyraldehyde	12.5[a]	134[f],0.6[g*]
	N-Chloro-2-methylbutan-1-imine	Not available	0.05[g*]
	2-Methylbutyronitrile	Not available	Not available
Leucine	3-Methylbutyraldehyde	0.15[c],0.2[d],2.0[a],5[b]	46[f], 0.6[g*]
	N-Chloro-3-methylbutan-1-imine	0.25[b]	0.4[g*]
	3-Methylbutyronitirile	210[b]	Not Available
Phenylalanine	Phenylacetaldehyde	4[e],30[b]	0.08[g*]
	N-Chlorophenylacetaldimine	3[b]	Not Available
	Benzyl Cyanide	1200[b]	
Valine	Isobutyraldehyde	0.9[c],1.0[d],2.3[a],4[b]	54[f], 0.2[g*]
	N-Chloroisobutyraldimine	0.20[b]	0.6[g*]
	Isobutyronitrile	430[b]	Not Available

[a]Ammore et. al. 1976, [b]Freuze et. al. 2005, [c]Guadagni et. al. 1963, [d]Guadagni et. al. 1972, [e]Buttery et. al. 1971, [f]Hrudey et. al. 1988, [g]Brosillon et. al. 2009

*These occurrences are presented as the average concentration at the outlet of three treatment plants over three seasons, calculated from the individual concentrations reported in Brosillon et. al., 2009

6.3 Materials and Methods

6.3.1 Chemicals

Amino acids (alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glycine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine) were purchased from Sigma Aldrich (New South Wales, Australia). The purity of all AA standards was greater than 97%. Aldehydes (2-methylbutyraldehyde, >90%; 3-methylbutyraldehyde, 97%; isobutyraldehyde, 99%, phenylacetaldehyde; >90%), nitriles (2-methylbutyronitrile,

98%; 3-methylbutyronitrile, 98%, isobutyronitrile, 99.6%; benzyl cyanide, 98%), aqueous sodium hypochlorite solution (10 – 15% chlorine) and isobutyric acid (99%) were also purchased from Sigma Aldrich. Surrogate standards, [^{2}H3] alanine (alanine-d_3), [^{2}H3] leucine (leucine-d_3), and [^{2}H3] glutamic acid (glutamic-d_3 acid), were purchased from CDN Isotopes (Quebec, Canada, distributed by SciVac, Hornsby, Australia), while [^{2}H2] glycine (glycine-d_2) and [^{2}H5] phenyl [^{2}H3] alanine (phenyl-d_5-alanine-d_3), were purchased from Sigma Aldrich. The internal standard, 1,2-dibromo-[^{2}H6]-propane (1,2dibromopropane-d_6) (99.7%), was purchased from CDN Isotopes. Methanol (HPLC grade), was purchased from Honeywell-Burdick & Jackson (Michigan, USA). Formic acid (99%) was purchased from Ajax FineChem (New South Wales, Australia). Analytical grade anhydrous sodium sulphate, purchased from Ajax FineChem, was baked at 400^{o}C for 4 hours prior to use, in order to remove organic impurities. The solid-phase microextraction fibre, carboxen/polydimethylsiloxane (CAR/PDMS) 75µm phase, was purchased from Supelco (Bellefonte, PA, USA). Ultrapure water was produced using an ion exchange system (IBIS Technology, Western Australia, Australia), followed by an Elga Purelab Ultra system with a 0.2 µm filter (Elga, High Wycombe, UK).

6.3.2 Sampling and Sampling Sites

Raw and post-disinfection waters were collected from four DWTPs, including two groundwater treatment plants, one surface water treatment plant and one mixed source (treated groundwater, untreated surface water and desalinated water) treatment plant. Influent and treated wastewater were collected from four WWTPs, including one oxidation ditch WWTP, and three waste stabilisation pond systems. Descriptions of the treatment processes used by each DWTP and WWTP are presented in Table 6-2.

Table 6-2: Drinking water and wastewater treatment plants, their treatment processes and chlorine residue at plant outlet

Plant	Treatment Process	Residual chlorine at plant outlet (mg L^{-1} as Cl)
Drinking Water Treatment Plant		
DWTP 1 (Groundwater)	Aeration, pre-chlorination, magnetic ion exchange and/or alum coagulation (2 separate trains), chlorination, multimedia filter	2
DWTP 2 (Groundwater)	Pre-chlorination, alum coagulation, multimedia filter, chlorination	2.5
DWTP 3 (Surface water)	Powdered activated carbon/alum coagulation, filtration, UV, chlorination	2
DWTP 4 (Mixed source)	Dissolved air flotation filtration, biological activated carbon filtration, UV, chloramination	4*
Wastewater Treatment Plant		
WWTP 1	One primary facultative waste stabilisation pond, and a storage basin where water remains for approximately 2 years before discharge	No disinfection
WWTP 2	Two primary facultative ponds in parallel, followed by one maturation pond	No disinfection
WWTP 3	Oxidation ditch and sedimentation tank	No disinfection
WWTP 4	Combined anaerobic and aerobic 'Smart' pond, followed by two maturation ponds in series	0.3

*Inorganic monochloramine is used as the disinfectant at DWTP 4, with chlorine added after the ammonia.

Samples were collected in 2 L amber glass bottles as grab samples in multiple sampling events. Dates of sampling are listed in Tables 6-3 and 6-5. As *N*-chloraldimines are destroyed upon quenching (How et al. submitted, Kimura et al. 2015), all chlorinated or chloraminated samples were collected in triplicate, both quenched (using two different quenching agents) and unquenched. Two quenching reagents were used. Ascorbic acid was used to quench samples for analysis of AAs and DBPs, as recommended by Kristiana et al. (2014). Sodium sulphite was used to quench samples for analysis of dissolved organic carbon (DOC) and total dissolved nitrogen (TDN), as ascorbic acid contains organic carbon and would contribute to the DOC concentration. Sample bottles were filled with no headspace and kept cool (in an ice box) during

transport to the laboratory. All samples were filtered through a 0.45 µm polyethersulfone membrane filter (Pall Life Science, Michigan, USA), with wastewater samples being pre-filtered through a 1.2 µm glass microfiber filter (Whatman, UK). Samples were then stored at 4 ºC until analysis, which was typically within 24 hours, especially for the DBP analysis. Trip and field blanks containing ultrapure water and quenching agents were also prepared for each sampling trip to identify if there was any contamination through the sampling process, storage and transport. Trip blanks remained unopened until analysis, while field blanks were opened at each sampling location.

6.3.3 Analysis of Dissolved Organic Carbon and Total Dissolved Nitrogen

Dissolved organic carbon and total dissolved nitrogen concentrations for DWTP and WWTP samples were measured using a high temperature combustion total organic carbon analyser with a total dissolved nitrogen analyser attachment (TOC-L and TNM-L analysers, Shimadzu Corporation, Kyoto, Japan), as per Standard Method 5310-B (APHA et al. 2005). A commercial laboratory measured ammonia using Standard Method 4500-NH_3-G (APHA et al. 2005), and nitrate and nitrite using Standard Method 4500-NO_3^--F (APHA et al. 2005). The dissolved organic nitrogen concentrations of the wastewater samples were calculated by subtracting the dissolved inorganic nitrogen from the total dissolved nitrogen.

6.3.4 Analysis of Free Amino Acids

The concentrations of 18 free AAs were determined using a modified liquid chromatography (LC)-high resolution mass spectrometric (HRMS) method described previously (How et al. 2014, How et al. 2016). The LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific Corporation, Waltham, USA) was operated in ESI positive mode; operating conditions can be found in Appendix 4 Table A4-1. Full-scan mode from 70-250 *m/z*, with a mass resolution of 30000 (at 400 *m/z*), was used for both screening and quantification. For increased mass accuracy, *n*-butyl benzenesulfonamide at 214.0896 *m/z*, a trace plasticiser present in the LC mobile phase, was used for the lock mass function. Data was processed using Xcalibur QualBrowser 2.0.7 SP1 and TraceFinder 3.1. Both the limit of detection (3 × signal to noise ratio) and the limit of quantification (10 × signal to noise ratio) were determined for each analytes.

6.3.5 Analysis of Aldehydes, Nitriles and *N*-Chloraldimines

Unquenched samples were extracted using automated headspace solid-phase microextraction (HS-SPME) (75 µm CAR/PDMS fibre) followed by gas chromatography-mass spectrometric (GC-MS) analysis, a method previously developed in our research group (Driessen submitted). The sample (10 mL) was transferred into a glass vial (20 mL), and sodium sulphate (3 g) and 1,2-dibromopropane-d_6 internal standard solution (2 µL of 100 µg L^{-1}) were added. After agitating each sample (500 rpm at 60 °C) for 10 minutes, the SPME fibre was inserted into the sample headspace for 15 minutes. The fibre was then transferred to the GC injector port for thermal desorption at 300 °C for 3 minutes. The operating conditions for the Agilent 6890N gas chromatograph (Agilent, Victoria, Australia), coupled with an Agilent 5975 mass selective detector, are reported in SI Table A6-2.The samples were analysed in both scan mode (*m/z* 20 to 320) for the identification of degradation products, and selected ion monitoring mode for quantification of the target aldehydes, nitriles and *N*-chloraldimines. The quantifying and qualifying ions monitored in selected ion monitoring mode are listed in Appendix A4 Table A4-3.

Aldehydes and nitriles were quantified using the ratio of the analyte peak area to the internal standard peak area, and an external calibration curve prepared from commercial standards (0.1 to 100 µg L^{-1}). *N*-Chloraldimines are unstable and analytical standards are not commercially available. Therefore, solutions of *N*-chloro-2-methylbutan-1-imine, *N*-chloro-3-methylbutan-1-imine and *N*-chloroisobutyraldimine were produced in-house immediately before each analytical run, by chlorination of their respective AAs (30 µM individually). Our previous study found the conversion of AA to *N*-chloraldimines after three hours was around 30% (How et al. submitted). Therefore, each *N*-chloraldimine in the in-house standard was measured by HS SPME-GC-MS after 3 hours and assumed to represent an approximate concentration of 9 µM. The concentrations of *N*-chloraldimines in the drinking water and wastewater samples and in the formation potential samples were then quantified from the in-house standard, again using the ratio of the analyte peak area to the internal standard peak area.

Method detection limits for the aldehydes and the nitriles were determined for every analytical run, based on triplicate measurements of low concentration standards

(USEPA 2005). Due to the lack of commercially available standards, the limits of detection of the *N*-chloraldimines were estimated by serial dilution of the *N*-chloraldimine in-house standard (9 µM) until each *N*-chloraldimine was not detected. The limit of detection was assigned to the lowest concentration that produced a signal that was 3 times the signal to noise ratio.

6.3.6 Formation Potential Experiments

All formation potential (FP) experiments were conducted on filtered (0.45 µm) drinking water or wastewater samples, with addition of aqueous sodium hypochlorite solution. The initial chlorine concentration required for each FP experiment was calculated using Equation 1 (Mitch et al. 2009) and a desired residual chlorine concentration of 2 or 10 mg L^{-1}. Preliminary tests using DWTP 1 (Apr-15) raw water and WWTP 3 (Jul-15) secondary effluent confirmed that the chlorine doses calculated using Equation 1 produced the desired residual chlorine concentrations after 7 days. The chlorine dose for FP experiments with a shorter contact time (e.g. 2 mg L^{-1} residual after 1 day) was adjusted based on chlorine decay experiments. The residual chlorine concentration was determined using the DPD colorimetric method as per Standard Method 4500-Cl-G (APHA et al. 2005).

Initial chlorine concentration (mg L^{-1} as Cl) = 3(TOC) + 7.6(NH_4) + desired residual chlorine (Equation 1)

Formation potential samples (500 mL) were chlorinated in duplicate, mixed thoroughly, and then subsampled with no headspace into duplicate 60 mL vials for each time point. The vials were stored in the dark at room temperature (20 to 25 ºC) until analysis. As the FP samples were not quenched (except for the samples used to test formation of isobutyric acid), they were analysed by HS SPME-GC-MS immediately after the desired length of time. The samples used to test formation of isobutyric acid were quenched with ascorbic acid, and analysed within 24 hours to avoid degradation. AAs (isoleucine, leucine and valine) were added to some drinking water and wastewater samples before undertaking FP experiments. The concentration of each AA in the samples was 30 µM, equivalent to a total DOC concentration of 6.12 mg L^{-1}.

6.4 Results and Discussion

6.4.1 Occurrence and Removal of Free Amino Acids in Water Treatment

The concentrations of DOC, TDN, target AAs (isoleucine, leucine, phenylalanine and valine) and total free AAs in raw and treated drinking water are presented in Table 6-3, while the concentrations of 14 other AAs are presented in Appendix 4 Table A4-4. The AA concentrations reported are above the limit of quantification. While only TDN was measured for each DWTP, previous studies have shown that ammonia contributes more than 65% of TDN in raw waters for both groundwater treatment plants (DWTP 1 and 2), but was below detection after treatment and disinfection (Liew et al. submitted). More than 80% of TDN in the raw surface water from DWTP 3 has been previously found to be organic nitrogen (Liew et al. submitted), but most DOC was high molecular weight material, of very hydrophobic character (Garbin et al. 2010). The total concentrations of free AAs in raw waters were between 0.7 and 2.8 μg L^{-1} as N, contributing between 0.06% and 2% of the total dissolved nitrogen. The total free AA concentrations were comparable to previous studies of surface waters, where the concentrations of free AAs were found to be between 0.7 to 4 μg L^{-1} as N (Chinn and Barrett 2000, Dotson and Westerhoff 2009). Considering all DWTPs, the average total free AA concentration in the raw water (1.9 ± 0.6 μg L^{-1} as N, 1σ) was statistically the same as the concentration in the treated water (1.7 ± 1 μg L^{-1} as N, 1σ), although generally concentrations decreased slightly after treatment.

Table 6-3: Dissolved organic carbon, total dissolved nitrogen and free amino acid concentrations from the plant inlet and outlet of four different drinking water treatment plants (DWTPs) at different sampling events. The concentrations of isoleucine, leucine, phenylalanine and valine (precursors to the odorous by-products analysed) are also presented. Limits of quantification are presented; the concentrations shown are above the limit of quantification.

	DWTP1						DWTP2		DWTP3				DWTP4*			
	Nov-14		Apr-15		Nov-15		Apr-15		Apr-15[a]		Nov-15		Nov-14		Feb-15	
	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet
DOC (mg L^{-1}-C)	3.0	1.4	4.6	2.1	2.0	1.4	10.3	1.7	11.6	1.3	12.7	3.1	1.2	1.2	1.7	1.3
TDN (µg L^{-1}-N)	160	<100	250	<100	360	<100	330	<100	-	-	1300	500	180	710	300	810
Isoleucine (µg L^{-1}-N)	<0.01	0.03	<0.01	<0.01	0.01	<0.01	<0.01	0.01	0.02	0.04	0.02	0.01	0.01	0.01	0.01	0.01
Leucine (µg L^{-1}-N)	<0.01	0.06	<0.01	<0.01	<0.01	<0.01	<0.01	0.01	0.04	0.08	0.03	0.01	0.01	<0.01	0.01	<0.01
Phenylalanine (µg L^{-1}-N)	<0.01	0.05	0.01	0.02	0.01	0.01	0.01	0.02	0.03	0.07	0.02	0.01	0.01	0.01	0.01	0.01
Valine (µg L^{-1}-N)	0.4	<0.01	2	<0.01	2	2	2	1	1	0.6	0.6	0.6	0.3	0.3	0.6	0.5
Total free AA (µg/L-N)	2.8	3.0	1.8	0.2	2.4	2.0	2.9	3.4	1.5	1.2	0.7	0.7	1.8	1.6	2.1	1.9

* Inorganic monochloramine is used as the disinfectant at DWTP4, with chlorine added after the ammonia.
[a]TDN was not measured for DWTP 3 Apr-15 samples.

Table 6-4: Frequency of amino acids reported above the limit of detection in either the inlet or outlet of the DWTPs at different sampling events. Target amino acids are in bold.

	DWTP1			DWTP2	DWTP3		DWTP4	
	Nov-14	Apr-15	Nov-15	Apr-15	Apr-15	Nov-15	Nov-14	Feb-15
Lysine	0%	0%	0%	0%	50%	0%	0%	0%
Histidine	0%	0%	0%	0%	100%	0%	0%	0%
Methionine	0%	0%	0%	0%	100%	50%	0%	0%
Asparagine	0%	0%	50%	50%	100%	100%	0%	0%
Aspartic Acid	100%	0%	0%	0%	50%	0%	100%	100%
Glutamic Acid	50%	0%	100%	100%	50%	0%	50%	50%
Alanine	50%	0%	50%	50%	100%	0%	100%	100%
Glutamine	0%	100%	100%	50%	100%	100%	0%	0%
Tyrosine	0%	100%	50%	100%	100%	100%	0%	0%
Glycine	100%	100%	0%	50%	100%	50%	50%	50%
Serine	0%	100%	100%	100%	100%	100%	0%	0%
Isoleucine	100%	0%	50%	50%	100%	100%	100%	100%
Threonine	50%	50%	100%	100%	100%	100%	100%	100%
Valine	50%	50%	100%	100%	100%	100%	100%	100%
Proline	100%	100%	100%	100%	100%	100%	100%	100%
Tryptophan	100%	100%	100%	100%	100%	100%	100%	100%
Leucine	100%	100%	100%	100%	100%	100%	100%	100%
Phenylalanine	100%	100%	100%	100%	100%	100%	100%	100%

When considering all concentrations measured above the limit of detection, the most frequently detected AAs, leucine, proline, tryptophan and phenylalanine, were detected in both raw water and treated water in all sampling events (Table 6-4). The least frequently detected AAs, lysine, histidine and methionine, were only detected in DWTP 3 (Apr-15) (Table A4-4). The target AAs (isoleucine, leucine, phenylalanine and valine) contributed an average 60% of the total concentration of free AAs in the raw waters, suggesting that the DBPs from the target AAs would be the most likely to have an impact on the treated water quality. Valine was the most abundant AA (48-95%) in eight of the 16 samples, while glycine (48-73%) and alanine (46-64%) were the most abundant AAs in four and three samples, respectively. One sample (DWTP 1 outlet Apr-15) had serine as the most abundant AA (48%), but the total AA concentration in this sample was very low (0.18 µg L^{-1} as N). The high abundance of valine measured in these samples supports its choice as a target AA in this study, which was based on a previous chlorination pathway study (McCormick et al. 1993) and studies of aldehydes and *N*-chloraldimines (Brosillon et al. 2009, Freuze et al. 2005). While odorous DBP formation from glycine has not been studied, we have previously identified chlorinated glycine (*N*-chloroglycine) to be the *N*-chloramino acid of highest health risk (How et al. 2016). Based on the reaction pathways between amino acids and free chlorine (Figure 6-1), alanine would be converted to acetonitrile, which has been found to be harmful to human health in drinking water (NRC 1982).

Changes in the AA concentration before and after drinking water treatment were variable, and without an obvious pattern. For example, the concentration of valine generally decreased after treatment, but was observed to remain constant for two sampling events (DWTP 1 and 3, Nov-15). There were also examples of AAs detected in treated water but not raw water at the same sampling event. For example, in two sampling events, glycine was not detected in raw water for DWTP 4, but was the major contributor to the total AAs concentration in the treated water. The change in AAs concentration and contribution to total AAs may be influenced by NOM removal, but may also be influenced by disinfection, as the disinfectant (chlorine or inorganic monochloramine) will have a different relative reactivity with each AA (How et al. 2016), which would result in a higher proportion of less reactive AAs in the treated water. In addition, the chlorination of more complex organic matter can result in the formation of simpler compounds (Richardson and Postigo 2012); in this case, the

oxidation of proteins might result in the formation of additional free AAs. The change in AA concentrations can also result from other treatment steps, such as sand filtration, after release of free AAs from the lysis of bacterial or algal cells (LeCloirec et al. 1986).

DOC concentrations also varied between water sources, with the highest DOC concentrations measured in the surface waters, DWTP 2 and DWTP 3. The DOC concentration in the raw water of DWTP 1 was variable, resulting from use of varying groundwater bore combinations for the source water. The lowest DOC concentrations were measured in DWTP 4 source water, which is a mix of three different sources, including previously treated groundwater and desalinated seawater, both of which have relatively low DOC concentrations. The ratio of each source water used in DWTP 4 is varied to ensure that the DOC of the inlet water is not above 2 mg L^{-1} as C. Some seasonal effects were seen for surface water DWTP 3, where the DOC concentration was lower in autumn (April) than in spring (November), which may be related to NOM inputs during winter rainfall (Garbin et al. 2010). In contrast, the total free AA concentration was higher in autumn than in spring, which may reflect increased algal activity in summer/autumn.

The DOC removal efficiencies of all DWTPs ranged between 0 and 90%. The lowest removal efficiencies (0 and 24%) were recorded for DWTP 4, which had the lowest source water DOC concentration, and contains water that has been previously treated for NOM removal. The DOC removal efficiency was correlated with DOC raw water concentration: raw waters with high DOC concentration also had high DOC removal. On 6 out of 8 sampling occasions, the DOC concentration was reduced to below 2 mg L^{-1} as C after treatment, regardless of the initial DOC concentration, indicating the presence of a fraction of DOC which is recalcitrant to conventional NOM removal techniques in all source waters.

6.4.2 Occurrence and Removal of DOC and Free Amino Acids in Wastewater

The concentrations of DOC, DON, ammonia, target AAs (isoleucine, leucine, phenylalanine and valine) and total free AAs in influent and treated wastewater are presented in Table 6-5, while the concentrations of 14 other AAs are presented in Appendix 4 Table A4-5. The AA concentrations reported are above the limit of quantification. Alanine was detected in all samples, but was not quantified, as the

concentrations were calculated to be more than 100 times the highest calibration point, and resulted in total free AAs concentrations that were higher than the DON concentration. The reanalysis of the samples showed similar concentration after quantification suggested that the wrong quantification was caused by either the signal of alanine in the samples were enhanced resulting in or the poor recovery of the surrogate standard for alanine, alanine-d_3. A false identification of the alanine is unlikely as the retention time alanine in the samples was similar to that of its standard and the mass error of alanine was in average -4.578 ppm. The WWTP 3 Feb-15 outlet sample was not included in the analysis of data as no AAs or DON were detected in the sample. It is not clear if this is a true result or from a sampling or analysis error. The concentration of free AAs in influent wastewaters ranged between 0.4 and 59 µg L^{-1} as N, contributing 0.06% to 19% of the DON, while the effluent free AAs ranged between 0.5 and 2 µg L^{-1} as N, contributing 0.06% to 0.9% of the DON.

Eleven of the 18 free AAs were consistently detected in both influent and effluent wastewaters (Table 6-6). Three of the 18 free AAs (arginine, aspartic acid and histidine) analysed were not detected in any of the samples (Table 6-6), and lysine was only detected in the influent to WWTP 4. Among the AAs, valine was the most abundant AA (35-71%) in nine of the 15 samples, while isoleucine (22-25%) and leucine (22-43%) were the most abundant AAs in two and three samples, respectively. The target AAs (isoleucine, leucine, phenylalanine and valine) contributed an average 62% of the total free AA concentration in the treated wastewater. This is consistent with previous research which has focussed on the study of these AAs in wastewaters (Conyers and Scully 1993, Conyers et al. 1993, McCormick et al. 1993, Nweke and Scully 1989).

While the DOC and ammonia concentrations in the wastewater influent did not vary seasonally, there was consistently higher DON measured in the wastewater influent in the summer (February 2015) compared to the winter (July 2015). However, the free AA concentrations of the wastewater influent did not show this trend, suggesting that the free AAs concentration in the wastewater influent is more influenced by the wastewater source, which will be independent of season.

Wastewater treatment was generally effective for DOC removal, which ranged between 36 and 79% for WWTPs 2, 3 and 4. The effect of treatment for DOC removal

could not be determined for WWTP 1 because the storage basin undergoes frequent algal blooms and is also open to run off from surrounding agricultural land. This resulted in increased DOC and free AA concentrations in the storage dam, compared to the wastewater influent. DWTP 3 utilises activated sludge wastewater treatment and had the highest decrease in total nitrogen and free AA concentrations. It is likely that microbial processes in the activated sludge step consumed organic compounds, including free AAs. The changes in concentration of free AAs in the waste stabilisation ponds (WWTP 1, 2 and 4) were generally more variable. While it is likely that microbial processes are also occurring in these systems, algal growth can also occur, and this can lead to higher free AA concentrations.

Table 6-5: Dissolved organic carbon, dissolved organic nitrogen, ammonia and free amino acids concentration from the plant inlet and outlet of four different wastewater treatment plants (WWTP) at different sampling events. The concentrations of isoleucine, leucine, phenylalanine and valine (precursors to the odorous by-products analysed) are also presented. Limits of quantification are presented; the concentrations shown are above the limit of quantification.

	WWTP1				WWTP2				WWTP3				WWTP4			
	Feb-15		Jul-15		Feb-15		Jul-15		Feb-15		Jul-15		Feb-15		Sep-15	
	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet
DOC ($mg\ L^{-1}$-C)	24	44	16	53	41	22	33	21	25	6	33	7	52	11	80	20
DON ($\mu g\ L^{-1}$-N)	1000	1400	0	500	3200	2900	400	100	2600	0	300	100	3200	1500	0	600
NH_4 ($\mu g\ L^{-1}$-N)	3300	200	4600	500	6800	3000	5000	3900	7400	50	6600	40	6300	600	4600	2700
Isoleucine ($\mu g\ L^{-1}$-N)	0.02	0.04	0.03	0.01	15	0.2	12	0.05	11	<0.01	11	0.02	0.4	0.1	0.03	0.07
Leucine ($\mu g\ L^{-1}$-N)	0.02	0.04	0.03	0.01	13	0.2	12	0.05	12	<0.01	11	0.03	0.4	0.2	0.03	0.1
Phenylalanine (µg/L-N)	<0.01	0.01	<0.01	0.01	12	0.2	10	0.07	2	<0.01	0.2	0.09	0.3	0.05	0.04	0.03
Valine ($\mu g\ L^{-1}$-N)	0.4	0.7	0.2	0.5	5	0.7	5	0.3	2	<0.01	0.4	0.2	1	0.2	0.2	0.2
Total free AA ($\mu g\ L^{-1}$-N)	0.6	1	0.4	0.7	59	2	51	0.9	27	<0.01	24	0.5	3	1	0.6	0.9

Table 6-6: Frequency of amino acids reported above the limit of detection in the influent and secondary effluent of all of the WWTPs in all sampling events. Target amino acids are in bold.

	Influent	Secondary Effluent
Asparagine	100%	100%
Glutamine	100%	100%
Glycine	100%	100%
Methionine	100%	71%
Proline	100%	100%
Serine	100%	100%
Threonine	100%	100%
Isoleucine	100%	100%
Leucine	100%	100%
Phenylalanine	100%	100%
Valine	100%	100%
Tryptophan	75%	86%
Glutamic Acid	38%	57%
Tyrosine	38%	71%
Lysine	25%	0%
Arginine	0%	0%
Aspartic Acid	0%	0%
Histidine	0%	0%

6.4.3 Potential for Disinfection By-product Formation in Drinking Water

All of the DBPs that were analysed by HS-SPME-GC-MS (i.e. aldehydes, *N*-chloroaldimines and nitriles) were below their respective detection limits (0.001 to 0.04 µM) in the treated (and disinfected) drinking waters (results not shown). This is probably due to the low concentration of free AAs found in the raw drinking waters (Table 6-3). Based on results from previous laboratory studies (How et al. submitted), the concentrations of target AAs measured in the raw waters (0.001 to 0.1 µM) were estimated to produce between 0.0003 and 0.03 µM of the analysed DBPs. Most of these estimated concentrations were below the respective limits of detection of the DBPs (0.001 to 0.04 µM), although this estimation ignores the potential for changes in AA concentrations during drinking water treatment. In order to investigate the maximum DBP formation from one of these raw waters, a FP experiment was conducted using DWTP 1 (Apr-15) raw water: adding aqueous sodium hypochlorite solution to achieve a desired residual chlorine concentration of 10 mg L^{-1} after 7 days. The concentrations of aldehydes, nitriles and *N*-chloraldimines were measured after 1 and 7 days (Table 6-7). Little or none of these DBPs were detected, consistent again with the low concentration of free AA precursors measured in this raw water.

To confirm that the lack of formation was due to low precursor concentrations, and not due to an unknown interference, a second FP of the DWTP 1 (Apr-15) raw water sample was undertaken, after addition of isoleucine, leucine and valine (30 µM each), and the DBP formation compared to the DBP formation in a FP experiment using the same AAs in ultrapure water. To ensure that the two FP experiments were comparable, and that sufficient chlorine was present to react with the added AAs, the target chlorine residual after 7 days was 10 mg L^{-1} as Cl_2. Overall, the concentrations of aldehydes, *N*-chloroaldimines and nitriles measured in these FP samples with AAs added were much higher than those measured in the raw water without added AAs (Table 6-7), confirming that very low DBP concentrations were the result of very low precursor concentrations in the raw water without added AAs experiment.

However, comparing day 1 and day 7, the concentrations of by-products increased in the FP of the raw water sample with added AAs, but decreased for the FP of ultrapure water with added AAs. The reduction in DBP concentrations in the ultrapure water with added AAs over 7 days was expected, as the same behaviour was observed in our

previous work studying DBP formation from valine (How et al. submitted). In our previous study using valine, we hypothesized that *N*-chloroisobutyraldimine would transform to isobutyraldehyde and isobutyronitrile, while isobutyraldehyde and isobutyronitrile would transform to isobutyric acid, over time (Figure 6-1) (How et al. submitted). These reactions are likely to occur with isoleucine and leucine as well.

The increase in concentrations over time observed in the raw water with added AAs might be due to the influence of the higher chlorine dose required (42 mg L^{-1} as Cl_2 in the raw water with added AAs compared to 24 mg L^{-1} as Cl_2 for raw water without added AAs), which may have caused degradation of proteins and peptides and production of more free AAs.

While the concentrations of aldehydes and nitriles measured in the raw water with added AAs and the ultrapure water with added AAs FP experiments were similar (Table 6-7), higher concentrations of *N*-chloraldimines were formed in the raw water with added AAs FP experiment than the ultrapure water with added AAs FP experiment. This difference in concentration might be due to the presence of natural organic matter, either through an increase in *N*-chloraldimine stability or an alteration to the reaction pathway to favour the formation of *N*-chloraldimines. Further study is required to understand this difference.

Table 6-7: Concentration ± standard deviation (µM) of disinfection by-products formed after 1 (n = 2) and 7 days (n = 2) from the chlorination (residual chlorine = 10 mg L^{-1} as Cl) of raw source water from DWTP 1 (Apr-15), raw source water with 30 µM each of isoleucine, leucine and valine, and ultrapure water with 30 µM each of isoleucine, leucine and valine.

Amino acid precursor	**Disinfection by-products**	**Raw water**		**Raw water with added AAs**		**Ultrapure water with added AAs**	
Days		1	7	1	7	1	7
Actual initial chlorine concentration (mg L^{-1} as Cl)		24		42		34	
Isoleucine	2-Methylbutyraldehyde	0.008±0.002	<0.003	0.5±0.2	0.9±0.3	1.0±0.3	2±1
	2-Methylbutyronitrile	<0.02	<0.02	6±2	9±3	9±3	8±3
	N-Chloromethyl-2-butaldimine	<0.003	<0.003	5±2	2±1	3±1	2±1
Valine	Isobutyraldehyde	<0.003	0.003	0.2±0.1	1.0±0.4	2±1	0.5±0.1
	Isobutyronitrile	0.04±.0.01	<0.01	8±2	10±2	14±4	10±2
	N-Chloroisobutyraldimine	<0.001	<0.001	7±2	0.8±0.3	3±1	1±0.3
Leucine	3-Methylbutyraldehyde	0.10±0.04	<0.001	0.9±0.3	1.0±0.2	1.0±0.3	3±1
	3-Methylbutyronitrile	0.02±0.01	<0.005	8±3	9±3	9±2	8±2
	N-Chloromethyl-3-butaldimine	<0.003	<0.003	18±5	2±1	4±1	2±1
Phenylalanine	Phenylacetaldehyde	<0.04	<0.04	Not added	Not added	Not added	Not added
	Benzyl Cyanide	<0.04	<0.04	Not added	Not added	Not added	Not added

6.4.4 Formation of Additional By-products from Aldehyde and Nitrile Transformation

To confirm our hypothesis that the observed reduction in aldehyde and nitrile concentrations observed in ultrapure water with added AAs FP experiments (Table 6-7) resulted in the formation of the corresponding carboxylic acid, additional experiments were conducted with valine. Valine (2 mg L^{-1}, 17 µM) was treated with chlorine to achieve a residual chlorine concentration of 2 mg L^{-1} after 1 day, and samples collected for analysis after 1, 7 and 14 days. The samples were quenched with ascorbic acid and analysed for isobutyric acid using a modified HS-SPME-GC-MS method previously reported for the analysis of volatile organic acids and sulphur compounds (Kim et al. 2002). The SPME conditions used were similar to those described in Section 2.5, with the only change being a reduction in the agitation temperature from 60 ºC to 40 ºC, to prevent damage to the SPME fibre. The GC conditions are presented in Table A4-2, and all data was acquired in scan mode (*m/z* 20 to 650). The identity of isobutyric acid in these FP experiments on valine was confirmed by comparing the mass fragmentation and retention times of the suspected isobutyric acid peak to a commercial isobutyric acid standard. Isobutyric acid was identified, but not quantified, in all samples from days 1, 7 and 14, confirming that isobutyric acid could be formed from the chlorination of valine. In addition, acetic acid was also detected in all samples, however, the pathway for the formation of acetic acid from the chlorination of valine remains unclear and further studies are required. The lack of an available analytical grade acetic acid standard means that the identity of acetic acid was only confirmed by comparison to the National Institute of Standards and Technology (NIST) mass spectral library databases, as available in the GC-MS data analysis software (Chemstation Enhanced Data Analysis, Agilent Technologies).

6.4.5 Potential for Disinfection By-product Formation in Wastewater

Disinfection by-products were not analysed in the treated wastewaters, as the treated wastewaters from WWTPs 1, 2 and 3 are not chlorinated. Therefore, 7 day DBP FP experiments were undertaken to explore the potential for the formation of aldehydes, *N*-chloraldimines and nitriles in treated wastewater, using water from WWTP 2 (Feb-15) and WWTP 3 (Jul-15), collected in the February sampling event. These WWTPs were chosen to compare treated wastewaters with high DON and ammonia (WWTP 2) and low DON and ammonia (WWTP 3). In particular, the presence of ammonia could

impact DBP formation if inorganic chloramines were preferentially formed over *N*-chloramino acids. Many of the aldehyde, nitrile and *N*-chloraldimine DBPs were formed and similar concentrations (aldehydes concentration ranged from 0.002 to 0.1 µM, nitriles concentration ranged from 0.2 to 0.6 µM and *N*-chloraldimines concentration ranged from 0.001 to 0.05 µM) were observed for both WWTP 2 and 3 treated wastewaters (Table 6-8), suggesting that the presence of ammonia had little impact on formation of these DBPs.

The effect of ammonia on the chlorination of AAs was investigated further by chlorination of valine (30 µM) with a fixed chlorine concentration of 10 mg L^{-1} as Cl_2 in the presence of ammonia, with ammonia concentrations chosen to simulate chlorination above breakpoint chlorination, at breakpoint chlorination and below breakpoint chlorination (1.25, 2 and 4 mg L^{-1} as N). Additionally, valine (30 µM) was chloraminated with preformed inorganic monochloramine (10 mg L^{-1} as Cl_2). The samples were analysed for DBPs at 3 hours and after 7 days. The concentrations of the DBPs are presented in Table 6-9. After 3 hours, FP experiments using chlorine without ammonia formed higher DBP concentrations than samples with ammonia present (Table 6-9); for example, the concentration of isobutyronitrile was 19 µM in the free chlorine FP experiment, but only 1 µM in the chloramine FP experiment. However, by day 7 (168 hours), similar concentrations of DBPs were formed in all experiments. Thus it appears that, while the presence of ammonia may influence short-term rates of DBP formation, overall it did not affect longer-term formation. Therefore, the use of chloramination instead of chlorination does not appear to reduce the formation of these DBPs from AAs. The presence of ammonia might have affected the formation of *N*,*N*-dichloramine (II→V in Figure 6-1) due to the competitive reaction of chlorine with ammonia instead of *N*-monochloramine, which would particularly affect the concentration of isobutyronitrile (VIII; R = propane) and *N*-chlorisobutyraldimine (VI; R = propane) formed. However, the presence of monochloramine could also promote alternative reaction pathways, such as the chloramination of aldehyde (IV) to form *N*-chloramino alcohol (IX), which provides an alternative pathway for the formation of *N*-chlorisobutyraldimine (VI) and isobutyronitrile (VIII) from valine. However, more studies are required to investigate the reaction rates of the proposed alternative pathways, to confirm that these could explain the differences in DBP concentrations measured between 3 hours and 7 days.

Table 6-8: Concentration (µM) of odorous by-products after 7 days from the chlorination (residual chlorine=10 mg L^{-1} as chlorine) using secondary wastewater from WWTP 2 and 3 from Feb-2015.

Analytes (µM)	WWTP 2	WWTP 3
2-methylbutyraldehyde	0.002±0.001	0.008±0.003
2-methylbutyronitrile	0.2±0.1	0.3±0.1
N-chloromethyl-2-butaldimine	0.008±0.003	0.05±0.03
Isobutyraldehyde	0.01±0.01	0.01±0.01
Isobutyronitrile	0.4±0.1	0.2±0.1
N-chloroisobutyraldimine	0.001±0.001	0.009±0.001
3-methylbutyraldehyde	0.01±0.01	0.02±0.01
3-methylbutyronitrile	0.4±0.1	0.5±0.1
N-chloromethyl-3-butaldimine	<0.003	0.005±0.002
Phenylacetaldehyde	<0.04	0.1±0.1
Benzyl Cyanide	0.05±0.01	0.6±0.2

Table 6-9: Concentration (µM) of isobutyraldehyde, isobutyronitrile and *N*-chloroisobutyraldimine at 3 and 168 hours after chlorination (10 mg L^{-1} as chlorine) with different concentration of ammonia in the water and a chloraminated standard. The ammonia to chlorine ratio were presented in the brackets.

Time (hours)	Concentration of ammonia (mg L^{-1} as N)				
	0(0.0)	4(1.7)	2(0.8)	1.25(0.5)	NH_2Cl(1.3)
Isobutyraldehyde (µM)					
3	3	1	1	1	0.5
168	2	2	3	5	3
Isobutyronitrile (µM)					
3	19	2	1	4	1
168	7	6	5	9	7.0
N-Chloroisobutyraldimine (µM)					
3	3	0.6	0.2	3	<0.001
168	2	2	0.1	5	2

6.4.6 Comparison of Laboratory Formation Studies and Real Systems

Our previous laboratory study using valine found that 30% of valine was converted to *N*-chloroisobutyraldimine when the chlorine to valine ratio was 2.8 (How et al. submitted). However, in most real systems, the chlorine to precursor (individual AA) ratio is likely to be two or three orders of magnitude higher. For example, at DWTP 1, the initial chlorine concentration required to achieve 2 mg L^{-1} as Cl_2 residual chlorine during the November 2015 sampling event would be around 6 mg L^{-1} as Cl_2 (85 µM). The valine concentration in the pre-chlorinated water was the same as that measured in the raw water (2.0 µg L^{-1} as N or 0.14 µM), the Cl: Val ratio was 600. Given that valine was the AA measured in the highest concentration in the raw water, and that this estimation assumes that no free AAs were removed or added before chlorination, the Cl:AA ratio of other AAs will be even higher. In the FP experiments investigating DBP formation from raw drinking water with added AAs (Table 6-7) and the impact of ammonia on DBP formation from ultrapure water with added AAs (Table 6-9), the chlorine to valine ratio is at least 4.4, and only 5-10% of valine was observed to convert to *N*-chloroisobutyraldimine. This suggests that higher chlorine to valine ratios suppress the conversion of *N,N*-dichlorovaline (V, Figure 6-1) to *N*-chloroisobutyraldimine (VI), or enhance the degradation of *N*-chloroisobutyraldimine (VI) to isobutyronitrile (VIII). The formation of isobutyraldehyde, previously proposed to predominantly result from the degradation of *N*-monochlorovaline (How et al. submitted), was similar in both the previous and current studies. This suggests that the formation of *N*-monochlorovaline and consequently *N,N*-dichlorovaline was also similar in both studies. The degradation of *N,N*-dichlorovaline into *N*-chloroisobutyraldimine and isobutyronitrile is independent of chlorine concentration (Figure 6-1). Therefore, given that the conversion of valine to isobutyronitrile was higher in this study (45-60%) than the conversion measured in our previous study (35%), it seems likely that the observed increase in isobutyronitrile concentration is most likely to result from the degradation of *N*-chloroisobutyraldimine rather than the enhance formation from *N,N*-dichlorovaline.

This result showed that at Cl:AA $\geq$ 4.4, the conversion of valine to isobutyraldehyde was 5%, to isobutyronitrile was 55% and to *N*-chloroisobutyraldimine was 10%, compared to isobutyraldehyde 5%, isobutyronitriles 35% and *N*-chloroisobutyraldimine 30% at Cl:AA = 2.8.

6.5 Conclusions

Although good removal of DOC was generally observed in both drinking water and wastewater treatment, the concentrations of free AAs were not significantly reduced. However, these free AAs contributed only a small fraction of DON or TDN in both drinking waters and wastewaters, and DBPs were not measured in actual drinking water systems. A study of total AAs, including proteins and peptides, is required to better understand the impact of all forms of AAs on water treatment. However, formation potential experiments to identify additional by-products showed that isobutyric acid can be formed from the chlorination of valine. Therefore, if the distributed water is expected to have a long retention time in the system, the formation of carboxylic acids from the degradation of aldehydes and nitriles (themselves derived from AAs) should also be considered.

Formation potential experiments at different ammonia concentrations showed that, while the presence of ammonia resulted in alteration of the formation pathways of the odorous by-products, overall there was little impact on the final concentration of these DBPs. Similarly, chlorination studies conducted for valine at Cl:AA ratios $\geq$ 4.4 showed that the proportion of aldehydes, nitriles and *N*-chloraldimines that formed was dissimilar to the controlled laboratory experiments undertaken with valine at Cl:AA = 2.8, where similar isobutyraldehyde conversion, lower *N*-chloroisobutyraldimine conversion and higher isobutyronitrile conversion were observed when comparing the DBPs from at Cl:AA ratios $\geq$ 4.4 to Cl:AA = 2.8. To date, most studies of DBP formation from AAs have been undertaken at controlled Cl:AA ratios. Additional experiments with conditions that are more similar to real water systems will improve understanding of the formation and speciation of these DBPs in distribution systems.

6.6 Acknowledgments

The authors acknowledge the Australian Research Council (LP110100548 and LP130100602), Water Research Australia and the Water Corporation of Western Australia (WCWA) for supporting this work. Curtin University (Curtin International Postgraduate Research Scholarship) and Water Research Australia (WaterRA PhD Scholarship) are also acknowledged for providing financial support for Z. How. The authors especially thank Fern Bradder, Arron Lethorn and operational staff from WCWA for assistance with sample collection.

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Chapter 7. Conclusions and Recommendations

This Thesis has shown that most amines and amino acids form organic chloramines upon chlorination. The organic chloramines produced from amines were found to be relatively stable with half-lives of more than three hours, while most organic chloramines produced from amino acids were less stable, with half-lives of less than 90 minutes. Amino acids like cysteine, methionine and tryptophan, which contain more reactive side groups (thiols for cysteine and methionine and indole ring for tryptophan), did not form organic chloramines at the chlorine to amino acid molar ratios examined (Cl:AA = 0.8 - 2.8), but potentially could form organic chloramines at the Cl:AA ratios usually found in drinking water. Based on a screening health risk assessment of organic monochloramines, *N*-monochloroglycine is proposed to be the organic monochloramine studied of highest health risk due to its stability, toxicity and precursor abundance. A more comprehensive health risk assessment would require further study of the toxicity and precursor abundance for other organic chloramines that are stable in water distribution systems (e.g., *N*-chloramines and *N*-chloraldimines). The degradation products of less stable organic chloramines should also be considered, particularly from amino acids that preferentially react with chlorine to form organic chloramines as compared to other less reactive amino acids.

Most of the amino acids investigated for differences in speciation of their organic chloramines formed upon chlorination followed the trend of formation of *N*-monochloramino acids at Cl:N $\leq$ 1 and *N*,*N*-dichloramino acids at Cl:N $\geq$ 2, except tyrosine. Tyrosine also formed *N*-monochlorotyrosine at Cl:N $\leq$ 1 but formed a mixture of dichlorotyrosine species at Cl:N $\geq$ 2. For tyrosine, further addition of chlorine at ratios of Cl:N > 2 led to chlorination of the phenol ring, ultimately resulting in degradation into other by-products at Cl:N = 12. This showed that the presence of a secondary functional group that is more reactive than the initially formed chloramine functional group will affect the speciation of organic chloramines formed from amino acids. However, since most amino acids do not have a reactive secondary functional group, it can be assumed that similar speciation and reaction pathways would be expected for most amino acids. Based on the study of formation and degradation pathways for the chlorination of valine, at Cl:N $\geq$ 2, amino acids react quickly with chlorine to form *N*-monochloramino acids. These *N*-monochloramino acids then either react with chlorine to form *N*,*N*-dichloramino acids or degrade to aldehydes. *N*,*N*-Dichloramino acids then degrade competitively to either nitriles or *N*-chloraldimines.

The conversion of degradation products from valine 3 hours after chlorination was 5% isobutyraldehyde, 35% isobutyronitrile and 30% *N*-chloroisobutyraldimine, with 30% of the by-products not identified. However, given that the reaction rates will differ for each individual amino acid, the final proportion of degradation products may vary. The formation of aldehyde and *N*-chloraldimine by-products is of concern as these compounds are odorous, with low odour threshold concentrations, while nitriles can be potentially harmful to human health.

From the occurrence study of free amino acids and dissolved organic carbon in both drinking water and wastewater treatment systems, it was found that good removal of dissolved organic carbon of up to 90% was achieved in both the drinking water and wastewater treatment and a general decrease in of free amino acids concentration after treatments for both drinking waters and wastewaters treatment. The concentration of free amino acids in raw drinking waters and secondary effluents were measured to determine the possibility of formation of disinfection by-products from free amino acids. The concentration of free amino acids ranged between 0.7 and 2.8 µg L^{-1} as N in raw drinking waters and between 0.5 and 2 µg L^{-1} as N in treated wastewaters. Free amino acids were found to contribute only a small fraction (0.06-2%) of total dissolved nitrogen in the drinking waters, and this was confirmed by the low formation of the odorous by-products from the chlorination of the raw waters samples. Studies of total amino acids, which includes proteins and peptides (combined amino acids), in drinking water and wastewater is recommended to further understand the impact of amino acids in water treatment.

When chlorination of amino acids was conducted at Cl:AA ratios relevant to drinking water applications (Cl:AA $\geq$ 4), the proportion of aldehydes, nitriles and *N*-chloraldimines that formed was dissimilar to the controlled lab experiments undertaken with valine at Cl:AA = 2.8. At Cl:AA $\geq$ 4, the conversion of valine to isobutyraldehyde was 5%, isobutyronitriles 10% and *N*-chloroisobutyraldimine 55%. Therefore, additional experiments with conditions that are more similar to 'real' drinking water systems would aid better understanding of the formation and speciation of these by-products in the distribution system. In addition, formation potential experiments to identify additional by-products showed that carboxylic acids can be formed from the chlorination of amino acids. Therefore, if the distributed water is

expected to have long retention times in the system, the formation of carboxylic acids from the degradation of aldehydes and nitriles should also be considered.

To consider the impact of ammonia on the formation of these three classes of disinfection by-products in wastewater, formation potential experiments at different ammonia concentrations were conducted. The results showed that, while the presence of ammonia resulted in alteration of the formation pathways of the odorous by-products and nitriles, overall there was little impact on the final concentrations of the odorous disinfection by-products and nitriles produced.

In summary, the chlorination of amino acids leads to the formation of *N*-chloramino acids which are generally not stable and the fact the concentration of free amino acids are typically very low in drinking water and thus are unlikely to pose a health risk to the consumer. However, the degradation products of *N*-chloramino acids, namely aldehydes, nitriles and *N*-chloraldimines, which some could result in odour problems in chlorinated water due to their low odour threshold concentrations. After formation, aldehydes and nitriles continue to degrade slowly into carboxylic acids, which can also be odorous and resulted in unknown health risk. Thus, for water distribution systems with short retention times, a greater focus should be placed on aldehydes, nitriles and *N*-chloraldimines, while for water distribution systems with long retention times, more focus should be directed to the formation of carboxylic acids. To date, little information is available on carboxylic acids in drinking water and so more studies on the occurrence and health impact of carboxylic acids in water is recommended. Given that the presence of ammonia had little impact on the concentration of the odorous disinfection by-products and nitriles formed, the use of chlorination or chloramination (in-line formation or preformed) would have little impact on the prevention of the formation of these odorous disinfection by-products and nitriles. Although, the three classes of disinfection by-products were not found in drinking waters, due to the low concentration of free amino acids in the waters, poor removal efficiency of free amino acids in the treatment processes was observed, which is of concern for any waters with high free amino acid concentrations. In addition, more information on removal of other sources of amino acids, such as peptides and proteins, is recommended to fully understand the removal efficiency of all amino acids (free and combined) in water treatment processes.

Appendix 1

Table A1-1: Basic characteristics of the drinking water source waters tested

	Surface water A	**Surface water B**	**Surface water C**
TOC (mg L^{-1})	4.378	5.868	2.854
DOC (mg L^{-1})	4.104	5.804	2.158
Br^- (µg L^{-1})	225	98	37
I^- (µg L^{-1})	<10	<10	<10
BrO_3^- (µg L^{-1})	<1	<1	<1
IO_3^- (µg L^{-1})	<1	<1	1.1
UV_{254} (filtered)	0.0836	0.0882	0.0663
SUVA	0.0204	0.0152	0.0307
NH_3 (mg L^{-1} as N)	0.01	<0.01	<0.01
NO_3 (mg L^{-1} as N)	<0.01	<0.01	0.8
NO_2 (mg L^{-1} as N)	<0.01	<0.01	0.01
TDN (mg L^{-1} as N)	0.4	0.32	1.0
DON (mg L^{-1} as N)	0.39	0.32	0.19

Table A1-2: Retention time (t_R), parent and product ions (m/z), optimised collision energy (CE) and surrogate standards used for analysis and quantification of amino acids. N.A. = not available.

Name	t_R (min)	Parent ion (m/z)	Product ions (m/z)	CE (eV)	Surrogate Standard
Window 1 (0-11 min)					
Lysine	6.1	147.2	84.3	15	Glutamic-d_3 acid
Arginine	6.3	175.2	70.3	15	Glutamic-d_3 acid
Histidine	6.3	156.2	110.3	12	Glutamic-d_3 acid
Glycine	7.2	76.3	76.29	1	Glycine-d_2
Glycine-d_2	7.2	78.3	78.29	1	N.A.
Serine	7.3	106.1	60.4	7	Glutamic-d_3 acid
Asparagine	7.4	133.1	74.2	15	Glutamic-d_3 acid
Alanine	7.5	90.1	44.5	10	Alanine-d_3
Glutamine	7.5	147.2	84.3	15	Glutamic-d_3 acid
Thereonine	7.5	120.1	102.2	7	Glutamic-d_3 acid
Alanine-d_3	7.5	93.3	47.5	10	N.A.
Glutamic Acid	7.6	148.1	102.2	11	Glutamic-d_3 acid
Glutamic-d_3 acid	7.6	151.2	86.3	15	N.A.
Aspartic Acid	7.8	134.2	88.3	10	Glutamic-d_3 acid
Cysteine	8.1	122.2	76.3	15	Glutamic-d_3 acid
Proline	8.4	116.1	70.2	12	Glutamic-d_3 acid
Valine	9.5	118.1	72.3	12	Glutamic-d_3 acid
Window 2 (8.5-25 min)					
Methionine	11.1	150.2	133.3 104.3	10 10	Leucine-d_3
Isoleucine	14.4	132.2	86.3 69.4	10 17	Leucine-d_3
Leucine-d_3	14.5	135.2	89.2 30.6	10 18	N.A.
Leucine	15.5	132.2	86.3 69.4	10 17	Leucine-d_3
Tyrosine	20.1	182.2	136.3	10	Leucine-d_3
Window 3 (24-35 min)					
Phenyl-d_5-alanine-d_3	27.9	174.3	128.0 157.1	15 10	N.A.
Phenylalanine	28.7	166.2	102.2 131.2	15 12	Phenyl-d_5-alanine-d_3
Tryptophan	29.9	205.2	146.0 188.0	12 10	Phenyl-d_5-alanine-d_3

Table A1-3: Linear range, regression, instrumental detection limit (IDL) and instrumental precision in term of retention time (t_R) and MRM ratio.

Name	t_R (min) ± SD	Linear range (ng µL^{-1})	IDL (pg on column)	R^2	MRM ratio ± RSD (%)
Lysine	6.18±0.08	0.002-5	2	0.993	N.A.
Arginine	6.30±0.09	0.002-5	3	0.990	N.A.
Histidine	6.34±0.08	0.002-5	1	0.991	N.A.
Glycine	7.24±0.08	0.002-5	32	0.994	N.A.
Glycine-d_2	7.23±0.08	N.A.	N.A.	N.A.	N.A.
Serine	7.43±0.07	0.002-5	7	0.997	N.A.
Asparagine	7.47±0.08	0.002-5	1	0.995	N.A.
Alanine	7.41±0.07	0.002-5	26	0.997	N.A.
Glutamine	7.58±0.08	0.002-20	1	0.991	N.A.
Threonine	7.62±0.08	0.002-20	6	0.994	N.A.
Alanine-d_3	7.39±0.08	N.A.	N.A.	N.A.	N.A.
Glutamic	7.75±0.09	0.002-20	2	0.990	N.A.
Glutamic-d_3 acid	7.73±0.09	N.A.	N.A.	N.A.	N.A.
Aspartic	7.77±0.08	0.002-10	4	0.993	N.A.
Cysteine	7.85±0.06	1-5	186	0.998	N.A.
Proline	8.37±0.15	0.002-5	3	0.998	N.A.
Valine	9.26±0.24	0.002-20	2	0.998	N.A.
Methionine	10.21±0.2	0.002-20	9	0.993	1.0±1.0
Isoleucine	13.34±0.5	0.002-5	2	0.997	7±2
Leucine-d_3	13.99±0.5	N.A.	N.A.	N.A.	70±2
Leucine	14.16±0.5	0.002-10	1	0.996	N.A.
Tyrosine	17.56±1.0	0.002-10	4	0.994	N.A.
Phenyl-d_5 - alanine-d_3	27.73±0.96	N.A.	N.A.	N.A.	33±1
Phenylalanin	28.12±0.7	0.002-5	1	0.996	30±2
Tryptophan	30.03±0.1	0.002-5	1	0.996	5±2

N.A. = not available.

Table A1-4: Conditioning, washing and elution of SPE cartridges.

SPE task	Stationary phase	
	Reversed phase	**Strong cation-exchange**
Conditioning	4.5 mL of methanol 4.5 mL of acetonitrile 9 mL ultrapure water	4.5 mL of methanol 9 mL ultrapure water
Washing	6 mL of ultrapure water 2.5 % methanol in ultrapure water	3 mL 0.1 mol L^{-1} of hydrochloric acid in water 3 mL 0.1 mol L^{-1} of hydrochloric acid in
Elution	6 mL of methanol 5.5 mL of acetonitrile	11.5 mL of 5 % ammonium hydroxide

Table A1-5: Analysis of washing solutions containing two different organic solvents in ultrapure water, isopropyl alcohol (IPA) and methanol (MeOH), in two different percentages at neutral pH. The recoveries of the analytes are reported as % recovery±RSD %.

Name	2.5 % IPA	4.5 % IPA	2.5 % MeOH	4.5 % MeOH
Lysine	10±14	81±22	<1	<1
Histidine	3±18	71±13	<1	<1
Arginine	<1	<1	<1	<1
Glycine-d_2	N.D.	24±50	N.D.	<1
Glycine	15±19	22±24	N.D.	<1
Serine	<1	12±24	N.D.	<1
Alanine-d_3	N.D.	28±9	N.D.	<1
Alanine	1±27	33±6	N.D.	<1
Asparagine	<1	8±1	N.D.	<1
Glutamine	1±28	3±10	N.D.	<1
Thereonine	1±25	14±10	1±29	<1
Glutamic – d_3	2±0	1±7	N.D.	<1
Aspartic Acid	1±19	1±31	<1	<1
Cysteine	N.D.	N.D.	N.D.	N.D.
Glutamic	2±17	4±8	<1	<1
Proline	21±21	81±22	<1	<1
Valine	10±82	95±10	<1	<1
Methionine	9±38	12±6	N.D.	32±9
Isoleucine	28±57	81±11	<1	59±5
Leucine	48±44	41±1	<1	24±3
Leucine-d_3	44±20	49±3	N.D.	107±6
Tyrosine	62±9	165±10	<1	98±19
Phenyl-d_5-alanine-d_3	57±14	61±10	1±14	58±2
Phenylalanine	56±12	86±12	2±10	103±0
Tryptophan	60±8	79±8	17±6	56±3

Table A1-6: Comparison of matrix interference on standards and their surrogate standard in Groundwater. Ion suppression is represented by a negative percentage, while ion enhancement is represented by a positive percentage.

Compound	Matrix Effect (%)
Glycine	-34
Glycine-d_2 (IS)	-66
Alanine-d_3	-56
Alanine-d_3 (IS)	-58
Glutamic Acid	-82
Glutamic-d_3- acid	-81
Leucine-d_3 (IS)	-35
Leucine	-31
Phenyl-d_5 - alanine-d_3 (IS)	116
Phenylalanine	90

Appendix 2

Liquid Chromatography-Ultraviolet Absorbance-High Resolution Mass Spectrometry Conditions

An Accela 600 LC system coupled to an Accela PDA detector (Thermo Fisher Scientific Corporation, Waltham, USA) set at λ=255 nm and a high resolution mass spectrometer (HRMS) (LTQ Orbitrap XL, Thermo Fisher Scientific Corporation, Waltham, USA) fitted with an electrospray ionisation (ESI) operated in positive ionization mode (+eV) was used for LC-UV-HRMS analysis. For increased mass accuracy on the LTQ Orbitrap XL, a plasticizer (n-butyl benzenesulfonamide) interference peak present in the LC mobile phase with *m/z* 214.0896 was used for the lock mass function. For screening and quantification, two different MS acquisition modes were combined in the same analytical run. The screening analysis was conducted with HRMS full-scan mode from 70-200 *m/z* with a mass resolution of 15000 (@ 400 *m/z*). The presence of the detected organic chloramines was confirmed using $HRMS^2$ mode, where the mass spectrometer was forced to isolate the parent compound, fragment it in the LTQ ion trap and then scan for the product ions in the Orbitrap mass analyser (50-200 *m/z*). A mass resolution of 7500 (@ 400 *m/z*) was used for the fragmentation experiments. Organochloramine identification used the chromatographic retention time, comparison of the measured masses (i.e. parent compound and fragments) to the theoretical masses (deviation < 5 ppm, relative error), and comparison of measured isotope patterns to simulated isotope patterns from the X-calibur software. The fragmentations of the parent ions of the organic chloramines were also analysed to act as a secondary confirmation of the structure. Data was processed using X-calibur QualBrowser 2.0.7 SP1 and TraceFinder 3.1. Chromatographic conditions were adopted from a LC-MS/MS method described previously (How et al. 2014). Briefly, chromatographic separation was achieved using an Accela 600 HPLC system. Amino acids and monochloramines were separated using a Gemini C18 column (250 mm x 3 mm I.D., 3 μm particle size) from Phenomenex (New South Wales, Australia) at a flow rate of 150 μL min^{-1}.

Table A2-1: Summary of ESI and $HRMS^2$ parameters used for target analysis of *N*-chloroisoleucine and *N*-chlorovaline.

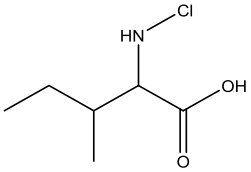
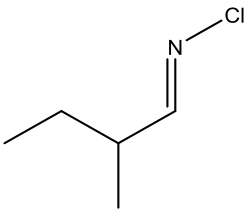
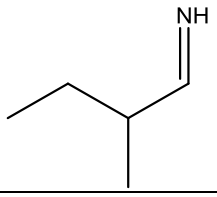
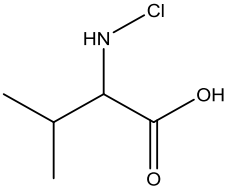
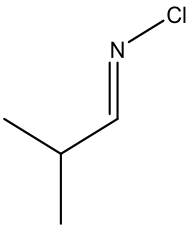
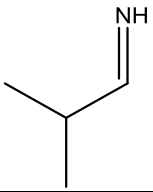
Parameter	Setting
Spray voltage (kV)	3.5
Capillary voltage (V)	-35
Capillary temperature (°C)	275
Sheath gas flow rate (Arb)	20
Aux gas flow rate (Arb)	0
Sweep gas flow rate (Arb)	0
Tube Lens (V)	-70
Scan range (*m/z*)	50-200
IT full MS AGC target	3E4
IT MS^n AGC target	1E4
FT full MS AGC target	2E5
FT MS^n AGC target	1E5
Ion trap and FT micro scans	3
IT full MS Max ion time (ms)	10
FT MS^2 Max ion time (ms)	100
MS^2 isolation window (*m/z*)	1

Arb: arbitrary units; ms: milliseconds; IT: Ion trap; FT Fourier Transform (Orbitrap mass spectrometer); AGC: Automatic Gain Control.

Table A2-2: List of organic chloramines with first-order degradation reactions where the R^2 value represents the fitting of the reaction to a first-order plot.

Organic chloramines	R^2 value
N-chloroglycine	0.9759
N-chloroisoleucine	0.9926
N-chlorovaline	0.9916
N-chloroalanine	0.9917
N-chloroserine	0.9916
N-chloroglutamic acid	0.9896
N-chlorothreonine	0.9914
N-chlorohistidine	0.9875
N-chloroarginine	0.9916
N-chloroleucine	0.9914
N-chlorolysine	0.9564
N-chloroproline	0.9981
N-chlorophenylalanine	0.9843
N-chlorotyrosine	0.9232
N-chloroasparagine	0.9841
N-chlorodimethylamine	0.9451
N-chlorodiethylamine	0.9607

Table A2-3: Predicted fragments from Massfrontier 7.0 software for *N*-chloroisoleucine and *N*-chlorovaline.

Fragment structure	**Theoretical mass $[M+H]^+$**
***N*-chloroisoleucine**	
	166.0629 (Parent ion)
	120.0582
	86.0972
***N*-chlorovaline**	
	138.0316 (Parent ion)
	106.5740
	72.0815

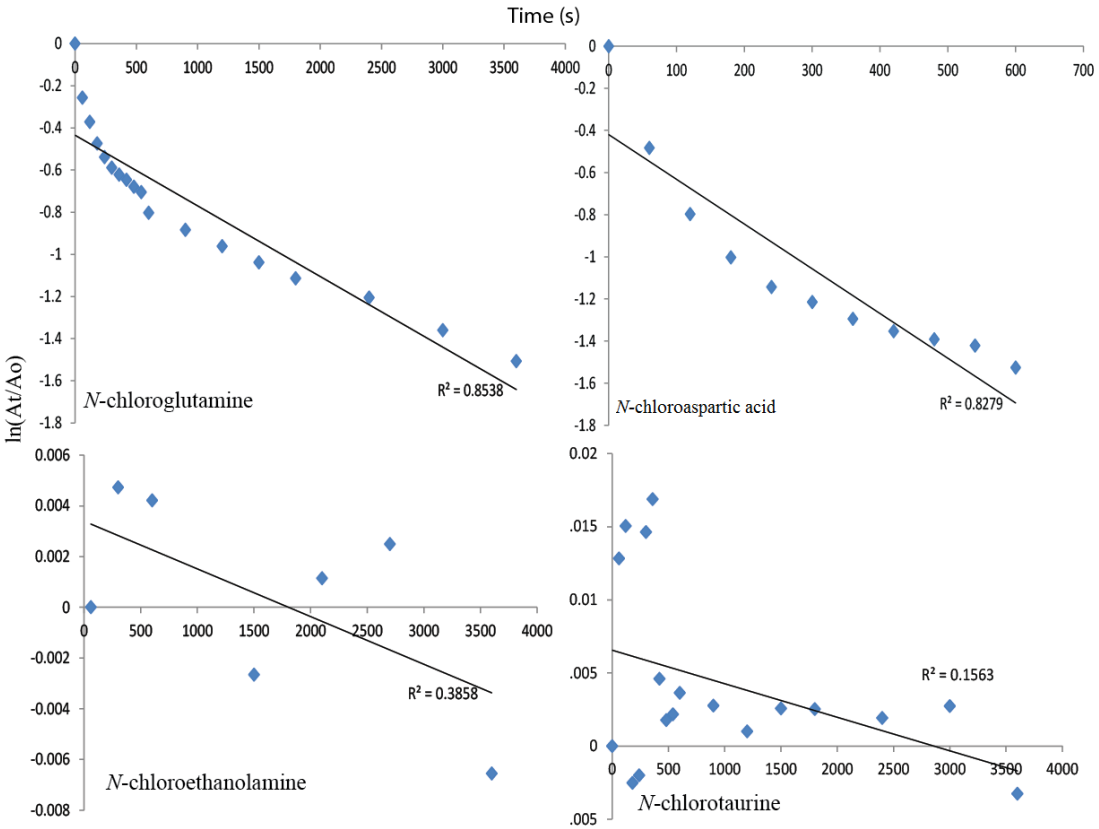


Figure A2-1: Pseudo-first order kinetic plots for organic chloramines where the degradation did not follow a first order reaction.

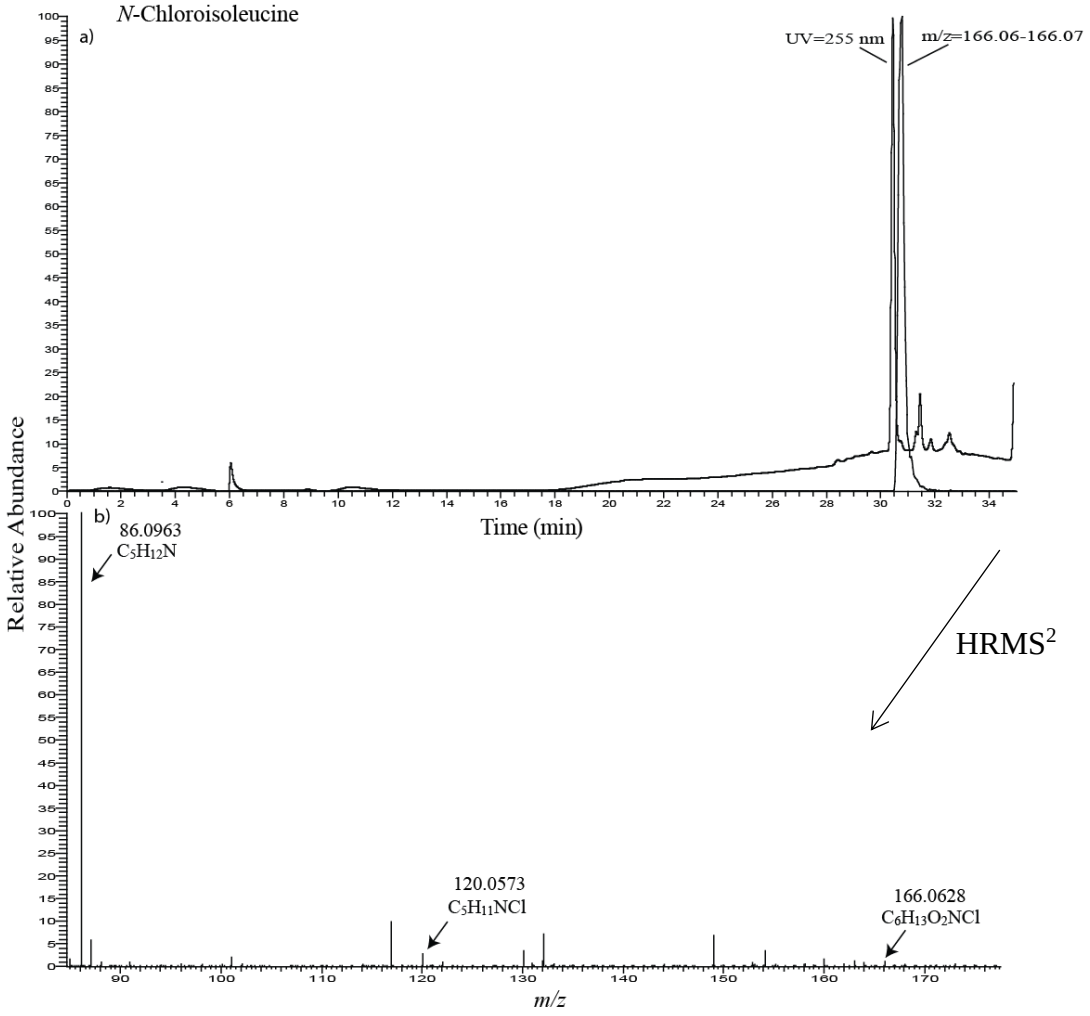


Figure A2-2: LC-UV-HRMS and HRMS^2 confirmation data for the formation of *N*-chloroisoleucine. a) The retention of the signal at UV= 255 nm and the signal for m/z 166.06 was similar suggesting that they were the same compound. b) The exact mass of the peak at t_R= 31 min and its fragments confirmed that the compound was *N*-chloroisoleucine.

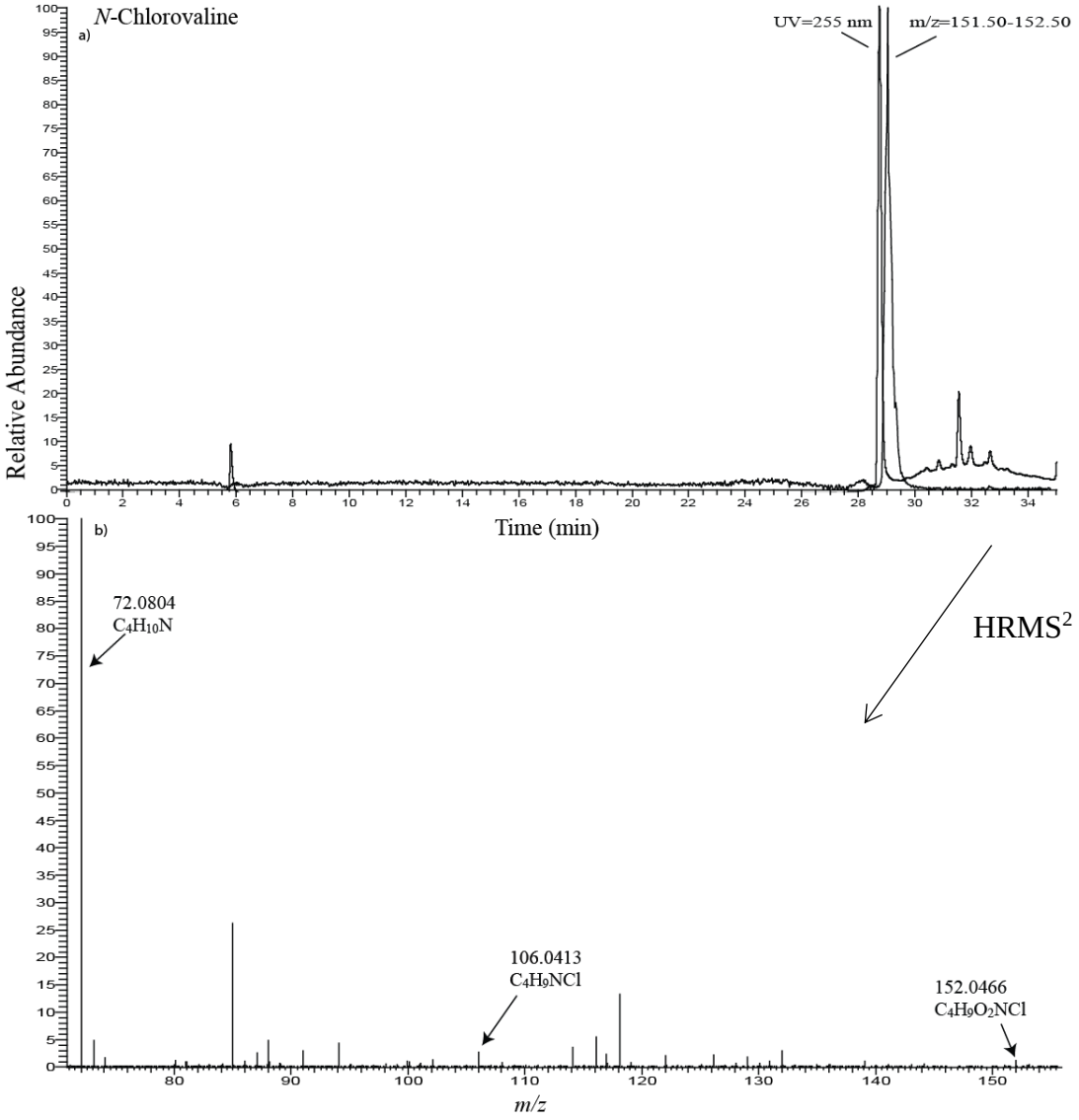


Figure A2-3: LC-UV-HRMS^2 confirmation for the formation of *N*-chlorovaline. a) The retention of the signal at UV= 255 nm and the signal for m/z 152.05 was similar suggesting that they were the same compound. b) The exact mass of the peak at t_R= 29 min and its fragments confirmed that the suspected compound was *N*-chlorovaline.

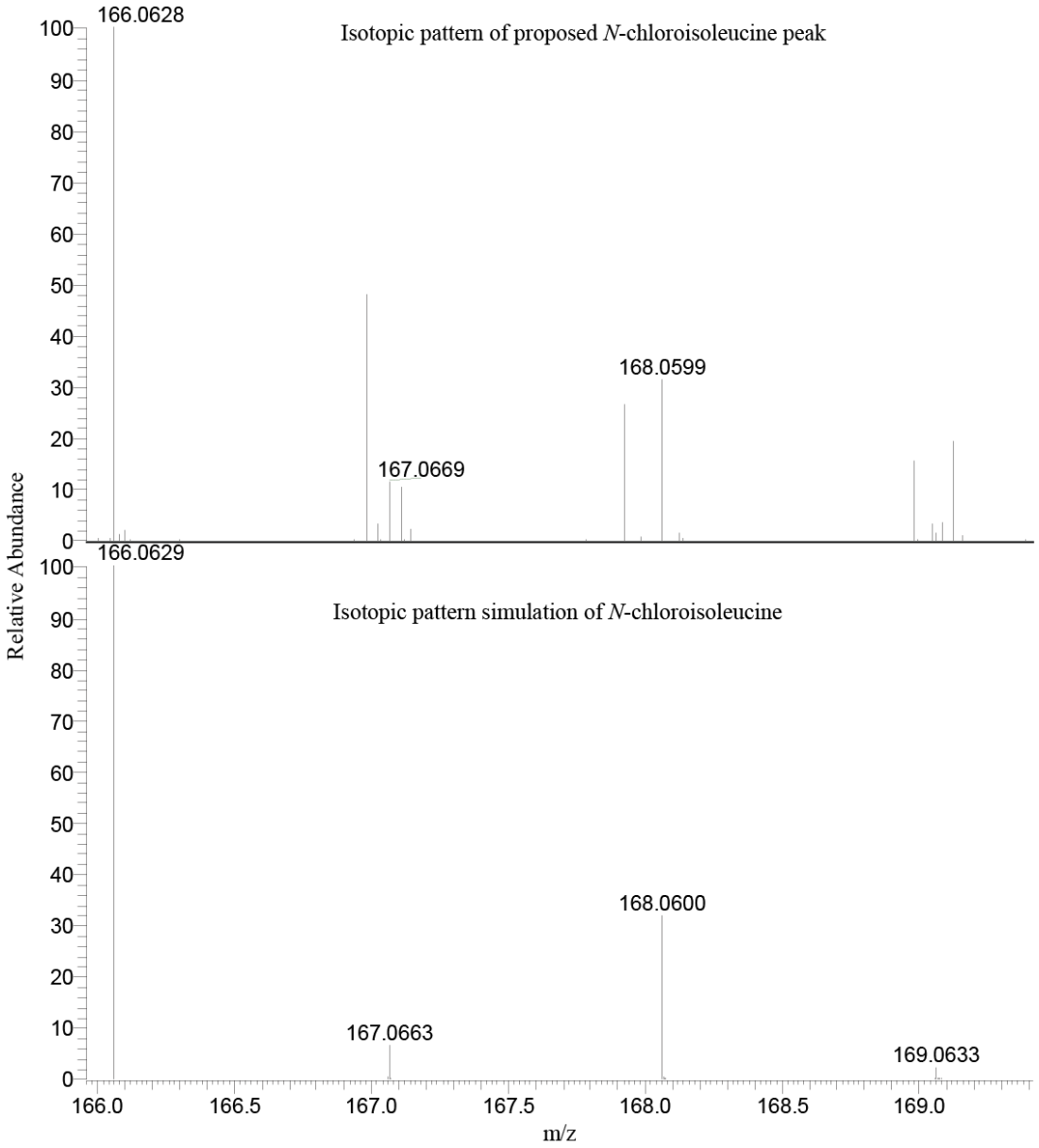


Figure A2-4: Isotopic pattern comparison between peaks proposed to be *N*-chloroisoleucine and simulation using X-calibur software.

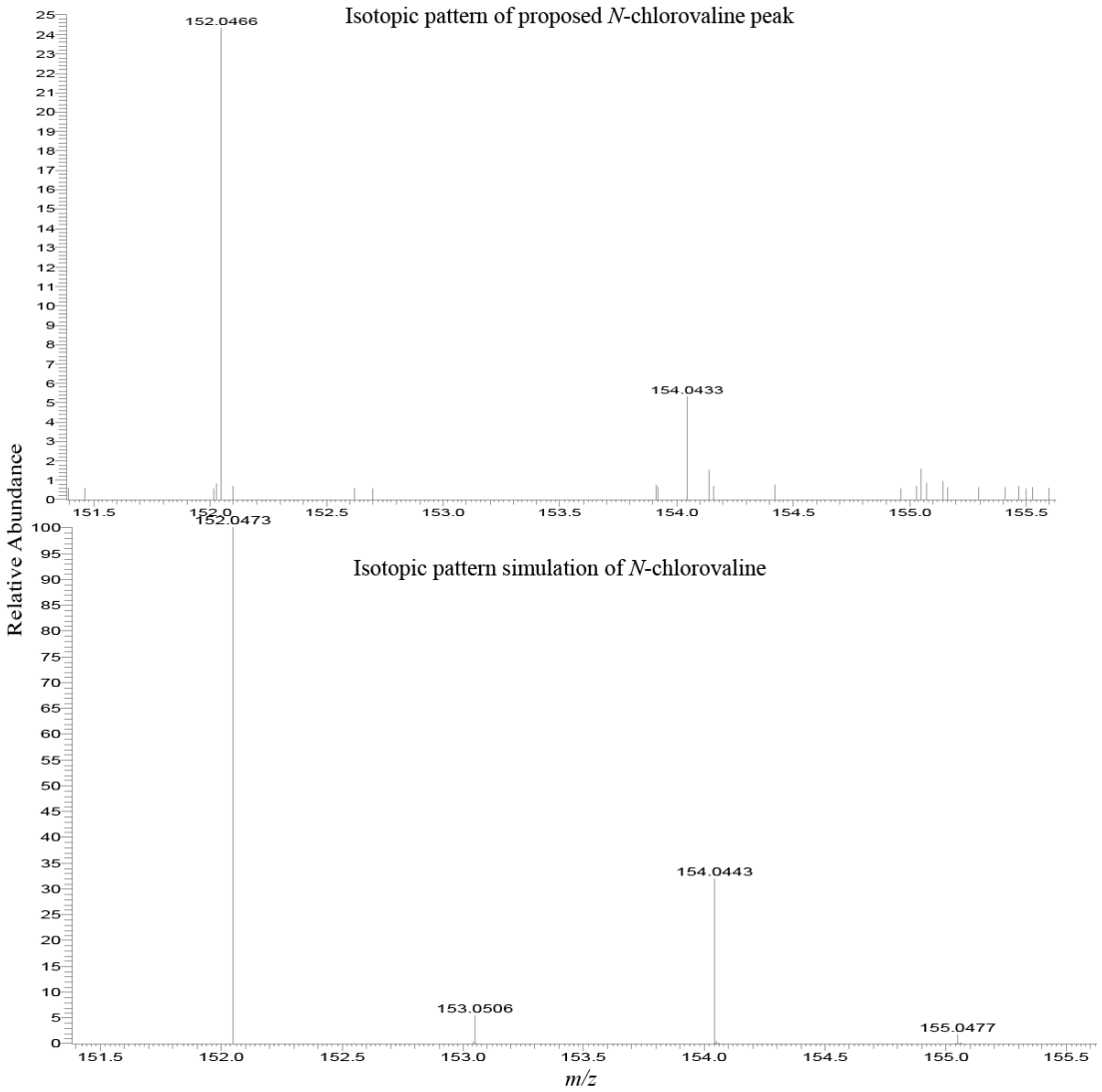


Figure A2-5: Isotopic pattern comparison between peaks proposed to be *N*-chlorovaline and simulation using X-calibur software.

Appendix 3

Text A3-1. Chemicals

Amino acids (glycine, isoleucine, leucine, lysine, phenylalanine, tyrosine and valine (purity of all amino acid standards ≥ 97%)), aldehydes (2-methylbutyraldehyde (>90%), 3-methylbutyraldehyde (97%), isobutyraldehyde (99%), phenylacetaldehyde (>90%)), nitriles (2-methylbutyronitrile (98%), isovaleronitrile (98%), isobutyronitrile (99.6%), benzyl cyanide (98%)), aqueous sodium hypochlorite (10 – 15% chlorine) and diethyl ether (HPLC grade) were all purchased from Sigma Aldrich (New South Wales, Australia). Taurine (97%) was purchased from AK Scientific (California, USA). The internal standard 1,2-dibromopropane-d_6 (99.7%) was purchased from CDN Isotopes (Quebec, Canada; distributed by SciVac, Hornsby, Australia). Methanol (HPLC grade) was purchased from Honeywell-Burdick & Jackson (Michigan, USA). Formic acid (99%) was purchased from Ajax FineChem (New South Wales, Australia). Analytical grade anhydrous sodium sulphate, purchased from Ajax FineChem, was baked at 400 ^{o}C for 4 hours prior to use, in order to remove organic impurities. The solid-phase microextraction fibre (Carboxen/Polydimethylsiloxane (CAR/PDMS) 75µm phase thickness) was purchased from Supelco (Bellefonte, PA, USA). Ultrapure water was produced using an ion exchange system (IBIS Technology, Western Australia, Australia), followed by an Elga Purelab Ultra system with a 0.2 µm filter (Elga, High Wycombe, UK).

Text A3-2. Kinetic Modelling of Formation and Degradation of Organic Chloramines Formed from Valine

Not all species from the formation and degradation pathways of the chlorination of valine could be detected, therefore it was not possible to experimentally determine the rate constants for all reactions. Therefore, the corresponding rate constants were modelled, i.e., the rate constants for the degradation of isobutyrimine and 2-chlorimino-3-methylbutanoic acid, and the formation of *N*,*N*-dichlorovaline, were modelled using the Kintecus software (Ianni 2015). The model conditions adopted were as follows: pH 8; chlorine to valine ratio 2.8; initial concentration of valine 30 µM. To investigate the rate constant for the formation or degradation of a compound that could not be measured experimentally, the model was populated with appropriate

reactions as listed in Table A3-8 (acronyms defined in Table A3-7). The initial proposed rate constant for each reaction was either the rate constant determined experimentally in this study, a reported rate constant value for a similar reaction, or a reasonable estimation based on available data. The rate constants for the dissociation of hypochlorous acid in water and its reaction with ammonia were adapted from the work by Valentine et al. (1986) The rate constants for the reactions from the chloramination of isobutyraldehyde (reactions 13 - 16, Table A3-8) were adapted from the work by Kimura et al. (2015) as they found that aldehydes could be chloraminated to form *N*-chloraldimines or *N*-chloramides. In addition, the formation of haloacetic acids have been observed from the chlorination or hydrolysis of haloacetonitriles (Yu and Reckhow 2015). Therefore the rate constant of the degradation of isobutyronitrile (reaction 17) was adapted from the work by Yu and Reckhow (2015). The proposed reaction pathways for the chlorination of valine used in the modelling are presented in Figure A3-4.

The rate constants, in the order listed in Table S8, were increased and decreased gradually by steps of 0.05. If changes to the initial proposed rate constant did not show any impact on the trends for the formation and degradation of isobutyraldehye, isobutyronitrile and *N*-chloroisobutyraldimine, the initial proposed rate constant was used. Fine adjustments of rate constants were then continued by trial and error until the initial simulated trend of formation and degradation of isobutyraldehyde, isobutyronitrile and *N*-chloroisobutyraldimine became similar to the trend observed experimentally.

Table A3-1: Structures of amino acids studied

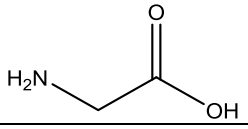
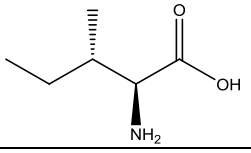
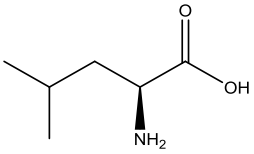
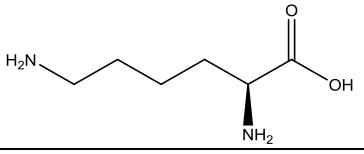
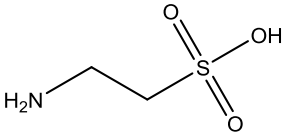
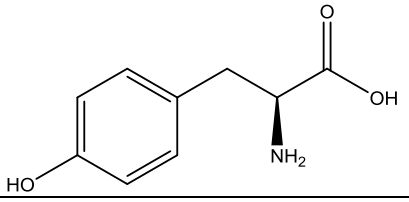
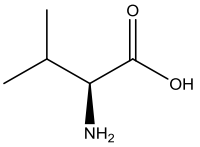
Glycine	
Isoleucine	
Leucine	
Lysine	
Taurine	
Tyrosine	
Valine	

Table A3-2: Summary of ESI and HRMS parameters used for screening analysis of organic chloramines in positive ionisation mode

Parameter	**Setting**
Spray voltage (kV)	3.5
Capillary voltage (V)	-35
Capillary temperature (°C)	275
Sheath gas flow rate (Arb)	20
Aux gas flow rate (Arb)	0
Sweep gas flow rate (Arb)	0
Tube Lens (V)	-70
Scan range (*m/z*)	70-300
IT full MS AGC target	3E4
IT MS^n AGC target	1E4
FT full MS AGC target	2E5
FT MS^n AGC target	1E5
Ion trap and FT micro scans	3
IT full MS Max ion time (ms)	10
FT MS^2 Max ion time (ms)	100
MS^2 isolation window (*m/z*)	1

Arb: arbitrary units; ms: milliseconds; IT: Ion trap; FT Fourier Transform (Orbitrap mass spectrometer); AGC: Automatic Gain Control.

Table A3-3: GC column and oven temperature programs for the analysis of aldehydes, nitriles and N-chloraldimines using headspace solid-phase microextraction (HS SPME) followed by GC-MS.

Analytical Column	ZB-5MS
Length (m)	30
Internal Diameter (mm)	0.25
Film Thickness (µm)	1
Carrier Gas	Helium
Carrier Flow (mL/min	0.7
Injector Temperature (ºC)	300 ºC
Oven Program	Hold at 35 ºC for 2 min Ramp to 105 ºC at 5 ºC/min and hold for 5 min Ramp to 300 ºC at 15 ºC/min and hold for 5 min

Table A3-4: List of quantifying (underlined) and qualifying ions monitored in SIM mode for the analysis of selected aldehydes, nitriles and *N*-chloraldimines.

Analytes	**Ions (m/z)**
Aldehydes	
2-Methylbutyraldehyde	<u>57</u>, 41, 29
Isobutyraldehyde	<u>43</u>, 72, 27
Isovaleraldehyde	<u>44</u>, 58, 39
Phenylacetaldehyde	<u>91</u>, 120, 65
Nitriles	
2-Methylbutyronitrile	<u>55</u>, 54, 29
Isobutyronitrile	<u>42</u>, 68, 54
Isovaleronitrile	<u>43</u>, 41, 39
Benzyl Cyanide	<u>117</u>, 116, 90
***N*-Chloraldimines**	
N-Chloroisobutyraldimine	<u>70</u>, 41, 62
N-Chloro-3-methylbutan-1-imine	<u>77</u>, 79, 55
N-Chloro-2-methylbutan-1-imine	91, <u>55</u>, 77
Internal Standard	
1,2-Dibromopropane-d_6	<u>127</u>, 129

Table A3-5: Retention time of selected amino acids and their monochloramino acids using a C-18 reverse phase column

Compounds	**Retention time (min)**	
	Amino acid	Monochloramino acid
Lysine	5.2	6.9 9.9
Taurine	7.8	47.0
Glycine	8.2	17.5
Valine	10.1	28.7
Isoleucine	12.3	31.5
Tyrosine	15.6	24.0

Table A3-6: Amino acid precursors and the corresponding aldehydes, nitriles and N-chloraldimines detected using headspace solid-phase microextraction-GC-MS.

Precursors	**Aldehyde**	**Nitrile**	***N*-Chloraldimine**
Glycine	Not Detected	Not Detected	Not Detected
Isoleucine	Detected	Detected	Detected
Leucine	Detected	Detected	Detected
Lysine	Not Detected	Not Detected	Not Detected
Phenylalanine	Detected	Detected	Not Detected
Taurine	Not Detected	Not Detected	Not Detected
Tyrosine	Not Detected	Not Detected	Not Detected
Valine	Detected	Detected	Detected

Table A3-7: List of assigned abbreviations of the chemicals used in the kinetic model

Abbreviations	**Full Name**
Val	Valine
Cl-Val	*N*-Monochlorovaline
IBI	Isobutyrimine
IBA	Isobutyraldehye
DiCl-Val	*N*,*N*-Dichlorovaline
NClIBA	*N*-Chlorisobutyraldimine
ClIA	2-Chlorimino-3-methylbutanoic acid
IBN	Isobutyronitrile
ClAA (alcohol)	1-(Chloroamino)-2-methylpropan-1-ol
ClA	*N*-Chlorisobutyramide
IBuA	Isobutyric acid

Table A3-8: Reactions for the formation and degradation pathways of the chlorination of valine and their proposed rate constants

No.	Reactions	Initial proposed rate constant	Final proposed rate constant	Source
1	$HOCl \rightarrow H^+ + ClO^-$	$3.20 \times 10^4\ s^{-1}$	$3.20 \times 10^4\ s^{-1}$	Valentine et al.
2	$H^+ + ClO^- \rightarrow HOCl$	$1.00 \times 10^{12}\ s^{-1}$	$1.00 \times 10^{12}\ s^{-1}$	Valentine et al.
3	$HOCl + Val \rightarrow Cl\text{-}Val$	$5.40 \times 10^4\ M^{-1}s^{-1}$	$5.40 \times 10^4\ M^{-1}s^{-1}$	Experimentally
4	$Cl\text{-}Val \rightarrow IBI$	$1.00 \times 10^{-4}\ s^{-1}$	$1.00 \times 10^{-4}\ s^{-1}$	Experimentally
5	$IBI \rightarrow IBA + NH_3$	$1.00 \times 10^2\ s^{-1}$	$5.00 \times 10^4\ s^{-1}$	Estimated
6	$HOCl + Cl\text{-}Val \rightarrow DiCl\text{-}Val$	$1.00 \times 10^4\ M^{-1}s^{-1}$	$4.85 \times 10^2\ M^{-1}s^{-1}$	Estimated
7	$DiCl\text{-}Val \rightarrow NClIBA$	$9.20 \times 10^{-5}\ s^{-1}$	$1.25 \times 10^{-4}\ s^{-1}$	Experimentally
8	$DiCl\text{-}Val \rightarrow ClIA$	$1.00 \times 10^{-4}\ s^{-1}$	$1.15 \times 10^{-4}\ s^{-1}$	Experimentally
9	$ClIA \rightarrow IBN$	$1.00 \times 10^{-3}\ s^{-1}$	$1.00 \times 10^{-2}\ s^{-1}$	Estimated
10	$NClIBA \rightarrow IBN$	$1.10 \times 10^{-6}\ s^{-1}$	$1.00 \times 10^{-6}\ s^{-1}$	Experimentally
11	$NClIBA \rightarrow IBA$	$1.10 \times 10^{-6}\ s^{-1}$	$1.00 \times 10^{-6}\ s^{-1}$	Experimentally
12	$HOCl + NH_3 \rightarrow NH_2Cl + H_2O$	$1.00 \times 10^4\ M^{-1}s^{-1}$	$1.00 \times 10^4\ M^{-1}s^{-1}$	Valentine et al.
13	$NH_2Cl + IBA \rightarrow ClAA$	$24.3\ M^{-1}s^{-1}$	$24.3\ M^{-1}s^{-1}$	Kimura et al.
14	$ClAA \rightarrow IBA$	$0.247\ M^{-1}s^{-1}$	$0.247\ M^{-1}s^{-1}$	Kimura et al.
15	$ClAA + NH_2Cl \rightarrow ClA$	$2.67 \times 10^4\ M^{-2}s^{-1}$	$2.67 \times 10^4\ M^{-2}s^{-1}$	Kimura et al.
16	$ClAA \rightarrow NClIBA$	$9.75\ M^{-1}s^{-1}$	$9.75\ M^{-1}s^{-1}$	Kimura et al.
17	$IBN \rightarrow IBuA$	$3.83 \times 10^{-8}\ s^{-1}$	$2.00 \times 10^{-6}\ s^{-1}$	Yu and Reckhow
18	$IBA \rightarrow IBuA$	$3.00 \times 10^{-5}\ s^{-1}$	$3.00 \times 10^{-5}\ s^{-1}$	Kimura et al.

Figures

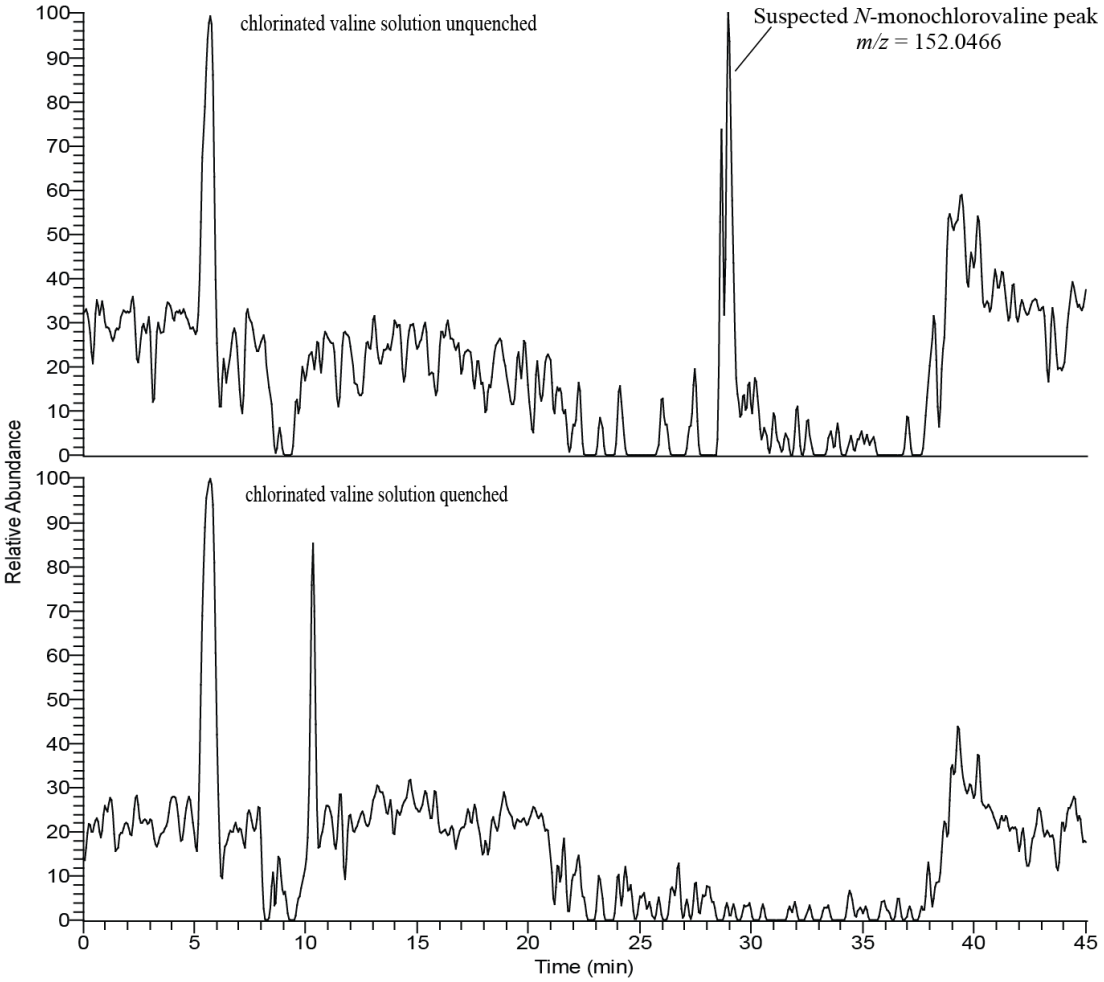
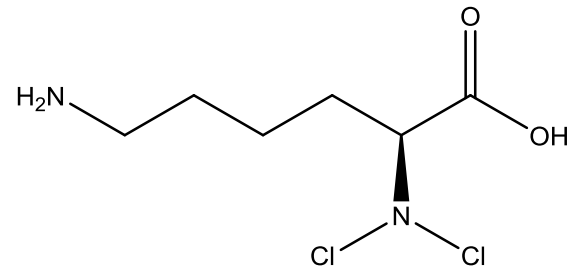
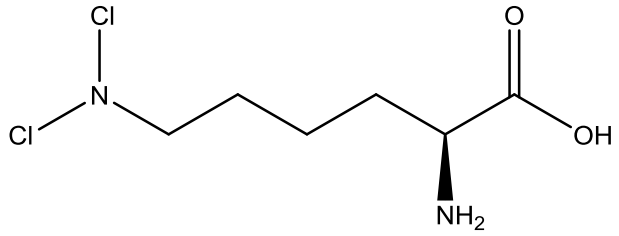


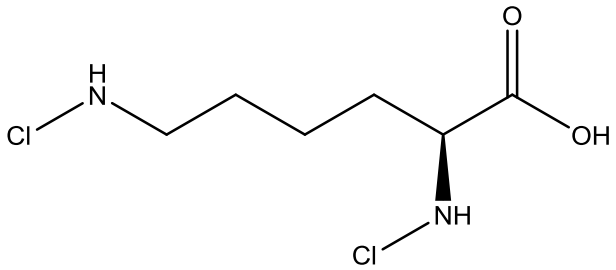
Figure A3-1: LC-MS total ion chromatograms of an unquenched chlorinated valine solution (top) and a quenched chlorinated valine solution (bottom) using MS scan. The disappearance of the suspected N-monochlorovaline peak in the quenched samples, along with the accurate mass of N-monochlorovaline (m/z=152.0466, at 5 ppm error), provides confirmation that the peak was N-monochlorovaline.



N,N-dichlorolysine



N',*N*'-dichlorolysine



N,*N*'-dichlorolysine

Figure A3-2: Possible dichlorolysine species at chlorine to lysine ratio of 2.8

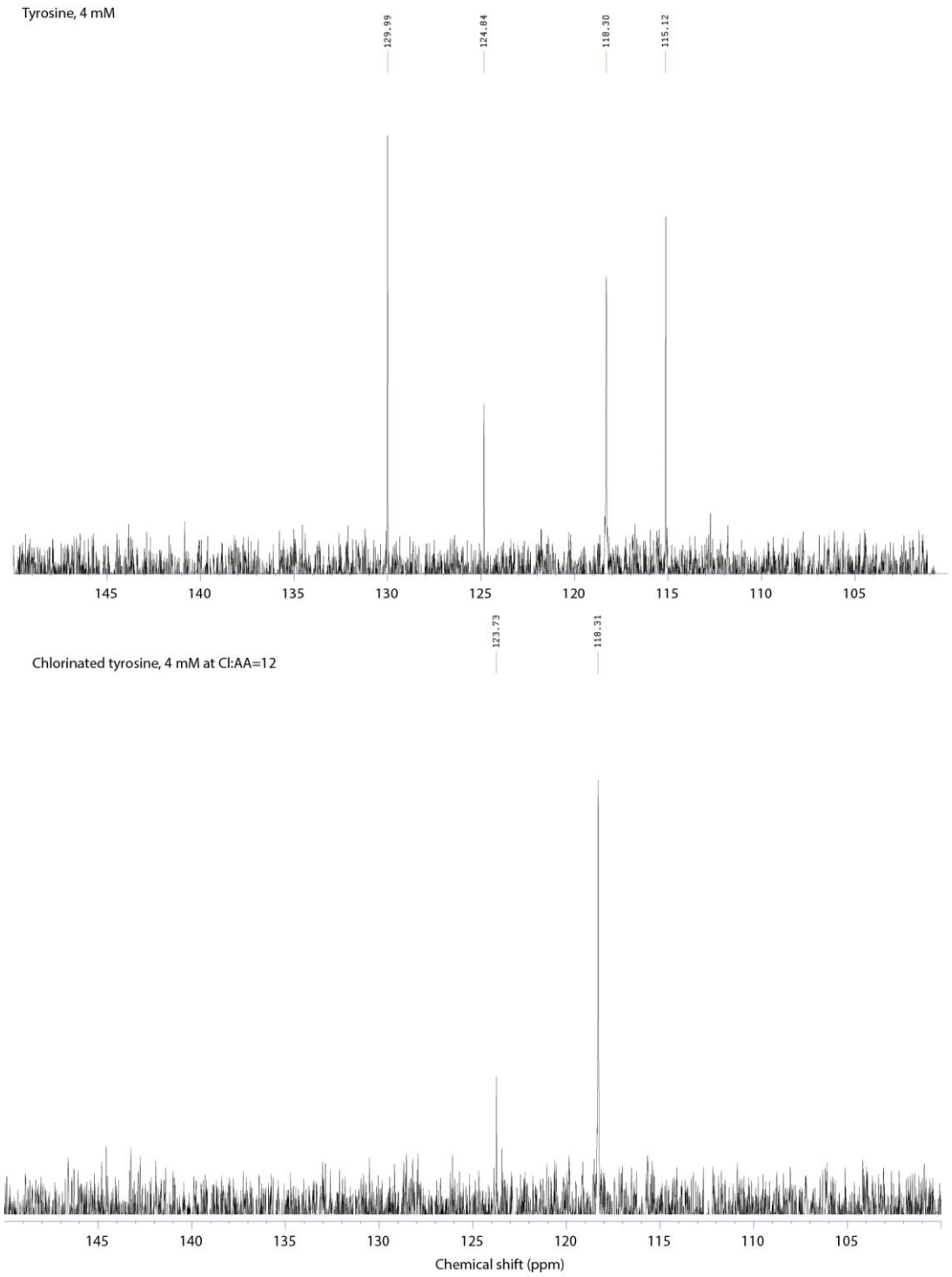


Figure A3-3: ^{13}C Nuclear magnetic resonance spectra (aromatic region only) of tyrosine standard (4 mM) (top) and chlorinated tyrosine (4 mM) at Cl:AA = 12 (bottom).

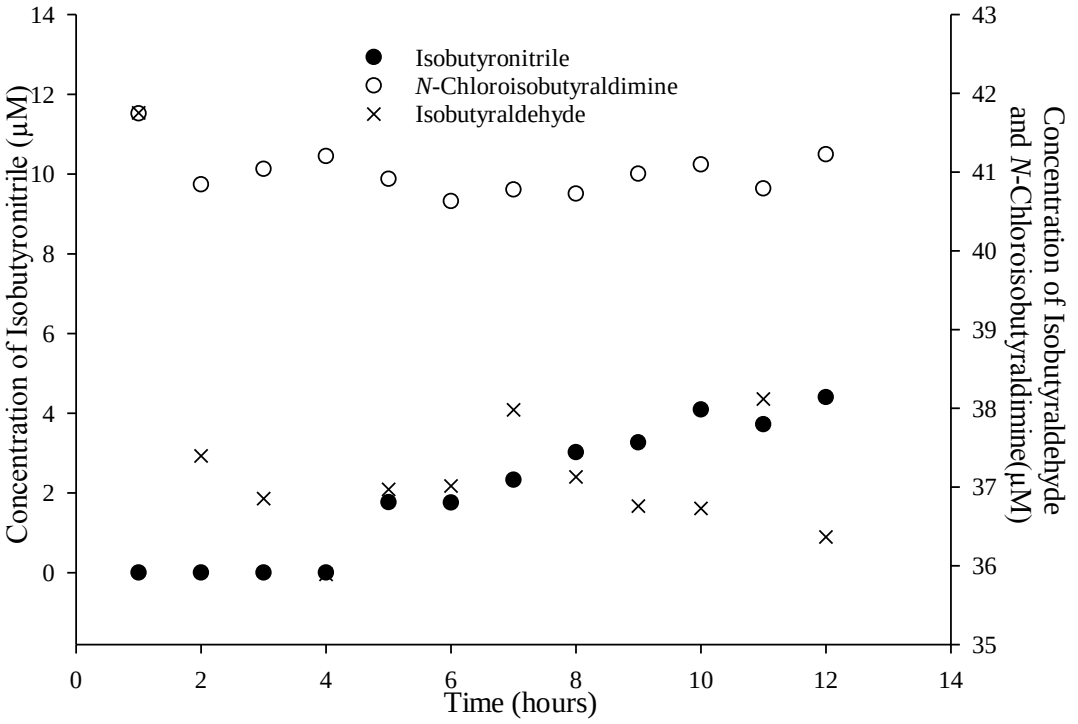


Figure A3-4: Formation of isobutyronitrile over 12 hours in anhydrous conditions from the degradation of *N*-chloroisobutyraldimine after its formation from the chloramination of isobutyraldehyde.

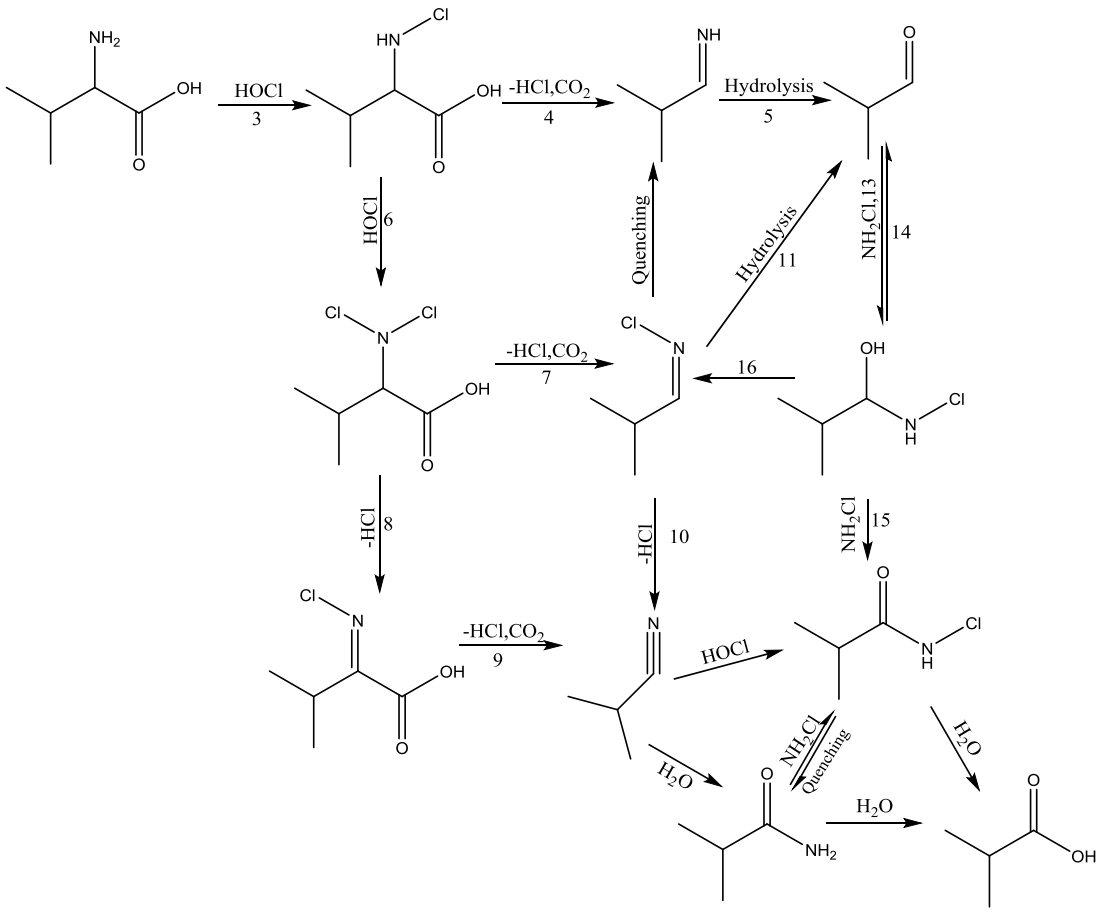


Figure A3-5: The proposed reaction pathways for the chlorination of valine used in the modelling studies. Numbers refer to reactions shown in Table S8.

References

Ianni, J.C. (2015) Kintecus, www.kinetecus.com.

Kimura, S.Y., Vu, T.N., Komaki, Y., Plewa, M.J. and Mariñas, B.J., 2015. Acetonitrile and N-Chloroacetamide Formation from the Reaction of Acetaldehyde and Monochloramine. Environ. Sci. Technol. 49(16), 9954-9963.

Valentine, R.L., Brandt, K.I. and Jafvert, C.T., 1986. A spectrophotometric study of the formation of an unidentified monochloramine decomposition product. Water Res 20(8), 1067-1074.

Yu, Y. and Reckhow, D.A., 2015. Kinetic Analysis of Haloacetonitrile Stability in Drinking Waters. Environ. Sci. Technol. 49(18), 11028-11036.

Appendix 4

Table A4-1: Summary of ESI and HRMS parameters used for screening analysis of amino acids in ESI positive mode

Parameter	Setting
Spray voltage (kV)	3.5
Capillary voltage (V)	35
Capillary temperature (°C)	275
Sheath gas flow rate (Arb)	20
Aux gas flow rate (Arb)	0
Sweep gas flow rate (Arb)	0
Tube Lens (V)	70
Scan range (*m/z*)	70-300
IT full MS AGC target	3E4
IT MS^n AGC target	1E4
FT full MS AGC target	2E5
FT MS^n AGC target	1E5
Ion trap and FT micro scans	3
IT full MS Max ion time (ms)	10
FT MS^2 Max ion time (ms)	100
MS^2 isolation window (*m/z*)	1

Arb: arbitrary units; ms: milliseconds; IT: Ion trap; FT Fourier Transform (Orbitrap mass spectrometer); AGC: Automatic Gain Control.

Table A4-2: GC column and oven temperature programs for the analysis of aldehydes, nitriles and N-chloraldimines (odorous by-products), and isobutyric acid, after headspace solid-phase microextraction (HS SPME) extraction**.**

	Odorous by-products	Isobutyric acid
Analytical Column	ZB-5MS	HP-Innowax
Length	30 m	30 m
Internal Diameter	0.25 mm	0.25 mm
Film Thickness	1 µm	1 µm
Carrier Gas	Helium	Helium
Carrier Flow	0.7 mL/min	0.7 mL/min
Injector Temperature	300 ºC	260 ºC
Oven Program	Hold at 35 ºC for 2 min Ramp to 105 ºC at 5 ºC/min and hold for 5 min Ramp to 300 ºC at 15 ºC/min and hold for 5 min	Hold at 35 ºC for 2 min Ramp to 200 ºC at 5 ºC/min and hold for 1 min

Table A4-3: List of quantifying (underline) and qualifying ions monitored in SIM mode for the analysis of selected aldehydes, nitriles and *N*-chloraldimines.

Analytes	**Ions (m/z)**
Aldehydes	
2-Methylbutraldehyde	57, 41, 29
Isobutyraldehyde	43, 72, 27
Isovaleraldehyde	44, 58, 39
Phenylacetaldehyde	91, 120, 65
Nitriles	
2-Methylbutronitrile	55, 54, 29
Isobutyronitrile	42, 68, 54
Isovaleronitrile	43, 41, 39
Benzyl Cynaide	117, 116, 90
N-chloraldimines	
N-Chloroisobutaldimine	70, 41, 62
N-Chloromethyl-3-butaldimine	77, 79, 55
N-Chloromethyl-2-butaldimine	91, 55, 77
Surrogate Standard	
1,2 Dibromopropane-d_6	127, 129

Table A4-4: Free amino acid concentrations at the plant inlet and outlet of four different drinking water treatment plants (DWTPs). Concentrations of isoleucine, leucine, phenylalanine and valine are presented in main paper.

	DWTP1						DWTP2		DWTP3				DWTP4*			
	Nov-14		Apr-15		Nov-15		Apr-15		Apr-15		Nov-15		Nov-14		Feb-15	
	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet
Alanine (µg/L-N)	0.5	<0.01	<0.01	<0.01	0.3	<0.01	<0.01	2	0.02	<0.01	<0.01	<0.01	1	0.2	1	0.2
Asparagine (µg/L-N)	<0.01	<0.01	<0.01	<0.01	0.01	<0.01	<0.01	0.03	<0.01	0.02	0.01	0.01	<0.01	<0.01	<0.01	<0.01
Aspartic Acid (µg/L-N)	0.2	0.3	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.3	0.1	0.3	0.1
Glutamic Acid (µg/L-N)	<0.01	0.1	<0.01	<0.01	0.1	0.01	0.05	0.2	0.03	<0.01	<0.01	<0.01	<0.01	0.07	<0.01	0.07
Glutamine (µg/L-N)	<0.01	<0.01	<0.01	<0.01	0.03	<0.01	<0.01	0.02	0.01	0.02	0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Glycine (µg/L-N)	2	2	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.9	<0.01	0.9
Histidine (µg/L-N)	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.02	0.02	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Lysine (µg/L-N)	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.05	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Methionine (µg/L-N)	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Proline (µg/L-N)	<0.01	0.1	0.01	0.03	0.02	<0.01	0.01	0.02	0.01	0.05	0.01	<0.01	<0.01	<0.01	0.01	<0.01
Serine (µg/L-N)	<0.01	<0.01	0.03	0.09	0.04	0.05	<0.01	0.04	0.1	0.1	0.09	0.01	<0.01	<0.01	<0.01	<0.01
Threonine (µg/L-N)	<0.01	0.08	<0.01	<0.01	<0.01	<0.01	<0.01	0.02	0.01	0.03	0.02	<0.01	<0.01	<0.01	<0.01	<0.01
Tryptophan (µg/L-N)	<0.01	0.01	0.02	0.02	0.02	0.01	0.01	0.02	0.05	0.04	0.01	<0.01	<0.01	<0.01	0.01	<0.01
Tyrosine (µg/L-N)	<0.01	<0.01	0.01	0.02	<0.01	<0.01	<0.01	<0.01	0.05	0.05	0.01	<0.01	<0.01	<0.01	0.01	<0.01

Table A4-5: Free amino acid concentrations in influent and secondary effluent from four different wastewater treatment plants (WWTP). Data for isoleucine, leucine, phenylalanine and valine is presented in main text.

	WWTP1				WWTP2				WWTP3				WWTP4			
	Feb-15		Jul-15		Feb-15		Jul-15		Feb-15		Jul-15		Feb-15		Sep-15	
	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet	Inlet	Outlet
Alanine* (µg/L-N)	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	<0.01	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.
Asparagine (µg/L-N)	0.01	0.02	0.02	0.01	0.06	0.02	0.05	0.02	0.1	<0.01	0.05	0.03	0.1	0.07	0.03	0.06
Aspartic Acid (µg/L-N)	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Glutamic Acid (µg/L-N)	0.01	0.1	<0.01	0.1	2	0.2	2	0.2	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Glutamine (µg/L-N)	0.03	0.01	0.02	0.01	0.07	0.02	0.05	0.01	0.1	<0.01	0.09	0.01	0.2	0.1	0.02	0.02
Glycine (µg/L-N)	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Histidine (µg/L-N)	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Lysine (µg/L-N)	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.06	<0.01	0.03	<0.01
Methionine (µg/L-N)	0.01	<0.01	0.01	<0.01	2	0.01	2	0.01	0.08	<0.01	0.02	<0.01	0.02	0.02	0.01	0.02
Proline (µg/L-N)	0.02	0.01	0.02	0.01	1	0.03	1	0.02	0.04	<0.01	0.03	0.02	0.08	0.08	0.04	0.06
Serine (µg/L-N)	0.01	<0.01	0.02	0.01	0.06	0.03	0.06	0.01	0.01	<0.01	0.1	0.07	0.2	0.2	0.06	0.2

Threonine (µg/L-N)	0.01	0.02	0.02	0.01	0.1	0.04	0.2	0.02	0.01	<0.01	0.1	0.03	0.08	0.09	0.06	0.08
Tryptophan (µg/L-N)	0.02	0.06	0.06	0.03	7	0.07	7	0.05	<0.01	<0.01	<0.01	<0.01	0.04	0.01	0.02	0.01
Tyrosine (µg/L-N)	<0.01	0.01	<0.01	0.01	<0.01	0.03	>0.01	0.04	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

*Concentration of alanine was not quantified due to possible false detection in the analysis, where the concentraion was found to be more than 100 times that of the standards.

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