Alcohol Congener Analysis in a Forensic Context: Detection of Iso-α-acids to Confirm Beer Consumption

A thesis submitted to the Faculty of Medicine, Nursing and Health Science, for the degree of DOCTOR OF PHILOSOPHY

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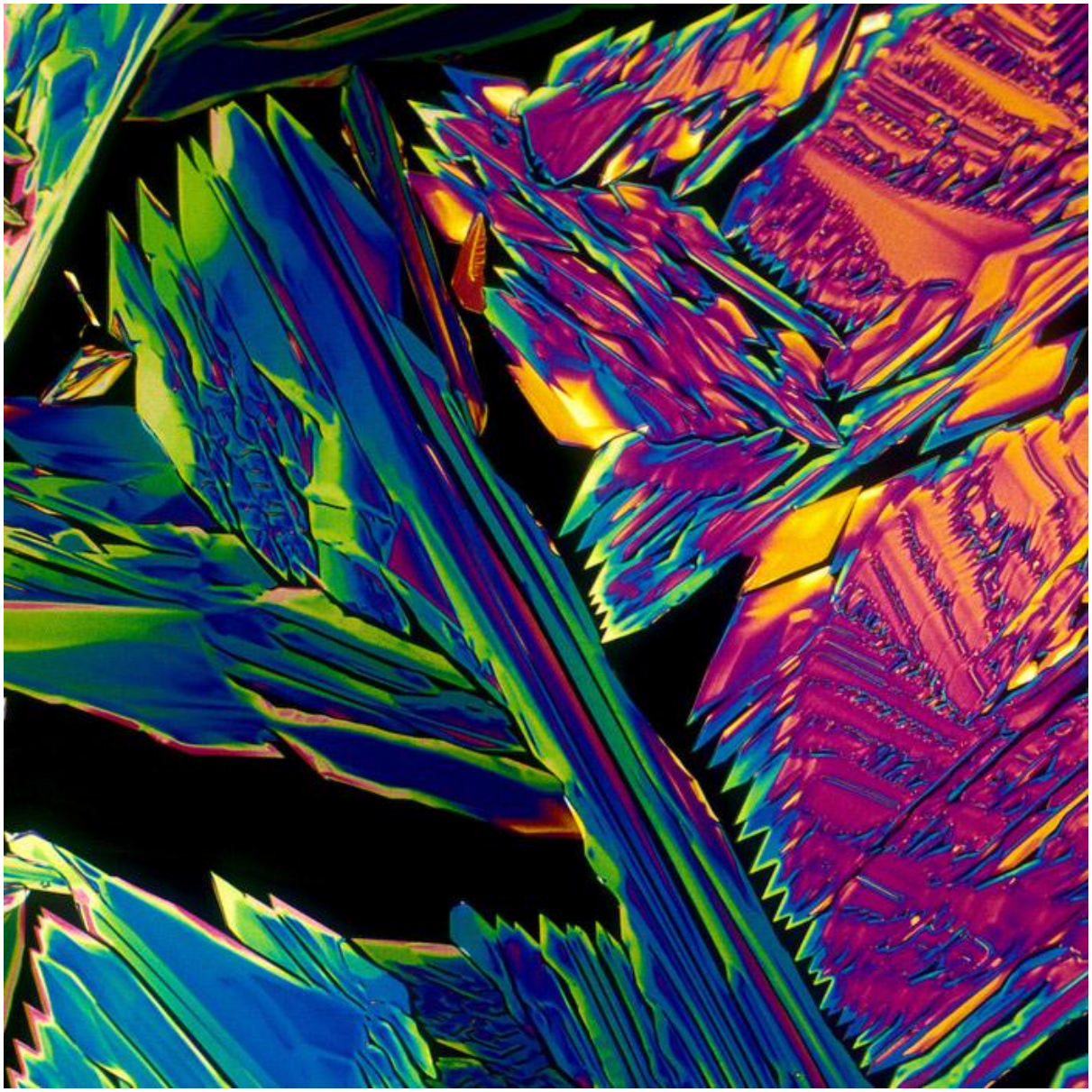

A Czech Pilsner crystalized and micrographed under a high-resolution polarised light microscope.
Source: BevShots®

"ἓν οἶδα ὅτι οὐδὲν οἶδα, hèn oîda hóti oudèn oîda"

"I know one thing: that I know nothing"

The Apology, Plato's account of Socrates

ABSTRACT

Alcohol abuse is a leading factor in many crimes and accidents with beer being the oldest and most widely consumed alcoholic beverage in the world. This research has focused on iso-α-acids (IAA) derived from the hop plant (*Humulus lupulus* L.) used in brewing and found in beer. Additionally, three structurally similar but chemically-altered IAA known as "reduced IAA" (rho-, tetrahydro- and hexahydro-IAA), are also beer-specific ingredient congeners found in beer, specifically used in green or clear bottled beer.

A protein precipitation extraction and ESI-UHPLC-MS/MS method was developed and validated for the detection of these compounds in biological specimens that can confirm beer consumption. The long-term stabilities of these analytes in stored blood specimens was assessed over 8 weeks with freezing (-20 °C) and refrigeration (4 °C) conditions determined as acceptable. The analysis of blood and urine collected over 6 hours from volunteers given five different beers in five drinking studies separated by at least one week. The natural and reduced IAA were found to be bioavailable, show small inter-variable differences in concentration-time profile, and possess pharmacokinetic data such as quick absorption rates and half-lives ranging between ~30-46 minutes. Furthermore, in the assessment of 130 postmortem cases, ~57% of positive BAC cases showed beer consumption prior to death, and an even higher prevalence (87%) in casework where "beer" was mentioned in the case circumstances. Considerable postmortem redistribution, a serum to blood ratio of ~3 and a weak association between BAC and IAA groups was observed. Vitreous humour and urine specimens contained very low concentrations and prevalence of IAA groups. Lastly, the analytical method was used to assess the IAA profiles for over 30 different brown, green and clear bottled, local and international beers. This reference catalogue assists forensic toxicologists in comparing and interpreting the results of authentic and unknown casework.

This novel approach can be used to provide important information on the drinking behaviour and circumstances surrounding *after-drinking (hip-flask)* defence cases and additional forensic applications such as coronial, drug facilitated sexual assault, research or clinical medico-legal casework. Thesis by publication is the primary structure of this PhD thesis with a total of seven peer-reviewed publications (three in review) resulting from this project that providing comprehensive knowledge for the use of this technique in forensic institutes locally and internationally.

TABLE OF CONTENTS

SUPERVISORS & CO-AUTHORS ..... **V**

ABSTRACT ..... **VII**

TABLE OF CONTENTS ..... **VIII**

LIST OF FIGURES ..... **XI**

LIST OF TABLES ..... **XII**

ABBREVIATIONS ..... **XIII**

ACKNOWLEDGEMENTS ..... **XIV**

PUBLICATIONS, PRESENTATIONS, AWARDS & PROFESSIONAL ROLES ..... **XVI**

GENERAL DECLARATION ..... **XIX**

CHAPTER 1. INTRODUCTION **1**

Chapter 1.1 Alcohol Congener Analysis Review **2**

1.1.1 Introduction 5
1.1.2 Application of ACA to Forensic Specimens 7
1.1.3 Fermentation By-product Congener ACA 9
1.1.4 Ingredient Biomarker Congener ACA 26
1.1.5 Conclusion 28

Chapter 1.2 Beer Summary and Thesis Aims **31**

1.2.1 Traditional ACA 32
1.2.2 Aims 33
1.2.3 Beer 34
1.2.4 Outline of Thesis 36

CHAPTER 2. ANALYTICAL METHODOLOGY **39**

Chapter 2.1 IAA by UHPLC-MS/MS Method Validation **40**

2.1.1 Introduction 43
2.1.2 Experimental 48
2.1.3 Results and Discussion 59
2.1.4 Conclusion 69

CHAPTER 3. STABILITIES.....71

Chapter 3.1 IAA Stability in Stored Spiked Blood 72

3.1.1 Introduction.....76
3.1.2 Materials and methods 79
3.1.3 Results.....82
3.1.4 Discussion 86
3.1.5 Conclusion 89

Chapter 3.2 IAA Stability in Stored Authentic Blood 90

CHAPTER 4. PHARMACOKINETICS 93

Chapter 4.1 Pharmacokinetics of Natural IAA.....94

4.1.1 Introduction.....97
4.1.2 Experimental 99
4.1.3 Results.....103
4.1.4 Discussion 107
4.1.5 Conclusion 110

Chapter 4.2 Pharmacokinetics of Reduced IAA 111

4.2.1 Introduction.....114
4.2.2 Experimental 117
4.2.3 Results.....123
4.2.4 Discussion 130
4.2.5 Conclusion 134

CHAPTER 5. POSTMORTEM PREVALENCE AND REDISTRIBUTION 136

Chapter 5.1 Specimen collection in Victorian Coronial Casework 137

Chapter 5.2 IAA PrevalEnce in Postmortem Specimens 140

5.2.1 Introduction.....143
5.2.2 Experimental 145
5.2.3 Results.....149
5.2.4 Discussion 160
5.2.5 Conclusion 165

Chapter 5.3 Postmortem Redistribution of IAA 166

5.3.1 Introduction.....169
5.3.2 Experimental 170
5.3.3 Results.....175
5.3.4 Discussion 179
5.3.5 Conclusion 182

CHAPTER 6. BEER PROFILES 185

Chapter 6.1 IAA in Beer Methodology 186

6.1.1 Introduction 187
6.1.2 Methodology 188
6.1.3 Results and Discussion 190

CHAPTER 7. CONCLUSIONS 195

7.1 Introduction 196
7.2 Review of Findings 197
7.3 Values of Research 202
7.4 Future Aspects 205
7.5 Conclusion 206

REFERENCES 208

APPENDICES 229

Appendix 1 Publications 230

Appendix 1.1: Chapter 1.1 Publication 231
Appendix 1.2: Chapter 2.1 Publication 247
Appendix 1.3: Chapter 3.1 Publication 262
Appendix 1.4: Chapter 4.1 Publication 270
Appendix 1.5: Chapter 4.2 Publication 278
Appendix 1.6: Chapter 5.2 Publication 308
Appendix 1.7: Chapter 5.3 Publication 335
Appendix 1.8: Chapter 6.1 Conference Poster 355

Appendix 2 Ethics Approvals 358

Appendix 2.1: Ec 04/2012 359
Appendix 2.2: Ec 04/2012 Amendment 361
Appendix 2.3: Ec 06/2013 362
Appendix 2.4: Ec 07/2013 363
Appendix 2.5: Ec 07/2013 Amendment 365

LIST OF FIGURES

Fig. 1 Botanical illustration of *Humulus lupulus* (L) 29

Fig. 2 The photolysis of IAA and 3-MBT formation scheme 35

Fig. 3 Isomerisation of α-acid to *trans*- and *cis*- iso-α-acids and three major analogs 45

Fig. 4 The synthesis of reduced IAA from natural IAA 47

Fig. 5 Chromatogram of blood spiked with IAA 60

Fig. 6 Chromatograph of blood taken 30 min after beer consumption in pilot study 68

Fig. 7 α-acid, iso-α-acids and reduced iso-α-acids scheme 76

Fig. 8 The stabilities of IAA groups in stored blood 82

Fig. 9 The stabilities of individual IAA analytes in stored blood 85

Fig. 10 *trans*-IAA concentration-time profiles in the natural IAA drinking studies 103

Fig. 11 *trans*/*cis*-IAA ratios at 0.5 and 2 h in the natural IAA drinking studies 104

Fig. 12 Reduced-IAA concentration-time profiles in the reduced IAA drinking studies 124

Fig. 13 Relative ratios of RIAA analytes to demonstrate variable elimination profiles 127

Fig. 14 Illustration of *Humulus lupulus* (L) and the structure of IAA groups 142

Fig. 15 Correlation between blood IAA and alcohol concentrations 160

Fig. 16 Correlation of postmortem redistribution and pre-autopsy interval 175

LIST OF TABLES

Table 1 Fermentation by-product congeners & ethanol concentrations in beverages.....13

Table 2 Properties and Widmark constants of fermentation by-product congeners 19

Table 3 Composition of beer 34

Table 4 Analyical method conditions of IAA analytes 52

Table 5 Concetrations of calibration and quality control standards 53

Table 6 Matrix effects and recoveries of IAA analytes.....65

Table 7 Accuracy, intermediate precision and repeatability of IAA method.....67

Table 8 The 12 month stability of *trans*-IAA in authentic blood specimens 90

Table 9 Beer profiles of the natural IAA drinking studies 99

Table 10 Measurements of volunteers in the natural IAA drinking studies.....99

Table 11 Blood IAA and alcohol concentrations in the natural IAA drinking studies 105

Table 12 Natural IAA and reduced IAA properties in beer 114

Table 13 Beer profiles of the reduced IAA drinking studies.....117

Table 14 Measurements of volunteers in the reduced IAA drinking studies 118

Table 15 Blood IAA and alcohol concentrations in the reduced IAA drinking studies.....125

Table 16 Urine IAA concentrations in the reduced IAA drinking studies 128

Table 17 Prevalence of natural and reduced IAA groups in specimens.....149

Table 18 Prevalence of natural and reduced IAA groups in different demographics 152

Table 19 Details and prevalence of IAA in beer-mentioned postmortem cases 156

Table 20 Comparison of paired mortuary admission and autopsy bloods 174

Table 21 The time and site collection dependence of postmortem redistribution.....176

Table 22 Serum to whole blood ratios of IAA 177

Table 23 Reference catalogue of IAA concentrations in beers 190

ABBREVIATIONS

3-MBT	3-methyl-2-butene-thiol
AA	α-acids
ABV	alcohol by volume percentage
ACA	alcohol congener analysis
ACN	acetonitrile
ADM	mortuary admission blood
ANOVA	one-way analysis of variance
AUT	autopsy blood
BAC	blood alcohol analysis
DCHA	dicyclohexylammonium salt
ESI	electron source ionisation
GC	gas chromatography
HIAA	hexahydro-iso-α-acids
IAA	iso-α-acids
IBU	international bitterness units
ICS	international calibration standards (beer industry)
I.D.	inner diameter
IS	internal standard
LC	liquid chromatography
LLE	liquid-liquid extraction
MRM	multiple reaction monitoring mode
MS	mass spectrometer
p	significance level
PK	pharmacokinetics
PMI	postmortem interval
PMR	postmortem redistribution
QC	quality control
RIAA	rho-iso-α-acids
S/B	serum-to-blood ratio
TIAA	tetrahydro-iso-α-acids
UHPLC-MS/MS	ultra performance liquid chromatography – tandem mass spectrometer
VIFM	Victorian Institute of Forensic Medicine

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Thank you,

Luke N. Rodda

…dedicated to Oma (7.2.24 – 31.1.14)

in loving memory of Karen (18.10.69 – 7.7.12)…

PUBLICATIONS, PRESENTATIONS, AWARDS &

PROFESSIONAL ROLES

Listed below are the candidate's: first-author and co-authored publications; domestic and international conference posters and presentations; awards and scholarships, and; professional roles; during the period of candidature.

Publications by the candidate relevant to the thesis:

1. **Rodda, L.N.**, J. Beyer, D. Gerostamoulos, and O.H. Drummer, *Alcohol Congener Analysis and the Source of Alcohol: A Review.* Forensic Sci Med Path, 2013. 9(2): p. 194-207. (Appendix 1.1)

2. **Rodda, L.N.**, D. Gerostamoulos, and O.H. Drummer, *The Rapid Identification and Quantification of Iso-α-acids and Reduced Iso-α-acids in Blood using UHPLC-MS/MS: Validation of a Novel Marker for Beer Consumption.* Anal Bioanal Chem, 2013. 405(30): p. 9755-9767. (Appendix 1.2)

3. **Rodda, L.N.**, D. Gerostamoulos, and O.H. Drummer, *The Stability of Iso-α-acids and Reduced Iso-α-acids in Stored Blood Specimens.* Forensic Sci Int, 2014. 239: p. 44-49. (Appendix 1.3)

4. **Rodda, L.N.**, D. Gerostamoulos, and O.H. Drummer, *Pharmacokinetics of Iso-α-acids in Volunteers following the Consumption of Beer.* J Anal Tox, 2014. ePub. (Appendix 1.4)

5. **Rodda, L.N.**, D. Gerostamoulos, and O.H. Drummer, *Pharmacokinetics of Reduced Iso-α-acids in Volunteers following Clear Bottled Beer Consumption.* Forensic Science International, 2014. (Under Review, Appendix 1.5)

6. **Rodda, L.N.**, D. Gerostamoulos, and O.H. Drummer, *Detection of Iso-α-Acids to Confirm Beer Consumption in Postmortem Specimens.* Drug Testing and Analysis, 2014. (Under Review, Appendix 1.6)

7. **Rodda, L.N.**, D. Gerostamoulos, and O.H. Drummer, *The Postmortem Redistribution of Iso-α-acids in Postmortem Specimens.* Forensic Science, Medicine, and Pathology, 2014. (Under Review, Appendix 1.7)

Additional peer-reviewed publications:

1. Chu, M., D. Gerostamoulos, J. Beyer, **L.N. Rodda**, M. Boorman, and O.H. Drummer, *The incidence of drugs of impairment in oral fluid from random roadside testing.* Forensic Science International, 2012. 215(1–3): p. 28-31.

2. Di Rago, M., E. Saar, **L.N. Rodda**, S. Turfus, A. Kotsos, D. Gerostamoulos, and O.H. Drummer, *Fast Targeted Screening of 132 Acidic and Neutral Drugs and Poisons In Whole Blood Using LC-MS/MS.* Forensic Science International, 2014. 243: p. 35-43.

Conference presentations:

1. **Rodda, L.N.**, D. Gerostamoulos, and O.H. Drummer. *An LC-MS/MS Method for the Detection and Quantification of Beer Congeners in Blood* in 51st Annual Meeting of the International Association of Forensic Toxicologists. 2013. Funchal, Madeira - Portugal. (Oral)

2. **Rodda, L.N.**, D. Gerostamoulos, and O.H. Drummer. *The Detection of Hop-Derived Compounds as a Novel Marker for Beer Consumption in Blood using UHPLC-MS/MS* in Forensic and Clinical Toxicology Association Inc. Conference. 2013. Sydney, Australia. (Oral)

3. **Rodda, L.N.**, D. Gerostamoulos, and O.H. Drummer. *The Quantification of Hop-Derived Iso-α-acid type Compounds in Beer using UHPLC-MS/MS* in Forensic and Clinical Toxicology Association Inc. Conference. 2013. Sydney, Australia. (Poster, Appendix 1.8)

4. **Rodda, L.N.**, D. Gerostamoulos, and O.H. Drummer. *The Detection of Hop-Derived Compounds as Novel Markers for Beer Consumption using µHPLC-MS/MS* in Australian and New Zealand Forensic Science Society Symposium. 2014. Adelaide, Australia. (Accepted Poster Abstract)

5. **Rodda, L.N.**, D. Gerostamoulos, and O.H. Drummer. *The Pharmacokinetics of Iso-α-Acids in Volunteers to Confirm Beer Consumption* in 52nd Annual Meeting of the International Association of Forensic Toxicologists. 2014. Buenos Aires, Argentina. (Submitted Oral Abstract)

Awards and Scholarships:

- **PhD Candidature Departmental Scholarship**, *DFM - Monash University* (2011-2014). ~$22,000/annum for 3 years 1 month

- **Postgraduate Travel Grant Award**, *Monash University* (2013). $2115

- **First Student Scholarship Award**, *Forensic and Clinical Toxicology Association Inc Meeting*. (2013). ~$1100

- **Best Peer-Reviewed Paper -Early Career Researcher Award**, *SPHPM - Monash University* (2013). For: Rodda, L.N. et al. ABC, 2013. 405(30): p. 9755-9767.

- **First Travel Victorian Award**, *Australian and New Zealand Forensic Science Society Symposium* (2014). ~$2200

Professional roles:

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Media and Candidature Lectures:

- **Rodda, L.N.** *The Science of Beer Explained* in Forensic Matters by VIFM. 2014: p.10-11

- **Rodda, L.N.** *Alcohol Congener Analysis in a Forensic Context*. 2012: SPHPM - Monash University.

- **Rodda, L.N.** *Confirming Beer Consumption* 2013: VIFM.

- **Rodda, L.N.** *How to Obtain a PhD using Beer: Alcohol Congener Analysis in a Forensic Context*. 2014: VIFM.

GENERAL DECLARATION

In accordance with Monash University Doctorate Regulation 17 / Doctor of Philosophy and Master of Philosophy (MPhil) regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university of equivalent institution and that, to the best of my knowledge and belief, this thesis contains not material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes seven original publications published in peer reviewed journals and no unpublished publications. The core theme of the thesis is the forensic science of confirming one's consumption of beer in toxicological specimens. The ideas, development and writing of the publications in this thesis were the principal responsibility of myself, the candidate, working within the Department of Forensic Medicine under the supervision of Professor Olaf Drummer, Doctor Dimitri Gerostamoulos and Doctor Jochen Beyer.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In peer-reviewed publications presented, my contribution to the work involved the following:

Thesis Chapter	**Publication Title** ***Journal (year)***	**Publication Status**	**Nature and extent of candidate's contribution**
1.1	Alcohol congener analysis and the source of alcohol: a review *Forensic Science, Medicine, and Pathology (2013)*	Published	85%
2.1	The rapid identification and quantification of iso-α-acids and reduced iso-α-acids in blood using UHPLC-MS/MS: validation of a novel marker for beer consumption *Analytical and Bioanalytical Chemistry (2013)*	Published	85%
3.1	The stability of iso-α-acids and reduced iso-α-acids in stored blood specimens *Forensic Science International (2014)*	Published	85%
4.1	Pharmacokinetics of iso-α-acids in volunteers following the consumption of beer *Journal of Analytical Toxicology (2014)*	Published	85%
4.2	Pharmacokinetics of reduced iso-α-acids in volunteers following clear bottled beer consumption *Forensic Science International (2014)*	Submitted, under peer-review	85%
5.2	Detection of iso-α-acids to confirm beer consumption in postmortem specimens *Drug Testing Analysis (2014)*	Submitted, under peer-review	85%
5.3	The postmortem redistribution of iso-α-acids in postmortem specimens *Forensic Science, Medicine, and Pathology (2014)*	Submitted, under peer-review	85%

Signed: ..... **Date:** *10.07.2014*

CHAPTER 1.

INTRODUCTION

The rational for the investigation into determining the source of alcohol derives from the supervisors and candidate who, with their knowledge and experience, were aware that the international forensic toxicology service was unable to provide such results. This PhD candidature attempts to enhance the understanding of the alcohol congener analysis field and the feasibility of its use in routine forensic services. The Victorian of Forensic Medicine who predominantly operates research through the onsite Department of Forensic Medicine, Monash University, houses a comprehensive toxicology laboratory sufficient to allow for such investigations to take place.

CHAPTER 1.1

ALCOHOL CONGENER ANALYSIS REVIEW

Rodda, L.N., J. Beyer, D. Gerostamoulos, and O.H. Drummer, *Alcohol Congener Analysis and the Source of Alcohol: A Review*. Forensic Sci Med Path, 2013. 9(2): p. 194-207.
(Appendix 1.1)

Monash University

Declaration for Thesis Chapter 1.1.

Declaration by candidate

In the case of Chapter 1.1., the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Located and translated articles to English, reviewed articles and wrote the paper	85%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Beyer, J.	*Advised candidate regarding direction and content*	
Gerostamoulos, D.	*Advised candidate regarding direction and content, reviewed article manuscript*	
Drummer, O.H.	*Advised candidate regarding direction and content, reviewed article manuscript*	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 26.6.14
Main Supervisor's Signature	[redacted]	**Date** 27/6/14

Abstract

For many decades, traditional alcohol congener analysis has provided the concentrations of fermentation by-product congeners found in blood, to ascertain if the alcoholic beverage(s) claimed to have been consumed by an individual was feasible in order to assist in after-drinking type cases. However, this technique does not provide information on the exact alcoholic beverage consumed. More recently, ingredient biomarker congeners specific to certain alcoholic beverages have been detected in blood that identify the particular alcoholic beverage consumed and therefore the source of alcohol (albeit for only a limited number of beverages). This novel approach may reduce current limitations that exist with traditional methods of detecting fermentation by-product congeners, which restrict the use of alcohol congener analysis internationally and for other medico-legal scenarios. This review of articles in all languages examines the forensic application of alcohol congener analysis in determining the source of alcohol and other techniques.

Keywords

Alcohol congener analysis; after-drinking; congener; fermentation by-products; ingredient biomarkers; review.

1.1.1 Introduction

The oldest evidence of alcohol production dates back to 10,000 B.C. [1]. Alcohol remains the most widely consumed drug in the world, with a worldwide annual average consumption of 6.5 litres (of pure ethanol) per person [2]. Alcohol is commonly abused, presenting a global health issue which contributes to over 2.5 million deaths annually in addition to a significant financial burden on society resulting from alcohol-related crimes and health care costs [2-4]. For many years, alcohol has been the most commonly detected substance in routine forensic analysis particularly those involving violence, sexual assault, and motor vehicle accidents [2, 5]. This has provided extensive knowledge for the interpretation of blood alcohol concentrations (BAC) [6, 7]. Widmark published the first detection method [8] and accompanying pharmacokinetics [9] of ethanol in the early 1920s and 1930s, advanced by Elbel in 1956 [10] who, established a forensic focus on blood alcohol research within the German speaking countries [11-13]. In the same decade, Machata developed the first gas chromatography with flame ionisation detection (GC-FID) method for analysis of simple volatiles in blood by direct [14] and headspace (HS-GC-FID) [15] injections. This subsequently led to the method for the quantitative analysis of ethanol, which remains fundamentally similar today [16].

Machata devised the initial workup and concept of investigating the source of alcohol by correlating detected *congeners* in blood with concentrations found in the beverage consumed [17], termed alcohol congener analysis (ACA). Congeners are all other compounds in an alcoholic beverage, other than water and ethanol, [1] that assist in the distinctive aroma, flavor and appearance of the beverage [18]. By the late 1970s, concentrations of a set target of fermentation by-product congeners were determined in a vast range of spirit [19], wine [20] and beer [21] type alcoholic beverages [22]. Throughout the 1980s and up and until

2000, Bonte advanced the technique of fermentation by-product congener detection by designing [20, 21] and then advancing a HS-GC-FID technique using the purge and trap technology [23-25]. The understanding of alcohol congener analysis (ACA) in blood was developed with the use of numerous drinking experiments and case studies [26-30]. In Germany, ACA has been useful to verify claims of *after-drinking* where determining the feasibility of the claimed consumption of alcohol can play a pivotal role in the case [28, 31]. However, this approach cannot determine the exact origin of the consumed ethanol. To date, ACA has not been applied in other medico-legal cases and only very minimally in post-mortem cases [32, 33].

In the last decade, Lachenmeier and Schulz targeted some ingredient biomarker congeners to detect congeners specific to a beverage type [34-38]. This approach identified the type of alcoholic beverage consumed, however this was limited to herbal flavored spirits.

This review aims to understand the significance of ACA in determining the source of alcohol, the analytical techniques used for quantitative detection of congeners and how the concentrations of these compounds can be applied to forensic cases.

1.1.1.1 Methodology

Papers were selected and reviewed based on a comprehensive PubMed search for articles of all languages published to date. Specific keywords were used to identify articles relating to congeners found in alcoholic beverages and biological specimens, analytic methodology, interpretation and medico-legal applications.

1.1.2 Application of ACA to Forensic Specimens

ACA has provided valuable information in certain types of forensic cases where understanding the source of alcohol is useful. Effective and more unique biomarkers of specific beverages may have wider applications in forensic science than the existing ACA methodology, which is limited only for use in after-drinking cases**.**

1.1.2.1 After-drinking

After-drinking (also known as the hip flask defence) refers to the situation where, after a motor vehicle accident, the driver flees from police and is later apprehended, returning a positive BAC which s/he claims is a result of alcohol consumption after the incident [28, 39, 40]. Drivers invariably claim to have been sober prior to the incident, maintaining the drink consumed post-offence (most commonly a strong spirit) was used to calm their nerves. This type of defence has been the top-ranked defence claim in Germany and in many parts of Europe, forming approximately 90% of all drink-driving defence claims [30]. Although this type of defence has also been used outside these countries [41], no data exists on the frequency of this claim outside of central Europe [39]. In Hampshire, England, there are procedures utilised by the local authorities to deal with drivers who have initiated a claim using the after-drink defence [42]. Local legislation can render these defence tactics unusable such as in Norway where it is illegal to consume alcohol within six hours after driving if there is reason to believe that the police may wish to investigate the driver [30]. This type of defence tactic may be used by a driver in countries such as Germany where the police can submit an individual to a BAC assessment after driving has ceased for a period of time. For example in Victoria, Australia, under the Road Safety Act (1986), police can submit a driver to an alcohol test within 3 hours of driving [43]. Furthermore, if heavier penalties described

in the Crimes Act are suspected, such as manslaughter, police may perform an alcohol analysis of the driver after the 3 hour period [44]. This scenario may also occur more frequently when the obligation lies with the prosecution to prove that the offence occurred, which is the case in Germany and many other countries.

Previous approaches and techniques to assist in the prosecution of these defences exist. Back-calculation of the BAC [45, 46] is generally acceptable [47]. However, this will result in wide ranges [12] and likely over- or under-estimations [48]. The technique of double blood sampling where the magnitude and direction of the ethanol concentration in the second blood sample is compared to the first (and similarly for blood to urine comparisons) has been utilised in the past [27]. However, it has been concluded that these methodologies have little advantage over traditional back-calculation from a single sample to support such evidence of after-drinking claims [26, 27] and generally require significant demand both from the police and the toxicologist. For this reason, ACA of fermentation by-products has been used consistently in Germany to examine such claims, supported by extensive literature and the ease of taking only one specimen [27, 28, 49]. The development of ingredient biomarker ACA techniques may provide supporting evidence for such claims.

1.1.2.2 Drug Facilitated Assaults

Ethanol is the most common drug detected, and more often than not; the only drug detected, in claims of drug facilitated sexual assault or "drink spiking" [50-52]. This has been demonstrated in countries including Australia [53, 54] and the UK [55, 56]. With data showing very few cases actually contain sedative or other stupefying drugs, it is clear that high alcohol consumption is an underlying factor for these cases [57, 58]. Evidence in a case indicating what beverages were consumed may assist in determining if a higher strength spirit

was added to a lower strength beverage, unsuspectingly increasing the ethanol intake of the victim. With a mean time of 5.9 h of sampling after an alleged spiking from one UK study [55], current ACA practices would be unable to provide assistance due to the three hour limit of the technique. Furthermore, drink spiking is regarded as the second most used defence against drink-driving in Germany and Sweden [30, 59]. Detection of alcohol sources other than what is expected may assist in prosecuting defendants in drug facilitated assault and drink spiking cases.

1.1.2.3 Post-mortem ethanol confirmation and reporting

It is possible to differentiate between ingested ante-mortem ethanol and post-mortem production [60]. This is feasible through ethyl glucuronide detection in blood, urine and hair as a marker of ante-mortem ethanol ingestion [61-63], although it has been found to be unstable under some post-mortem putrefactive conditions [64]. 1-Butanol is a marker for putrefaction [60] particularly when detected in concentrations above 0.03 mg/L [65]. However, it has been detected consistently in fruit spirits and in wine at low concentrations [22, 66] limiting the application of current ACA practices in decomposed cases. Detection of ingredient biomarkers may be more useful to determine the source of ethanol in decomposed cases. Additionally, it may offer insight into a case otherwise not possible where there are coronial or legal outcomes relating to the type of alcoholic beverage consumed.

1.1.3 Fermentation By-product Congener ACA

Although fermentation by-product congeners are found in nearly all alcoholic beverages, different quantities of each congener detected are dependent on the amount and type of the

beverage consumed. In order to determine a likely source, or to refute a claim made by the defendant, scientists compare the actual with theoretical blood congener concentrations under a specified set of circumstances [20, 21, 34]**.**

1.1.3.1 Congener Content of Alcoholic Beverage

Beer commonly contains approximately 800 congeners [67], wine: 600 [68] and spirits: 800 [69] at generally very low concentrations (~ 1:1000) compared with the corresponding ethanol content [1, 35]. Analysis has been undertaken on different types of alcoholic beverages [19-21, 70-76]; Table 1 describes the concentrations of the common fermentation by-product type congeners found in a range of alcoholic beverages. However, the claimed consumed beverage may contain slightly different amounts of congeners depending on the batch and storage conditions. This suggests that analysis of the beverage itself may be necessary, albeit not always possible. The production techniques described below aim to alter the ethanol, water and congener content to that of the desired final product, including the concentrations of fermentation by-products that are targeted by ACA.

Fermentation

Under anerobic conditions, sugars are converted into ethanol and carbon dioxide by particular strains of yeasts. However, other volatile substances are also produced by this fermentation process. These targeted fermentation by-products typically consist of methanol and the ‘fusel alcohols’ (a German term for bad liquor) 1-propanol, isobutanol, 1-butanol, 2-methyl-1-butanol or 3-methyl-1-butanol, amongst others. The amount of these congeners produced is largely subject to availability of amino acids during production, known as the Ehrlich

mechanism [77]. Yeast replaces the amino moiety with a hydroxyl group to form the alcohol [78], except in the case of methanol, where pectins are the source for its production [79]. For example, amino acids such as threonine, leucine, isoleucine and valine have been shown to produce 2-methyl-1-butanol, 3-methyl-1-butanol, 1-propanol and isobutanol [18, 80, 81]. Other factors affecting the production of alcohol congeners are the presence of other carbon sources such as carbohydrates [1]. The source of these ingredients is contained in the plant materials used to produce the range of fermented alcoholic beverages available, i.e. beer (cereal grains), wine (grapes), sake (rice) and cider (apple or pear); resulting in varying congener content for each. In addition, different strains of yeast ferment at variable rates, consequently producing different congener profiles [82].

Distillation

Distillation is not only capable of increasing the alcohol by volume percentage (ABV) [83], but can also alter the relative congener concentrations produced during fermentation. Distillation separates ethanol and volatile congeners based on their boiling points, with congeners of similar or lower boiling points to ethanol also undergoing distillation (particularly methanol). This concentration effect is demonstrated by fermented beverages generally containing lower concentrations of total congeners per volume when compared with distilled beverages [22]. However, when congener concentrations are compared to the concentration of ethanol (i.e. congener / ABV), the relative difference in congener concentration between fermented and distilled beverages is reduced (**Table 1**). For example, wine and the spirit produced from distilling wine, brandy, contain ~ 24-109 and ~ 58-441 mg/L of isobutanol, respectively, demonstrating a significant difference between the two beverages. However, when the ABV is taken into account, these become ~ 2-10 and ~ 2-14

mg of isobutanol per standard drink (10 g of ethanol) for wine and brandy, respectively. This indicates a more similar ingestion of isobutanol content in order to consume the same amount of ethanol, providing less differentiation of isobutanol ingestion between the two beverages to distinguish between them for ACA purposes.

Further Production Techniques

Later production practices have been designed to alter the congener content further in order to produce the final drinking product. Maturation can also remove, change or add congeners, due to interactions with the barrel material, chemical reactions in the liquid and/or evaporation [1]. Secondary fermentation may take place as yeast acts on the remaining sugars in the liquid, further modifying the fermentation by-product congener content of the beverage [1]. Variable conditions such as oxygen levels, temperature and duration of production can also vary the composition of congeners [18, 84, 85]. Additionally, the blending of beverages can create new products, such as blended whiskeys or fortified wines (wine and brandy) providing new congener profiles.

Manufacturers of Alcoholic Beverages

Large variations in congener content may exist within a type of beverage. It is common for the same type of beer to be produced by breweries in several countries under license. Hence, results from a batch produced in another country may not be representative of a beverage produced elsewhere, particularly when production techniques have changed [86]. Additionally, individuals producing alcoholic products using simple grains, fruits, potatoes and/or rice [87], have less control of fermentation by-product congener concentrations. This

Table 1

Published common fermentation by-product congeners and ethanol concentrations in alcoholic beverages in mg/L (top row) compared to milligrams of congener per standard drink (using the ABV – bottom row). Demonstrating the difference of congener content between beverages becomes less distinct when ABV is taken into account.

Alcoholic Beverage	n	Ethanol (ABV)[b]	Concentration	Methanol	1-Propanol	1-butanol	2-butanol	Isobutanol	2-methy-1-butanol	3-methy-1-butanol	Reference
Beer	653	5	mg/L	1-27	10-124	0-6	0	9-109	37-124	10-248	[31, 70]
			mg/std drink[a]	3-7	3-6	0-2	0	2-28	10-32	3-64	
Wine	813	14	mg/L	8-151	15-63	0-9	0-1	24-109	34-314	4-183	[31, 70]
			mg/std drink[a]	1-14	1-6	0-<1	0-<1	2-10	3-30	<1-17	
Fortified Wine	4	20	mg/L	125-329	54-63	4-15	N/A	60-95	N/A	115-166	[70]
			mg/std drink[a]	8-21	3-4	<1-1	N/A	4-6	N/A	<1-11	
Brandy	120	40	mg/L	176-4766	79-3445	0-359	0-1088	58-441	678-961	28-158	[31, 70]
			mg/std drink[a]	60-153	3-110	0-12	0-35	2-14	22-31	1-5	
Whiskey	113	40	mg/L	6-328	22-205	0-5	0	20-487	102-1247	26-1200	[31, 70, 75]
			mg/std drink[a]	<1-11	<1-70	0-<1	0	<1-16	33-40	1-39	
Rum	28	40	mg/L	6-131	34-3633	0-1	0-126	8-455	0-788	0-219	[31, 70]
			mg/std drink[a]	<1-4	1-116	0	0-4	<1-15	0-25	0-7	
Vodka	31	40	mg/L	0-170	0-102	<1	0	<1-164	0	0-90	[31, 70, 75]
			mg/std drink[a]	0-5	0-3	<1	0	<1-5	0	0-3	

[a] 10 grams of ethanol (Australian standard drink) [88].
[b] typical ABV for beverage class [70].

can cause differences in congener concentration of up to ten-fold higher when compared with their commercial counterparts [89, 90]

.1.1.3.2 Other Sources of Congeners

Fermentation by-product congeners may also be sourced from intentional or accidental consumption of products of industry, medicines or the home; albeit generally at relatively low levels. Absorption of ethanol through the skin from use of antiseptics or hand sanitisers has not been shown to raise ethanol levels sufficiently in order to affect BAC implications [91, 92] with the products regarded as safe for everyday use [93, 94]. However, congener absorption from the use of hand sanitisers has been demonstrated with concentrations of 0.2 mg/L and 2 mg/L of 1-propanol and isopropanol detected in blood, respectively [95]. Another study revealed isopropanol concentrations of up to 12 mg/L in serum after using pre-surgical skin disinfection [96]. In a 'worst-case model' of repeated applications with a commercially available hand sanitiser, concentrations of 18.0 mg/L and 10.0 mg/L of 1-propanol and isopropanol, respectively, were detected [97]. Other occupational absorption of congeners has been shown to occur in workers exposed to 1-butanol in their workplace, where concentrations of up 0.2 mg/L of free 1-butanol were detected in urine [98]. Fruit juices have also been shown to contain methanol derived from some fruits, nectars and syrups, ranging from low concentrations to those in excess of 100 mg/L [99]. Furthermore, these refreshments have also been shown to raise the concentrations of methanol in blood due to the pectin within the cells of the fruit undergoing metabolism to methanol in the colon [100]. Drinking experiments with fruit juices have shown methanol concentrations of up to ~ 0.5 mg/L in blood [101]. Similarly, a large variety of chocolates containing predominantly alcoholic fruit brandies and liqueurs showed considerable concentrations of ethanol and

congeners [102]. Although experiments involving ingestion of the chocolates have not been performed, it would be unlikely that sufficient blood concentrations of congeners would occur with reasonable consumption of the chocolates. After-drinking claims involving circumstances such as these described above have not been documented. Nonetheless, absorption or ingestion of congeners through sources other than alcoholic beverages requires consideration as a potential misrepresentation to the sensitive nature of ACA interpretation.

1.1.3.3 Pharmacokinetics of Fermentation By-product Congeners

Considerable investigation has been undertaken surrounding the pharmacokinetics of the targeted congeners, in order to determine the theoretical congener concentrations in blood. Although similar to ethanol, the pharmacokinetics of low and higher alcohols do not completely fit the Widmark model, designed originally for ethanol. Drinking experiments with isolated congeners [103-105] and whole beverages [106-108] helped develop blood congener concentration curves with the use of previously established data on ethanol. This provided a better understanding of the pharmacokinetics of congeners, when combined with data on their physicochemical properties (**Table 2**). However, caution is still necessary when interpreting the fermentation by-product congener results.

Absorption

Compared with ethanol, alcohol congeners have a slower absorption rate [70]. Differences in beverage absorption occur largely due to Fick's Law, where higher concentrated beverages such as spirits diffuse across membranes faster than lower concentration beverages, such as

beer. Food can delay the absorption, whilst an empty stomach and beverages containing high amounts of carbon dioxide (carbonated beverages), accelerate absorption [109].

Distribution

The reduction factor (r_A) described for each congener in ACA literature is comparable to the volume of distribution and is used for extrapolating the theoretical blood concentration using the back calculation in a revised Widmark formula (equation 1). Like ethanol; methanol, 1-propanol, isopropanol and 2-butanol are water soluble and are readily distributed into the body water. The higher alcohols of 1-butanol and isobutanol are less hydrophilic; whilst 2- and 3-methyl-1-butanol are approximately 50% soluble in body water, have a higher $\mathbf{r_A}$ **(Table 2**) and consequently penetrate more rapidly into tissues. The proportion of the body available for distribution of the congeners varies considerably throughout the population, affecting the r_A for each congener. Anthropometric parameters (body fat, body mass, age and gender) have led to much discussion regarding the potential to increase the accuracy of BAC detection [110]. However, ACA research is not as comprehensive. There have been improvements for ethanol using updated anthropometric measurements that take into account body mass index (BMI) or total body water (TBW) [111]. For example, overweight and obese individuals may not fit the r_A regressions currently set for ACA. People with a BMI over 30 (clinically obese) result in abnormally low $r_{A.}$ and those with a BMI over 40 (grossly obese) would have unattainable r_A values [47]. As demonstrated by Watson for ethanol [112], the TBW must also be taken into account. This is achieved by using updated population data for both men and women, utilising modern anthropometric measurements. This formula provides a more accurate r_A when incorporated into the Widmark formula for ethanol [113]. There have been many updated versions since, with the most recent account presenting a

mathematical model of five previously published updated versions of the Widmark model [114, 115]. Here, Posey [110] recognised that having several different populations with more variety in anthropometric measures, better represents the general population for ethanol back-calculations. However, the fermentation by-product ACA methodology utilises mean values from drinking experiments without incorporating any additional parameters, except for weight. Some use three levels to allow for variation (minimum, medium and maximum) [31]; whilst others suggest using a variation of ±30% [28]. This may be due to the complexities of having multiple compounds of interest that increase the drinking experiment size and also the relatively low amount of ACA performed when compared to ethanol back-calculations internationally. Nonetheless, this presents potentially significant inaccuracies when performing ACA in the general population and particularly in individuals who do not accurately fit the r_A regression patterns.

Metabolism

The congener alcohols can remain unchanged, or undergo phase I (oxidation) or phase II (conjugation) metabolism [116]. The lower alcohol congeners predominantly favor phase I metabolism in the liver where competition at the alcohol dehydrogenase (ADH) enzyme binding sites occurs with the highly abundant ethanol. To explain the influence that relatively high concentrations of ethanol have on the metabolism of the congeners, as well as competition between congeners themselves, animal [117] and human [23, 118] trials were conducted, with and without ethanol. Further drinking trials comprising of congener(s) vs congener(s) of methanol [119], 1-propanol [104, 119], isopropanol [119] and isobutanol [105], were performed, with and without concurrent administration of ethanol to demonstrate how the pharmacokinetics of each congener were altered. Phase II conjugation involved

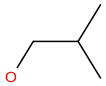
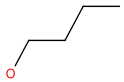
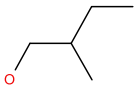
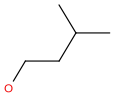
reactions with glucuronic acid alongside the carbon chain length, as seen with higher alcohols [120], to the extent that 2- and 3-methyl-1-butanol were almost completely conjugated [31].

The phenomenon of continual methanol build up has been heavily investigated, as methanol metabolism is inhibited by ADH when ethanol levels are above approximately 0.2 g/kg [119, 121]. This is regularly seen in alcoholics allowing methanol concentrations to build up to potentially toxic levels, well above the endogenous levels of 0.86 ± 0.76 mg/kg that are found to be in the blood of non-alcoholic drinkers [119]. For this reason, measurement of blood methanol has been shown to be useful as a diagnostic tool to distinguish acute from chronic alcoholism [122-124]. However, this methanol accumulation then also requires consideration when evaluating ACA as it has the potential to alter the expected methanol concentration.

Excretion

With the exception of methanol, the elimination of alcohol congeners generally follows first-order kinetics when ethanol levels are high, due to their lower blood concentrations and other pathways of metabolism [31, 121]. Excretion of the unchanged congener alcohols through urine and expired air and has been suggested to be from 1-2% [28] and up to 5-10% [125]. In general, the higher alcohol congeners are excreted faster, due to the increasing affinity of ADH to longer carbon-chained alcohols. It has also been suggested that 2- and 3-methyl-1-butanol have less importance than lower alcohol congeners as their metabolism and excretion occur relatively rapidly [28]. If the ethanol concentration is low enough and does not alter hepatic metabolism, excretion for all alcohol congeners is increased [31].

Table 2
Physicochemical properties of congeners and associated Widmark constants [126]

Compound CAS #	**Structural Formula**	**Formula**	**Molecular Weight**	**Boiling Point (°C)**	**Density (g/mL at 25°C)**	**Vapor Pressure (kPa at 20°C)**	**Water Solubility (g/L at 20°C)**	**Volume of Distribution [31] (r_A)**	**Congener concentration (C_{time}) [31]**
Ethanol 64-17-5		C_2H_6O	46.068(2)	78.3	0.785	5.90	soluble	N/A	N/A
Methanol 67-56-1		CH_4O	32.0419(9)	64.7	0.787	12.8	soluble	$r_{A\ min} = 0.6$ $r_{A\ med} = 0.7$ $r_{A\ max} = 0.8$	$C_{30} = 0.79 \times C_0 + 0.01 \pm 0.58$ $C_{90} = 0.89 \times C_0 + 0.08 \pm 0.44$ $C_{150} = 0.95 \times C_0 + 0.16 \pm 0.28$
Isopropanol 67-63-0		C_3H_8O	60.095(2)	82.5	0.781	4.4	soluble	N/A	N/A
1-Propanol 71-23-8		C_3H_8O	60.095(2)	97.2	0.800	1.90	soluble	$r_{A\ min} = 0.6$ $r_{A\ med} = 0.7$ $r_{A\ max} = 0.8$	$C_{30} = 0.72 \times C_0 \pm 0.05$ $C_{90} = 0.59 \times C_0 + 0.01 \pm 0.07$ $C_{150} = 0.48 \times C_0 + 0.01 \pm 0.12$
2-Butanol 78-92-2		$C_4H_{10}O$	74.122(3)	99.5	0.802	1.65	260 [127]	N/A	N/A
Isobutanol 78-83-1		$C_4H_{10}O$	74.122(3)	107.9	0.798	1.06	80	$r_{A\ min} = 1.1$ $r_{A\ med} = 1.3$ $r_{A\ max} = 1.5$	$C_{30} = 0.56 \times C_0 + 0.03 \pm 0.11$ $C_{90} = 0.40 \times C_0 + 0.03 \pm 0.09$ $C_{150} = 0.30 \times C_0 \pm 0.04$
1-Butanol 71-36-3		$C_4H_{10}O$	74.122(3)	117.7	0.806	0.67	79	N/A	N/A
2-methyl-1-butanol 137-32-6		$C_5H_{12}O$	88.148(4)	128.0	0.815	0.43	36	N/A	N/A
3-methyl-1-butanol 123-51-3		$C_5H_{12}O$	88.148(4)	131.1	0.805	0.31	25	$r_{A\ min} = 1.6$ $r_{A\ med} = 2.0$ $r_{A\ max} = 2.4$	$C_{30} = 0.32 \times C_0 \pm 0.05$ $C_{90} = 0.15 \times C_0 \pm 0.04$ $C_{150} = 0.07 \times C_0 \pm 0.06$

1.1.3.4 Analytical Methodology for Fermentation By-product ACA

The current analytical technique to determine the fermentation by-product congeners in beverages and blood is by HS-GC-FID and can include trapping and/or cryofocusing techniques to increase the sensitivity**.** Often ~ 0.1–0.3 mL of blood is used in order to achieve the required limits of detection (LOD) of ~ 0.1 mg/L for methanol and ~ 0.01 mg/L for the remaining alcohol congeners [28, 31]. Calibration curves are not linear for all congener alcohols and are matrix dependent [28] emphasising the need for internal standards. T-butanol (*tert*-butanol) is the most commonly used internal standard as it is not found in beverages, nor is it produced endogenously or from bacterial putrefaction [15, 128-130]. Matrix effects have been demonstrated for the HS-GC-FID method; these may be reduced with dilution when analysing alcoholic beverages [35], however, due to the required sensitivity, dilutions of biological matrices is limited. The addition of perchloric acid in order to precipitate proteins and minimise matrix effects has been trialed, but is problematic as it also dilutes the congener concentrations [28, 131]. Other pretreatment techniques available to increase the sensitivity of the analysis include employing solid-phase microextraction **(**SPME) or in-tube extraction (ITEX) that enhances the recovery of certain analytes. However, competitive binding may be problematic as the high ethanol content decreases the recovery of other analytes of lower concentrations [132]. Competitive binding was not detected using trapping techniques supporting it as the preferred current technique [35]. Chromatographically, large glass columns packed with absorbent material (Carbowax 20M / Carbopack B) [123, 133] were traditionally used, however more recently, laboratories have achieved better chromatography with capillary columns (CP-Sil-19, CP-Wax-52, Rtx-BAC1, Rtx-BAC2 etc.) [31, 130]. However, care is required to minimise the water content entering the capillary column, as degradation of chromatography can occur even at low levels in the

headspace [134]. Problems exist where co-elution of peaks occurs, particularly when a small peak of a congener is on the tail of the highly abundant ethanol peak [28].

1.1.3.5 Stability of Congeners

The stability of congeners in blood has also been investigated and, like ethanol, they have shown to alter with inappropriate storage conditions. Long term (12 month) storage of blood at room temperature show considerable changes in congener concentrations [65] and even some bacterial production of congeners [135]. When refrigerated at 4°C, up to a 10% loss of congeners has been demonstrated, compared with no change to congener concentrations when stored frozen at -27°C for the same 12 month period [65]. However, refrigeration is generally accepted as a suitable storage temperature for after-drinking claims, where freezing is not possible. Even with appropriate storage conditions, correct antiseptic techniques are also required, as congener production by bacteria is possible if specimens are not handled aseptically. Correct handling of the specimen, sufficient preservative (e.g. sodium fluoride) and refrigeration, is adequate to inhibit ethanol and congener bacterial artifact production, whilst minimising losses in blood concentrations [65, 135]. Bacterial ethanol production can be prevented by either refrigeration and/or addition with at least 1% (w/v) of sodium fluoride as a preservative in urine [136] and blood [137]. However, one study showed that even with similar preservative concentrations, contamination most likely from blood diluters resulted in ethanol degradation by bacteria growth when left at room temperature [138]. This suggests that a preservative (such as sodium fluoride) at least 1% (w/v) under refrigerated temperatures is required for sufficient inhibition of bacteria and to increase the stability of congeners.

1.1.3.6 Evaluation of ACA

The evaluation process involves the back-calculation of each targeted congener to provide the theoretical concentrations of the congeners in blood using the claimed circumstances surrounding consumption, in conjunction with the alcoholic beverage congener content, anthropometric measurements, and known pharmacokinetic parameters of the congeners. The following brief ACA workflow for fermentation by-product congeners has been extrapolated from the review by Bonte where the congeners primarily targeted were: methanol; 1-propanol; isobutanol; 2-butanol; 1-butanol; and 3-methyl-1-butanol [31]. This is achieved by determining the congener concentrations in the beverage(s) that were claimed to be consumed either from published literature, or analysis of the beverage itself. The amount of each congener consumed is determined by multiplying the volume of beverage consumed with the concentration of that congener in the beverage. As the pharmacokinetics of each congener differ and with competition between ethanol occurring at the ADH binding site, each congener must be considered individually when performing the interpretation. The final theoretical concentration of the congener in the blood at a certain time of drinking cessation (C_0) is attained using body weight of the individual and the r_A placed in the Widmark formula (equation 1). Only for the methanol, isobutanol, 1-propanol and 3-methyl-1-butanol congeners are the revised version of the Widmark formula provided, described as:

$$C_0 = \frac{\text{amount of the congener consumed (mg)}}{\text{body weight (kg)} \times r_A}$$

Equation 1

Drinking experiments have determined three parameters of the confidence for the r_A of each congener based on pharmacokinetic variability (i.e. minimum, medium and maximum). The use of the appropriate r_A is important as variation occurs between the ranges. However, details on how to select an appropriate r_A are not described, suggesting that it is left to the judgment of the evaluating analyst.

Obtaining the C_{time} is achieved by subtracting the cessation of drinking time from the time of sampling , in minutes. This is required to provide the appropriate formula for each specific congener, as shown in **Table 2**. Using the C_0 calculated using the above Widmark formula, a congener concentration can then be calculated for each congener at the time of drinking cessation. Interpolation can then assist for times in-between these time points by estimating the concentration of the congener. Extrapolation is required if blood was taken outside of the one to three hour time points. However naturally, this increases the uncertainty and creates limitations for use of the analysis. Relatively wide standard deviations provide confidence ranges for the expected congener(s) blood concentrations which the actual blood congener(s) concentration is compared with [28]. If the actual congener concentrations fall within the theoretical congener ranges, the claimed alcoholic beverage consumption is feasible and an opinion regarding the case can be made.

It is suggested that this methodology is most useful for 1-propanol and isobutanol and refers to these congeners as the most important of all the congeners in the evaluation of ACA. Due mainly to the very low concentrations in blood after alcohol consumption, 2- and 3-methyl-1-butanol are considered to be of minor importance in ACA and accordingly, methodology on how to evaluate the concentrations of these congeners has not been provided.

There are anomalies with fermentation by-product ACA in regards to the congener content in specific beverage types. For example, specifying that a certain beverage was consumed is usually not possible; only if the claim is feasible or not. However, as fruit brandies contain relatively high amounts of 1-butanol (**Table 1**), it may indicate that this type of beverage was consumed should the congener be present. In contrast, beverages containing an extremely low congener content such as vodka [22], gin and clear rum [1], will provide little or no detectable congeners in the blood, consequently complicating evaluation. Additionally, isopropanol is not found in alcoholic beverages unless intentionally added as an adulterant, as seen in some designer drinks [109]. However, detectable isopropanol levels may be due to endogenous production associated with acetone formation in diabetic ketoacidosis, vigorous exercise or prolonged alcohol abuse [119, 139-141], potentially making confirmation of an isopropanol laced drink difficult. Consumption of multiple different beverage types throughout a drinking session can cause issues with evaluating each congener accurately. Also, ACA is of little use if the same drink is allegedly consumed before and after an incident. Finally, in order to obtain sufficient congener concentrations to meet sensitivity requirements and accurate evaluations, a BAC of 0.08 and above must be present [31]. This may not be suitable in Australia and most other countries where the legal limit is 0.05% or lower.

Additional Evaluating Techniques

There are different approaches in the calculation of likely congener content. Iffland and Jones [28] and Krause [11] use exponential elimination of the congeners (i.e. half-life) to determine time dependent changes [105]. The drinking cessation (C_0) is calculated as for Bonte and then used in the formula below (equation 2). However, the mean r_A is used for each congener,

referenced by Bonte as r_A medium. This differs by the approach used by Bonte where specific formulae (r_A = minimum, medium or maximum) and a standard deviation with each drinking cessation time (**Table 2**) for each congener were given. Instead, a 30% variation in distribution provides a confidence range for each congener.

$$C_t = C_0 \times e^{-k \times t}$$

Equation 2

The elimination constant (k) is given by substituting the half-life ($t_{½}$) of the congener as such.

$$k = \frac{0.693}{t_{\frac{1}{2}}}$$

Equation 3

Interpretations and opinions are handed to the courts referring to the claims of the after-drink defence equating from "almost certainly excluded" to "irrefutable" conclusions. In 2000, a German institute reported issuing up to a hundred expert opinions for ACA annually [31]. These opinions contained conclusions that confirmed only around 1% of the defence's claims, rejected 75%, ruled 14% as improbable and were unable to determine in 15% of cases.

1.1.3.7 ACA Quality Assurance

In Germany, a working group called “Alcohol consumption and after-drinking" was founded in 2001 by the German Society of Toxicological and Forensic Chemistry (GTFCh) [142]. In 2006 the group showed the methodology for satisfactory proficiency testing of 16 participating German laboratories performing ACA on fermentation by-products [143]. Prior to this, Iffland and Jones suggested that due to some unsatisfactory external proficiency results, additional investigation was required to standardise the analysis and interpretation throughout laboratories conducting ACA [28]. Nonetheless, Bonte had high confidence in his approach which appeared to be accepted by the courts, at least in the German jurisdiction [31]. This is supported with a recent retrospective study that indicates that German courts strongly refer to the ACA expert opinions in 80.6% of after-drinking type offence cases, concluding that this technique is accepted in forensic practice [144].

1.1.4 Ingredient Biomarker Congener ACA

Other than from by-products of fermentation, congeners also exist in beverages as a result of the ingredients and materials used during production. These other substances includes aldehydes, esters, histamines, additives, coloring agents, tannins, phenols and other organic and inorganic compounds [1, 70] and are often beverage-specific. To date, the detection of ingredient biomarker congeners that are uniquely present in certain alcoholic beverage types has only been explored in some herbal spirits for the detection of eugenol [36], anethole [37] and menthone [38]. The detection of these ingredient biomarkers may indicate the consumption of herb, aniseed and peppermint-type liqueurs, since they appear are not present in other alcoholic beverages. There are no other ingredient biomarker congeners identified specifically for other alcoholic beverages including beer and wine thus far. The identification

of such substances may prove useful in the future to determine if beer, wine or some other beverage was consumed.

Unique substances can potentially be identified from manufacturers that determine certain congeners as part of their legislative responsibility [66, 145, 146] or for research purposes in the pursuit of a better product [76, 147-149]. Additionally, profiling of alcoholic beverages has been performed to assist in age markers [150] and authentication claims [76], with some achieving this by principal component analysis [150, 151]. The knowledge obtained by the alcohol industry will assist in discovering potential targets as ingredient biomarkers for a specific alcoholic beverage.

Whatever substance(s) is targeted as a potential unique marker, it is mandatory that research studies assess how unique this marker(s) may be and of course the bioavailability and pharmacokinetics in humans under various simulated conditions [1]. The targeted congener must also be detectable using conventional techniques.

1.1.4.1 Analytical Methodology for Ingredient Biomarker ACA

Analytical techniques for the detection of ingredient biomarker congeners are capable of utilising wider techniques than current ACA practices of HS-GC-FID since they possess larger molecular weights. There has been recent use of MS in the detection of higher molecular weight congeners other than fermentation by-products (such as aromatic congeners) [152], and with SPME pretreatment to detect congeners sourced from herbs [36-38]. The use of high performance liquid chromatography coupled with tandem mass spectrometry (LC-MS) has only been used for flavonoids in beer [153, 154]. Ultimately MS

detection is necessary to gain the sensitivity and selectivity required for medico-legal purposes.

1.1.5 Conclusion

Fermentation by-product ACA is a technique used in Germany to investigate after-drinking defense claims by examining the feasibility of the claimed alcoholic beverage consumption. This review expresses concerns surrounding the current ACA approach due to the limitations, uncertainties, the difficult nature of carrying out the evaluation, varying methodologies used by experts and the limited knowledge of the variation in pharmacokinetics of these congeners in humans. For example, many of the fermentative by-product congeners can either be produced endogenously, by bacterial putrefaction during storage, or obtained from sources other than consumption of the claimed alcohol beverage. Additionally, blood sampling must occur within 1 to 3 hours of cessation of drinking and a significant BAC of 0.08 must be present. Furthermore, there is a reliance on information from the defense such as body weight, length of drinking times and case circumstances. Individual pharmacokinetic variability and the consumption of vastly different alcoholic beverage types alter the expected congener concentrations. It is difficult to obtain a universally accepted evaluation method for ACA, which also requires careful interpolation that allows for potentially wide uncertainty ranges. Although these factors exist, it has shown to be a prominent tool used for after-drink type cases in Germany; although it is inadequate for determining the alcohol source in other scenarios.

Although minimally researched to date, ingredient biomarkers offer an alternative and potentially more defendable approach to examine after-drink claims, having the potential for application in other cases by determining the specific source of alcohol. This review

highlights a considerable opportunity for investigations into the area of developing methodology for the detection of ingredient biomarker congeners for a range of commonly consumed alcoholic beverages in biological specimens.

Online Graphical Abstract

Fig. 1

Botanical illustration of *Humulus lupulus* (L). By permission of Puple Sage Botanicals [155]

Key Points

1. Besides ethanol and water, alcoholic beverages contain a number of compounds called congeners that can be used to determine the type(s) of beverage consumed.
2. Providing proof that a particular alcoholic beverage is the source of alcohol, may assist in cases where the type of beverage consumed is at question.
3. Fermentation by-product ACA may assist in determining the feasibility of claimed alcoholic beverage(s) consumption however, it does not indicate the source of alcohol.
4. Detection of ingredient biomarkers from alcoholic beverages may provide a means to identify the source of alcohol consumed.

CHAPTER 1.2

BEER SUMMARY AND THESIS AIMS

1.2.1 Traditional ACA

In light of the comprehensive review of literature in Chapter 1.1, the following limitations of traditional alcohol congener analysis included:

- the time of sampling should be within 1-3 hours after the incident [31];
- the interpretation commonly requires information such as gender, age, body weight, type and how much beverage was ingested, length of drinking times, and accurate time of sample collection;
- the interpretation is formula-heavy and time consuming requiring the utilisation of constants for each given congener in an attempt to counteract for person to person variations and each case requires and individual approach to a certain extent [31];
- careful interpolation is sometimes required that can result in considerably wide uncertainty ranges;
- different alcoholic beverages types consumed throughout a drinking session, or the same drink consumed before and after an incident, can alter the expected congener concentrations and make it difficult or impossible for ACA to be used;
- the minimal amount of beverage consumed should be at least 1 L of beer, 500 mL of wine or 60 mL of spirits [28, 31].
- mainly only beverages local to Germany have been investigated with published congener concentrations [22];
- as fermentation products will vary slightly between batch to batch and sometimes considerably over time making a database of congener concentrations in beverages potentially not as useful over time and the analysis of the actual claimed beverage itself sometimes required [86];
- beverages containing an extremely low congener content such as Vodka [22]; Gin and clear Rums [1] will provide little to no congeners being able to be detected in the blood and as such, difficult to identify;

- it cannot be applied in a post mortem scenario since both ethanol and the targeted congener analysis compounds can be produced during putrefaction as artefacts [60]. It also makes it unsuitable as a tool to confirm if the deceased's ethanol level is genuine; and,

- specifying that a certain beverage was consumed is not possible, only if 2-butanol is present can it be suggested that certain spirits such as fruit brandies may have been ingested.

Essentially, these limitations are why the alcohol congener analysis of fermentation by-product congeners has received skepticism and has not been widely applied in medico-legal casework in other parts of the world. Although this traditional ACA has proved useful in after-drinking casework, it became apparent that the use of ingredient biomarkers may help in alleviating some of these limitations and in addition, be a more appropriate approach that may be used more widely in forensic services.

1.2.2 Aims

As the most widely consumed alcoholic beverage, the aims of this thesis are to examine the feasibility of determining if beer has been consumed. This will require the identification of an ingredient marker that is principally found in beer. The targeted compound(s) must be able to be identified and measured in practical forensic specimens such as blood post-consumption of beer. Consequently, such an outcome may suggest the likely source of an elevated blood alcohol concentration and thus, the source of alcohol ingestion.

1.2.3 Beer

The examination of beer for the identification of an ingredient maker demonstrated the requirement to incorporate knowledge and literature from the food and beverage industry into this thesis. Like many other beverages, beer contains up to 800 congeners [78, 156]. However the examination of the composition beer more closely, showed that of all the classes, the hop derivatives are the only group that are sourced from one ingredient (**Table 3**). This demonstrated a good target to investigate as yeast and malt are also commonly used in food and beverages products.

Table 3

Composition of beer detailing the class of substance, typical concentration, number of, and the source, of the congeners [78].

Substances	Concentration	No. of Congeners	Source
Water	90-94%	1	-
Ethanol	3-5% v/v	1	Yeast, Malt
Carbohydrates	1- 6% w/v	~100	Malt
Carbon dioxide	3.5-4.5 g/L	1	Yeast, Mal
Inorganic salts	0.5-4 g/L	~25	Water, Malt
Total nitrogen content	0.3-1 g/L	~100	Yeast, Malt
Organic acids	50-250 mg/L	~200	Yeast, Malt
Higher alcohols	100-300 mg/L	~ 80	Yeast, Malt
Aldehydes	30-40 mg/L	~50	Yeast, Hops
Esters	25-40 mg/L	~150	Yeast, Malt, Hops
Sulphur compounds	1-10 mg/L	~40	Yeast, Malt, Hops
Hop derivatives	20-60 mg/L	>100	Hops
Vitamin B compounds	5-10 mg/L	13	Yeast, Malt

The hop derivatives are compounds sourced from the hop cone, used to principally provide bitterness to the beer. The compounds found in beer that are predominantly responsible for the bitterness are known as iso-α-acid or isohumulones (IAA). Additional hop products such as pre-isomerised hops, and isolation of pure IAA, have been available for over 50 years and were developed in order to save costs [157]. However they still contain the same natural IAA compounds that are found specifically in beer.

The term lightstruck is given to beer when exposed to ultraviolet and visible light [158]. This results in the break down of IAA with a cleaved side chain reacting with proteins that results in the formation of the 3-methylbut-2-ene-1-thiol (3-MBT) molecule and "skunky" smelling beer (**Fig. 2**). To combat this photolytic cleavage, chemicall altered "reduced IAA" were sysnthesised from the original IAA compounds. Information of these hop derived IAA and reduced IAA are given in detail in **Chapter 2.1**. Importantly, these compounds presented as superior targets to be utilised as ingredient biomarkers, specifically found in beer.

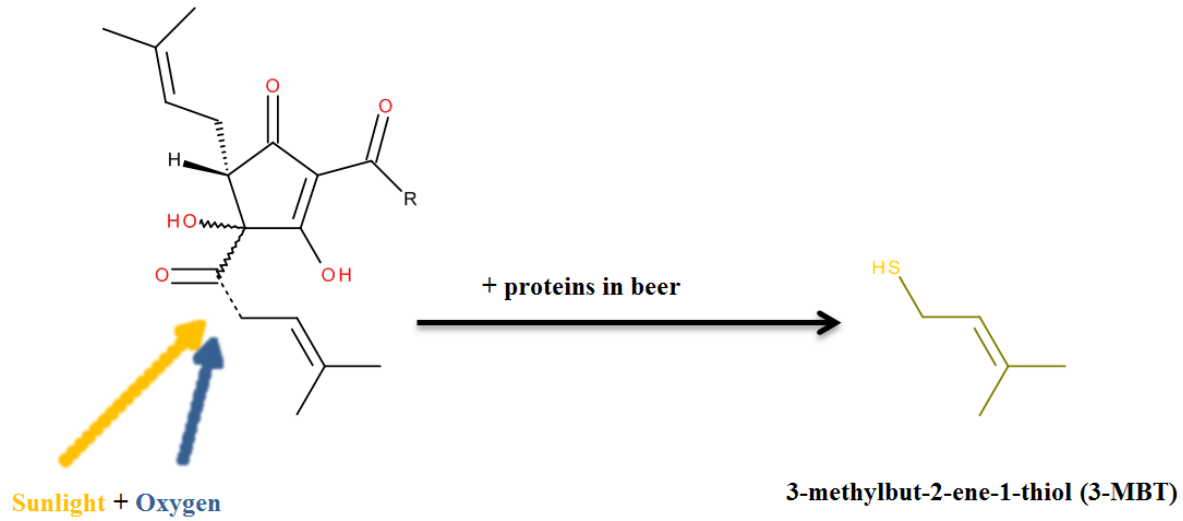


Fig. 2

The photolysis of iso-α-acids (IAA) scheme showing the cleavage of the isohexenoyl side chain and subsequent formation of 3-MBT.

1.2.4 Outline of Thesis

Each chapter will assess separate studies and provide knowledge that will assist in the overall PhD project and provide peer-reviewed literature for reference and use in authentic toxicological case work. To address these aims and comprehensively examine the use of IAA to confirm beer consumption, the following research activities were undertaken:

Chapter 1. A review of the literature to investigate and describe

a. the feasibility of identifying the source of alcohol by traditional ACA.
b. the current limitations of traditional ACA.
c. possible alternative approaches that identify the consumed alcoholic beverage.

Chapter 2. The development and validation of an analytical method for the detection and quantification of IAA providing

a. the sufficient separation of the IAA analogs and isomers
b. analytical details of the developed method
c. the validation data to demonstrate the accuracy and precision of results
d. the bioavailability of the IAA in the biological specimens in humans

Chapter 3. The stability of IAA in blood and describe

a. the optimal storage conditions of specimens for future IAA analysis
b. any unacceptable degradation of IAA groups
c. the individual stabilities of IAA analogs and isomers

Chapter 4. Investigate the pharmacokinetics of IAA by volunteer drinking studies and describe

a. the ability of the method to detect low level beer consumption
b. the PK of natural IAA with brown bottled beer consumption.
c. the PK of reduced IAA with clear bottled beer consumption.
d. any inter-individual or gender differences.
e. the concentration-time profiles of IAA following controlled beer administration.

Chapter 5. Apply IAA ingredient biomarker analysis to postmortem cases to show

a. beer confirmation and source of ethanol ingestion in authentic casework.
b. IAA concentrations and the prevalence of beer in cases received.
c. the ability to detect IAA in other postmortem specimens.
d. .the postmortem redistribution of IAA.

Chapter 6. Develop a reference catalog of beer IAA profiles from

e. a range of popular local and international varieties.
f. a range of brown, green and clear bottled beers.

Chapter 7. To interpret the collected knowledge of previous chapters and the outcomes of this PhD research as a whole and discuss their impact and suggest possible future research.

CHAPTER 2.

ANALYTICAL METHODOLOGY

Conventional congener analysis is somewhat complicated, difficult to interpretate and limited in its applicability in other forensic scenarios. A simplified methodology is required that provides forensic analysis of one's alcohol consumption as discussed in **Chapter 1**. It has been identified that iso-α-acids are somewhat specific to beer and their determination in blood may indeed demonstrate beer consumption. The development of an analytical method that sufficiently separates and identifies the analogs and isomers was undertaken, in addition to a validation to international guidelines.

CHAPTER 2.1

IAA BY UHPLC-MS/MS METHOD VALIDATION

Rodda, L.N., D. Gerostamoulos, and O.H. Drummer,
The Rapid Identification and Quantification of Iso-α-acids and Reduced Iso-α-acids in Blood using UHPLC-MS/MS: Validation of a Novel Marker for Beer Consumption.
Anal Bioanal Chem, 2013. 405(30): p. 9755-9767.
(Appendix 1.2)

Monash University

Declaration for Thesis Chapter 2.2.

Declaration by candidate

In the case of Chapter 2.2., the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Located articles, reviewed articles and wrote the paper	85%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Gerostamoulos, D.	*Advised candidate regarding direction and content, reviewed article manuscript*	
Drummer, O.H.	*Advised candidate regarding direction and content, reviewed article manuscript*	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 26.6.14
Main Supervisor's Signature	[redacted]	**Date** 27/6/14

Abstract

A method for the detection of iso-α-acid (IAA) type ingredient congeners that are derived from the hop plant (*Humulus lupulus* L.) was developed to detect recent consumption of beer in blood. Three structurally similar but chemically-altered IAA, also used as beer-specific ingredients, are known as "reduced IAA", consisting of the rho-, tetrahydro- and hexahydro-IAA were also targeted. The use of a simple protein precipitation extraction and UHPLC-MS/MS system enabled detection of these analytes in both ante-mortem and post-mortem blood. Extracts were injected onto a C_{18} solid-core column under gradient elution to achieve separation of isobaric analogs and isomers within a 10 min run-time. Electrospray ionisation in negative multiple reaction monitoring mode was used to monitor three transitions for each of the analytes that were ultimately grouped together to form a calibration curve for quantification of each of the four IAA groups. The method was fully validated according to international guidelines that included extraction efficiency, matrix effects, process efficiency, ion suppression/enhancement of co-eluting analytes, selectivity, crosstalk, accuracy and precision, stabilities, and lower limits of quantification. Finally, applicability of the method described was demonstrated by the detection of IAA ingredient congeners in the blood of a volunteer following the consumption of a relatively small amount of beer in a pilot study.

Keywords

Alcohol congener analysis; beer; ingredient congener; hop-derived iso-α-acids; UHPLC-MS/MS; validation

2.1.1 Introduction

Compounds other than ethanol and water that are present in alcoholic beverages are termed congeners and may be detected in blood and urine following alcoholic beverage consumption. The detection and interpretation of these congeners is termed alcohol congener analysis (ACA) and can provide information for after-drinking (or hip-flask) defense cases regarding the feasibility of claimed alcohol consumption prior and/or subsequent to a motor vehicle incident [11, 28, 31]. Traditionally, ACA has been used to detect levels of fermentation by-product congeners (typically alcohols) that are found in nearly all alcoholic beverages [31, 70, 75]. However, the exact origin of the consumed ethanol cannot be determined and consequently, its use in a post-mortem setting has been minimal [32, 33, 159].

Congeners may also exist in a beverage as a result of the ingredients used during beverage production, in the form of different classes of compound (i.e. not only alcohols) [1]. Ingredient congeners may often be beverage-specific and present as targets for detection in biological fluid to determine consumption of specific alcoholic beverages [159]. To date, the detection of ingredient congeners present in alcoholic beverage has only been explored in some herbal spirits (e.g. eugenol, anethole and menthone) [36-38]. Furthermore, ACA that targets ingredient congeners may have fewer limitations to that of the fermentation by-products and can potentially be utilised in a broader range of medico-legal cases where determining the source of consumed alcohol is in dispute. There are no beer ingredient congeners thus far identified as targets for ingredient congener ACA, indicating the potential to identify targets in this common beverage.

Beer is the oldest and most widely consumed alcoholic beverage; just short of 2 billion hectoliters were produced in 2012 worldwide [160]. Beer traditionally utilizes the four

ingredients water, yeast, malt and hops; and may contain other ingredients for preservation or specific flavoring [78, 161]. These ingredients provide up to 800 congeners consisting of not only alcohols, but also, aldehydes, esters, histamines, additives, tannins, phenols and other organic and inorganic compounds [28, 67, 78, 156]. Of the range of congeners in beer, those identified as specific to beer and potential targets as ingredient congeners for ACA are those derived from the hop plant.

Traditionally in the boiling phase of beer brewing, the female cones (strobiles) from the hop plant (*Humulus lupulus* L.) are added to the wort (a liquid containing malt-derived sugars). Initially hops were used to impart the desired bitterness and aromatic *hoppy* properties to the beer. It was later established that hops also stabilizes the beer, including the foam or head [78, 162, 163] and protects the beer principally against gram-positive bacteria and/or microbial damage [164, 165]. Dried hops contain approximately 2-17% of alpha acids (AA), also known as α-acids or humulones, that consist of three major analogs (defined as; n-, co- and ad-AA) which are almost bitterless [67]. However, after the wort containing the hops is boiled for a prolonged time, the AA molecules are converted into the intensely bitter isomerised-α-acids (IAA) with *cis* and *trans* configurations (**Fig. 3**) [166-168]. The wort is then cooled and yeast is added, and following fermentation the beer is filtered and packaged into the final product.

Whole extract of the hop cone is also used as a natural herbal remedy commonly in combination with Valerian *(Valeriana officinalis*) for similar complementary properties as a mild sedative in humans for anxiety, insomnia, nervousness, memory and mood disorders [169-171]. However, as this is a whole extract and does not undergo a boiling (isomerisation) process, IAA are not present. Other than for beer, there are no other known uses for hops that undergo isomerisation and develop IAA, presenting a specific ingredient congener for beer.

Six stereoisomers of IAA are produced from the three predominant AA analogs that isomerise into diasterisomers [172]. The compounds are structurally very similar, only differing in the nature of the saturated acyl side-chain and the absolute configuration of one of the chiral centers (**Fig. 4**). The IAA are water soluble, tensioactive, have low pK_a values of ~3.5 and are responsible for the bitter character of beer and foam stabilisation [67].

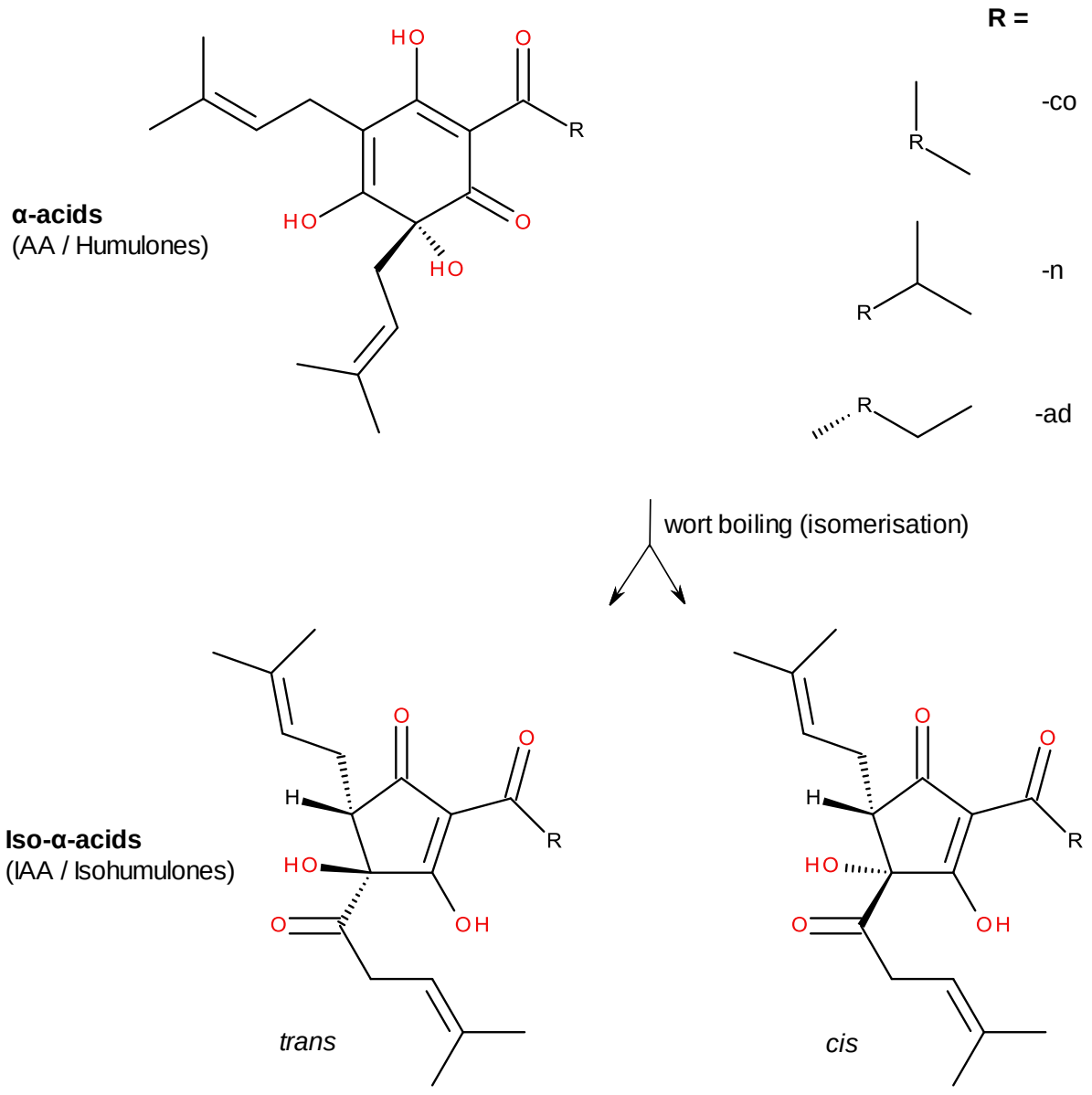


Fig. 3

Isomerisation of α-acid to iso-α-acids in diasteroisomeric *cis* and *trans* forms after boiling of the wort, including the three major analogs.

International bitterness units (IBU) are used by the alcohol industry as a measurement of the bitterness profile of beer with one unit being approximately equal to one mg/L of total IAA [173]. Beers historically ranged from 20-60 IBU however currently, they range from ~6-30 IBU, with the exception of some craft beers [165]. It is known that the *cis*:*trans* ratios in beer are 68:32 [174], however, the *trans*-IAA are present in the foam in greater proportion than the *cis*-IAA due to their lower water solubility. This hydrophobic property also explains the lower half-life of the *trans*-IAA (~1 year) compared to the *cis*-IAA (>5 years) [175], of which a variety of degradation products are formed [176]. Following the isomerisation, minor analogs post-, pre- and adpre-AA are also converted into their respective isomers, however they are generally regarded as insignificant due to their relatively low concentrations [153]. Although co-elution may be problematic particularly with ultraviolet detection [177], the use of mass spectrometry potentially solves this issue due to the differences of selection based on molecular weights and detected ions.

The IAA are prone to becoming light-struck in the presence of sunlight (near ultraviolet, blue light) and oxygen resulting in familiar and undesirable "skunky" aroma producing compounds, of which 3-methyl-2-butene-thiol (3-MBT) being the most offensive [174]. This historically led to beer being bottled in brown or green, lightproof glass. In recent years, a range of so-called light-stable "reduced IAA" were developed from the naturally available IAA, namely rho-IAA (RIAA), tetrahydro-IAA (TIAA) and more recently, hexahydro-IAA (HIAA). By reducing the relatively weak double bonds or carbonyl group in the side chains of IAA to stronger single bonds as seen with the reduced IAA, photolytic cleavage is unable to occur on that side chain and the production of 3-MBT is prevented from developing (**Figs. 2 and 4**).

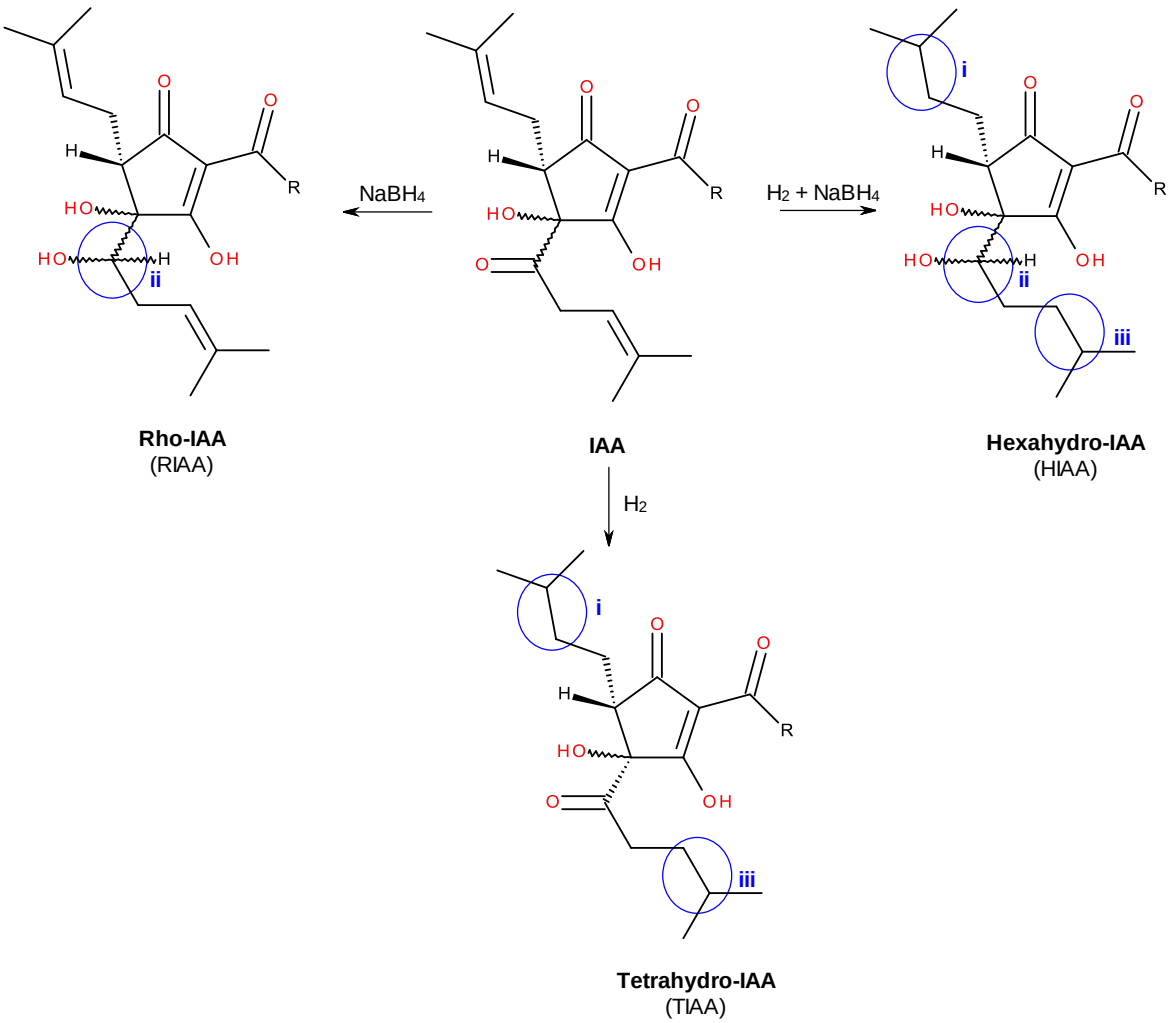


Fig. 4

The synthesis of reduced-IAA and properties of increased hydrophobicity (i and iii) and photolytic cleavage prevention (ii and iii). The carbonyl group in the side chain of IAA is reduced with sodium borohydride to produce rho-IAA. Reduction by hydrogenation of both side-chains double carbon bonds within the IAA produce the tetrahydro-IAA derivative. Execution of both the sodium borohydride and hydrogenation processes yields the hexahydro-IAA derivative of IAA.

This reduction process provides altered intensities of bitterness [165]. Coincidently, due to increasing hydrophobicity of the compounds (in order of RIAA to HIAA to TIAA), enhanced beer foam stability, appearance and "cling" are observed. As 2.4 and 4.2 ppm (for TIAA and HIAA, respectively) have been sufficient to show foam stabilization to that of the natural IAA; reduced IAA used for the purpose of foam improvement are added in lower concentrations [178].

The reduced IAA products are available as potassium salts preparations ready to be added directly to the finished beer (post-fermentation) [165, 179]. Among other non-natural additives, the non-natural reduced forms of IAA are not allowed in beers for the German market due to the "Reinheitsgebot" law stating that only natural hop products, water, malt and yeast, may be used in the brewing process [180].

The aim of this study was to develop a fully validated method for the detection of IAA and reduced IAA in human blood as potential specific ingredient congeners for the confirmation of beer consumption using an ultrahigh-performance liquid chromatography system coupled with a tandem mass spectrometer **(**UHPLC-MS/MS). This may also alleviate some issues and limitations of current methodologies that detect only fermentation by-product congeners. The detection of reduced IAA in blood may also provide information on the type of beer consumed, for example, beer within a clear glass bottle.

2.1.2 Experimental

2.1.2.1 Chemicals and Reagents

Reference standards for: DCHA-Iso, ICS-I3 (containing 62.3% w/w of *trans*-IAA); DCHA-Rho, ICS-R2 (containing 65.3% w/w of *cis*-RIAA); Tetra, ICS-T2 (containing 99.4% w/w of TIAA); DCHA-Hexa, ICS-H1 (containing 65.7% w/w of *cis*-HIAA), and; ICE-3 (containing 44.64% w/w of AA); were obtained from Labor Veritas (Zurich, Switzerland). The isotope labeled internal standard (IS) nimodipine-d_7 was purchased from PM Separations (Brisbane, Australia). Acetonitrile (ACN), methanol and formic acid were purchased from Merck (Melbourne, Australia). Ammonium formate was purchased from Sigma Aldrich (Sydney,

Australia). All chemicals were of analytical grade or better and water was purified using a Milli-Q Ultrapure Water System from Waters (Sydney, Australia).

2.1.2.2 Specimens

Preserved blank blood 10 mL samples (containing 200 mg sodium fluoride and 30 mg potassium oxalate) for calibration purposes and validation experiments were obtained from a local blood bank (Melbourne, Australia). Blood of the volunteer in the application to authenticity study was collected in sterile 5 mL Venosafe blood tubes containing 9 mg sodium fluoride and 9 mg potassium oxalate purchased from Hazpak (Melbourne, Australia). Concentrations of the preservatives are therefore at least 2.25% of each and blood samples were stored at -20 °C, sufficient to inhibit bacterial alteration of ethanol and congeners [137, 138].

2.1.2.3 Apparatus

The UHPLC-MS/MS system comprised of a Shimadzu MS 8030 quadrupole mass spectrometer (Melbourne, Australia) operated in the electrospray ionisation (ESI) in negative mode and a Shimadzu Nexera UHPLC system (Melbourne, Australia) that consisted of a degasser, two eluent pumps, a column oven and a chilled autosampler. For MS data evaluation, Shimadzu Postrun and Shimadzu Browser Analysis (Melbourne, Australia) softwares were used. GraphPad Prism 5.04 from GraphPad Software (San Diego, USA) was used for statistical analysis.

2.1.2.4 UHPLC conditions

Gradient elution was performed on a Kinetex C_{18} (3.0 × 150 mm, 2.6 μm) column coupled with a SecureGuard C_{18} Ultra guard-column (3.0 × 10 mm, 2.6 μm), both purchased from Phenomenex (Melbourne, Australia). The mobile phases consisted of 50 mmol/L aqueous:ACN (90:10) ammonium formate pH 2.8 (eluent A) and ACN containing 0.1% formic acid (eluent B). The flow rate of the mobile phase was 0.5 mL/min and was degassed by the integrated Shimadzu Nexera degasser during use. The gradient was programmed as follows: 0-0.5 min hold at 50% eluent B; 0.5-6.0 min eluent B increasing to 60%; 6.0-9.5 min eluent B increasing to 75 %; 9.5-10 min eluent B hold at 75 %. Before the start of batch analysis and before each injection, the UHPLC system was flushed for 2 min (90% eluent B) and equilibrated at starting conditions (50% eluent B) for 3 min. The column oven was maintained at 30 °C, the autosampler was operated at 4 °C and the autosampler needle was rinsed before and after aspiration of the sample using methanol.

2.1.2.5 MS/MS conditions

The MS data were acquired with the following ESI inlet conditions: nebulizing gas and drying gas were nitrogen at a flow rate of 3.0 and 12.5 L/min, respectively; the interface voltage was set to 4.8 kV; desolvation line (DL) temperature was 190 °C, and; the heat block temperature was 500 °C. The mass spectrometer was operated in negative multiple reaction monitoring mode (MRM) with argon as the collision induced dissociation gas (CID) at a pressure of 230 kPa; the detector voltage was set to 1.72 kV.

Specific MRM conditions for nimodipine-d_7 were auto-optimized by direct flow injection of 1 μL of a 1 mg/mL solution in acidic methanol. However, as the IAA and reduced IAA compounds are only available as a combined mixture of analogs, each analyte was manually-

optimized by injecting 10 µL of a 1 mg/mL solution of the respective IAA group in acidic methanol onto the column. With the system in Q1 scan mode, the retention time (RT) and a precursor mass (Q1 Mass) were obtained for each analytes. Using product ion scan mode (PIS) in a series of repeated injections over a range collision energies (CE) from 10-50 V at increments of 2 V, the three most abundant product ions and optimal CE for each individual analyte were obtained. The most abundant product ion was selected as the quantifier ion and subsequent two ions as suitable qualifiers**.** The results of the auto- and manual-optimizations are summarised in **Table 4**. All analyte dwell times were set at 25 msec.

Table 4

Groups and analytes, retention times (RT) [min], multiple reaction monitoring (MRM) transitions Q1 and Q3 masses [Da], Q1 pre-bias [V], collision cell energy (CE) [V], and Q3 pre-bias [V], used in LC-ESI-MS/MS.

Group	Analyte	(RT [min])	Ions	Q1 mass [Da]	Q3 mass [Da]	Q1 pre-bias [V]	CE [V]	Q3 pre-bias [V]
IAA	**I1** *trans*-isocohumulone	(2.70)	Quant	347.30	251.05	12	12	20
	I2 *cis*-isocohumulone [a]	(2.95)	Qual		182.05		16	14
			Qual		329.10		18	26
	I3 *trans*-isohumulone	(3.20)	Quant	360.90	264.95	16	14	22
	I4 cis-isohumulone [a]	(3.45)	Qual		195.95		16	24
	I5 *trans*-isoadhumulone	(3.75)	Qual		291.95		14	22
	I6 *cis*-isoadhumulone [a]	(4.05)						
RIAA	**R1** *cis*-rho-isocohumulone 1	(4.25)	Quant	348.90	251.10	16	16	20
	R2 *cis*-rho-isocohumulone 2	(4.90)	Qual		181.95		16	24
			Qual		233.00		20	10
	R3 *cis*-rho-isohumulone 1	(5.55)	Quant	362.90	265.25	16	16	20
	R4 *cis*-rho-isohumulone 2	(5.70)	Qual		196.20		16	24
	R5 *cis*-rho-isoadhumulone 1	(6.30)	Qual		247.00		18	20
	R6 *cis*-rho-isoadhumulone 2	(6.55)						
TIAA	**T1** *trans*-tetrahydro-isocohumulone	(4.55)	Quant	350.90	239.05	16	16	14
	T2 *cis*-tetrahydro-isocohumulone	(4.90)	Qual		253.00		14	20
			Qual		235.10		30	18
	T3 *trans*-tetrahydro-isohumulone	(5.10)	Quant	364.90	267.10	16	14	22
	T4 *cis*-tetrahydro-isohumulone	(5.75)	Qual		321.10		16	18
	T5+6 *trans*+*cis*-tetrahydro-isoadhumulone	(6.50)	Qual		125.00		42	40
HIAA	**H1** *cis*-hexahydro-isocohumulone 1	(6.90)	Quant	353.00	253.00	16	16	20
	H2 *cis*-hexahydro-isocohumulone 2	(7.45)	Qual		235.00		18	18
			Qual		209.10		16	16
	H3 *cis*-hexahydro-isohumulone 1	(7.80)	Quant	367.00	267.10		16	22
	H4 *cis*-hexahydro-isohumulone 2	(8.45)	Qual		249.00		32	30
	H5+6 *cis*-hexahydro-isoadhumulone 1+2	(9.10)	Qual		223.05		14	12
	IS nimodipine-d_7	(4.55)	Quant	424.20	122.10	10	22	14
			Qual		92.30		33	12
			Qual		301.20		21	23

[a] Residual analytes from IAA standard, not quantified.

2.1.2.6 Preparation of stock solutions, control samples and calibration standards

Individual stock solutions of each IAA group and nimodipine-d_7 were prepared at a concentration of 1 mg/mL using acidic methanol (0.05% formic acid) and methanol, respectively. The working solutions of IAA were prepared by pooling IAA stock solutions to make a single 0.1 mg/mL working solution with subsequent dilutions at the following concentrations: 0.01, 0.001 and 0.0001 mg/mL; using acidic methanol. Stock and working solutions were stored at -20 °C for a maximum time frame of six and one month, respectively.

The calibration standards were prepared using pooled blank blood spiked with the working solutions to obtain the final concentrations of 0.001, 0.01, 0.05, 0.15, 0.06, 2, 5 and 10 mg/L. These calibrations provided the levels required for varying calibration models for each IAA group (**Table 5)**. The quality control samples (QC) were prepared using pooled blank blood spiked with the working solutions to obtain the final concentrations of 0.1 mg/L (low), 0.8 mg/L (med) and 6 mg/L (high) (**Table 5**), and were stored at -60 °C before analysis.

Table 5

Concentrations of calibration standards and quality control samples of all studied grouped analytes in mg/L.

Group	**Weighting**[a]	**Calibration standards**								**Quality controls**		
		1	2	3	4	5	6	7	8	Low	Med	High
trans-IAA	1/x	0.001	0.01	0.05	0.15	0.6	2	5	10	0.1	0.8	6
RIAA	1/x	n/a	0.01	0.05	0.15	0.6	2	5	10	0.1	0.8	6
TIAA	$1/x^2$	n/a	0.01	0.05	0.15	0.6	2	5	10	0.1	0.8	6
HIAA	$1/x^2$	n/a	0.01	0.05	0.15	0.6	2	5	10	0.1	0.8	6

[a] Weighting was used for analysis using calibration standards 1- 6 for quantification of samples below 2 mg/L. Otherwise no weighting was applied when analysis using full curve to quantify higher concentrations from 2-10 mg/L.

2.1.2.7 Sample preparation

Briefly, 100 µL of blood underwent protein precipitation in a 2 mL Eppendorf tube from Eppendorf (Sydney, Australia) using 200 µL of cold (-20 °C) ACN containing the IS nimodipine-d_7 (0.5 mg/L) for 5 min on a shaker at 2500 rpm. After a 10 min rest and subsequent centrifugation at 15,000 rpm for 10 min, the supernatant was transferred to an autosampler vial and evaporated to dryness under nitrogen using a Ratek dry block heater DBH10 (Melbourne, Australia) operated at room temperature. The residue was reconstituted in 50 µL of a mixture of eluent A and eluent B (60:40, v:v). Twenty microliters of the final extract was injected into the UHPLC-MS/MS system.

2.1.2.8 Validation

Full validation was performed as per international guidelines [181, 182]. Although results are reported as the concentration of the groups (rather than individual analytes), individual analytes were validated for such parameters as selectivity, the stabilities and matrix effects, as they each have their own chemical properties. Accuracy and precision was based on the calibration model of total area of the analytes consisting in that group.

Selectivity and Crosstalk

Selectivity experiments were carried out using ten ante-mortem and ten post-mortem blood samples obtained by the authors' laboratory for routine toxicological requirements. In total, the twenty different blood samples were analyzed to demonstrate no interference with endogenous peaks and targeted ions, including potential crosstalk of ions within the MS. An additional two zero samples (blank sample + IS) were analyzed to check for the absence of

analyte ions in the respective peaks of the IS. Furthermore, pooled blank blood was spiked with either *trans*-IAA, RIAA, TIAA, HIAA or AA in duplicates at concentrations of the upper limit of calibration curve (ULOQ) 10 mg/L using individual stock solutions, to check for the absence of interference with IS ions. This also ensured there was no interference between IAA groups as the IAA/AA family of compounds are structurally similar and include similar fragmented product ions. Pooled blank blood was also spiked with a mix of ~350 common therapeutic drugs ranging from levels 1-10 mg/L to determine any interference with individuals taking medications concurrent to beer consumption.

Extraction efficiencies, matrix effects and process efficiencies

The extraction efficiencies, matrix effects and process efficiencies were estimated with a set of three different samples at two concentrations (low and high QC levels) with five samples each, according to the simplified approach described by Matuszewski et al. [183]. The IS was estimated concurrently at a concentration used in the described method. Sample set 1 represented the neat standard, sample set 2 represented blank matrix spiked after extraction, and sample set 3 consisted of blank matrix spiked before extraction. Extraction efficiencies were estimated by comparison of the peak area of the samples of set 2 to those of set 3. For the matrix effects, the peak area of the samples of set 2 was compared to those of set 1. For process efficiencies, the peak area of the samples of set 3 was compared to set 1. All values are reported in percentage. General acceptability levels of 50% extraction efficiency or more were applied. Values over 100% for matrix effects indicate ion enhancement, while values below 100% indicate ion suppression.

Linearity

The calibration model was based on the total area of the analytes consisting in that group, as ratio to the IS area. Blank blood aliquots were spiked at concentrations given in **Table 5** and extracted as described previously to obtain calibration standards. Replicates (n=6) at each of the eight concentration levels were analyzed. As the expected concentrations of IAA in blood are unknown, the calibration model was tiered into two calibration levels based on the QC to be analyzed. Quality controls low and medium were analyzed using calibration standards 1-6 whilst the high QC was analyzed using the full (1-8) calibration curve. All groups were visually checked for a linear or quadratic fit and weighting (none, 1/x or $1/x^2$). Daily calibration curves using the same concentrations (single measurements per level) were prepared with each batch of validation and authentic samples.

Lower Limit of Quantification

The lower limit of quantification (LLOQ) was defined as the lowest point of the calibration curve of the method (**Table 5**) and fulfilled the requirement of LLOQ, signal-to-noise ratio ≥10:1 for quantifier and qualifier of the lowest abundant peak in the respective group. Furthermore, it was tested whether the quantifier/qualifier ratio of the MRM signals was within the acceptable limits and if there were at least 12 data points available at this concentration for each analyte of the group. Limit of detection (LOD) values were not systematically evaluated due to the multiple analytes present in each group, a signal-to-noise ratio ≥3:1 of at least one analyte is sufficient to confirm detection of the respected group [181, 182].

Processed Sample Stability

The stability of the processed samples during batch analysis under the conditions of the described method were estimated. Quality control samples at low and QC high concentrations (n=9 of each) were extracted as described previously and resulting extracts pooled. Aliquots of these pooled extracts at each concentration level were transferred to autosampler vials and injected into the UHPLC-MS/MS system and analyzed under the described method. The time intervals between the analyses of the QC samples were extended to 3.1 h by the injection of eleven blank samples repeated over a 24.8 h period. Stability of the extracted analytes was tested by regression analysis plotting absolute peak areas of each analyte at each concentration versus injection time. The instability of the processed samples was indicated by a negative slope, significantly different from zero ($P \leq 0.05$) [182, 184].

Freeze/thaw and Bench Top-Stability

Combined freeze/thaw and bench-top stability were evaluated by analysis of low and high QC samples (n=6 of each) before (control samples) and after four and eight freeze/thaw cycles (stability samples) for evaluation of freeze/thaw stability. Samples completed in total eight freeze/thaw cycles by undergoing 22 h freezing period (-60 °C) and a thawing period of 2 h at room temperature to incorporate bench-top stability. The experiments were carried out together with the accuracy and precision experiments and the concentrations of the control and stability samples were calculated via daily calibration curves. For stability, there are two criteria which have to be fulfilled: the ratio of means (stability/control) has to be within 90-110%, and the 90% confidence interval has to be within 80-120% from the control sample [181].

Accuracy and Precision

Low, medium and high quality control samples were prepared at the previously described concentrations and in duplicate each QC concentration was analyzed over a period of eight consecutive days using the described method using daily calibration curves. Accuracy was calculated for each analyte and bias determined by calculating the percent deviation of the mean of all calculated concentration values at a specific level from the respective nominal concentration. Repeatability (within-day precision) and time-different intermediate precision were calculated as relative standard deviation (RSD) [185] using one-way analysis of variance (ANOVA) with the grouping-variable "day". For low and medium concentrations, the acceptance limit was set to 20% CV and RSD, respectively; and 15% CV and RSD, respectively, for high concentrations.

2.1.2.9 Application to Authentic Samples

A normal healthy male volunteer was administered ~570 mL of West Coast IPA from Green Flash Brewery (San Diego, USA) described as 95 IBU and therefore assumed that ~55 mg of total IAA was consumed. The volunteer consumed this quantity of beer at 7.3% alcohol by volume (ABV), in order to obtain ~0.05 blood alcohol concentration (BAC), the legal limit in Australia and many other countries. Blood was sampled prior (zero), 0.5, 2, and 6 h post-consumption where the zero sample acted as the control and blank blood of the participant.

2.1.2.10 Ethics

Approval for the human consumption of beer and subsequent specimen retrieval and analysis was obtained from the Human Research Ethics Committee at the Victorian institute of Forensic Medicine.

2.1.3 Results and Discussion

2.1.3.1 Separation and Detection

Sample Preparation and Methods of Detection

Historically the bitter content of beer was estimated by a broad photometric analysis to provide an IBU rating [186] and has shown to suffice for the brewing industry to attribute a general bitterness profile for beers. Currently, the technique predominately used in routine beer and hop product analysis ensures consistent testing using a standardised HPLC-UV method by the European Brewery Convention (EBC method 7.8) [187]. Other analytical techniques utilizing capillary electrophoresis [188] and HPLC-UV [167, 177] showed improvement, however accurate and specific determination of IAA and reduced IAA content was not possible until the emergence of LC-MS(MS) [153, 177, 189]. These LC-MS(MS) methods are highly selective and sufficiently sensitive for the analysis of beer that contain a relatively high concentration of IAA. Our method describes an extraction with sufficient recovery to allow for the analysis of IAA compounds in human blood after consumption of beer. A typical sample chromatogram acquired in ESI negative mode shows the separation of the analytes **(Fig. 5)**. Liquid-liquid extraction was found to be inappropriate when in combination with the highly acidic buffers required to improve extraction of the acidic IAA compounds. The combination would lyse the red blood cells and cause intra-cellular matrix to

fall into the extracted sample and interfere with analysis. Protein precipitation was demonstrated as a sufficient clean-up step that did not degrade the biological matrix or result in a loss of recovery of the analytes. The extraction was found to be further optimized with the use of cold (-20 °C) ACN and the supernatant allowed to rest for 10 min after centrifugation.

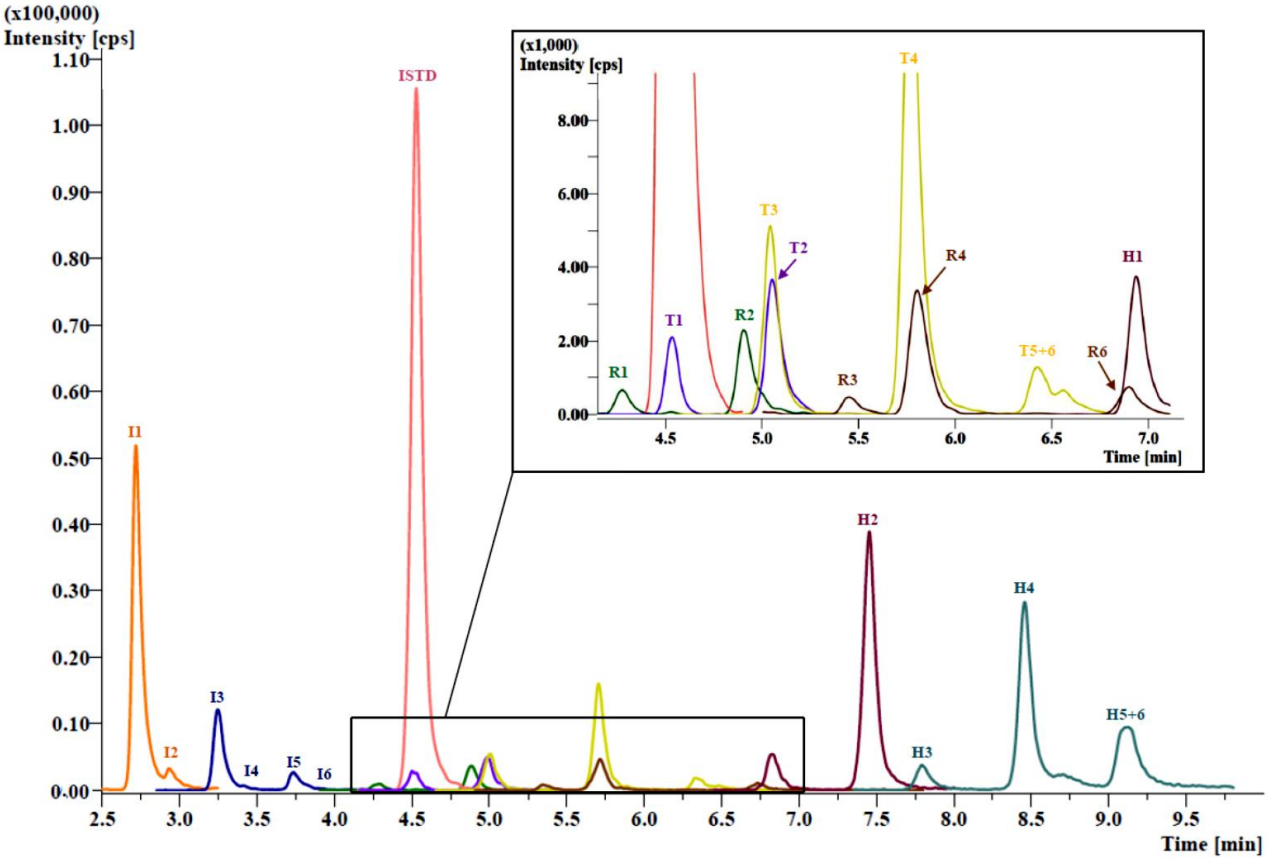


Fig. 5

Chromatograph of individual analytes at spiked blood concentrations of 1 mg/L for the *trans*-IAA, *cis*-RIAA, TIAA and *cis*-HIAA groups (0.1 mg/L for IS) using the validated LC-MS/MS method presented. Residual *cis*-IAA (mainly analyte I2, but also I4 and I6 in higher concentrations) remain in the *trans*-IAA reference standard and as carry-over during production.

Only in recent years have methods (using typically C_{18} columns) shown near complete separation and selectivity of individual IAA [153, 177] and reduced-IAA analytes [173], requiring a run-time of 20-50 min. After extraction from blood, our method separated the analytes using a gradient elution on a core-shell C_{18} column within 10 min**.** Preliminary experiments showed increased chromatographic selectivity and sensitivity using this column with considerably lower flow rates under high pressure (data not shown). The decrease of flow rate over the run improved the separation and peak shape of late eluting compounds. This chromatographic system was further optimized by applying a lower pH to eluent A (preferably at least 1 pH unit below the ~3.5 pK_a of IAA) in order to obtain improved ionization of acidic compounds. However, phosphate buffers are not compatible with MS detection and ammonium acetate buffer systems allowing for relatively low pH ranges were tested with poor chromatography results. Although the lowest capacity of the chosen ammonium formate buffer system is pH 2.8, this pH demonstrated the best separation of nearly all analytes. The class of IAA compounds contains two analogs (n- and ad-IAA) with the same molecular weight and there are structural isomers within these analogs (*cis* and *trans*), see **Fig. 3** and **Fig. 4**. It is therefore expected that separation difficulties with compounds of related chemistries and selectivity issues may be encountered with isobaric compounds with similar fragmentation. To avoid misidentifications, chromatographic separation of these isobaric compounds needed to be achieved. The determination of retention times within a batch of analysis compared to the IS in positive samples was necessary to avoid misidentifications of isobaric compounds. Some isobaric structural isomers (T5 and T6, as well as H5 and H6) were not baseline separated. However, as they are present in their respective standards and in the reduced hop products used, the total area of the combined peaks was sufficient to achieve reasonably accurate quantification under the described conditions.

Stability

Information from the IAA manufacture Labor Veritas and EBC indicate that IAA standards are unstable after a day in methanol but the use of phosphoric acid in methanol for stock and working solutions was sufficient to stabilize most of the compounds. However degradation of *trans*-IAA in the DCHA-Iso, ICS-I1 standard has still been demonstrated [177]. Due to the known deterioration with MS metallic parts following the continual use of the non-volatile phosphoric acid, formic acid was substituted. A 0.05% concentration of formic acid in methanol was found to stabilize the IAA sufficient for stock and working solutions to be stored at -20 °C over a six and one month period, respectively. As reduced on-column stability of the analytes was observed with increases in temperatures (data not shown), the column oven was maintained at a minimal temperature above ambient (30 °C).

Grouped Standards

Due to the unavailability of individual analyte standards and as each analyte has variable abundance within the respective group, the LLOQ of the group was restricted to the analyte with the lowest abundance with a signal-to-noise ratio of 10:1. However, detection of individual analytes with a signal-to-noise ratio of 3:1 [181], was sufficient to report beer consumption as detected. This also proves problematic for the determination of MRM transitions which resulted in the precursor and product ions meticulously selected manually by repeated injections of standards at different conditions described in **Table 5**. This review process ensured that all fragment ions were explainable as possible fragments of the respective chemical structure. To improve overall sensitivity of IAA compounds, a thorough source optimization was undertaken improving all groups of analytes with the IAA group recording the greatest gains. This reflects the relatively lower LLOQ for this group

specifically and results in better sensitivity for the majority of beer consumption. The dwell times were optimized depending on the signal response of each individual analyte by repeated injection; all analytes obtained >30 points across the peaks. Although there are more theoretical reduced IAA isomers present in brewing products and reference standards (i.e. the *trans* isomers of the RIAA and HIAA groups), the extremely low relative abundance of these analytes did not provide sufficient detection limits. These analytes were also unable to be detected at high (10 mg/L) concentrations and would therefore not interfere with quantification even with large beer consumption. Although the manufacturer of the IAA standards claim that residual pre-, post- and adpost-IAA may be present from the production process [187], the difference in precursor ions to that of the scheduled MRMs allows for accurate selectively of targeted IAA using MS.

Calibration Model

As a deuterated derivative of IAA is currently unavailable, the chemical and structural similarities of various compounds were examined. Nimodipine-d_7 was selected as a suitable IS for the calibration model. Other techniques have used an *ECHO* technique consisting of a shortly followed second injection containing the IAA group as the IS [153]. However, a closely followed injection of IS does not compensate for injection volume variability and is not wholly appropriate when gradient elution chromatography is undertaken. The relatively low abundance of R5 only produced a signal-to-noise ratio of 10:1 at total RIAA concentrations of 2 mg/L. Therefore the R5 analyte does not contain a peak in **Fig. 5** and validation data using QCs at lower concentrations were also unable to be performed. For this reason it was not used for calculation of the concentration curve or for any samples. As mentioned earlier, the calibration models involved two curves for analysis. The same

calibrations standards were used for quantification, the two curves only differed on whether point 7 and 8 were included when quantifying high quality controls. All IAA groups were spiked into all standards, irrespective of whether the particular group would be included in the calibration level (i.e. groups RIAA, TIAA and HIAA were not examined at calibration level 1 due to their LLOQ, see **Table 5**; however, the groups were still present in the extracted samples). Ante-mortem blood was chosen as the matrix for the calibration standards, matrix effects and quality controls rather than post-mortem blood. Excess blank postmortem blood from deceased persons is difficult to obtain ethically for assay calibration purposes and matrix effects studies, whereas ante-mortem blood was readily available through blood donor banks.

2.1.3.2 Validation

The described procedure was validated according to internationally accepted recommendations [181, 182] For the detection of the IAA and reduced IAA, three MRM transitions were used for each analyte; their use and their respective peak area ratios enabled unambiguous identification of all IAA and reduced IAA compounds included in the assay. The selectivity studies showed no interference or crosstalk in 20 different clinical and post-mortem samples.

Table 6 shows mean values of extraction efficiencies and matrix effects of the corresponding variation over five different blood samples. Datasets in which the variation (minimum and maximum values in percentage) is greater than 20% difference of the mean value are marked in bold type. Overall, the method showed satisfactory extraction efficiencies for most analytes however variation occurred for many analytes at low concentrations. **Table 6** also shows the mean values of matrix effects and the corresponding variation over five different

Table 6

Matrix effects and recoveries in % [range] of all targets and IS, n=5.

Analyte		Matrix effects		Extraction efficiency	
		QC low	QC high	QC low	QC high
I1	*trans*-isocohumulone	139 [133-150]	77 [70-85]	75 [63-90]	68 [60-77]
I3	*trans*-isohumulone	195 [179-207]	84 [79-88]	97 [87-110]	69 [59-78]
I5	*trans*-isoadhumulone	287 [266-310]	107 [101-114]	**78 [47-101**]	76 [65-89]
R1	*cis*-rho-isocohumulone 1	272 [257-297]	143 [133-155]	**92 [52-109]**	72 [61-85]
R2	*cis*-rho-isocohumulone 2	313 [254-345]	123 [118-130]	**66 [30-97]**	71 [61-82]
R3	*cis*-rho-isohumulone 1	214 [200-226]	98 [95-102]	71 [65-80]	65 [58-73]
R4	*cis*-rho-isohumulone 2	537 [525-550]	135 [126-143]	**86 [57-102]**	67 [59-78]
R6	*cis*-rho-isoadhumulone 2	[b]	187 [171-217]	[b]	68 [53-82]
T1	*trans*-tetrahydro-isocohumulone	**174 [95-232]**	128 [122-134]	**139 [98-165]**	86 [74-96]
T2	*cis*-tetrahydro-isocohumulone	265 [238-284]	82 [78-89]	100 [83-117]	79 [71-89]
T3	*trans*-tetrahydro-isohumulone	[b]	90 [85-95]	^	81 [71-91]
T4	*cis*-tetrahydro-isohumulone	209 [199-223]	87 [80-94]	**121 [75-147]**	**73 [54-84]**
T5+6	*tran*+*cis*-tetrahydro-isoadhumulone	[b]	86 [72-93]	[b]	83 [72-93]
H1	*cis*-hexahydro-isocohumulone 1	**208 [147-240]**	87 [77-95]	**117 [98-141]**	71 [63-78]
H2	*cis*-hexahydro-isocohumulone 2	**238 [148-341]**	102 [92-111]	83 [51-97]	59 [50 -69]
H3	cis-hexahydro-isohumulone 1	[b]	95 [86-106]	[b]	63 [53-74]
H4	*cis*-hexahydro-isohumulone 2	**257 [146-277]**	105 [96-115]	**73 [63-94]**	60 [51-71]
H5+6	*cis*-hexahydro-isoadhumulone 1+2	**209 [158-315]**	104 [96-115]	116 [97-135]	66 [56-79]
IS	nimodipine-d_7 [a]	83 [79-90]		68 [58-76]	

Datasets with variations (minimum and maximum value in %) greater than 20% difference of the mean value (not acceptable) are marked in bold type.

[a] The IS nimodipine-d_7 was analyzed at a concentration of 0.1 mg/L during the sample preparation of the described method.

[b] Abundance of analyte relatively low in quality control, subsequent area unattainable.

blood samples, with strong ion enhancement demonstrated. The described extraction procedure showed no significant matrix effects over five different blank blood samples in

high concentrations. However, nearly half of the analytes demonstrated variation in matrix effects at low concentrations. Matrix effect studies of IS nimodipine d_7 were also performed at the target concentration with no significant matrix effects observed over five different blank blood samples with satisfactory results. The described method demonstrated satisfactory process efficiency of 50% or more for all analytes.

Linear regression was applied to all studied groups and no weighting applied when using the full calibration curve for high QC analysis. As a result of heteroscedasticity that is commonly encountered for calibrations ranges spanning more than one order of magnitude [181]; a weighted model (1/x for IAA and RIAA, $1/x^2$ for TIAA and HIAA, **Table 5**) was used for the calibration curve using standards 1-6 for lower and medium QC analysis. The calibration fit showed a coefficient of determination of r^2 >0.95 for all groups and calibration ranges.

In the freeze/thaw and long-term stability experiments, the ratio of means (stability versus control samples) was within 90-110%, whereas the 90% CIs for stability samples were within 80-120% of the respective control means. Stability issues have demonstrated degradation of the *trans*-IAA standard when stored at 20 °C for 24 h *without* light exposure [177], potentially problematic for processed sample stability. However, in the processed sample extracts, the acidic eluent conditions that the samples were reconstituted with assisted in sufficiently stabilizing the analytes for over 24 h analytes at low and high concentrations. Stability was potentially assisted as the described method utilises a chilled autosampler for storage of processed samples at 4 °C.

Accuracy data was within the acceptance interval of ±15% (±20% for low and medium) of the nominal values for all IAA groups excluding TIAA at low QC concentrations. Within-day (repeatability) and intermediate precision required limits of ±15% RSD (±20% for low and medium) of which all repeatability data was within. However, the results summarised in

Table 7 demonstrates variability in intermediate precision for low and high concentrations in most groups when criteria above is applied. The calibration model incorporating total peak areas as a single group may induce greater variation in accuracy and precession results. The synthesis of a deuterated IAA may help alleviate some of the problems. Overall, the grouped calibration using the described IS provides an approximate quantification for *trans*-IAA, RIAA, TIAA and HIAA and is a good representative value for the amount of IAA, RIAA, TIAA and HIAA, respectively, in blood post-consumption of beer.

Table 7
Accuracy [%], intermediate (time-dependent intermediate precision) [% RSD] and repeatability (within-day precision) [% RSD] of the UHPLC-MS/MS assay for IAA type groups in blood.

Group	Quality Control	QC LOW (0.1 mg/L)	QC MED (0.8 mg/L)	QC HIGH (6 mg/L)
trans-IAA	Repeatability	7.0	5.8	7.7
	Precision	**31.1**	17.8	**17.2**
	Accuracy	19.0	5.4	9.2
RIAA	Repeatability	7.4	12.7	9.3
	Precision	**23.0**	12.7	**15.4**
	Accuracy	18.1	0.9	5.6
TIAA	Repeatability	5.0	8.0	8.0
	Precision	15.9	16.5	**17.4**
	Accuracy	**34.7**	12.0	11.4
HIAA	Repeatability	9.6	16.5	6.1
	Precision	**24.3**	**27.3**	**18.7**
	Accuracy	3.3	10.6	5.7

Datasets outside required limits are marked in bold type, n=2 (over 8 consecutive days).

2.1.3.3. Application to Authentic Samples

Detection of all IAA analytes were present in the 0.5 and 2.0 h duplicate bloods at *trans*-IAA concentrations of ~0.2 (**Fig. 6**) and ~0.02 mg/L, respectively, of the volunteer. Blood taken 6 h post-consumption detected the I1 analyte only and therefore accurate quantification was not possible. This is most probably due to the increased sensitivity of this presented method for that particular analyte, compared to analogs I3 and I5 at the similar concentrations. However,

detection of solely I1 at 6 h demonstrates how detection of individual analytes is sufficient to show beer consumption. Similarly, detection of the I2, I4 and I6 analytes of the *cis*-IAA group that were not quantified due to unavailability of a reference standard was also able to confirm beer consumption. Furthermore, should *trans* isomers undergo a more rapid metabolism and/or have since degraded at the time of sampling, the *cis* isomers have a much longer half-life and may still be used to indicate beer consumption over a greater detection window. The detection of beer ingredient congeners in the participant of the pilot study at a relatively low amount of beer consumption than what might be typical, demonstrates high sensitivity and consequently, applicability of the method.

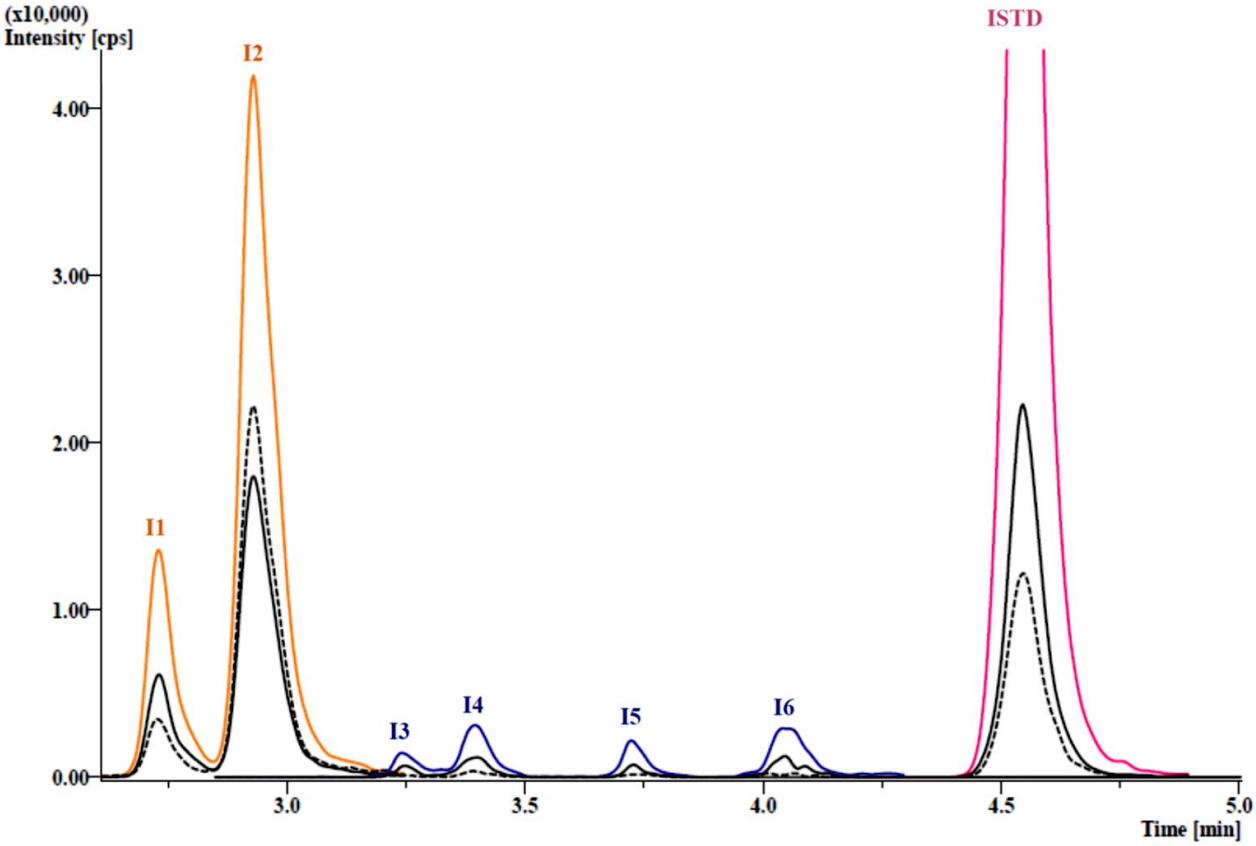


Fig. 6

Chromatograph of blood taken 30 min after consumption of ~570 mL beer containing ~55 g of IAA (*trans* and *cis*) from a volunteer. The concentration of the *trans*-IAA (analytes I1, I3 and I5) was ~0.2 mg/L. The *cis*-IAA group (analytes I2, I4 and I6) are unable to be quantified due to the unavailability of a reference standard; however, they do provide evidence for the consumption of beer. All IAA analytes demonstrate baseline separation as not to interfere with quantification results. Colored lines demonstrate quantification ions; black full and dashed lines indicate the two other MRMs.

2.1.4 Conclusion

The UHPLC-MS/MS assay presented is a suitable procedure for the separation, detection, and quantification of IAA, RIAA, TIAA and HIAA compounds as beer-specific ingredient congeners in blood samples to confirm beer consumption. Validation has proven to be selective, linear, accurate, and precise for the range of beer ingredient congeners at concentrations expected to be found in blood. The *trans*-IAA were quantified in a volunteer post-consumption of beer and demonstrates possible applicability for clinical and forensic toxicological casework.

Highlights

- The confirmation of beer as the source of alcohol in blood is currently unavailable
- Hop derived iso-α-acids are targeted in blood as ingredient congeners specific to beer
- We describe a novel UHPLC-MS/MS method validated for use in forensic casework
- Iso-α-acid bioavailability is demonstrated in the blood of a participant consuming a known amount of beer

CHAPTER 3.

STABILITIES

International guidelines for the forensic toxicology community describe the required parameters for validating developed methods, including the processed sample, freeze thaw and bench-top stabilities, as discussed in **Chapter 2**. However, the examination of any analyte degradation in stored specimens is also crucial in order to determine the accuracy of IAA concentrations when specimens are subjected to prolonged, and variable, storage conditions.

CHAPTER 3.1

IAA STABILITY IN STORED SPIKED BLOOD

Rodda, L.N., D. Gerostamoulos, and O.H. Drummer,
The Stability of Iso-α-acids and Reduced Iso-α-acids in Stored Blood Specimens.
Forensic Sci Int, 2014. 239: p. 44-49.
(Appendix 1.3)

Monash University

Declaration for Thesis Chapter 3.1.

Declaration by candidate

In the case of Chapter 3.1., the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Located articles, reviewed articles and wrote the paper	85%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Gerostamoulos, D.	*Advised candidate regarding direction and content, reviewed article manuscript*	
Drummer, O.H.	*Advised candidate regarding direction and content, reviewed article manuscript*	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 26.6.14
Main Supervisor's Signature	[redacted]	**Date** 27/6/14

Abstract

The long-term stability of the iso-α-acids, and three structurally similar but chemically-altered iso-α-acids (known as 'reduced iso-α-acids' and consisting of the rho-, tetrahydro- and hexahydro-iso-α-acid groups) were investigated in whole blood. Pools of blank blood spiked with the four beer-specific ingredient congener groups at two different concentration levels were stored at 20 °C, 4 °C and -20 °C; and extracted in duplicate in weeks 1, 3, 5 and 8, using a previously published method. A loss of 15 % of the initial concentration was considered to indicate possible instability and losses greater than 30 % demonstrated significant losses. The individual analytes within the four iso-α-acid groups were also measured to determine which iso-α-acids were subject to greater degradation and were responsible for the overall group instability. All four iso-α-acid groups showed significant losses after 8 weeks of storage under room temperature conditions in particularly the natural iso-α-acid group where major losses were observed (96% and 85% losses for low and high concentrations, respectively). Some degradation in all iso-α-acid groups were seen at 4 °C samples predominantly due to the 'n' analogs of the groups showing an increased instability in blood. The -20 °C storage conditions resulted in minimal changes in concentrations of all analytes. Higher than frozen storage temperatures can result in substantial changes on the stability of the iso-α-acid type groups in blood. The aim of this study was to highlight the stabilities of the IAA analytes in order to assist in the interpretation of IAA in stored blood specimens.

Keywords

Stability; Ingredient congener; Beer; Iso-α-acids; Blood; LC-MS/MS

3.1.1 Introduction

Methodology for novel alcohol congener analysis that allows detection of ingredient congeners specific to beer has recently been developed in blood [190]. Detection of these compounds in blood allows for the confirmation of beer ingestion which is potentially useful in cases where an after-drinking (hip-flask) defense is being used [28, 31], but also in other medico-legal casework where the source of alcohol is sought [159]. These beer-specific ingredient congeners consist of the hop-derived iso-α-acids (IAA) type compounds that are formed following the isomerization of α-acid (AA) contained within the hops that are added during the boiling process of brewing beer (**Fig. 7**). Furthermore, derivatives of the natural IAA group exist and are termed 'reduced IAA', consisting of the rho-, tetrahydro-, and hexhydro-IAA (RIAA, TIAA and HIAA, respectively). The three reduced IAA groups are used for their light protective and foam stabilizing properties (**Fig. 7**), can also be detected post-consumption of particular beers containing these types of compounds.

During the boiling phase of brewing beer, the three predominant AA analogs (co, n and ad) isomerize into diasteromers to produce six IAA stereoisomers [172], differing only in the absolute configuration of one of the chiral centers and saturated acyl side-chain (**Fig. 7**). The tensioactive, water soluble and low pK_a (~3.5) properties of the IAA compounds lead to the intense bitterness of beer and also provide foam stabilization [67, 172]. The IAA are subject to radical-assisted oxidation [191] and furthermore, in the presence of sunlight and oxygen, the undesirable "skunky" smelling 3-methyl-2-butene-thiol (3-MBT) is produced when beer is not protected by lightproof brown-bottled glass (or less-so green) [174, 192]. However in recent years, the development of a range of light-stable reduced IAA allows for clear glass bottles to be used. By reducing the susceptible carbonyl group in the side chains or double

bonds of IAA to less susceptible single bonds (i.e. reduced IAA), the production of 3-MBT by photolytic cleavage is prevented (**Fig. 7**).

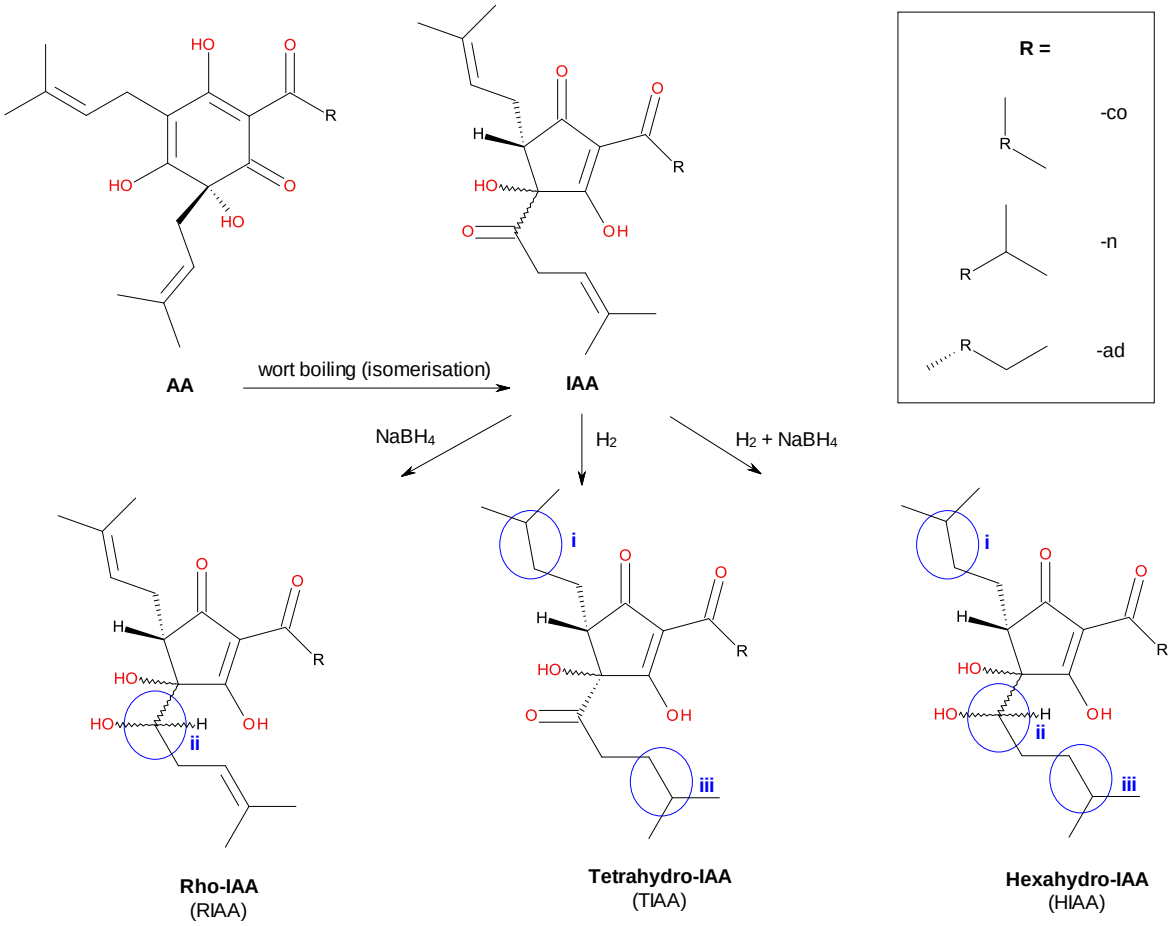


Fig 7.

The isomerization of the α-acid (AA) structure including the three major analogs (co, n and ad) into the iso-α-acid (IAA) diasteromers and subsequent synthesis of the three reduced IAA groups. Sites of increased hydrophobicity (i and iii) and photolytic cleavage prevention (ii and iii) properties are highlighted in the reduced IAA. The reduced carbonyl group produces rho-IAA. Reduction of both side-chains double carbon bonds within the IAA produces the tetrahydro-IAA derivative. Execution of processes yields the hexahydro-IAA derivative of IAA. Modified from Figs. 1 and 2 from Rodda et al. [190].

Assessment of analyte stability in bioanalytical methods is regularly achieved during method validation using experiments: freeze/thaw stability, bench-top stability, stability in the processed samples, and long-term stability in the sample matrix [181]. All but the long term-stability was performed for the method validation of an UHPLC-MS/MS method for the determination of these compounds in blood [190]. Therefore the stability of the analytes in blood during common storage conditions (e.g. ambient temperature, refrigeration and frozen) should be evaluated over a time period that includes at least the length of typical sample collection, sample handling and analytical preparation for authentic samples [184, 193].

Degradation experiments targeting IAA have only been carried out in aqueous solutions, beer wort or in finished beer [153, 166, 174, 191, 192, 194-199]. This is generally performed to demonstrate how to most efficiently isomerize the AA to IAA during brewing and to measure the loss of beer quality during storage, a known issue with the light sensitive natural IAA compounds. The varying stabilities of these compounds has been shown where certain analogs and isomers, in particular the *trans*-IAA, have been shown to degrade more so than others, into the non-bitter final degradation product, humulinic acid [200].

The aim of this study is to determine the pattern of stability of the four groups of IAA in stored blood samples at different temperatures at a number of time intervals over an eight-week period and to investigate if particular analogs and/or isomers are subject to degradation more so than others.

3.1.2 Materials and methods

3.1.2.1 Chemicals and Reagents

Reference standards for: DCHA-Iso, ICS-I3 (containing 62.3 % w/w of *trans*-IAA); DCHA-Rho, ICS-R2 (containing 65.3 % w/w of *cis*-RIAA); Tetra, ICS-T2 (containing 99.4 % w/w of TIAA), and; DCHA-Hexa, ICS-H1 (containing 65.7 % w/w of *cis*-HIAA); were obtained from Labor Veritas (Zurich, Switzerland). The monitored analogs and isomers of the four IAA groups were: ***trans*-IAA** (I1 = *trans*-isocohumulone; I2 = *cis*-isocohumulone; I3 = *trans*-isohumulone; I4 = *cis*-isohumulone; I5 = *trans*-isoadhumulone; I6=*cis*-isoadhumulone); ***cis*-RIAA** (R1 = *cis*-rho-isocohumulone 1; R2 = *cis*-rho-isocohumulone 2; R3 = *cis*-rho-isohumulone 1; R4 = *cis*-rho-isohumulone 2; R6 = *cis*-rho-isoadhumulone 2); **TIAA** (T1 = *trans*-tetrahydro-isocohumulone; T2 = *cis*-tetrahydro-isocohumulone; T3 = *trans*-tetrahydro-isohumulone; T4 = *cis*-tetrahydro-isohumulone; T5+6 = *trans*+*cis*-tetrahydro-isoadhumulone); and ***cis*-HIAA** (H1 =*cis*-hexahydro-isocohumulone 1; H2 = *cis*-hexahydro-isocohumulone 2; H3 = *cis*-hexahydro-isohumulone 1; H4 = *cis*-hexahydro-isohumulone 2; H5+6 = *cis*-hexahydro-isoadhumulone 1+2).

The isotope labeled internal standard (IS) nimodipine-d_7 was purchased from PM Separations (Brisbane, Australia). Acetonitrile (ACN), methanol and formic acid were purchased from Merck (Melbourne, Australia). Ammonium formate was purchased from Sigma Aldrich (Sydney, Australia). All chemicals were of analytical grade or better and water was purified using a Milli-Q Ultrapure Water System from Waters (Sydney, Australia).

3.1.2.2 Specimens

Whole blood for stability experiments was obtained from the local Red Cross Blood Bank (Melbourne, Australia) in lithium heparin-coated plastic bags (500 mL). The blood was aliquoted into 10 mL polypropylene tubes containing 200 mg sodium fluoride and 30 mg potassium oxalate. Blood was analysed using a previously published method [190] and was found to be free of IAA. Preserved blood samples were stored at -20 °C prior to analysis, preservative was sufficient to inhibit bacterial alteration of ethanol and fermentation by-product congeners [137, 138].

3.1.2.3 Apparatus and Analytical Conditions

The UHPLC-MS/MS system comprised of a Shimadzu MS 8030 quadrupole mass spectrometer (Melbourne, Australia) operated in the electrospray ionisation (ESI) in negative mode and a Shimadzu Nexera UHPLC system (Melbourne, Australia) that consisted of a degasser, two eluent pumps, a column oven (30 °C) with a Kinetex C_{18} (3.0 mm × 150 mm, 2.6 μm) column, and a chilled autosampler. For MS data evaluation, Shimadzu Postrun and Shimadzu Browser Analysis (Melbourne, Australia) softwares were used. GraphPad Prism 5.04 from GraphPad Software (San Diego, USA) was used for statistical analysis. The mobile phases consisted of 50 mmol/L aqueous:ACN (90:10) ammonium formate pH 2.8 (eluent A) and ACN containing 0.1 % formic acid (eluent B). The apparatus and analytical conditions were as described in the previously validated method [190].

3.1.2.4 Preparation of Stock Solutions and Extraction

Preparation of stock solutions, calibration standards, quality controls, stability samples and extraction procedures were performed as published previously [190]. The extraction consisted of a protein precipitation of the whole blood using ACN with the resulting supernatant dried under nitrogen and the residue reconstituted with 50 µL of a mixture of eluent A and eluent B (60:40, v/v).

3.1.2.5 Long-term Group Stability

'Low' (0.1 mg/L) and 'High' (6 mg/L) concentrations of the IAA groups were prepared in 50 mL volumetric flasks using pooled blank blood and inverted on a rotary wheel for 1 h at room temperature to ensure homogeneity, 500 µL aliquots was transferred into 2 mL Sarsteadt tubes (Sarsteadt, Adelaide, Australia). The batch of low and high aliquots were then divided into 4 groups of different storage temperatures: 20 °C, 4 °C and -20 °C (stability samples); and -60 °C (reference samples). All specimens were housed in light-proof boxes during storage. Furthermore, previous bench-top stability experiments showed the IAA analytes to be stable for up to 2 h [190], therefore degradation of analytes during the process of developing stability samples does not affect experimental results. These samples of were evaluated in duplicates at weeks 1, 3, 5 and 8.

The concentrations of the IAA groups were calculated using the daily calibration curves included in each assay as per previously described methodology [190]. Importantly, a relative concentration calculated as the ratio of the means (stability samples *vs.* reference samples), in percent was used to compare stabilities. Stability of the IAA groups was measured as 'acceptable' for <15 % variation, 'some losses' determined by ≥15 to <30 % degradation and 'significant losses' with degradation of ≥30 %.

3.1.2.6 Individual Analyte Stability

The specific stabilities of the individual analytes of the four IAA groups were determined in order to demonstrate if certain analytes were particularly responsible for the loss in total group stability. The analyte peak areas of the high concentration stability samples were examined in the final week of the long-term stability experiment (week 8) for the 20 °C, 4 °C and -20 °C storage temperatures. The relative response ratio for each analyte was calculated as the ratio of the peak area response (analyte/internal standard) of the stability samples duplicates *vs.* the mean area of the reference samples, in percent.

Multiple unpaired *t*-tests using the Holm-Sidak method assessed the storage temperatures of 4 °C and 20 °C against -20 °C to demonstrate if a significant degradation occurred ($p < 0.05$) with each analyte.

3.1.2.7 Ethics

This research was approved by the Victorian institute of Forensic Medicine's Human Research Ethics Committee (E04/12).

3.1.3 Results

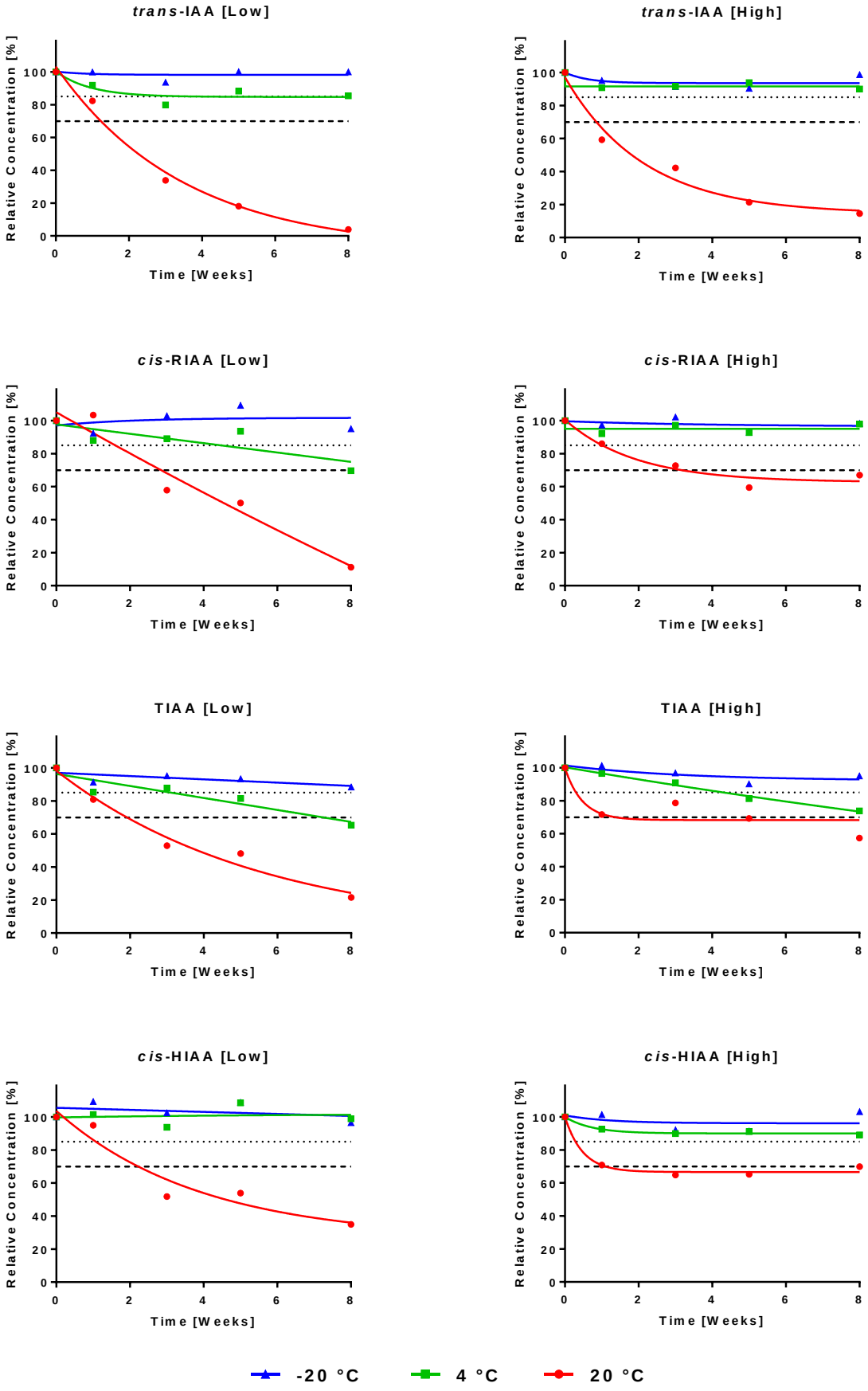
3.1.3.1 Long-term Group Stability

The relative degradation patterns of the four IAA groups in the long-term stability experiment are shown in **Fig. 8**. The mean of the duplicate results were plotted and an exponential one-phase decay regression model applied that best profiles natural chemical and biological processes. Dotted and dashed lines outline 15 % and 30 % variations, respectively, and

demonstrate that acceptable losses of < 15 % were seen for all groups at -20 °C temperatures in both low and high concentrations. Although generally showing a slightly less stable profile compared to -20 °C, most IAA groups at 4 °C also showed acceptable losses at conclusion of the study. However, some losses > 15 % were seen with *cis*-RIAA (29 %) at low concentrations and high concentrations of TIAA (26 %) at 4 °C storage conditions. Furthermore, the low levels of TIAA resulted in significant losses of 35 % at the same temperature, after 8 weeks of storage.

Fig. 8 (overleaf)

The relative concentration (%), as a ratio compared to the reference sample (-60 °C), of 'Low' (0.1 mg/L) and 'High' (6 mg/L) levels of the four IAA groups in blood in long-term stability samples stored for 8 weeks at 20 °C, 4 °C and -20 °C. Symbols represent the mean of the duplicates with an exponential one phase decay regression model applied. Dotted and dashed lines outline 15 % and 30 % variations, respectively.


trans-IAA [Low]
trans-IAA [High]
cis-RIAA [Low]
cis-RIAA [High]
TIAA [Low]
TIAA [High]
cis-HIAA [Low]
cis-HIAA [High]
Relative Concentration [%]
Time [Weeks]
-20 °C
4 °C
20 °C

At 20 °C, the four IAA groups showed further losses below the 30 % range at both low and high concentrations. The reduced IAA groups of *cis*-RIAA, TIAA and *cis*-HIAA, showed similar degradation profiles; after initial losses at ~30 % variation, the concentrations then plateaued surprisingly at ~1-3 weeks of storage. However, the *trans*-IAA group continued to degrade throughout the eight weeks with only 4% and 15% of the initial concentration remaining at the conclusion of the stability experiment at both concentrations.

3.1.3.2 Individual Analyte Stability

The individual analyte stabilities of the four IAA groups that are comprised of analogs and isomers are presented in **Fig. 9**. The *cis* isomers of the IAA group (I2, I4 and I6) are residual by-products of the *trans*-IAA production and their concentrations are unable to be calculated due to the lack of a '*cis'* reference standard. However; the sufficient abundance of I2 and I4 allowed for the peak areas to be monitored in order to demonstrate degradation. Notably, these *cis*-IAA do not contribute to the *trans*-IAA group instability as demonstrated in **Fig. 8**.

At -20 °C storage little degradation was observed for all analytes (**Fig. 9**). At 4 °C a significant loss was seen for I3 and a visible loss also for the equivalent *cis* isomer, I4. Remarkably, all the 'n' analogs (i.e. I3, I4, R3, R4, T3, T4, H3 and H4; see **Fig. 9** for analog definition) degraded to a larger extent than the corresponding 'co' and 'ad' analogs. There was a major and significant loss at 20 °C for these analogs, with some losses also seen at 4 °C in all but the RIAA 'n' analogs (R3 and R4). It was also evident that all the *cis*- and *trans*-IAA analogs resulted in major significant losses when subject to 20 °C storage conditions, as verified in both low and high concentrations in **Fig. 8**.

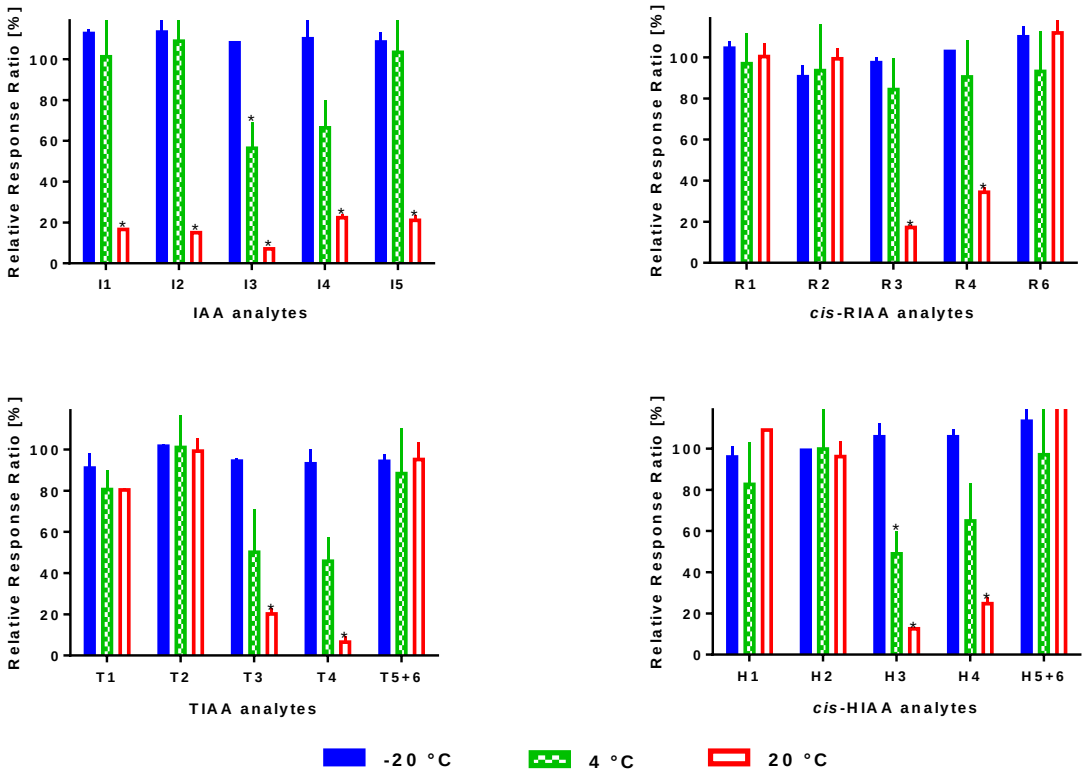


Fig. 9

The peak area response (analyte/internal standard) as a ratio compared to the reference sample [%] in the four IAA groups stability samples in 'High' concentrations in duplicate after 8 weeks of storage at 20 °C, 4 °C and -20 °C. I6 (*cis*-isoadhumulone) and R5 (*cis*-rho-isoadhumulone 1) are not included due to extremely low abundance in the commercial reference standards. I2 (*cis*-isocohumulone) and I4 (*cis*-isohumulone) are not included in the stability of the total *trans*-IAA group presented in **Fig. 8**. Error bars demonstrate the standard deviations. Multiple unpaired t-tests using the Holm-Sidak method assessed the storage temperatures of 4 °C and 20 °C against -20 °C to demonstrate significant degradation (*), $p < 0.05$, with each analyte.

3.1.4 Discussion

The recent development and validation of methodology to detect these compounds in blood now enables their detection in biological specimens as well as in beer. Freeze/thaw and bench-top stabilities of the compounds were accessed after 8 freeze/thaw cycles of 22 h freezing periods (-60 °C), followed by thawing periods of 2 h at ambient temperatures [190].

However, their long-term stabilities in whole blood required investigation. The storage temperatures were chosen as these are regular temperatures that toxicological specimens are exposed to.

The assessment and definition of 'stability' varies throughout the literature as demonstrated by Shah et al. who defined stability as the "The chemical stability of an analyte in a given matrix under specific conditions for given time intervals" [184]; whilst Hartmann et al. gave the definition "Absence of an influence of time on the concentration of the analyte in a sample" [201]. Although there are varied definitions and parameters to assess the long-term stability of a compound in matrices [184, 193, 201-203], they are generally similar in design and outcomes. These experimental setups commonly recommend analyzing 'reference samples' at time zero and a set of 'stability samples' after just one certain time-point of storage, in replicates of ~5 in both low and high therapeutic concentrations [201, 203]. Suggestion of stability issues is determined by statistical tests that assess if drug concentrations degrade significantly between reference and stability samples. However, as the IAA compounds have not been assessed previously in a biological matrix for stability, a degradation profile with fewer replicates and more time points is advantageous. In order to determine instabilities of the targeted groups, the autosampler stability approach first used by Wieling et al. in 1996 [204] and subsequently modified to demonstrate when the losses occurred [205], was used in these experiments.

The individual analyte stability was also investigated at high concentrations that provided a better indication of losses than at lower concentrations. The exacerbated losses in IAA groups shown at low concentrations may be more likely due to the lower limit of quantification of the experimental methodology, rather than true instability. The individual analyte stability

was also assessed using -20 °C samples as a control (as it showed almost no degradation for all analytes).

The possibility of analytes degrading into related compounds within the group seems unlikely as it has been shown that during prolonged beer storage, the isomers do not epimerize into another isomer [206]. Literature suggests these compounds degrade into other products (e.g. humulinic acid; 3-MBT, and; tri- and tetra-cyclic *trans*-IAA degradation products) [174, 194, 195, 199, 200, 206].

The *cis*-IAA isomers I2 and I4 were shown to degrade at a similar rate as the corresponding *trans* isomers. This differs in brewing chemistry literature that shows that the *trans*-IAA are more hydrophobic which consequently results in the lower half-life in beer of the *trans*-IAA (~9-12 months) compared to the *cis*-IAA (> 5 years) [175, 195, 206], leading to range of degradation products to be formed in beer [176, 206]. A possible explanation for this may be the acidic nature of beer (pH ~4), compared to that of blood (pH ~7.4), better stabilizes the *cis* isomer, where the near neutral blood does not support *cis* stability. Interestingly, data showed that the degradation in blood was analog based, rather than by isomer and furthermore that both *cis*- and *trans*-IAA analytes are subject to major losses at room temperature, irrelevant of isomeric or analog configuration.

The thermodynamic stability of IAA has also been investigated in order to determine kinetic models and efficient utilization of hop products [194] and demonstrated although boiling temperatures are required for the isomerization of AA to IAA, the prolonged exposure to such temperatures degrades IAA rapidly [198]. It has been shown that handling, storage and aging of beer affects *trans*-IAA to degrade by ~70 % after 8 months of storage at 28 °C in a glass bottle [176]. Huang et al. demonstrated that temperature has the most profound influence in IAA degradation where a recovery of ~85 % of IAA was seen at temperatures of

90 °C after 6 h, whilst all IAA were degraded at 130 °C for the same time period [194]. Not surprisingly, this correlates with results from this study where a decrease in temperature showed a consistent increase in the stabilities of the IAA groups.

Iso-α-acid degradation studies investigating the effect pH of the aqueous matrices demonstrated that increasing basic matrices was detrimental to IAA stability [194]. At 100 °C and over 6 h, an increase of pH from 4.5 to 5.5 resulted in losses of IAA from 36 % to ~45 %; however, an increase to pH 6.5 increase the rate of degradation giving a loss of ~70 % IAA. As the pH of blood is ~7.4, it can be expected that the near neutral nature of this biological matrix leads to a similar degradation rate of the IAA groups. Furthermore, blood extract samples containing IAA that were reconstituted in acidic eluent conditions stabilized the IAA analytes, at least for 24 h [190].

3.1.5 Conclusion

In summary, even though IAA and reduced IAA compounds appear to be stable over lengthy storage times in beer, instability was demonstrated in blood over 8 weeks of storage at warmer than frozen temperatures. Although the overall stability of the IAA and reduced IAA groups were acceptable during refrigerated storage (4 ^{o}C), increased degradation of the ‘n’ analogs was observed. Significant losses were seen at ambient storage temperatures for all groups, particularly with the natural IAA group.

CHAPTER 3.2

IAA STABILITY IN STORED AUTHENTIC BLOOD

Briefly, additional stability studies were performed on authentic blood from five volunteers in a drinking study described later in **Chapter 4.1**.

IAA analysis was immediately performed on collection of the blood from volunteers participating in the high-hopped study where beer containing only natural IAA was consumed. These specimens were then immediately stored at -20 °C for approximately 12 months, where they were then re-analysed.

Table 8 shows that following repeat analysis, two of the results are the same with insignificant changes in the other three. Furthermore, no reduced IAA analytes or MRM transitions were present. This confirms that natural IAA due not degrade into their reduced derivatives, demonstrating specificity for this beer consumption technique.

This validates the sufficient stability of the most significant iso-α-acid, *trans*-IAA, in authentic blood specimens when frozen for a period of at least a year.

Table 8

The stability of *trans*-IAA (mg/L) concentrations in blood analysed at collection, and after 12 months storage at -20 °C, in specimens of authentic beer consumption.

Volunteer[a]	**A**	**B**	**C**	**D**	**E**
At collection	0.10	0.12	0.11	0.10	0.11
12 months at -20 °C[b]	0.10	0.12	0.10	0.09	0.10

[a] five volunteers in the high-hopped drinking study (**Chapter 4.1**)

[b] blood specimen was promplty analysed following collection and then immediately stored at -20 °C for 12 months

CHAPTER 4.

PHARMACOKINETICS

The detection of blood IAA indeed provides the confirmation of beer consumption. However to better understand how the IAA are absorbed, distributed, metabolised, and eliminated by the body, pharmacokinetic studies must be performed, particularly for newly identified or monitored xenobiotics. This also provides additional knowledge that supports medico-legal investigations for judicial processes. Moreover, such experiments may allow for the discriminate confirmation of either natural IAA or reduced IAA from brown or transparent bottled beers, respectively.

CHAPTER 4.1

PHARMACOKINETICS OF NATURAL IAA

Rodda, L.N., D. Gerostamoulos, and O.H. Drummer,
Pharmacokinetics of Iso-α-acids in Volunteers following the Consumption of Beer.
J Anal Tox, 2014. ePub
(Appendix 1.4)

Monash University

Declaration for Thesis Chapter 4.1.

Declaration by candidate

In the case of Chapter 4.1., the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Located articles, reviewed articles and wrote the paper	85%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Gerostamoulos, D.	*Advised candidate regarding direction and content, reviewed article manuscript*	
Drummer, O.H.	*Advised candidate regarding direction and content, reviewed article manuscript*	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 26.6.14
Main Supervisor's Signature	[redacted]	**Date** 27/6/14

Abstract

Hop-derived iso-α-acids (IAA) ingredient congeners are specific to beer. Concentrations of IAA were determined in blood of five volunteers over 6 hours following consumption of small volumes of beer containing relatively high (Pale Ale beer) or low (Wheat beer) concentrations of IAA. IAA were quickly absorbed with peak *trans*-IAA concentrations at 0.5 h followed by a drop of generally tenfold at 2 h and low or not detectable *trans*-IAA levels at 6 hours. However, the qualitative monitoring showed that the *cis*-IAA were detected at all time-points. Preliminary pharmacokinetics of these compounds in humans shows relatively small inter-individual differences and an estimated short half-life of ~30 minutes. Comparison of 0.5 and 2 hour blood specimens demonstrated that the *trans* isomers were eliminated faster than the *cis* counterparts. Preliminary urine analysis showed only unmodified 'co' analytes detectable throughout the 6 hours. In authentic forensic casework where typically large amounts of conventionally hopped beer are consumed this approach may provide a novel method to target ingredient congeners consistent with beer ingestion.

Keywords

Alcohol congener analysis; beer; ingredient congener; iso-α-acids; pharmacokinetics; blood; urine

4.1.1 Introduction

Iso-α-acids (IAA) are derived from the hop plant (*Humulus lupulus* L.) and are present in beer following the isomerisation of α-acids (AA) during the boiling phase of the brewing process. Six stereoisomers of IAA are produced from the three predominant AA analogs (co, n and ad) that each isomerize into diasterisomers (**Fig. 3 of Chapter 2.1**). The compounds are structurally very similar, only differing in the nature of the saturated acyl side-chain and the absolute configuration of one of the chiral centers [172].

Beers commonly contain ~6-30 international bitterness units (IBU, one unit being approximately equal to one mg/L of total IAA), however certain styles of 'highly-hopped' craft beers often contain much higher amounts [165, 173]. The *cis*:*trans* ratios in beer are usually ~68:32 [174]. However, the *trans*-IAA are present in the foam in greater proportion than the *cis*-IAA due to their lower water solubility which also explains the lower half-life of the *trans*-IAA (<1 year) compared to the *cis*-IAA (~5 years) during beer storage [175]. The long-term stabilities of the IAA in stored blood recently concluded that the IAA 'n' analogs were most susceptible to degradation, however refrigeration and freezing provided acceptable stability [207].

It has been proposed that due to the IAA content, beer may also have medicinal properties, including weight loss, anti-diabetic, anti-carcinogenic and anti-inflammatory [156, 208-214]; and, animal models show that diets enriched with IAA and administered orally to mice [208-210] and rats [211], led to physiological changes; suggesting these compounds may be bioavailable. Furthermore, IAA were dosed to New Zealand white rabbits where it was determined that the total bioavailability of IAA was 13.0%, of which, diastereomerization showed no influence however the more lipophilic 'n' analogs showed greater bioavailability compared to other analogs [212]. More recently using human *in vitro* models, IAA were

shown to be highly permeable across caco-2 intestinal cells [215], whilst also shown to promote gastric acid secretion in human gastric cancer cells [216]. The AA compounds, of which IAA originate from and are structural similar, were shown to permeate epithelial membranes and demonstrate absorption also in human *in vitro* models [215].

Recently, these IAA have been detected in blood of one volunteer following the consumption of a high-hopped beer using a UHPLC-MS/MS procedure [190]. This confirmation of beer consumption method specifically targets and quantifies the *trans* stereoisomers of the IAA group, whereas the *cis* counterparts are qualitatively monitored (**Fig. 3 of Chapter 2.1**). Detection of all IAA analytes were present at 0.5 and 2 h post-dose with *trans*-IAA concentrations of ~0.2 and ~0.02 mg/L, respectively This pilot study demonstrated that these compounds were bioavailable and suggested the possible applicability for clinical and forensic toxicological casework.

This may represent another method of performing alcohol congener analysis (ACA) to confirm the consumption of beer in routine forensic casework and in after-drinking (or hip-flask) defense cases where the feasibility of claimed alcohol consumption prior and/or subsequent to a motor vehicle incident is in question [11, 28, 31, 159].

The aim of this study was to detect and determine the pharmacokinetics of IAA in the blood and urine of human volunteers given controlled doses of two types of beer containing different amounts of IAA.

4.1.2 Experimental

4.1.2.1 Drinking Study Design

Five healthy male volunteers were administered specific volumes of a relatively high-hopped beer (Little Creatures Pale Ale from Perth, Australia) and a relatively low-hopped beer (Erdinger Weissbier from Erding, Germany), see **Table 9**. Studies were performed separately over a day each with a wash-out period of at least one week between studies and no alcohol was permitted for 48 h prior to each of the study days.

To estimate blood alcohol concentration (BAC), a revised version of the Widmark formulae by Posey et al. was employed that mathematically combines recent variations by other authors into a single formulae to provide an accurate 'Widmark factor' for each individual and subsequently the volume of beer required to produce a peak BAC of 0.05 g/100mL, the legal limit in Australia [9, 110, 159]. The anthropometric measurements (i.e. height, weight and age) of each participant are described in **Table 10**.

Blood was obtained by a registered phlebotomist and urine by the volunteers themselves at prior (zero), 0.5, 2 and 6 h post-consumption time-points. The zero hour specimens acted as the control and blank blood and urine of the respective participant. In order to minimalize absorption variables, all participants fasted from food on the morning of the study and the administered beer was required to be consumed within 10 min. No drink and food was permitted until two hours post consumption.

Table 9

Details of the beers consumed during the two drinking studies of relatively high-hopped and low-hopped beers, the class and style of that beer, location of the brewery, the batch number of the beers, alcohol content, the described international bitterness units and approximate total iso-α-acid content.

Beer consumed	Class	Style	Brewery	Location	Batch (Expiry)	Ethanol (ABV%)	IBU / IAA (mg/L)
High-hopped	Craft	Pale Ale	Little Creatures	Perth, Australia	10174/1 (Exp 3/2014)	5.2	~40
Low-hopped	Popular	Weissbier	Erdinger	Erding, Germany	L221311 (Exp 11/2013)	5.3	~10

ABV% = alcohol by volume (volume / volume %)

IBU = approximately one mg/L of total IAA

Table 10

Anthropometric measurements (height, weight and age) for the volunteers and volumes of high-hopped (Little Creatures Pale Ale) and low-hopped (Erdinger Weissbier) beers consumed.

Volunteer	Gender	Height (cm)	Weight (kg)	Age (y)	Widmark factor [110]	High-hopped beer consumption (mL)	Low-hopped beer consumption (mL)
A	Male	179	105	37	0.594	802	787
B	Male	186	80	32	0.675	694	681
C	Male	175	72	25	0.675	624	613
D	Male	178	98	44	0.611	770	755
E	Male	182	90	39	0.641	741	727

Widmark factor = an estimate of volume of distribution calculated using the anthropometric measurements of the volunteers.

4.1.2.2 Specimens

Preserved blank blood (10 mL samples containing 200 mg sodium fluoride and 30 mg potassium oxalate) for instrument calibration purposes were obtained from a local blood bank (Melbourne, Australia). Blood of the participants in the drinking studies were collected in sterile 5 mL Venosafe blood tubes containing 9 mg sodium fluoride and 9 mg potassium oxalate purchased from Hazpak (Melbourne, Australia).

Blank urine for instrument calibration purposes was obtained from the authors after abstinence from beer and other alcoholic beverages for one week. Blank and volunteer urine specimens were contained in 50 mL urine pots and stored at -20 °C until analysed.

4.1.2.3 Chemicals and Reagents

The *trans*-IAA reference standards was DCHA-Iso, ICS-I3 (containing 62.3 % w/w of *trans*-IAA), obtained from Labor Veritas (Zurich, Switzerland) and contains primarily *trans*-isocohumulone, *trans*-isohumulone and *trans*-isoadhumulone that were grouped and quantified together (*trans*-IAA). However during commercial production, residual *cis*-IAA remain and were utilised to qualitatively monitor *cis*-isocohumulone, *cis*-isohumulone and *cis*-isoadhumulone (**Fig. 3 of Chapter 2.1**).

The isotope labeled internal standard nimodipine-d_7 was purchased from PM Separations (Brisbane, Australia). Acetonitrile (ACN), methanol and formic acid were purchased from Merck (Melbourne, Australia). Ammonium formate was purchased from Sigma Aldrich (Sydney, Australia). All chemicals were of analytical grade or better and water was purified using a Milli-Q Ultrapure Water System from Waters (Sydney, Australia).

4.1.2.4 IAA Analytical Method

IAA determination was performed using a previously published ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method that was validated for blood analysis [190]. Briefly, the extraction consisted of a protein precipitation of 200 µL of whole blood using -20 °C ACN with the resulting supernatant dried under nitrogen and the residue reconstituted with 50 µL of a mixture of eluent A and eluent B (60:40, v:v). Urine analysis was performed using this method, replacing blood for urine in the calibration model.

The UHPLC-MS/MS system comprised of a Shimadzu MS 8030 quadrupole mass spectrometer (Melbourne, Australia) operated in the electrospray ionisation (ESI) in negative mode and a Shimadzu Nexera UHPLC system (Melbourne, Australia) that consisted of a degasser, two eluent pumps, a column oven (30 °C) with a Kinetex C_{18} column (3.0 × 150 mm, 2.6 µm), and a chilled autosampler. For MS data evaluation, Shimadzu Postrun and Shimadzu Browser Analysis (Melbourne, Australia) softwares were used. GraphPad Prism 5.04 from GraphPad Software (San Diego, USA) was used for statistical analysis. The mobile phases consisted of 50 mmol/L aqueous:ACN (90:10) ammonium formate pH 2.8 (eluent A) and ACN containing 0.1 % formic acid (eluent B).

Preparation of stock solutions, calibration standards, quality controls, stability samples and extraction procedures were performed as published previously [190].

4.1.2.5 Blood Alcohol Analysis

Blood alcohol analysis was undertaken in conjunction with routine blood alcohol analysis within the toxicology department at the Victorian Institute of Forensic Medicine. Twenty-five microliters of blood was diluted with 1000 µL of deionized water and directly injected into a

gas chromatograph coupled with flame ionization detection and a packed glass column (0.2 % carbowax 1500 on carbopack C 80/100 mesh, Sigma–Aldrich, Sydney, Australia) for separation and quantification. This method has been in use for over two decades and utilizes daily calibrations and ensures all quality controls are within range.

4.1.2.6 Ethics

Approval for the human consumption of beer and subsequent specimen retrieval and analysis was obtained from the Ethics Committee of the Victorian institute of Forensic Medicine (EC 04/2012).

4.1.3 Results

The IAA content of the two beers and their sources are shown in **Table 9**. Neither alcohol nor IAA analytes were detected in any of the pre-dose blood or urine specimens.

Following the consumption of ~600-800 mL of relatively high-hopped beer the *trans*-IAA were detected in all post-dose blood specimens (**Fig. 10**). IAA concentrations peaked at 0.5 h in all volunteers. Results were similar between participants with the *trans*-IAA reaching ~0.1 mg/L at 0.5 h and dropping to ~0.01-0.02 mg/L by 2 h with only one participant having *trans*-IAA detected at the final 6 h time-point.

Consumption of the low-hopped beer showed *trans*-IAA levels also peaking at 0.5 h, although at a lower ~0.02 mg/L concentration compared to higher hopped beer. At 2 h, these concentrations had dropped to ~0.002 mg/L, just above the lower limit of quantification. In

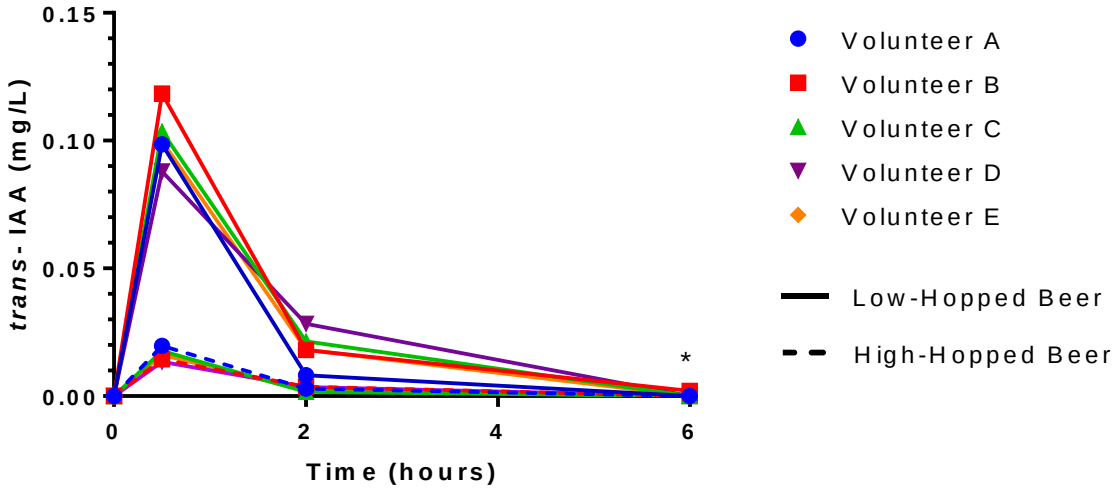


Fig. 10

The *trans*-IAA (mg/L) blood concentration-time profiles of the five volunteers at 0, 0.5, 2 and 6 h post consumption of Little Creatures Pale Ale (*high-hopped* beer) and the Erdinger Weissbier (*low-hopped* beer). *Cis*-IAA were detected in all volunteers at the 0.5, 2 and 6 h time-points. * denotes *trans*-IAA was only detected in volunteer B at the 6 h time-point of the high-hopped study.

both studies the *trans*-IAA levels fell ~10-fold from 0.5 h to 2 h. This rate of decrease in both studies after 90 min suggests a *trans*-IAA half-life (t½) of about 30 min.

Cis-IAA were detected in the 0.5, 2 and 6 h blood specimens of all participants in both high- and low-hopped studies, of which the 'co' analog was consistently most abundant (data not shown).

Since the 'co' analogs were the most abundant ions monitored and were therefore the best analogs to demonstrate variations in elimination between stereoisomers. **Fig. 11** shows the two 'co' analytes within the IAA group (*trans*- isocohumulone and *cis*-isocohumulone) and compares the areas of these analytes at the 0.5 and 2 h time-points. Results show that levels of the *trans* isomer fell ~5-10 % more in the 90 min timespan in both studies, suggesting that

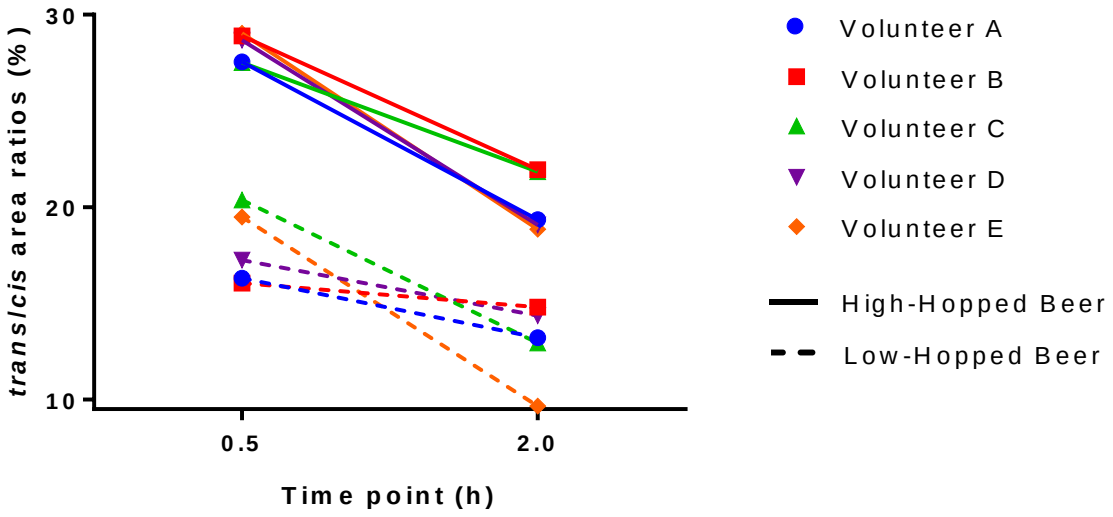


Fig. 11

The comparison of *trans*-isocohumulone / *cis*-isocohumulone area ratios in the blood of the five volunteers at 0.5 and 2 h post-consumption of beer in the high- and low-hopped beer drinking studies in order to demonstrate metabolism differences between *trans* and *cis* isomers.

the *trans* isomers may be eliminated more rapidly. Notable differences in the ratios between studies of about 10 % were also observed in the low-hopped study.

The mean and standard deviation of *trans*-IAA, BAC and the comparison of *trans* and *cis* metabolism results, for the five participants in both studies are shown in **Table 11**.

Analysis of urine for *trans*-IAA showed only the ‘co’ analogs detected in volunteers at all time-points in the high-hopped study. Although quantification is not possible due to the detection of only one analog, there was an noticeable variation in the area responses between volunteers. Only one volunteer in the low-hopped beer study had a detectable *trans* ‘co’ analog (at 0.5 h). No ‘n’ or ‘ad’ analogs were detected in any volunteer at any time-point. However, the *cis*-IAA ‘co’ analog was detected in all volunteers in both studies at all time-points post consumption.

Table 11

The mean and stardard devations of the blood alcohol concentration (BAC g/100mL), blood *trans*-IAA (mg/L) and *trans*-IAA / *cis*-IAA area ratios of the five volunteers in the high- and low-hopped drinking studies at three time points post-drinking.

Analysis	**n**	**High-hopped**			**Low-hopped**		
		Time-point (h)			Time-point (h)		
		0.5	**2**	**6**	**0.5**	**2**	**6**
BAC (g/100mL)	5	0.034±0.005	0.028±0.004	< 0.01	0.032±0.008	0.024±0.005	< 0.01
***trans*-IAA (mg/L)**	5	0.102 ± 0.011	0.019 ± 0.007	0.004 ± 0.001	0.016 ± 0.003	0.003 ± 0.001	< 0.001
***trans*- / *cis*-IAA**	5	28.332 ± 0.739	20.212 ± 1.542	n/a	17.890 ± 1.941	12.998 ± 2.016	n/a

n/a = not performed at 6 h time-point due to area response below limit of detection (signal:noise < 3).

4.1.4 Discussion

Although one volunteer had given detectable concentrations of IAA after consumption of a high-hopped beer in a pilot study [190], the pharmacokinetics of IAA in the blood of humans have not yet been established. This current study provided some basic pharmacokinetic information on IAA, largely in agreement with previous animal models that demonstrated that IAA was rapidly absorbed, had a t½ of 32 ± 1.8 min and only trace amounts of unmodified IAA were excreted in the urine [212].

Inter-individual differences in pharmacokinetics were small as *trans*-IAA concentration over time profiles between participants demonstrated no obvious outliers. The low- and high-hopped beers were described as ~10 and ~40 IBU, respectively; therefore although similar volumes of the beer were consumed, participants in the low-hopped beer study consumed approximately four fold less IAA. This correlated well as the *trans*-IAA levels between the beer studies showed a similar profile with differences in *trans*-IAA concentrations of ~4 times. Furthermore, the IBU of the Indian Pale Ale which was consumed in the pilot study was approximately double that of the Little Creatures Pale Ale used in this study [190]. This compared well with the *trans*-IAA concentrations at 0.5 and 2 h from this current study being approximately half to that of the pilot study.

Although differences between individuals were shown to be small, this data only provides a limited understanding of the pharmacokinetics of these compounds and does not currently permit correlations of *trans*-IAA and BAC levels to be made or estimate when alcohol consumption occurred. Furthermore, future studies should account for potential gender differences with the inclusion of female volunteers.

As the absorption of ethanol is commonly accepted to provide peak BAC at ~1 h post-consumption [47], the 0.5 and 2 h time points most likely do not represent the peak BAC and explains lower than BAC 0.05 g/100 mL target levels observed.

Since the low-hopped wheat beer was described as only ~10 IBU and beers naturally contain less *trans*-IAA than *cis*, it is expected that the concentrations of *trans*-IAA in blood to be only a few mg/L. With participants consuming between ~600-800 mL of Erdinger Weissbier, only ~1-3 mg of *trans*-IAA was estimated to have been consumed thus making blood IAA detection more difficult. However, confirmation of beer ingestion almost throughout the 6 h demonstrates the sensitivity of the method and possible applicability for detection of different styles of beers.

Notable differences in the overall *trans*:*cis* ratios between studies of ~10 % less in the low-hopped (Erdinger Weissbier) were observed. This is most likely due the *trans* isomer being less stable in beer long-term and a slight loss occurring during the transportation from Germany to Australia, rather than any pharmacokinetic variation between beer studies taking place.

Although no significant differences were detected between stereoisomers during long-term storage in blood [207], this study demonstrated that the *trans* isomers may be more subject to metabolism *in vivo*. Furthermore, the *cis* isomers are also approximately ~1.5 times more abundant in beer to that of the *trans* counterpart [174] and as previously mentioned, are stable for longer in beer during storage. Finally, the detection of *cis*-isocohumulone at 6 h post consumption was possible where *trans* isomers were not detected. Therefore it is noteworthy, that although the *cis* isomers were unable to be quantified, they may present as possibly important qualitative markers when greater detection windows of beer consumption are required.

Additional pharmacokinetic information can be obtained with use of the *in silico* metabolism pathway prediction modeling software such as 'MetaPred' which suggested that the cytochrome P450 (CYP) 2C9 was primarily responsible for the oxidation of the IAA compounds [217]. Furthermore, 'SmartCyp', a CYP-mediated metabolism prediction tool suggested that the most probable site of metabolism would occur at the end of both side chains on the four methyl groups [218]. Similarly, Cattoor et al. demonstrated that IAA conjugates were not significantly present in rabbits post-ingestion of large amounts of IAA and also proposed that Phase I metabolism was the primary pathway for the elimination of IAA [212].

Preliminary urine analysis showed a significant difference in the elimination of unmodified IAA analogs with the 'co' being the only detectable analog. Although *cis*-IAA 'co' analytes were able to be abundantly detected, the development of an analytical method for metabolites or conjugates of IAA may allow for longer windows of detection whilst also providing further information on the metabolism pathways of IAA.

Reduced-IAA are chemically synthesized from IAA and are commonly used in either green or clear glass bottles for their photolytic protective properties [165, 173, 174, 212, 213]. They may be used in isolation or in conjunction with traditional IAA hopping techniques. Detection of reduced-IAA in blood and urine following the consumption of these beers may allow for further discrimination between the ingestion of different beers. Additionally, determination of accurate IAA concentrations in a range of popular, craft and homemade beers would benefit forensic toxicologists in correlating blood IAA results with the beer(s) suspected to have been consumed.

4.1.5 Conclusion

although outside the scope of this study, more IAA pharmacokinetic data may be achieved with the oral and intravenous dosing of pure IAA formulations and with specimens collected more often. Whilst these studies are limited to five volunteers and two different types of beer, the detection of IAA in blood and perhaps in other specimens may be possible in forensic casework particularly where typically large amounts of conventionally hopped beer are consumed and the confirmation of beer consumption is valuable.

CHAPTER 4.2

PHARMACOKINETICS OF REDUCED IAA

Rodda, L.N., D. Gerostamoulos, and O.H. Drummer,
Pharmacokinetics of Reduced Iso-α-acids in Volunteers following Clear Bottled Beer Consumption.
Forensic Science International, 2014.
(Under Review, Appendix 1.5)

Monash University

Declaration for Thesis Chapter 4.2.

Declaration by candidate

In the case of Chapter 4.2., the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Located articles, reviewed articles and wrote the paper	85%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Gerostamoulos, D.	*Advised candidate regarding direction and content, reviewed article manuscript*	
Drummer, O.H.	*Advised candidate regarding direction and content, reviewed article manuscript*	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 26.6/14
Main Supervisor's Signature	[redacted]	**Date** 27/6/14

Abstract

Reduced iso-α-acids (reduced IAA) consisting of the rho-, tetrahydro- and hexahydro-IAA groups are ingredient congeners specific to beer generally found in clear and also occasionally green bottled beer. Concentrations of reduced IAA were determined in the blood and urine of five volunteers over 6 hours following consumption of small volumes of beer containing each of the reduced IAA. The reduced IAA were quickly absorbed with peak concentrations at 0.5 h followed by a drop of generally fivefold by 2 h. Preliminary pharmacokinetics of these compounds in humans shows relatively small inter-individual differences and an estimated short half-life varying between ~38-46 for the three groups. Comparison of RIAA analyte ratios within the group indicate that some analytes eliminate relatively faster than others and the observation of metabolite products. Preliminary urine analysis showed only unmodified RIAA analytes were detectable throughout 6 h and suggests extensive phase I metabolism of TIAA and HIAA analytes. In authentic forensic casework where clear or green bottled beers are consumed, the identification of reduced IAA groups may provide a novel method to target ingredient congeners consistent with beer ingestion and suggest the type of beer consumed.

Keywords

Alcohol congener analysis; Reduced iso-α-acids; beer; pharmacokinetics; blood; urine

4.2.1 Introduction

Traditionally, iso-α-acids (IAA) that have been derived from the hop plant (*Humulus lupulus* L.) have been used in beer for their bitterness, bacteriostatic and hydrophobic properties [165, 219, 220]. However IAA are prone to becoming light-struck in the presence of sunlight (near ultraviolet, blue light) and oxygen resulting in familiar and undesirable "skunky" aroma producing compounds, of which 3-methyl-2-butene-thiol (3-MBT) being the most offensive [174]. This historically led to beer being packaged in lightproof glass such as brown, and to some extent green, bottles.

Over the last five decades, a series of "light-stable" derivatives called reduced IAA have been chemically synthesised from the naturally available IAA; namely rho-IAA (RIAA), tetrahydro-IAA (TIAA) and more recently, hexahydro-IAA (HIAA) [172, 219, 221]. By reducing the relatively weak double bonds or carbonyl group in the side chains of IAA to stronger single bonds, the photolytic cleavage that produces 3-MBT is unable to occur [165], see **Fig. 4 of Chapter 2.1**. These photolytic protective properties allows for the use of these products to be commonly used in isolation in unprotected clear glass bottles [165, 174, 212, 213]. However in order to stablise the bitterness of the beer, they may be used also in conjunction with traditional IAA hopping techniques in green bottles that are subject to low levels of light where some photolytic degradation will still occur to the natural IAA [180].

This reduction process provides altered intensities of bitterness and sensory properties [219, 222]. Additionally, due to increasing hydrophobicity of the compounds (in order of RIAA to HIAA to TIAA), enhanced beer foam stability, appearance and "cling" are observed (**Table 12**). Concentrations of 2.4 and 4.2 ppm have been sufficient to show foam stabilisation for TIAA and HIAA, respectively, demonstrating that reduced IAA used for the purpose of foam improvement only are added in lower concentrations [178].

Table 12

Summary of the natural and reduced IAA products used in brewing for their desriable bittering [195, 222-225], foam stabilising [178], light protective [195] and sensory [222] properties.

Reduced Iso Product	**Bittering Power (%)**	**Foam Stability**	**Light Stable** [a]	**Sensory Analysis**
Iso-α-acids (IAA)	100	++	No	astringent, chalky, less fruity and vegetative
Rho-iso-α-acids (RIAA)	60-80	+	Very Good	medicinal, metallic, sharp, astringent
Tetrahydro-iso-α-acids (TIAA)	110-180	++++	Good	less medicinal, more vegetative and fruity, flat, dull
Hexahydro-iso-α-acids (HIAA)	100-170	+++	Very Good	medicinal, metallic, aspirin, vegetative, green

[a] the prevention of only the degradation product 3-methyl-2-butene-1-thiol (3-MBT) occurring after photolytic cleavage of side chains [158] (see **Fig. 2 in Chapter 1.2**)

Reduced IAA hop-products such as Redihop® (RIAA), Tetrahop Gold® (TIAA) and Hexahop® 95 (HIAA) [223-225] are just some examples of the commercially available reduced IAA products that are available as potassium salt preparations ready to be added directly to the finished beer (post-fermentation) [165, 179]. Amongst other non-natural additives, the reduced forms of IAA are prohibited in beers for the German market due to the "Reinheitsgebot" law that states only natural hops, water, malt and yeast, may be used in the brewing process [180]. A range of lager beers have shown to contain ~3-6 mg/L and ~5-28 mg/L of TIAA and RIAA, respectively [180]. Following the manufacture process, residual levels of up to 0.1 – 0.2% of the parent IAA have been found in reduced IAA products [192], this may lead to trace amounts of IAA in some beers.

Recently, natural IAA have been detected in the blood and urine of volunteers for up to six hours following the consumption of natural hopped brown bottled beers using a newly developed ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) procedure [190, 226]. These early IAA pharmacokinetic studies

demonstrated that IAA compounds can be detected and suggests that the structurally similar reduced IAA may also be able to be targeted. Furthermore, there have been toxicity [227] and pharmacokinetic studies investigating reduced IAA, particularly RIAA and TIAA, due to their possible medicinal properties such as weight loss, anti-diabetic, anti-carcinogenic and anti-inflammatory [212-214, 228-230]. Of these, two studies detected RIAA and TIAA in New Zealand white rabbits [212], and one measured RIAA in humans [213], confirming those reduced IAA groups examined were indeed bioavailable. The long-term stabilities of the reduced IAA groups in stored blood recently concluded that they were less susceptible to degradation than the natural IAA and refrigeration or freezing conditions provided acceptable stability [231].

This suggests that reduced IAA would be able to be detected in blood post consumption and therefore present as beer-specific ingredient congeners that if detected, can confirm beer ingestion. This may represent another method of performing alcohol congener analysis (ACA) to assist investigations into the source of alcohol in routine forensic casework and in after-drinking (or hip-flask) defence cases where the feasibility of claimed alcohol consumption prior and/or subsequent to a motor vehicle incident is in question [11, 28, 31, 159].

The aim of this study was to detect and determine the pharmacokinetics of reduced IAA in the blood and urine of human volunteers given controlled volumes of three different types of beer containing different amounts of RIAA, TIAA and HIAA.

4.2.2 Experimental

4.2.2.1 Drinking study design

Five healthy volunteers were administered specific volumes of beers (**Table 13**) targeting RIAA (Amstel), TIAA (Hahn Premium Light) and HIAA (Coopers Clear). Beers were selected due to the levels of reduced IAA detected in preliminary investigations [232]. Coopers Clear was found to contain the highest levels of HIAA, however as it also contained relatively high levels of RIAA, this reduced IAA group was also examined in detail.

Studies were performed separately over a day each with a wash-out period of at least one week between studies. No alcohol was permitted for 48 h prior to each of the study days. In order to minimalise absorption variables, all volunteers fasted from food on the morning of the study and the administered beer was required to be consumed within 10 min. No drink and food was permitted until two hours post consumption.

To estimate blood alcohol concentration (BAC), a revised version of the Widmark formulae was employed that mathematically combines recent variations by other authors into a single formulae to provide an accurate 'Widmark factor' for each individual and subsequently the volume of beer required to produce a peak BAC of 0.05 g/100mL [9, 110], the legal limit in Australia. The anthropometric measurements (i.e. height, weight and age) and subsequent required volume of beer consumed for each volunteer were described (**Table 14**). Due to volunteer unavailability, volunteer A1 was replaced by A2 for the last two drinking studies.

Blood was obtained by a registered phlebotomist and urine by the volunteers themselves at prior (zero), 0.5, 2, 4 and 6 h post-consumption time-points. Due to delays in the phlebotomy, the 0.5 h time point bloods were collected between 0.5-1.25 h and at 0.75 h for the Amstel

Table 13

Details of the clear-bottled lager beers consumed during the three drinking studies targeting beers containing each of the three reduced-IAAs (RIAA, TIAA and HIAA) including the variety, brewer, location of brewery, batch or expiry, alcohol content, and the approximate IAA and reduced IAA content of the beers [232].

Name	Variety	Brewery	Location	Batch or Expiry	Ethanol (ABV%)	IAA (mg/L)	RIAA (mg/L)	TIAA (mg/L)	HIAA (mg/L)
Amstel	Low Carb	Amstel Brouwerij B.V.	Amsterdam, Holland	April 14 3119528R1826	4.7	Trace < 0.1	~5	~1	-
Hahn Premium Light	Low Alchol	Hahn	Sydney, Australia	19 March 14 44:57 142	2.6	-	-	~6	-
Cooper Clear	Low-Carb	Coopers	Adelaide, Australia	05 Nov 14 13:41	4.5	Trace < 0.1	~19		~0.3

ABV% = alcohol by volume (volume / volume %)

Table 14

Anthropometric measurements (height, weight and age) that provide a Widmark factor required to estimate the volumes of the three beers that were consumed in the drinking studies.

Volunteer	Gender	Height (cm)	Weight (kg)	Age (y)	Widmark factor [110]	Amstel beer consumption (mL)	Hahn Premium Light beer consumption (mL)	Coopers Clear beer consumption (mL)
A1	Male	186	80	32	0.675	768	n/a	n/a
A2	Male	182	90	39	0.641	n/a	1483	857
B	Male	175	72	25	0.675	691	1249	722
C	Female	173	73	36	0.510	530	958	554
D	Female	168	68	28	0.514	497	899	519
E	Female	166	64	33	0.521	475	858	496

Widmark factor: an estimate of volume of distribution calculated using the anthropometric measurements of the volunteers.

NB: Volunteer A1 was replaced by volunteer A2 in the two final drinking studies.

and Hahn Premium Light drinking studies, respectively. The zero hour specimens acted as the control and blank blood and urine of the respective volunteer.

4.2.2.2 Specimens

Preserved blank blood (10 mL samples containing 200 mg sodium fluoride and 30 mg potassium oxalate) for instrument calibration purposes were obtained from a local blood bank (Melbourne, Australia). Blood from the volunteers in the drinking studies were collected in sterile 5 mL Venosafe blood tubes containing 9 mg sodium fluoride and 9 mg potassium oxalate purchased from Hazpak (Melbourne, Australia). Volunteer blood specimens were analysed on the day of study and blank bloods were stored at -20 °C until analysed.

Blank urine for instrument calibration purposes was obtained from the authors after abstinence from beer and other alcoholic beverages for one week. Blank and volunteer urine specimens were contained in 50 mL urine pots and stored at -20 °C until analysis.

4.2.2.3 Chemicals and reagents

Reference standards for: DCHA-Rho, ICS-R2 (containing 65.3 % w/w of *cis*-RIAA); Tetra, ICS-T2 (containing 99.4 % w/w of TIAA); DCHA-Hexa, ICS-H1 (containing 65.7 % w/w of *cis*-HIAA), and; DCHA-Iso, ICS-I3 (containing 62.3 % w/w of *trans*-IAA); were obtained from Labor Veritas (Zurich, Switzerland).

The monitored analogs and isomers of the three reduced IAA groups were: **RIAA** (R1 = *cis*-rho-isocohumulone 1; R2 = *cis*-rho-isocohumulone 2; R3 = *cis*-rho-isohumulone 1; R4 = *cis*-rho-isohumulone 2; R5 = *cis*-rho-isoadhumulone 1; R6 = *cis*-rho-isoadhumulone 2); **TIAA** (T1 = *trans*-tetrahydro-isocohumulone; T2 = *cis*-tetrahydro-isocohumulone; T3 = *trans*-tetrahydro-isohumulone; T4 = *cis*-tetrahydro-isohumulone; T5+6 = *trans*+*cis*-tetrahydro-

isoadhumulone), and; **HIAA** (H1 = *cis*-hexahydro-isocohumulone 1; H2 = *cis*-hexahydro-isocohumulone 2; H3 = *cis*-hexahydro-isohumulone 1; H4 = *cis*-hexahydro-isohumulone 2; H5+6 = *cis*-hexahydro-isoadhumulone 1+2).

The monitored analogs and isomers of the natural **IAA** group were: I1 = *trans*-isocohumulone; I2 = *cis*-isocohumulone; I3 = *trans*-isohumulone; I4 = *cis*-isohumulone; I5 = *trans*-isoadhumulone; I6=*cis*-isoadhumulone.

The isotope labeled internal standard nimodipine-d_7 was purchased from PM Separations (Brisbane, Australia). Acetonitrile (ACN), methanol and formic acid were purchased from Merck (Melbourne, Australia). Ammonium formate was purchased from Sigma Aldrich (Sydney, Australia). All chemicals were of analytical grade or better and water was purified using a Milli-Q Ultrapure Water System from Waters (Sydney, Australia).

4.2.2.4 Reduced IAA Analytical Methodology

Reduced IAA determination was performed using a previously published UHPLC-MS/MS method that was validated for blood IAA and reduced IAA analysis [190]. Briefly, the extraction consisted of a protein precipitation of 200 µL of whole blood using -20 °C ACN with the resulting supernatant dried under nitrogen and the residue reconstituted with 50 µL of a mixture of eluent A and eluent B (60:40, v:v). Urine analysis was performed using this method, replacing blood for urine in the calibration and quality control models.

The UHPLC-MS/MS system comprised of a Shimadzu MS 8030 quadrupole mass spectrometer (Melbourne, Australia) operated in electrospray ionisation negative mode. A Shimadzu Nexera UHPLC system (Melbourne, Australia) consisted of a degasser, two eluent pumps, a column oven (30 °C) with a Kinetex C_{18} column (3.0 × 150 mm, 2.6 µm from

Phenomenx, Melbourne, Australia), and a chilled autosampler. For MS data evaluation, Shimadzu Postrun and Shimadzu Browser Analysis (Melbourne, Australia) softwares were used. GraphPad Prism 5.04 from GraphPad Software (San Diego, USA) was used for statistical analysis. The mobile phases consisted of 50 mmol/L aqueous:ACN (90:10) ammonium formate pH 2.8 (eluent A) and ACN containing 0.1 % formic acid (eluent B).

Preparation of stock solutions, calibration standards, quality controls, stability samples and extraction procedures were performed as published previously [190].

4.2.2.5 Blood alcohol analysis

Blood alcohol analysis was undertaken in conjunction with routine blood alcohol analysis within the toxicology department at the Victorian Institute of Forensic Medicine. Twenty-five microliters of blood was diluted with 1000 µL of deionised water and directly injected into a gas chromatograph coupled with flame ionisation detection and a packed glass column (0.2 % carbowax 1500 on carbopack C 80/100 mesh, Sigma–Aldrich, Sydney, Australia) for separation and quantification. This method has been in use for over two decades and utilises daily calibrations and ensures all quality controls are within range.

4.2.2.6 Ethics

Ethics was obtained from the Human Research Ethics Committee at the Victorian Institute of Forensic Medicine (E06/2013).

4.2.3 Results

The IAA and reduced IAA content of the three beers and their sources are shown in **Table 13**. **Table 14** shows the mean and standard deviation BAC for the studies, lower than 0.05 g/100mL results suggest the peak BAC was not in correlation with the studies' time points. Neither alcohol nor IAA and reduced IAA analytes were detected in any of the pre-dose blood or urine specimens. Metabolism and/or modification of reduced IAA into other reduced IAA or IAA groups was not observed.

4.2.3.1 Blood reduced IAA concentration-time profiles

The reduced IAA blood concentration-time profiles of the three beer studies are shown in **Fig. 12**. **Table 15** shows the blood quantifiable mean and standard deviations values of the IAA and reduced IAA levels, or the detected result if only some of the IAA or reduced IAA analyte(s) of the group could be detected. All profiles showed reduced IAA concentrations peaking at maximum absorption times (T_{max}) of 0.5 h and decreased rapidly by 2 h with further elimination continuing throughout the remaining time points. Due to the high concentration of RIAA in the Coopers Clear beer, this group is also shown in additional to the originally targeted HIAA group in **Fig. 12**.

Following the consumption of ~470-770 mL of Amstel beer, RIAA peak blood concentrations (C_{max}) of ~0.17 and ~0.05 mg/L were observed in 0.5 and 2 h bloods, respectively. However by the forth hour, the R2 analyte was the only detected analyte. In comparison and even though similar beer volumes were consumed, all RIAA analytes were able to be detected throughout the Coopers Clear study do to the higher content of RIAA in that beer. The half-life (t½) of RIAA remained constant between studies at ~46 minutes

reflected by the similar four- to five-fold reduction in concentrations from the 0.5 to 2 h time points. With the exception of volunteer D in the Amstel drinking study due to phlebotomy delays, the RIAA profiles were similar between all volunteers in both studies.

All TIAA analytes were seen in the 0.5 h blood after ~850-1500 mL of Hahn Premium Light beer was consumed. Although a larger volume of beer was consumed due to the low alcohol content, elimination of analytes T3 to T6 only allowed for analytes T1 and T2 to be quantified at the later 2, 4 and 6 h time points. Examination of the 0.75 and 2 h time points showed a t½ of ~ 45 min for the TIAA group. As Amstel contained ~1 mg/L of TIAA, that drinking study showed detection of the T2 analyte in all 0.5 h bloods also. In **Fig. 12**, male (A and B) and female (C, D and E) volunteers showed different TIAA profiles to each other with males generally showing higher concentrations, this is also reflected by the larger group standard deviation in **Table 15**.

After ~500-850 mL of Coopers Clear beer consumed, very low levels of blood HIAA were detected at only 0.5 and 2 h bloods of which a t½ of ~ 38 min can be estimated. No HIAA analytes were detected at 4 and 6 h time points. Although Hahn Premium Light contains trace levels of HIAA, no HIAA analytes were detected in the bloods from this study.

All 0.5 h bloods were able to detect traces of just I2 and all the IAA analytes in the Amstel and Coopers Clear beer studies, respectively.

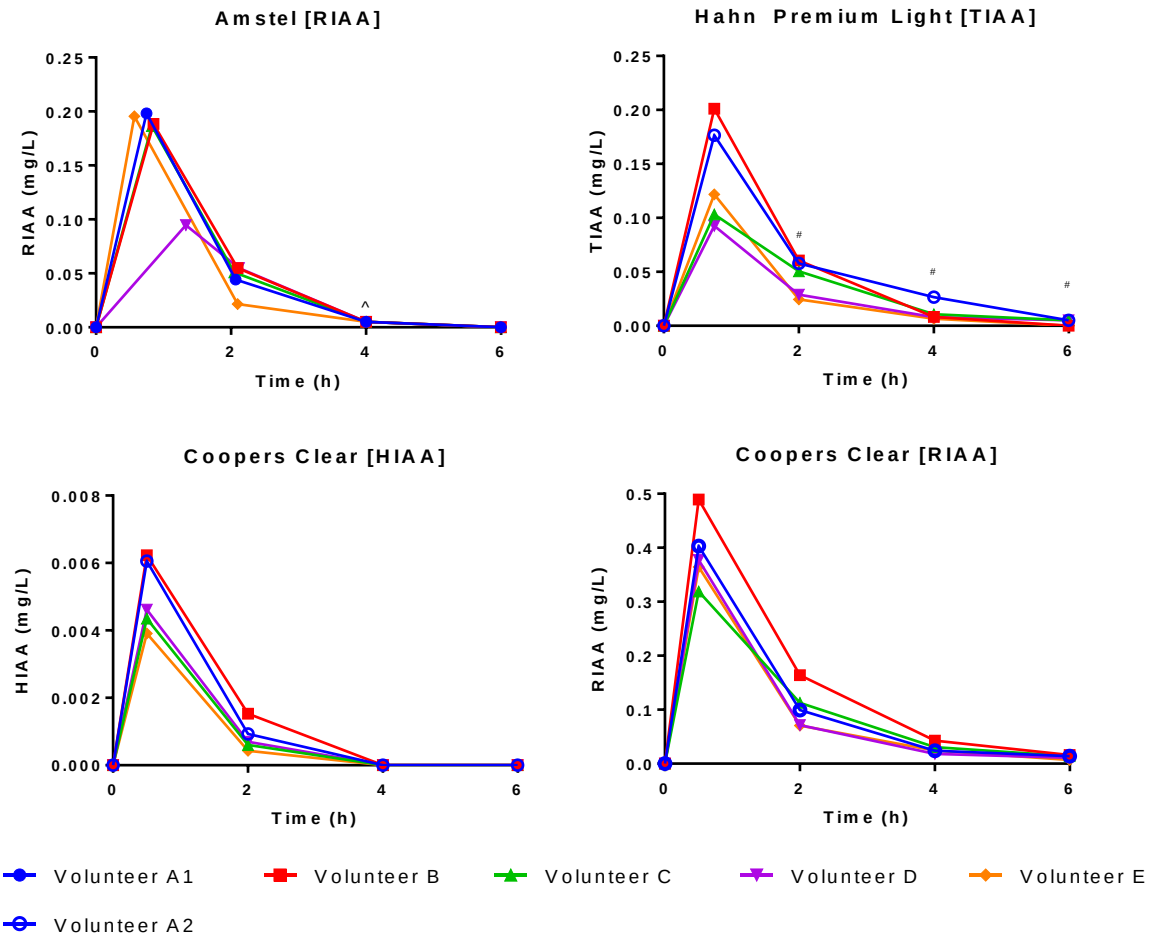


Fig. 12

The reduced IAA groups blood concentration-time profiles (mg/L) of the five volunteers at 0, 0.5, 2, 4 and 6 h post consumption of the Amstel (RIAA), Hahn Premium Light (TIAA) and Coopers Clear (RIAA and HIAA) drinking studies. Due to delays in the phlebotomy, the 0.5 h time point bloods were collected between 0.5-1.25 h and at 0.75 h for the Amstel and Hahn Premium Light drinking studies, respectively.

^ = Only R2 was detected in all 4 h bloods.

= volunteers only had T1 and T2 detected at 2, 4 and 6 h time points.

Table 15

The mean and stardard devations of the blood alcohol concentration (BAC g/100mL), and blood IAA, RIAA, TIAA and HIAA (mg/L) results of the five volunteers in the Amstel, Hahn Premium Light and Coopers Clear drinking studies at four time points post-consumption. If analytes were only detected then the indiviual analytes are indicated.

Analysis	n	Amstel				Hahn Premium Light				Coopers Clear			
		Time-point (h)				Time-point (h)				Time-point (h)			
		0.5-1.25	**2**	**4**	**6**	**0.75**	**2**	**4**	**6**	**0.5**	**2**	**4**	**6**
BAC	5	0.033 ±0.006	0.016 ±0.006	-	-	0.030 ±0.013	0.015 ±0.008	-	-	0.030 ±0.006	0.018 ±0.005	-	-
IAA	5	I2	-	-	-	-	-	-	-	I2, I4, I6	-	-	-
RIAA	5	0.173 ±0.044	0.045 ±0.014	R2	-	-	-	-	-	0.391 ±0.063	0.103 ±0.038	0.028 ±0.009	0.012 ±0.004
TIAA	5	T2	-	-	-	0.139 ±0.047	0.044 ±0.017	0.012 ±0.008	T1, T2	-	-	-	-
HIAA	5	-	-	-	-	-	-	-	-	0.005 ±0.001	0.001 ±0.001	-	-

4.2.3.2 RIAA analyte metabolism profile

Since RIAA in the Coopers Clear beer study was the only group where analytes were quantified throughout the six hours in blood (excluding R5 and R6 at 4 and 6 h), the relative metabolism rates of the analytes within the RIAA group were further examined to demonstrate variations in elimination (**Fig. 13**). Producing the same monitored ions to that of R3, R4, R5 and R6 (362.90>265.25; 362.90>196.20, and; 362.90>247.00 m/z), unknown peaks at 5.75 and 6.00 min (U/K 1 and U/K 2, respectively) were observed at the first 0.5 h time point and monitored thereafter. These demonstrate as metabolites of one, or more, of the RIAA analytes as they are not present in the control bloods, reference standard or the beer. The U/K1 peak initially is the more abundant of the two, however it gradually was also eliminated at a relatively quicker rate and eventually the U/K2 metabolite was more prominent by 2-4 h and onwards. The increasing relative abundance of the R3 analyte suggests it may be eliminated at a relatively slower rate. Similarly, the relative decreases in the R1, R4, R5 and R6 analytes may imply that they are subject to more extensive elimination. The R2 showed no obvious overall change in direction inferring an average type elimination profile relative to that of the collective RIAA group.

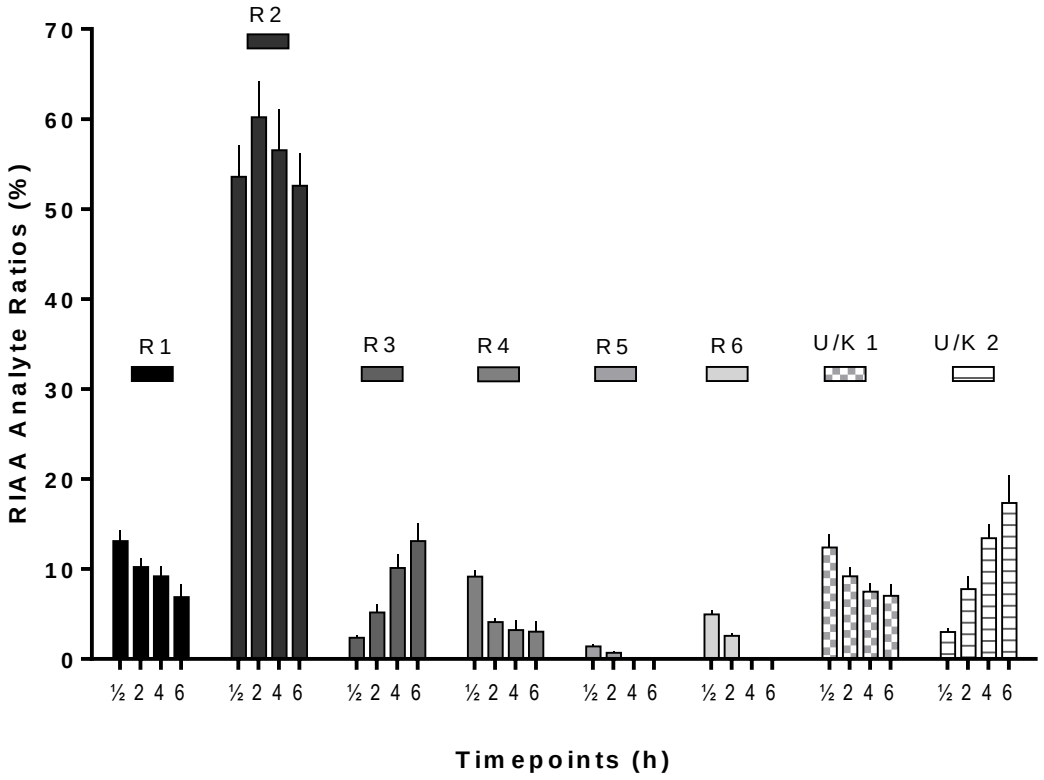


Fig. 13

Values represent the mean RIAA analyte/internal standard area ratios, as a percentage of the sum of the total RIAA group of analytes, at each 0.5, 2, 4, and 6 h time points post consumption of Coopers Clear. Error bars represent the standard deviation. U/K 1 and U/K 2 represent unknown metabolite peaks observed at 5.75 and 6.00 min, respectively. Values represent elimination (metabolism an excretion) rates that are proportionate to other analytes within the RIAA group.

4.2.3.1 Urine reduced IAA concentration-time profiles

Table 16 shows the mean and standard deviations values of the reduced IAA levels and the detected IAA analytes in the urine of the drinking studies. Notably RIAA was the only reduced IAA group to be detected in urine post consumption. Urine RIAA concentrations in both Amstel and Coopers Clear studies fluctuated through the 6 hours and was moderately varied between volunteers as demonstrated by the relatively wide standard deviations in **Table 16**.

Table 16

The mean and stardard devations of the urine IAA, RIAA, TIAA and HIAA (mg/L) results of the five volunteers in the Amstel, Hahn Premium Light and Coopers Clear drinking studies at four time points post-consumption. If analytes were only detected then the indiviual analytes are indicated.

Analysis	n	Amstel				Hahn Premium Light				Coopers Clear			
		Time-point (h)				Time-point (h)				Time-point (h)			
		0.5-1.25	**2**	**4**	**6**	**0.75**	**2**	**4**	**6**	**0.5**	**2**	**4**	**6**
IAA	5	I2	-	I2 [a]	-	-	-	-	-	I2, I4	I2, I4	I2	I2
RIAA	5	0.018 ±0.010	0.012 ±0.007	0.041 ±0.041	-	-	-	-	-	0.054 ±0.052	0.043 ±0.016	0.151 ±0.163	0.122 ±0.128
TIAA	5	-	-	-	-	-	-	-	-	-	-	-	-
HIAA	5	-	-	-	-	-	-	-	-	-	-	-	-

[a] detected in only volunteers B and C

Similar to the blood results, trace levels of the *cis*- isomers of the IAA group could be detected in the urine specimens of the Amstel and Coopers Clear beers. Only I2 could be detected in the 0.5 and 4 h urines in the Amstel study, whilst I2 was detected throughout the six hours in the Cooper Clear study in addition to I4 at 0.5 and 2 h time points.

4.2.4 Discussion

Previous studies have shown some pharmacokinetics of natural IAA in human volunteers [190, 226], RIAA and TIAA in animal models [212], and in humans dosed with large amounts of RIAA [213]. However the pharmacokinetics of all the reduced IAA in the blood and urine of humans following consumption of beer have not yet been established. This current study provided some basic pharmacokinetic information on reduced IAA, largely in agreement with previous studies [212, 213], in addition to providing HIAA pharmacokinetic data for the first time.

4.2.4.1 Pharmacokinetics

Cattoor *et al*. showed that RIAA and TIAA was 28% and 23% bioavailable, respectively, in New Zealand white rabbits [212]. This study also showed varied T_{max} of 4-12 h (RIAA) and 0.5-6 h (TIAA) following oral consumption, in comparison to this current study that demonstrated rapid absorption. The calculated human equivalent dose (HED) of oral administration of RIAA and TIAA in that study equated to ~500 mg for a 60-70 kg human and provided plasma C_{max} of ~6-8 mg/L [212]. Furthermore in another study by Hall *et al*., two individual RIAA analytes each reached Cmax of ~1-3 mg/L 4 h after oral dosing of 700

mg of total RIAA to 2 healthy human volunteers [213]. This report also showed RIAA levels were ~70% less in plasma to that of whole blood and goes further to suggest that total RIAA levels in whole blood would be higher than the reported plasma values. Our study had volunteers consume ~10-16 mg of RIAA in the Coopers Clear study and produced Cmax values of ~0.4 mg/L. The relatively large dose of 700 mg of RIAA orally in combination with food *ad libitum* may have delayed the Tmax and reduced the Cmax in comparison to our current study where absorption was potentially aided due to volunteers were fasted and lower doses given.

In general, the reduced IAA exhibit longer t½ to that of the natural IAA where it was determined that *trans*-IAA had a ~30 min ½ life [212, 226]. Our study showed reduced IAA groups had similar elimination slopes and comparable t½ (~45-46 min) to those found when rabbits were dosed (t½ = ~43 and ~41 min for RIAA and TIAA, respectively) [212].

Urine RIAA and TIAA was excreted 12% and 1%, respectively, unmodified following the rabbit model [212]. Comparably, our study also showed significant unchanged urine RIAA concentrations in both the Amstel and Coopers Clear studies, however our study did not detect TIAA in any urine samples. Enzymatic hydrolysis of plasma samples revealed that the presence of TIAA conjugates could not be significantly demonstrated indicating phase I metabolism the most likely limiting factor of bioavailability. However, it was estimated that 50% (plasma) and 22% (urine) of RIAA was either a sulphate or a glucuronide conjugate undergoing phase II metabolism [212].

Hall e*t al*. examined the inhibition of cytochrome P450 (CYP) isoenzymes by RIAA with CYP2C9 found to be the most strongly inhibited by RIAA followed by moderately inhibition of CYP2C19 [213]. Furthermore, Cattoor *et al*. examines the probable sights of metabolism for the RIAA and TIAA in detail and suggests phase I (IAA and TIAA) and phase II (RIAA)

processes [212]. Additional pharmacokinetic information can be obtained with use of the *in silico* metabolism pathway prediction modeling software which suggested that the CYP2C9 was primarily responsible for the oxidation of the reduced IAA compounds [217]. Furthermore, a CYP-mediated metabolism prediction tool proposed the most probable sites of metabolism occurred at the end of both side chains on the four methyl groups for reduced IAA groups [218]. Further human *in vitro* and *in vivo* modelled studies are required to confirm such predictions.

Similar to previous IAA pharmacokinetic studies suggesting that *trans* isomers are metabolised at a quicker rate that the *cis* counterparts [226], altered ratios between analytes in the RIAA group were observed also, however no isomer or analog trend was obvious. As the RIAA concentrations peaked rapidly at 0.5 h, it is implied that the differences in the ratio profiles of analytes over the time points is most probably due to metabolism and excretion differences rather than variable absorption rates. Further studies may include larger doses of TIAA and HIAA in order to provide elimination profiles of the individual analytes contained within those groups also.

Although outside the scope of this study, more reduced IAA pharmacokinetic data may be achieved with the oral and intravenous dosing of pure reduced IAA formulations to humans with additional time points.

4.2.4.2 Applicability

Importantly, metabolism and modification of reduced IAA into other reduced IAA or IAA groups was not observed, indicating the specificity of the reduced IAA hop-derived beer marker model suitable. Besides possibly TIAA where males showed slightly higher levels, no

obvious or significant differences in concentration-time profiles were observed between genders in these preliminary drinking studies.

The RIAA study using LC-MS methodology to analyse the plasma of humans post-dosing with 700 mg of RIAA has not progressed onto other IAA or reduced IAA analytes and does not achieve the sensitivity required for application in forensic casework [213]. This current analytical technique has shown suitable sensitivity for the confirmation of relatively low levels of beer ingestion up to six hours post consumption of certain reduced IAA containing beers. The small number of beers containing HIAA and low concentrations of HIAA in those beers suggests that RIAA and TIAA are of the more important reduced IAA groups to monitor.

The development of an analytical method for the metabolites and/or conjugates of reduced IAA may allow for TIAA and HIAA determination when similar beer consumption volumes take place. Additionally, longer windows of detection and further information on the metabolism pathways of reduced IAA in humans may also be achieved. Urine methodology was not creatinine corrected, quantified results are approximate and as RIAA concentrations fluctuated throughout the six hours, caution should be applied when reporting urine results.

Presumably due to residual deposits following reduced IAA manufacture and consequently trace IAA levels in certain beers [192], some IAA analytes were detected in the Amstel and Coopers Clear beer studies in trace amounts. Following consumption of natural hopped beer it was shown that *trans*-IAA concentrations well above the lower limit of quantification were possible [226], this distinction may assist in determining if beer consumed is from brown or clear bottles.

As a result of a study performed where osteoarthritis, rheumatoid arthritis and fibromyalgia patients were prescribed a combination of reduced-IAA and other anti-inflammatory

substances [214, 230], there have been some supplementary medicines produced that contain RIAA and TIAA [233]. The particular doses of each are not described only that up to 225 – 370 mg of total reduced IAA content are in each tablet. To the best of the authors' knowledge these are not commonly used supplements and is the only use of reduced IAA outside of brewing. For these reasons, the use of these medicines as a defence tactic to mask authentic beer consumption seems unlikely.

Reduced IAA may be used in isolation or in conjunction with traditional IAA hopping techniques. Detection of reduced IAA in blood and urine may allow for discrimination between the ingestion of different bottled beers. Furthermore, determination of accurate IAA and reduced IAA concentrations in a range of popular, craft and homemade beers additional to current preliminary studies [232], would benefit forensic toxicologists in correlating blood IAA and reduced IAA results with the beer(s) suspected to have been consumed.

4.2.5 Conclusion

This study showed that the reduced IAA groups detected in the blood and urine correlated with the profiles of the beers consumed. The analytical methodology demonstrated the suitable sensitivity to confirm beer ingestion several hours post consumption of a variety of clear bottled beers. Whilst these studies are limited to five volunteers and three different types of beer, the detection of RIAA, TIAA and HIAA in blood and perhaps in other specimens is possible in forensic casework where typically large amounts of beer are consumed and the confirmation of beer and type of beer consumption is valuable.

CHAPTER 5.

POSTMORTEM PREVALENCE AND REDISTRIBUTION

Postmortem redistribution has been described as a “toxicological nightmare” [234]. Indeed, the assessment of any possible fluctuations in compound concentrations from body tissue to tissue is important to understand in order to interpret postmortem toxicological results. The analysis of a postmortem cohort, that also includes a range of specimen types, sites and collection times, can shed light on such phenomena. In addition, the prevalence of IAA, and therefore the prevalence of beer consumption, in authentic casework can be observed. The comparison of blood IAA and alcohol concentrations may demonstrate any correlation and allow for additional conclusions to be drawn.

CHAPTER 5.1

SPECIMEN COLLECTION IN VICTORIAN CORONIAL CASEWORK

In Victoria, a coroner's investigation is conducted in all unnatural, unexpected and suspicious deaths, to establish wherever possible the identity of the deceased, the circumstances surrounding the death and ultimately the cause of death. The Victorian Institute of Forensic Medicine (VIFM) is a statutory body providing the essential medical death investigation and forensic services roles on behalf of the coroner for the state. The autopsy includes full macroscopic and microscopic examination of the body and all major organs, anthropometric measurements, and observation of other distinguishing features identifying the state of disease or trauma, to assist in determine the cause of death. For further investigation to indicate the presence of alcohol, drugs or poisons, body fluids and tissues are collected for toxicological analysis.

Of the roughly 5000 medico-legal death investigations carried out at VIFM per year, toxicology is conducted in nearly 4500 of those cases. To expedite the death investigation process and optimise time and resources, the toxicology laboratory has recently reconstructed its systematic toxicological approach and offered a greater accepted more responsibility in the high throughput of results. Immediately upon admission of a deceased person to the mortuary, a mortuary admission blood specimen is taken from the femoral vein. By the following morning, the toxicology laboratory provides a semiquantitative result of drugs detected in this specimen. In light of these results and along with details of the medical history of the deceased and the circumstances surrounding the death, this plays an important role in determining whether or not a full autopsy or external examination is required. If an autopsy is required, an autopsy blood specimen is taken from preferably the femoral vein, along with a more comprehensive suite of postmortem tissue. When performing alcohol and drug analysis, testing and the reporting of results is generally given to the mortuary admission specimen, as compound concentrations are likely to be more closely representative of concentrations at the time of death.

Similar to conventional coronial processes, the autopsy can take up to a week until it is undertaken and allows for the comparison of different specimen collection times, sites and types. Importantly, this presents a unique situation that was exploited in subsequent chapters. The detection of the IAA compounds in different specimens and prevalence of beer consumption for the case can be performed, **Chapter 5.2**. Whilst the capacity to analyse multiple specimens from the same case provides the ability to comprehensively describe any postmortem redistribution phenomena, **Chapter 5.3**.

CHAPTER 5.2

IAA PREVALENCE IN POSTMORTEM SPECIMENS

Rodda, L.N., D. Gerostamoulos, and O.H. Drummer,
Detection of Iso-α-Acids to Confirm Beer Consumption in Postmortem Specimens.
Drug Testing and Analysis, 2014.
(Under Review, Appendix 1.6)

Monash University

Declaration for Thesis Chapter 5.2.

Declaration by candidate

In the case of Chapter 5.2., the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Located articles, reviewed articles and wrote the paper	85%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Gerostamoulos, D.	*Advised candidate regarding direction and content, reviewed article manuscript*	
Drummer, O.H.	*Advised candidate regarding direction and content, reviewed article manuscript*	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 26.6.16
Main Supervisor's Signature	[redacted]	**Date** 27/6/16

Abstract

Iso-α-acids (IAA) can be used as markers for the consumption of beer. Postmortem specimens from a range of coronial cases were analyzed for IAA in order to determine the prevalence of beer consumption and any correlation to blood alcohol concentrations (BAC). A total of 130 cases were included in this study including those where beer was mentioned in the case circumstances, cases where beer was not mentioned specifically but alcohol was detected, and cases where neither beer was mentioned nor a positive BAC was present. Available blood, serum, vitreous humor and urine specimens were analyzed. Of the 50 cases where beer was mentioned, 87% had one or IAA detected. In cases that only had a positive BAC (n = 60), 57% of these cases also showed the presence of these beer markers. Iso-α-acids were detected in specimens obtained from traumatized, burnt and decomposed cases with a mention of beer consumption or where BAC was positive in blood. No IAAs were detected in cases where BAC was negative. There was little or no correlation between blood IAA concentrations and BAC. This study demonstrates the possible detection of IAA as a marker for beer consumption.

Keywords

beer; ingredient congener; iso-α-acids; postmortem blood, urine, vitreous humor

Online Graphical Abstract

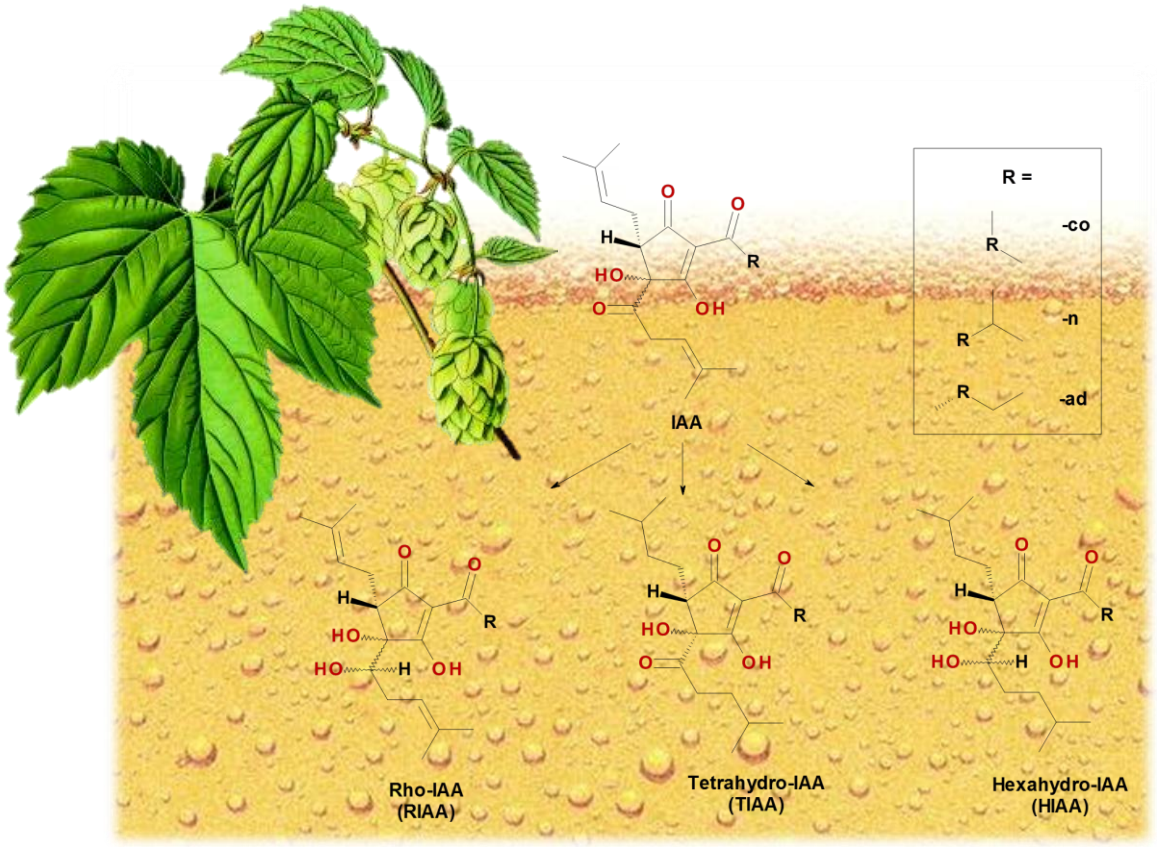


Fig. 14

Illustration of *Humulus lupulus* (L) and the structure of IAA groups

5.2.1 Introduction

Alcohol is the most widely consumed drug in the world with approximately two billion hectoliters produced worldwide in 2012 [160, 235]. Alcohol abuse is at hazardous and harmful consumption levels in many countries resulting in a global health issue and is one of the most common analytes in forensic case work including motor vehicle crashes and assaults [2, 4, 236, 237]. In some situations, the identification of which alcoholic beverage was consumed can assist the investigation and associated court case [159].

Compounds other than ethanol and water that are present in alcoholic beverages are termed congeners and may be detected in blood and urine following alcohol consumption, i.e. alcohol congener analysis (ACA). For many decades traditional ACA has determined the fermentation by-product congeners in blood to ascertain if the claims of alcoholic beverage(s) consumption by an individual was feasible, assisting in cases where after-drinking (or hip flask) defenses are involved [28, 31]. However this does not always determine the origin of the consumed ethanol and requires circumstantial evidence, generally provided by the offender [32, 33, 60, 159]. In death investigations this approach is limited to those compounds not being produced postmortem [238].

Recently, the authors developed a ultra high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) technique that was validated for the detection and quantification of several hop-derived iso-α-acids (IAA) and reduced IAA compounds [190]. These compounds are used in brewing for their bittering, bacteriostatic and foam stabilizing properties [165, 172]. However, they can also be used as beer-specific ingredient congeners that could confirm beer consumption. These substances have been detected in blood and urine of volunteers following controlled beer drinking studies [226, 239]. Depending on the type of IAA detected the type of beer consumed can also be identified [232].

This methodology has not yet been applied to authentic casework where such an approach may have advantages over, or compliment, traditional ACA techniques. Investigation into a variety of case scenarios that forensic toxicology laboratories are commonly presented (e.g. indication of beer consumption and positive BAC) is necessary in order to indicate IAA prevalence in different body tissues. Additional information such as any association between blood alcohol concentrations (BAC) and IAA concentrations is also required.

This study examines the presence of several IAA compounds in blood and other specimens taken from a selection of postmortem cases.

5.2.2 Experimental

5.2.2.1 Case and specimen selection

The Victorian Institute of Forensic Medicine performs medico-legal investigations of deceased cases reported to the Coroners Court of Victoria. The toxicology laboratory within the Institute receives specimens on admission of the body to the mortuary (generally femoral whole blood and serum) and also following an autopsy when it is conducted with a larger range of body specimens including blood, serum, urine and vitreous humor. Coronial, autopsy and toxicological data (e.g. demographics, cause of death, drug exposure, BAC etc.) was obtained using the Institute's case management system.

There were a total of 130 cases of varying cause of death that were selected and placed into one of three groups based on the following criteria:

A. beer mentioned in case circumstances (n = 50);
B. positive BAC (ethanol > 0.01 g/100mL) and beer not mentioned (n = 60), and;
C. cases where neither beer was mentioned nor alcohol was detected (n = 20).

The mortuary admission blood of the cases was analyzed first (or autopsy blood if mortuary admission was not available). If any IAA analytes were detected, subsequent testing of all other blood (mortuary admission and autopsy), serum (mortuary admission and autopsy), vitreous humor and urine specimens were conducted.

5.2.2.2 Ethical Approvals

Permission for this research was obtained by the Research Advisory Committee (RAC 013/13) and Human Research Ethics Committee (EC 07/2013) of the Victorian Institute of Forensic Medicine.

5.2.2.3 Specimens

For mortuary admission specimens, blood (and often serum) was collected as soon as practicable after a body was admitted to the mortuary. At autopsy, additional blood and serum specimens from the same deceased person were collected, along with vitreous humor and urine specimens. Mortuary admission blood was refrigerated (~4 °C) prior to analysis, all other specimens were stored frozen (-20 °C). All blood specimens were collected in 10 mL polypropylene tubes containing 1 % sodium fluoride/potassium oxalate. Serum was obtained by centrifugation for 10 min at 2400 × g. Unless otherwise stated all blood specimens were collected from the femoral region.

Antemortem specimens were obtained by the laboratory if collected when the deceased was hospitalized prior to death.

Drug-free specimens were used for instrument calibration and quality control purposes. Preserved blank blood (10 mL samples containing 200 mg sodium fluoride and 30 mg potassium oxalate) was obtained from a local blood bank (Melbourne, Australia). Blank urine was obtained from the authors after abstinence from beer and other alcoholic beverages for one week. A blank postmortem serum and vitreous humor was obtained from a case previously analysed that showed no IAA analytes. All blank specimens underwent additional screening to ensure there were no IAA analytes or other interferences. All blank specimens

were collected in polypropylene tubes (or containers for urine) and immediately frozen (-20 °C).

5.2.2.4 Chemicals and reagents

Reference standards for the natural IAA and reduced IAA were obtained from Labor Veritas (Zurich, Switzerland): IAA DCHA-Iso, ICS-I3 (containing 62.3 % w/w of *trans*-IAA); rho-IAA (RIAA), DCHA-Rho, ICS-R2 (containing 65.3 % w/w of *cis*-RIAA); tetrahydro-IAA (TIAA), Tetra, ICS-T2 (containing 99.4 % w/w of TIAA), and; hexahydro-IAA (HIAA), DCHA-Hexa, ICS-H1 (containing 65.7 % w/w of *cis*-HIAA).

The isotope labeled internal standard nimodipine-d_7 was purchased from PM Separations (Brisbane, Australia). Acetonitrile (ACN), methanol and formic acid were purchased from Merck (Melbourne, Australia). Ammonium formate was purchased from Sigma Aldrich (Sydney, Australia). All chemicals were of analytical grade or better and water was purified using a Milli-Q Ultrapure Water System from Waters (Sydney, Australia).

5.2.2.5 IAA and Reduced IAA Analytical Methodology

Natural IAA (*trans*-IAA and *cis*-IAA) and reduced IAA (RIAA, TIAA and HIAA) determination was performed using a previously published validated UHPLC-MS/MS method that was validated for blood analysis [190]. Briefly, the extraction consisted of a protein precipitation of 200 µL of whole blood using -20 °C ACN with the resulting supernatant dried under nitrogen and the residue reconstituted with 50 µL of a mixture of eluent A and eluent B (60:40, v:v). Preparation of stock solutions, calibration standards, quality controls, stability samples and extraction procedures were performed as published

previously [190]. For the analysis of serum, vitreous humor and urine, blood was replaced by the respective blank matrix in the calibration and quality control models. The availability of IAA reference standards allowed for the quantification of *trans*-IAA, RIAA, TIAA and HIAA groups. Residual *cis*-IAA in the *trans*-IAA reference standard was used to allow for qualitative *cis*-IAA results.

The UHPLC-MS/MS system comprised of a Shimadzu MS 8030 quadrupole mass spectrometer (Melbourne, Australia) operated in electrospray ionization negative mode. A Shimadzu Nexera UHPLC system (Melbourne, Australia) consisted of a degasser, two eluent pumps, a column oven (30 °C) with a Kinetex C_{18} column (3.0 × 150 mm, 2.6 μm from Phenomenx, Melbourne, Australia), and a chilled autosampler. For MS data evaluation, Shimadzu Postrun and Shimadzu Browser Analysis (Melbourne, Australia) softwares were used. The mobile phases consisted of 50 mmol/L aqueous:ACN (90:10) ammonium formate pH 2.8 (eluent A) and ACN containing 0.1 % formic acid (eluent B).

The *cis*-IAA group was unable to be quantified due to lack of specific reference standard. However to allow for comparisons to be made, the internal standard/area ratios of each *cis*-IAA analyte were summed to provide a total are ratio for the *cis*-IAA group and given the following qualitative ratings (area ratio: < 0.01 = +; 0.01-0.09 = ++; 0.10-0.49 = +++; > 0.5 = ++++).

5.2.2.6 BAC:IAA Correlation

The association between BAC and IAA concentrations was determined using a two-tailed Pearson test. All positive BAC cases from both Group A and B that contained a matching pair with either *trans*-IAA, *cis*-IAA, RIAA, TIAA and/or HIAA in non-decomposed whole

blood femoral specimens were considered. Additionally, correlation data were visually shown in a scatter plot to show if any obvious correlation existed between BAC and IAA results in these matching pairs.

5.2.2.7 Descriptive and Statistical analysis

All statistical analysis was performed using GraphPad Prism 5.04 from GraphPad Software (San Diego, USA). Descriptive statistics were performed on all specimens and cases in all cohorts, and summarized.

5.2.3 Results

Based on the described criteria there were 50, 60 and 20 cases analyzed for Groups A, B and C, respectively.

Table 17

A summary of the mean concentration and prevalence of the natural and reduced IAA groups in the whole blood, serum, vitreous humor and urine of cases in Groups A & B.

	Beer Mentioned (Group A)			**BAC Positive and Beer not Mentioned (Group B)**								
	Blood[1]		**Case**	**Blood[1]**		**Serum[1]**		**Vitreous Humor**		**Urine**		**Case**
	mean	Prevalence	Prevalence	mean	Prevalence	mean	Prevalence	mean	Prevalence	mean	Prevalence	Prevalence
trans-IAA (mg/L)	0.011	41 (72%)	38 **(76%)**	0.013	47 (58%)	0.027	20 (100%)	0.002	7 (35%)	0.002	10 (59%)	26 **(43%)**
cis-IAA (area ratio)	+++	50 (88%)	43 **(86%)**	+++	53 (65%)	+++	20 (100%)	+	18 (90%)	++	16 (94%)	32 **(53%)**
Natural IAA		50 (88%)	43 **(86%)**		53 (65%)		20 (100%)		18 (90%)		16 (94%)	32 **(53%)**
RIAA (mg/L)	0.005	7 (21%)	7 **(14%)**	0.006	5 (7%)	0.008	7 (35%)		0 (0%)		0 (0%)	7 **(12%)**
TIAA (mg/L)	0.010	21 (37%)	20 **(40%)**	0.019	26 (32%)	0.033	15 (75%)	Det[2]	1 (5%)		0 (0%)	18 **(30%)**
HIAA (mg/L)	0.020	1 (2%)	1 **(2%)**		0 (0%)	Det[2]	1 (5%)		0 (0%)		0 (0%)	1 **(25%)**
Reduced IAA		24 (48%)	23 **(52%)**		27 (33%)		16 (80%)		1 (5%)		0 (0%)	17 **(28%)**
Beer Positive[3]	50 (88%)		43 **(86%)**	54 (67%)		20 (100%)		19 (95%)		16 (94%)		34 **(57%)**
Total Tested	57		**50**	81		20		20		17		**60**

[1] mortuary admission and/or autopsy.

[2] Detected = Det

[3] Beer Positive = the detection of one or more of the natural and/or reduced IAA groups in any specimen provides the prevalence of beer in that case. Note: Available additional blood, serum, vitreous humor and urine specimens were only analyzed if the initial blood (generally the mortuary admission) for that case was positive for natural IAA and/or reduced IAA.

5.2.3.1 Prevalence

Specimens

The twenty specimens analyzed that had neither mention of beer consumption nor a positive BAC result (Group C) were all negative for natural IAA and reduced IAA analytes.

Table 17 shows the prevalence and mean concentrations of natural and reduced IAA by Groups. The concentration ranges for the IAA groups in all types of specimens ranged from trace levels to ~0.08 mg/L.

In all 125 instances when *trans*-IAA was detected in any blood specimen (both Group A and B), *cis*-IAA was also present. However, *cis*-IAA was detected in an additional 35 cases. This held true for all specimens; if *trans*-IAA was detected, *cis*-IAA was also present. The mean *trans*-IAA blood concentrations in Group A and B were not dissimilar, 0.011 and 0.013 mg/L, respectively ($P > 0.05$). Similarly, the average blood *cis*-IAA area ratio (+++) was qualitatively similar across the two groups of cases.

Of the cases where reduced IAA was detected, TIAA was shown to be the most prevalent followed by RIAA, with only one case in each cohort containing detectable HIAA. Only in one case of any group was reduced IAA detected without the presence of natural IAA; this case only contained a mortuary admission and autopsy blood specimen. On all other occasions at least *cis*-IAA were detected when RIAA, TIAA and/or HIAA were present.

The concentrations in serum were always higher than the corresponding blood specimen. All serum specimens analyzed contained natural IAA and had positive BAC. All bloods that were positive for a reduced IAA, also contained the appropriate reduced IAA in the serum specimen. However, two additional RIAA and an additional HIAA were detected during the serum screening.

There was a marked reduction in the prevalence and concentration of *trans*-IAA and more so for the reduced IAA groups in the vitreous humor specimens. Although the ability to detect *cis*-IAA in the vitreous humor specimens remained high (90%), the qualitative mean also decreased to trace levels (+). Similar prevalence and concentrations were seen in the urine specimens for the natural IAA. However, there was no reduced IAA detected in any of the urine specimens.

Overall, comparisons between the individual IAA groups were relatively similar to each other throughout the specimens in both groups (i.e. in order of prevalence *cis*-IAA > *trans*-IAA > TIAA > RIAA > HIAA). The prevalence and concentrations of all IAA groups between specimens decreased in order of serum > blood > vitreous humor > urine.

Cases

In Group A, natural IAA and reduced IAA were present in 86% and 52% of cases, respectively. In Group B, 53% and 28% of cases contained natural IAA and reduced IAA, respectively, and suggested that beer was consumed in 57% of these cases.

Table 18 summarizes the prevalence of natural and reduced IAA by age, gender, intent and condition of body in the two positive groups.

Both groups of cases tended to have the highest prevalence of beer consumption from below 30 years olds to the 40 – 49 age strata where 100% and 90% of cases were positive for beer consumption in Groups A and B, respectively. The consumption of beer decreased with age above 49 years old, although there were only a small number of cases in the over 70 age range. Natural IAA followed a similar trend however reduced IAA was more prevalent in younger ages (>30 – 39 years), close to similar levels to that of natural IAA.

Table 18

Prevalence of the natural and reduced IAA groups by age, gender, intent and condition of body in Groups A and B.

Characteristics		**Group A** (n = 50)				**Group B** (n = 60)				**Total** (n = 110)			
		n	Natural IAA (%)	Reduced IAA (%)	Beer Positive[1] (%)	n	Natural IAA (%)	Reduced IAA (%)	Beer Positive[1] (%)	n	Natural IAA (%)	Reduced IAA (%)	Beer Positive[1] (%)
Age (years)	<30	5	80	60	**80**	7	57	57	**57**	12	67	58	**67**
	30-39	5	80	20	**80**	12	67	50	**67**	17	71	41	**71**
	40-49	11	100	64	**100**	10	80	40	**90**	21	90	52	**95**
	50-59	13	92	54	**92**	8	38	0	**38**	21	71	33	**71**
	60-69	12	83	33	**83**	14	50	14	**50**	26	65	23	**65**
	≥70	4	50	25	**50**	9	33	11	**33**	13	38	15	**38**
Gender	Male	46	85	43	**85**	48	65	39	**70**	92	75	41	**77**
	Female	4	100	75	**100**	14	14	7	**14**	18	33	22	**33**
Intent	Natural	23	83	30	**83**	29	50	11	**50**	41	68	22	**68**
	Accident	15	100	60	**100**	18	67	50	**67**	33	82	55	**82**
	Suicide	5	60	20	**60**	9	44	56	**56**	14	50	43	**57**
	Undetermined	7	86	71	**86**	15	47	20	**47**	22	59	36	**59**
Body Condition	Normal	38	87	45	**87**	37	68	38	**73**	75	77	41	**80**
	Decomp	8	75	25	**75**	15	7	0	**7**	23	30	9	**30**
	Trauma	3	100	100	**100**	7	86	86	**86**	10	90	90	**90**
	Burnt	1	100	100	**100**	0				1	100	100	**100**
	Antemortem	3	67	0	**67**	1	0	0	**0**	4	50	0	**50**

[1] Beer Positive = the detection of one or more of the natural and/or reduced IAA groups in any specimen provides the prevalence of beer in that case.

A quarter of the BAC positive cohort were females (n = 14) however only 14% (n = 2) were positive for IAA. In contrast, 70% of the 48 males showed beer consumption through detection of IAA.

There was no apparent difference in prevalence of IAA by intent (natural, accident, suicide or undetermined).

IAA analytes were also detected in antemortem (clinical) specimens and in cases that were traumatized or burnt as well as in cases that showed significant decomposition (although there was a marked difference of IAA prevalence between the groups in the decomposed cases).

Group A Case Details

Table 19 details all of the individual results such as BAC and IAA content by age, sex, cause of death and a brief account of the relevant circumstances in all 50 Group A cases. Forty three (86%) of these cases detected natural and/or reduced IAA beer markers with circumstances indicating a range of bottled and canned beers.

The seven cases not positive for beer consumption had circumstances that described beer being located only in the vicinity of the deceased and may simply be associated with the case but not necessarily directly consumed prior to death. Of these seven cases, 4 had negative BAC and 3 contained positive BAC (cases 6, 7 & 49). Cases 7 and 49 were decomposed and with BAC of 0.01 and 0.04 g/100mL, respectively, where postmortem bacterial ethanol production was a likely reason for the BAC.

There were 16 cases that had a negative BAC, 13 of these were found to have beer consumed with *cis*-IAA detected in all of those cases. However, only 8 of these detected *trans*-IAA with a relatively low mean concentration of 0.002 mg/L. TIAA was detected in two cases at low concentrations. The highest of these concentrations was Case 46 having a *trans*-IAA of just 0.005 mg/L.

Case 13 was a male with brown bottled glass present around a chest injury that occurred during a motor vehicle collision. This may possibly indicate contamination of bodily tissue with beer contained within the bottle. The femoral blood was both positive for BAC and natural IAA beer markers. Toxicology testing had confirmed a similar BAC level (to that of the blood) in the vitreous humor confirming alcohol consumption prior to death. Furthermore, TIAA was also present, a reduced IAA used for green or clear bottled consumption. In conjunction with the positive natural IAA determination, this suggests consumption of at least colored bottled beer prior to death. Unfortunately, the vitreous humor was unavailable for IAA analysis.

In Cases 34 and 43,antemortem specimens obtained prior to death (in hospital). showed trace levels of natural IAA. The circumstances associated with these two cases described heavy drinking the night before (Case 34) and just one beer consumed ~6 h prior to death (Case 43).

The highest *trans*-IAA concentration was 0.056 mg/L for case 11 where the deceased was said to have consumed ~12 long neck beers (a 750 mL bottle) a day. It was also described that of the day of death a brown bottled beer that utilizes traditional natural IAA hopping techniques was consumed. As such, additional beer consumption from beer containing reduced IAA was likely to have also been consumed as 0.003 mg/L of TIAA was also detected.

Eight cases also mentioned other types of alcoholic beverages possibly indicating co-consumption with beer, specifically: wine (Cases 36, 45 and 48), whiskeys including "scotch" and "bourbon" (Cases 20, 21, 33, 37), and rum (case 23). This may demonstrate the ability to detect IAA even if co-consumption of other beverages has taken place.

Table 19

Details of 50 cases where beer was mentioned in the case circumstances (Group A).

ID	Age/sex	Intent	Cause of Death	Quality	Femoral Blood	BAC (g/100mL)	Natural IAA		Reduced IAA			Beer Positive[2]	Circumstances[3]
							trans-	*cis-*	RIAA	TIAA	HIAA		
1	F /61	Acc	Alcohol & drug toxicity	N	Admin	0.02	**0.010**	+++				✓	Alcoholic. 1 x brown bottled beer located
2	M /62	Nat	Heart disease	N	Admin	0.03	**0.008**	+++		**0.008**		✓	Consumed 3 heavy beers night before death
3	M /53	Nat	Heart disease	N	Admin	0.02	**0.007**	+++		**0.001**		✓	Alcoholic. Consumed ½ to 1 cartons of beer per day
4	M /51	Nat	Heart disease	N	Admin	ND	**0.002**	++				✓	Consumes 24 beers per day
5	M /58	Unk	Heart disease	N	Admin	0.08	**0.026**	+++ +	**0.001**	**0.001**		✓	Alcoholic. 3 slabs of brown bottled beer[3] per week
6	M /28	Sui	Hanging	N	Admin	0.08							Beer cans located
7	M /37	Sui	CO poisoning	D	Admin	0.01							Half empty beer bottle located
8	M /60	Unk	Heart disease	B	Autopsy Heart	0.01	**0.006**	++	**0.004**	**0.014**		✓	1 x empty brown bottled mid-strength beer[3] located
9	M /48	Sui	CO poisoning	D	Admin	0.10	**0.015**	+++	**0.011**	**0.001**		✓	3 x empty 750 mL bottles of brown bottled beer[3] located
10	F /52	Nat	Alcohol & drug toxicity	N	Admin	0.19	**0.014**	+++		**0.001**		✓	Alcoholic. Consumed several stubbies and perhaps other type of alcohol
11	M /71	Unk	Heart disease	N	Admin	0.11	**0.056**	+++ +		**0.003**		✓	Alcoholic. Consumes ~12 long neck beers a day. Day of death = brown bottled beer
12	M /65	Nat	Heart disease	N	Admin	0.09	**0.021**	+++		**0.031**		✓	Consumed 7 x mid-strength beers
13	M /42	Acc	Motor vehicle collision	T	Admin	0.19 (0.17)[#]	**0.025**	+++ +		**0.003**		✓	Brown beer bottle glass present around chest injury
14	M /25	Unk	Drug toxicity	N	Admin	0.02	**0.003**	+++		**0.003**		✓	Regularly consumes ~8 beers a day
15	M /47	Sui	Hanging	N	Admin	ND		**(+)**				✓	Beer cans & bottles located
16	M /45	Unk	Alcohol & drug toxicity	D	Admin	0.23	**0.005**	++	**0.001**			✓	Consumes a minimum of 6 beers per day
17	M /42	Acc	Haemoperitoneum & liver cirrhosis	N	Admin	0.11	**0.003**	++				✓	Alcoholic. ~20 empty brown bottled beer[3] cans & empty wine cask located
18	F /19	Acc	Drug toxicity	N	Admin	0.08	**0.001**	++		**0.001**		✓	Empty beer bottle located
19	M /57	Nat	Liver cirrhosis	D	Autopsy Cavity	0.07	**0.002**	++				✓	Alcoholic. Beer cans located near deceased
20	M /58	Nat	Heart disease	N	Admin	0.27	**0.009**	+++				✓	Consumed 4 beers & unknown quantity of scotch during the 6 h prior to death
21	M /77	Nat	Aortic aneurysm	N	Admin	ND							Known to drink beer & bourbon

ID	Age/sex	Intent	Cause of Death	Quality	Femoral Blood	BAC (g/100mL)	Natural IAA		Reduced IAA			Beer Positive[2]	Circumstances[3]
							trans-	*cis-*	RIAA	TIAA	HIAA		
22	M /60	Nat	Heart disease	N	Admin	0.02	**0.008**	++				✓	Can of beer on side table
23	M /77	Nat	Heart disease	N	Admin	ND		**(+)**				✓	Empty beer stubby & ¾ full bottle of rum located
24	M /49	Nat	Heart disease	N	Admin	ND	**0.001**	++		**0.002**		✓	Consumes 2 beers each day
					Autopsy			**(++)**		**0.001**		✓	
25	M /60	Nat	Heart disease	N	Admin	0.04	**0.005**	++		**0.004**		✓	Consumes 20-30 pots of beer daily
26	M /45	Nat	Acute pancreatitis	N	Admin	ND	**0.003**	++				✓	Consumed 10 x 375 mL brown bottled beer[3] cans a day prior to death
27	M /40	Acc	Drug toxicity	N	Admin	0.07	**0.021**	+++ +	**Det[1]**			✓	Consumed beer
28	M /51	Acc	Alcohol & drug toxicity	N	Admin	0.13	**0.009**	+++		**0.020**	**0.020**	✓	Stubby of beer located
29	M /60	Nat	Chronic alcoholism	N	Admin	ND	**0.001**	+				✓	Alcoholic. Empty beer cans located
					Autopsy		**0.001**	+				✓	
30	M /69	Nat	Abdominal aortic aneurysm	D	Admin	0.05		**(+)**				✓	Beer cans located
					Autopsy			**(++)**				✓	
31	M /52	Sui	Hanging	N	Admin	0.18	**0.017**	+++ +	**0.010**			✓	Beer cans located
32	M /55	Acc	Drug toxicity	N	Admin	0.07	**0.013**	+++ +	**Det[1]**	**0.030**		✓	30 empty beer cans located
33	M /85	Nat	Spine operation complications	N	Ante-mortem	ND							2 beers & 5 whiskeys 48 h prior to death due to fall & subsequent surgery
34	M /53	Acc	Head injury	N	Ante-mortem	0.22	**Det[1]**	++				✓	Consumed 1 slab of beer within day, seizing at 16:30, hospitalised at 18:30, died next day
35	M /35	Acc	Aviation accident	T	Autopsy	0.06	**0.008**	+++		**0.010**		✓	Beer consumed for some time prior to piloting plane
36	M /49	Acc	Drug toxicity	N	Admin	0.08	**0.002**	++		**Det[1]**		✓	Consumed ~6 beers & 2 L of cask wine
37	M /29	Acc	Train incident	T	Admin	0.26	**0.043**	+++ +		**0.040**		✓	Consumed a large quantity of beer & whiskey 5 h prior to death
38	M /28	Acc	Drug toxicity	N	Admin	ND	**Det[1]**	++				✓	Consumed ~11 stubbies of beer over a 12 h period prior to bed, died ~14 h later
39	M /64	Nat	Heart disease	N	Admin	ND							Light drinker. Consumed beer once a week
40	M /55	Nat	Heart disease	N	Admin	ND	**0.002**	++		**0.002**		✓	½ glass of beer ~8 hours prior to death
41	F /48	Acc	Alcohol & drug toxicity	N	Admin	0.17	**0.004**	+++		**0.010**		✓	Consumed at least ½ a slab of beer during day prior to falling over & hitting head

ID	Age/sex	Intent	Cause of Death	Quality	Femoral Blood	BAC (g/100mL)	Natural IAA		Reduced IAA			Beer Positive[2]	Circumstances[3]
							trans-	*cis-*	RIAA	TIAA	HIAA		
42	M /58	Nat	Heart disease	N	Admin	0.03		**(++)**				✓	Alcoholic. 1 empty slab & 3 unopened slabs located
					Autopsy		**0.001**	++				✓	
43	M /33	Unk	Unascertained	N	Ante-mortem	ND		**(+)**				✓	Consumed 1 heavy beer 355 mL bottle with meal ~6 h prior to death
					Admin	ND		**(+)**				✓	
44	M /57	Nat	Heart disease	N	Autopsy	ND							Consumed 1 light beer with meal ~2 h prior to death
45	M /48	Nat	Pulmonary embolism	N	Autopsy	ND	**0.001**	++				✓	Consumed beer & wine night prior to death
46	M /67	Nat	Heart disease	D	Admin	ND	**0.005**	++				✓	Alcoholic. Many empty beer bottles located
47	M /68	Nat	Heart disease	D	Admin	0.05	**0.004**	+++				✓	Beer can & bag of empty cans located
					Autopsy		**0.015**	+++				✓	
48	M /33	Acc	Drug toxicity & anabolic steroid use	N	Admin	ND		**(+)**				✓	Beer & wine bottles located
49	M /64	Nat	Heart disease	D	Autopsy	0.04							Empty beer bottles located
50	M /33	Acc	MVA pass	N	Admin	0.13	**0.039**	+++ +				✓	~3-4 beers claim to have been consumed
					Autopsy		**0.030**	+++ +				✓	

[1] Detected = Det

[2] Beer Positive = the detection of one or more of the natural and/or reduced IAA groups in any specimen provides the prevalence of beer in that case.

[3] Specific brands of beer have been de-identified

Intent (Nat = natural, Acc = accident; Unk = undetermined);

Cause of Death (Carbon monoxide = CO; Motor vehicle accident unrestrained passenger = MVA pass);

Quality = Quality of body/specimens (N = normal; D = decomposed; T = traumatized; B = burnt);

Femoral Blood (Admin = collection on admission to mortuary);

BAC = blood alcohol concentration (ND = ethanol not detected < 0.01 g/100mL; # = Vitreous Humor result);

parenthesis = *cis*-IAA only detected

"Stubby" is colloquially known in Australia as a small 375 mL bottle of beer; "long neck" = 750 mL bottle; Carton/slab = pack of 24 beers;

5.2.3.2 Correlation of IAA:BAC

Fig. 15 shows a plot of individual BAC and matched *trans*-IAA, *cis*-IAA, RIAA and TIAA results for all cases analyzed in non-decomposed femoral whole blood specimens. These data showed essentially no correlation between IAA groups, although there was a weak correlation for TIAA (r^2 0.169, p = 0.02).

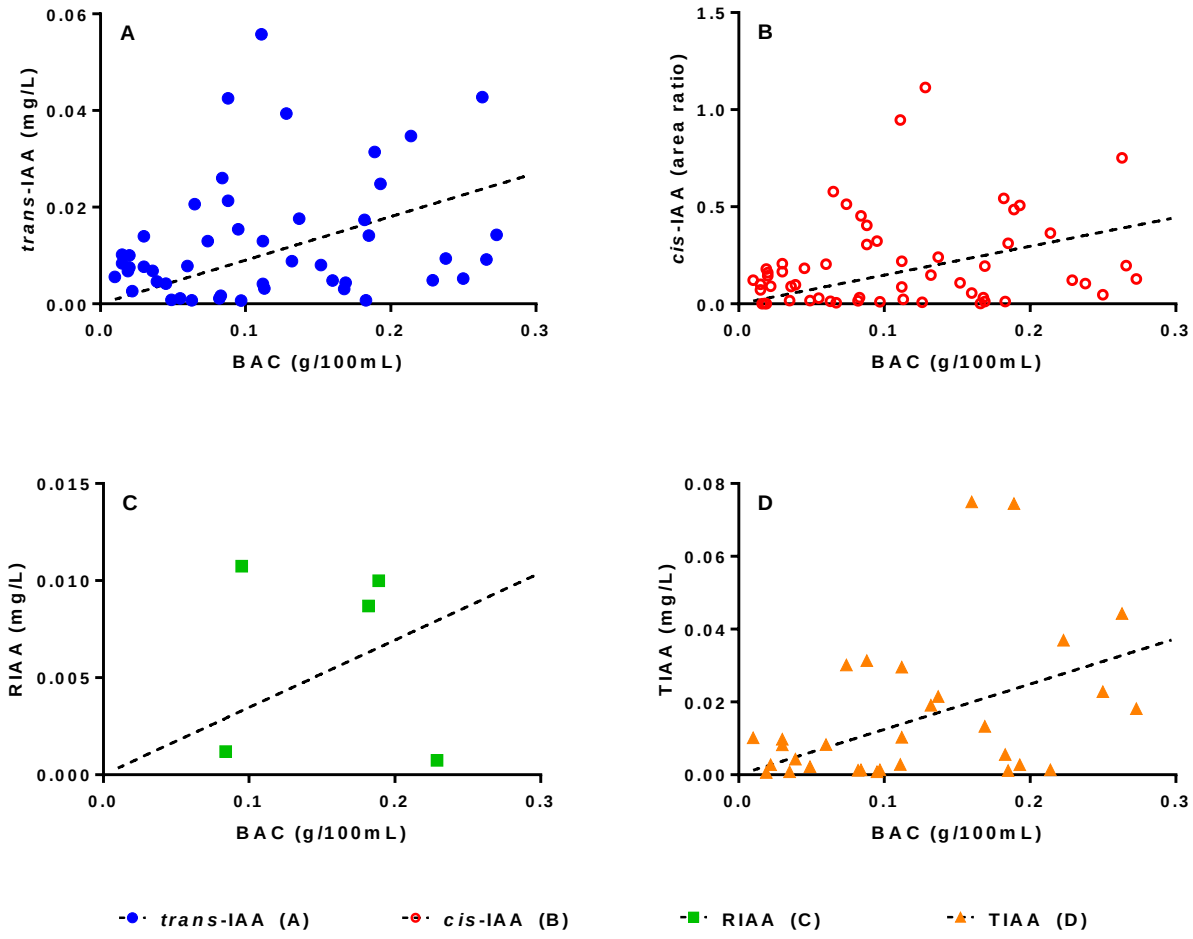
There were no substantial *trans*-IAA and TIAA concentrations (> ~0.01 mg/L) when BAC was ~0.05 g/100mL or less. In contrast, it is possible to have low IAA concentrations even if BAC are high. This is demonstrated with the clustering of points along the x-axis.

5.2.4 Discussion

This validated methodology that uses natural IAA and reduced IAA groups as ingredient congeners to confirm beer consumption, has analyzed two controlled beer pharmacokinetic studies, and stability investigations [190, 226, 231, 239]. This technique can be important in forensic science when a confirmation of circumstances is required or even to complement existing congener analysis to determine what beverage may have been consumed after an incident. The detection of *trans*-IAA, *cis*-IAA, or both, demonstrates natural IAA presence. Similarly, the detection of RIAA, TIAA and/or HIAA determines the presence of reduced IAA. If a case had natural IAA, reduced IAA, or both, in one or more specimens, it has been shown that beer had been consumed at some time prior to death (Beer Positive). This approach also may be used in broader applications. The routine reporting of beer consumption in clinical and forensic toxicology may assist in the circumstances, and outcomes, of many types of medico-legal situations.

Fig. 15

Scatter plot and correlation analysis[1] of BAC with presence of either *trans*-IAA, *cis*-IAA, RIAA and/or TIAA in non-decomposed femoral whole blood specimens for all cases. A weak positive association is shown between increasing BAC and IAA results.



	BAC vs. *trans*-IAA	BAC vs. *cis*-IAA	BAC vs. RIAA	BAC vs. TIAA
Pairs	48	56	5	31
R^2	0.064	0.051	0.010	0.169
p value[1]	0.082	0.096	0.873	0.022*

R^2 = coefficient of determinations

[1]two-tailed Pearson matched pairs correlation test. *$p < 0.05$

Note: HIAA had too few pairs (n = 1) to perform correlation analysis.

Besides ACA, other related drink driving scenarios such as claims to have one's drink laced (i.e. drink spiking), is another highly used defence in Germany and Sweden to explain an elevated BAC [30, 59]. Similarly, in drug facilitated sexual assaults where drink spiking has been claimed, ethanol is the most common drug detected, and more often than not the only drug detected [53, 55, 56]. It is not uncommon for additional alcohol to be added to the beverage of an unsuspecting victim and the excess alcohol consumption to be the underlying factor for assisting in the assault [50-52, 57, 240]. Should a considerable amount of IAA be shown to be present, this may verify a high rate of beer consumption and assist in such cases.

It is currently possible to differentiate antemortem ethanol obtained by ingested of alcoholic beverages to that of post-mortem bacterial production through ethyl glucuronide detection [60]. However this marker of antemortem ethanol ingestion has been found to be unstable under post-mortem putrefaction conditions [64]. This study shows the ability to confirm the presence of IAA in decomposed specimens and may provide an additional tool to confirm if the ethanol is genuine or a postmortem artifact.

The circumstances provided in this study do not explicitly distinguish between acute beer intake and beer consumption for possibly days prior to death. It could be feasible that a higher than 86% agreement rate for beer consumption in Group A could be achieved using only cases when acute beer ingestion was confirmed. Nonetheless these results show the ability to detect the beer consumption when beer is indeed mentioned in the case circumstances and suggests applicability in authentic settings.

Our study showed that over half of the cases (57%) that were recruited only on the basis of having a positive BAC showed markers of beer consumption. This is comparable to the current trend of apparent alcohol beverage consumption that suggests that ~41% of all alcoholic beverages consumed in Australia are beer products which reflects beer being the

most popular alcoholic beverage, ranked fourth internationally for beer consumption (~110 L per capita annually) [235, 241].

The qualitative analysis of the *cis*-IAA group is shown here to be advantageous as they were detected in ~10% of all beer positive cases, when no other IAA was detected. However, due to the sensitivity for *cis*-IAA detection, the prolonged window of detection for this group may therefore not definitively demonstrate acute beer consumption, particularly if at trace levels.

A high number of cases that had a positive BAC and aged between 40 and 49 years were also shown to have consumed beer (90%), considerable higher than the other age groups. Reduced IAA were more common in younger years, a possible direct reflection of how clear bottled beer is marketed to these generations [242].

This study shows that free and unchanged IAA are also found in high concentrations in serum and low concentrations in vitreous humor and urine specimens, which therefore can be used to compliment any blood-derived results in postmortem cases.

Reduced IAA are commonly used in clear, and in in conjunction with natural hopping techniques in green bottles [232]. Their presence in the demonstrated casework suggests either clear and/or green bottled consumption. The single case where only reduced IAA was detected suggests it was unlikely that brown bottled beer was consumed, at least in significant amounts.

Trace levels of natural IAA can also be found in beers using only reduced IAA bittering products due to residual amounts remaining following the synthesis of reduced IAA [232]. It was recently shown that volunteers who consumed only clear bottled beer that contained trace amounts of natural IAA, resulted in trace levels of natural IAA (mainly *cis*-IAA) in their blood in the following hours [239]. Additionally, many green bottled beers contain significant

levels of both natural and reduced IAA [232] which may explain the high rate of natural IAA detection when reduced IAA was also detected in these studies, particularly for very low natural IAA levels. Although the co-consumption of brown and clear (or green) bottled beer cannot be ruled out if both natural and reduced IAA are detected, it at least confirms the consumption of clear or green bottled beer.

Of the reduced IAA groups, monitoring of TIAA seems to be of most importance followed by RIAA, with only one case in each cohort detecting HIAA analytes. These profiles are a reflection by the prevalence of reduced IAA in a range of common beers, of which TIAA and RIAA are the most commonly used reduced hop products [232].

RIAA and TIAA have been used to treat patients suffering rheumatic disorders [214, 230]. A supplementary medicine has been produced that containing a combined total of 225-370 mg of RIAA and TIAA [233]. This is not a commonly used supplement and is the only apparent use of reduced IAA outside of brewing.

Previous natural IAA and reduced IAA pharmacokinetic studies described a relatively similar concentration-time profiles to that of alcohol [226, 239]. These controlled drinking studies involved measured quantities of beer and showed good correlations between BAC and IAA concentrations when measured over several hours post consumption. Not surprisingly on a population scale where such controls are not possible there are only (at best) weak associations between blood alcohol and IAA concentrations. This will be caused by factors such as concurrent use of other alcoholic beverages, variable ethanol and IAA congener profiles between beers, and varying pharmacokinetics (e.g. adsorption, half-life) between ethanol and IAA [226, 239]. Additionally, the possible alcohol intake from multiple sources (that do not contain IAA), may explain the tendencies of the correlations to trend towards the x-axis where higher BAC but low IAA concentrations lie. Such variables will not allow back-

calculations to be performed. Additionally, there may be other changes in a postmortem context such as postmortem redistribution that may affect tissue concentrations. At least in this cohort, it was shown that IAA concentrations higher that ~0.01mg/L contained significant BAC levels.

5.2.5 Conclusion

The analytical methodology demonstrated the suitable sensitivity and selectivity to detect IAA in postmortem specimens and help to confirm beer ingestion. Using this novel approach, the high prevalence of beer consumption in society was demonstrated with over half of all alcohol positive cases confirming beer intake sometime prior to death. Such examination of cases provides a greater understanding of the typical detection rates of IAA type compounds in authentic casework. Although serum showed higher concentrations, the analysis of whole blood also proved suitable. As is common in many aspects of forensic toxicology, postmortem phenomena demand further investigations and make back-calculations of drugs, or alcohol, a challenging task.

CHAPTER 5.3

POSTMORTEM REDISTRIBUTION OF IAA

Rodda, L.N., D. Gerostamoulos, and O.H. Drummer,
The Postmortem Redistribution of Iso-α-acids in Postmortem Specimens.
Forensic Science, Medicine, and Pathology, 2014.
(Under Review, Appendix 1.7)

Monash University

Declaration for Thesis Chapter 5.3.

Declaration by candidate

In the case of Chapter 5.3., the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Located articles, reviewed articles and wrote the paper	85%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Gerostamoulos, D.	*Advised candidate regarding direction and content, reviewed article manuscript*	
Drummer, O.H.	*Advised candidate regarding direction and content, reviewed article manuscript*	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 26.6.14
Main Supervisor's Signature	[redacted]	**Date** 27/6/14

Abstract

Iso-α-acids (IAA) and reduced IAA can be used as beer-specific ingredient congeners to confirm beer consumption when detected in blood and other specimens using a UHPLC-MS/MS method. Recent analysis of postmortem casework demonstrated a high prevalence of beer consumption and the possibility of providing the source of alcohol in forensic casework. Research outlined in this manuscript has examined the degree to which the interval after death and quality of blood affects the concentration of IAA in postmortem cases. Postmortem whole blood and serum were analysed in cases where natural or reduced IAA groups were detected. The *trans*-IAA, *cis*-IAA and TIAA groups were subject to postmortem redistribution (PMR), although only weakly associated with the length of time from death to collection of specimens. Serum had 3-fold higher concentrations than blood for *trans*-IAA, *cis*-IAA and TIAA. These studies confirm that although postmortem concentrations cannot be easily compared to concentrations found in living persons but the presented findings do provide some understanding to assist the interpretation where the confirmation of beer consumption is required in forensic casework.

Keywords

beer; ingredient congener; iso-α-acids; postmortem; postmortem redistribution; blood, serum

5.3.1 Introduction

Alcohol consumption results in a significant increase in deaths, hospitalizations and alcohol-related crimes and is reflected domestically with the recent findings that spotlight the dangers of alcohol-fuelled violence [2, 243]**.** Responsible for ~4.7 L of the pure alcohol ingestion per capita annually, beer is the most commonly consumed alcoholic beverage and third most popular overall beverage following water and tea [235, 244]**.**

Iso-α-acids (IAA) and three structurally similar but chemically-altered IAA known as reduced IAA provide the bitter properties in beer. However they can also be used as beer-specific ingredient congeners to confirm beer consumption. In the authors' laboratory, a protein precipitation extraction and ESI-UHPLC-MS/MS method was developed and validated for the detection of these compounds in blood [190]. The long-term stabilities of these analytes in stored blood specimens was assessed over 8 weeks with freezing (-20 °C) and refrigeration (4 °C) conditions determined as acceptable [231]. The analysis of the blood and urine from volunteers consuming a range of brown and clear bottled beers in small amounts in controlled drinking studies demonstrated successful bioavailability of these compounds [226, 239]. Additional pharmacokinetic data demonstrated small inter-variable differences in concentration-time profile, quick absorption rates and half-lives ranging between ~30-46 minutes.

Recently this methodology was applied to the human tissue from forensic postmortem cases to determine the ability to detect natural IAA and reduced IAA groups and ultimately to demonstrate the prevalence of beer consumption [245]. Nearly 90% of all cases that had "beer" mentioned in the circumstances or autopsy report of the cases, contained an IAA beer marker. It was further shown in a separate cohort 57% of cases that had no mention of beer

ingestion but contained a positive alcohol concentration, demonstrated beer consumption prior to death. Such data demonstrates the high prevalence of beer consumption in Australia.

Further investigation of postmortem casework is **n**ecessary in order to obtain information such as postmortem redistribution (PMR) [246] and the comparison between serum and whole blood concentrations. This study aims to examine such postmortem phenomena to provide the relevant toxicology data required in order to interpret and confirm beer consumption when IAA type compounds are detected in casework.

5.3.2 Experimental

5.3.2.1 Case and Specimen Selection

The Victorian Institute of Forensic Medicine performs medico-legal investigations of deceased cases reported to the Coroners Court of Victoria. The toxicology laboratory within the Institute receives specimens on admission of the body to the mortuary (generally femoral whole blood and serum) and also following an autopsy when it is conducted with a larger range of body specimens including another whole blood and serum. Coronial, autopsy and toxicological data (e.g. demographics, cause of death, drug exposure, BAC etc.) were obtained using the Institute's case management system. No decomposed cases were considered for analysis.

5.3.2.2 Ethical Approvals

Permission for this research was obtained by the Research Advisory Committee (RAC 013/13) and Human Research Ethics Committee (EC 07/2013) of the Victorian Institute of Forensic Medicine.

5.3.2.3 Specimens

For mortuary admission specimens, blood (and often serum) was collected as soon as practicable after a body was admitted to the mortuary. At autopsy, additional blood and serum specimens from the same deceased person were collected. Mortuary admission blood was refrigerated (~4 °C) prior to analysis, all other specimens were stored frozen (-20 °C). All specimens were collected in polypropylene tubes with all whole blood specimens containing 1% sodium fluoride/potassium oxalate preservative. Serum was obtained by centrifugation for 10 min at 2400 × g. Blood was collected from the femoral region unless otherwise indicated.

Drug-free specimens were collected for instrument calibration and quality control purposes. Preserved blank blood (10 mL samples containing 200 mg sodium fluoride and 30 mg potassium oxalate) was obtained from a local blood bank (Melbourne, Australia). A blank postmortem serum was obtained from a case previously analyzed that showed no IAA analytes. All blank specimens underwent additional screening to ensure there were no IAA analytes or other interferences. All blank specimens were collected in polypropylene tubes and immediately frozen (-20 °C).

5.3.2.4 Chemicals and reagents

The details of the reference standards for the natural IAA and reduced IAA obtained from Labor Veritas (Zurich, Switzerland) are: IAA DCHA-Iso, ICS-I3 (containing 62.3 % w/w of *trans*-IAA); rho-IAA (RIAA), DCHA-Rho, ICS-R2 (containing 65.3 % w/w of *cis*-RIAA); tetrahydro-IAA (TIAA), Tetra, ICS-T2 (containing 99.4 % w/w of TIAA), and; hexahydro-IAA (HIAA), DCHA-Hexa, ICS-H1 (containing 65.7 % w/w of *cis*-HIAA).

The isotope labeled internal standard nimodipine-d_7 was purchased from PM Separations (Brisbane, Australia). Acetonitrile (ACN), methanol and formic acid were purchased from Merck (Melbourne, Australia). Ammonium formate was purchased from Sigma Aldrich (Sydney, Australia). All chemicals were of analytical grade or better and water was purified using a Milli-Q Ultrapure Water System from Waters (Sydney, Australia).

5.3.2.5 IAA and reduced IAA analytical methodology

Natural IAA (*trans*-IAA and *cis*-IAA) and reduced IAA (RIAA, TIAA and HIAA) determination was performed using a previously published UHPLC-MS/MS method that was validated for blood analysis [190]. Briefly, the extraction consisted of a protein precipitation of 200 µL of whole blood using -20 °C ACN with the resulting supernatant dried under nitrogen and the residue reconstituted with 50 µL of a mixture of eluent A and eluent B (60:40, v:v). Preparation of stock solutions, calibration standards, quality controls and extraction procedures were performed as published previously [190]. Blank serum replaced whole blood in the calibration and quality control models **for** the analysis of serum casework. The availability of IAA reference standards allowed for the quantification of *trans*-IAA, RIAA, TIAA and HIAA groups. Residual *cis*-IAA in the *trans*-IAA reference standard was used to allow for qualitative *cis*-IAA results.

The *cis*-IAA group was unable to be quantified due to lack of specific reference standard. However to allow for comparisons to be made, the internal standard/area ratios of each *cis*-IAA analyte were summed to provide a total are ratio for the *cis*-IAA group.

The UHPLC-MS/MS system comprised of a Shimadzu MS 8030 quadrupole mass spectrometer (Melbourne, Australia) operated in electrospray ionisation negative mode. A Shimadzu Nexera UHPLC system (Melbourne, Australia) consisted of a degasser, two eluent pumps, a column oven (30 °C) with a Kinetex C_{18} column (3.0 × 150 mm, 2.6 μm from Phenomenex, Melbourne, Australia), and a chilled autosampler. For MS data evaluation, Shimadzu Postrun and Shimadzu Browser Analysis (Melbourne, Australia) softwares were used. The mobile phases consisted of 50 mmol/L aqueous:ACN (90:10) ammonium formate pH 2.8 (eluent A) and ACN containing 0.1 % formic acid (eluent B).

5.3.2.6 Statistical analysis

All statistical analysis was performed using GraphPad Prism 5.04 from GraphPad Software (San Diego, USA).. The median, range and population size (n) was reported and $p < 0.05$ was considered statistically significant for all analyses

Postmortem redistribution

To demonstrate PMR any changes in natural and reduced IAA median concentrations between mortuary admission (ADM) and autopsy (AUT) bloods was determined. The median postmortem interval (time of death – time of collection, PMI), median concentrations and their ranges, and the AUT/ADM ratio were examined. The Wilcoxon matched-pairs signed rank test ($p < 0.05$) was performed on the concentrations of natural and reduced IAA in

femoral whole bloods from cases that had five or more paired mortuary admission and autopsy specimens.

Additionally any correlation between the pre-autopsy interval (collection time difference between mortuary admission and autopsy specimens) and changes in IAA concentrations for the same cases were plotted to demonstrate if PMR was influenced by a prolonged or delayed specimen collection. The following formula was used to evaluate the change in concentration [%] between ADM and AUT specimens:

$$\frac{\mathrm{Conc(AUT)} - \mathrm{Conc(ADM)}}{\mathrm{Conc(ADM)}} \times 100 = \Delta\mathrm{Conc}\ [\%]$$

If ΔConc [%] > 0 an increase in concentration was observed between ADM and AUT blood specimens, if ΔConc [%] < 0 a decrease in concentration was observed between ADM and AUT blood specimens.

Finally, any influence of different blood collection sites on the concentrations of natural and reduced IAA were accessed. Femoral and non-femoral (heart, cavity and subclavian) specimens were compared in paired specimens with positive IAA groups.

Serum/blood ratios

The serum to blood ratios (S/B) for the natural and reduced IAA groups were estimated. Comparison of whole blood and serum specimens collected only from the femoral region at admission to the mortuary admission were considered in order to determine if a significant difference exists in the IAA median concentrations by utilizing a Wilcoxon two-tailed matched-pairs test.

5.3.3 Results

The analysis of a large cohort of authentic casework provided for the statistical examination for *trans*-IAA, *cis*-IAA and TIAA in most test parameters. Unfortunately, the low prevalence of the RIAA and HIAA ($n < 5$) did not allow these IAAs to be considered. However, these three IAA groups were largely similar in results between each of the test parameters.

Table 20 shows the *trans*-IAA, *cis*-IAA and TIAA median concentrations for ADM and AUT bloods. There was a significant difference between the specimens for *trans*- and *cis*-IAA which had AUT/ADM ratios of 1.6 and 1.7, respectively. The median PMI (time of specimen collection - time of death) for the natural IAA were similar for the ADM and AUT specimens at ~0.4 and 4.8 days, respectively. However, TIAA showed no difference in median concentrations and had a median AUT collection time approximately a day earlier than the natural IAA group.

Table 20

Comparison of mortuary admission (ADM) and autopsy (AUT) whole blood femoral specimens for *trans*-IAA, *cis*-IAA and TIAA concentrations.

		***trans*-IAA (mg/L)**	***cis*-IAA (area ratio)**	**TIAA (mg/L)**
Mortuary Admission femoral blood (ADM)	**Median PMI (days)**	0.3	0.2	0.4
	Median concentration	0.008	0.059	0.010
	[min - max]	[0.001 – 0.043]	[0.010 – 1.114]	[0.001 – 0.030]
Autopsy femoral blood (AUT)	**Median PMI (days)**	4.9	4.7	3.7
	Median concentration	0.013	0.099	0.010
	[min - max]	[0.001 – 0.068]	[0.008 – 1.105]	[0.001 – 0.058]
Pairs		18	22	9
Ratio (AUT/ADM)		1.6	1.7	1.0
p value		0.008*	0.034*	0.844

PMI = Postmortem Interval; *significant difference ($p < 0.05$) using a Wilcoxon two-tailed matched-pairs test.

The influence of the pre-autopsy interval was also examined by comparing against the difference in specimen collection time of a case with the change in IAA concentration in the same case, **Fig. 16**. There were 18, 22 and 9 cases for *trans*-IAA, *cis*-IAA and TIAA, respectively. The pre-autopsy interval ranged up to ~8.5 days with each case plotted in sequence from lowest to highest pre-autopsy interval. The largest ΔConc was ~1000%, whilst there were also some cases where concentrations were considerably lower than at mortuary admission. Although there appears to be a slight relationship for AUT concentrations to rise with prolonged collection times, there were also cases where all IAA concentrations decreased, particularly for cases collected between ~4-5 days difference.

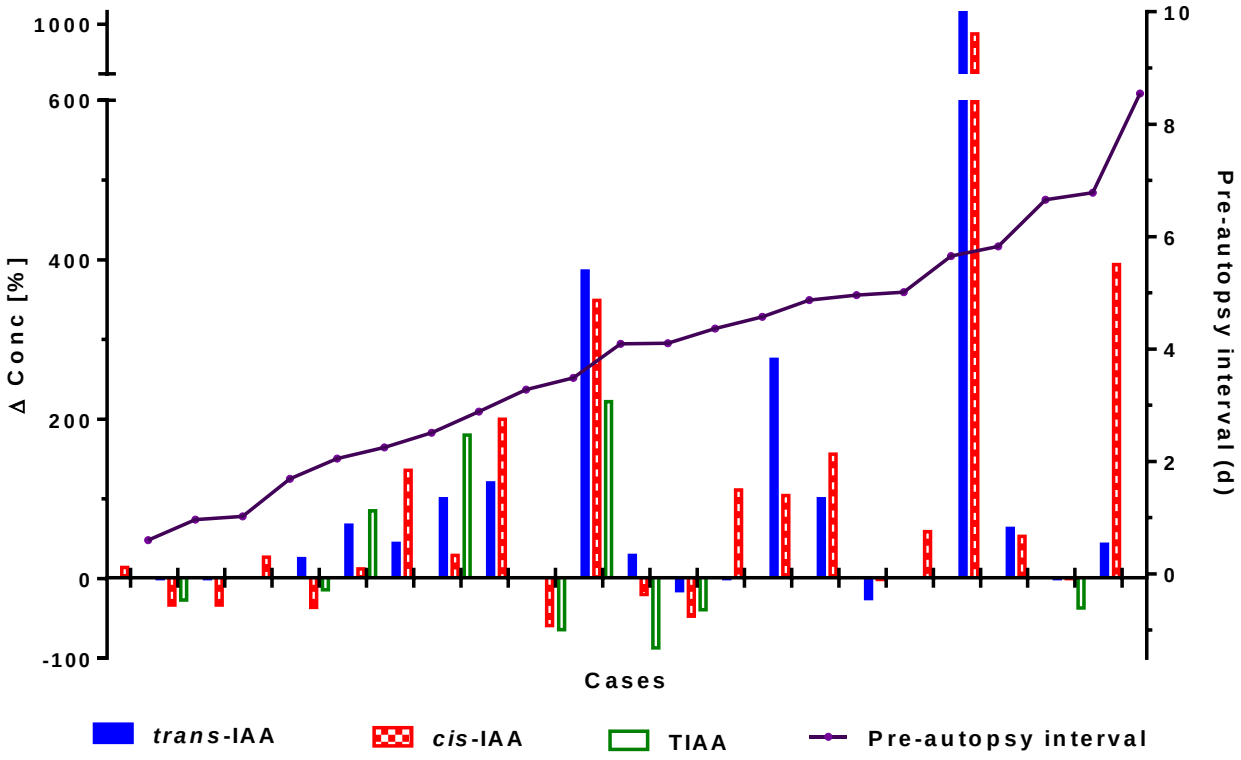


Fig. 16

Changes in IAA concentrations were compared to the pre-autopsy interval (collection time difference between mortuary admission and autopsy) in femoral whole bloods from non-decomposed cases that had paired mortuary admission and autopsy samples that were positive for *trans*-IAA, *cis*-IAA and/or TIAA

There were insufficient non-femoral (heart, cavity and subclavian) whole blood specimens that were positive IAA groups to suitably compare to matched femoral blood concentrations. However, a single case study describes the difference in natural IAA concentrations from multiple bloods taken at mortuary admission and at autopsy, where three autopsy bloods were collected from different sites, **Table 21**. Redistribution was observed between bloods collected at the same time at autopsy with 0.010, 0.010 and 0.026 mg/L *trans*-IAA concentrations in the femoral, subclavian and cavity bloods, respectively. Similar redistribution of analytes was observed with the *cis*-IAA area ratios. This analysis demonstrates the potential fluctuations in IAA redistribution between specimens collected from femoral and non-femoral blood collection sites. Following a pre-autopsy interval length of ~8.5 days, there was also a slight increase in natural IAA in the autopsy femoral blood, when compared to the mortuary admission.

Table 21

The time and site collection dependent PMR in a 66 year old male who died of natural causes with untraumatized body specimens collected.

	Mortuary admission	**Autopsy**		
	Femoral	**Femoral**	**Subclavian**	**Cavity**
***trans*-IAA (mg/L)**	0.007	0.010	0.010	0.026
***cis*-IAA (area ratio)**	0.090	0.245	0.230	0.523
PMI (days)	1.1	9.6	9.6	9.6

An assessment of the *trans*-IAA, *cis*-IAA and TIAA median concentrations, S/B ratios and p-values of cases with both serum and blood specimens are given in **Table 22**. A significant and marked difference was demonstrated between the matched serum and whole bloods for the three IAA concentrations and resulted in an average S/B ratio of consistently ~3 for the analyzed IAA groups. In addition, linear regression analysis (serum vs. blood concentrations) was performed on the plotted S/B ratios (data not shown) that demonstrated a uniform trend between matched pairs. This demonstrated that the increase in concentrations did not show any changes in ratios over the concentration range for the IAA groups under investigation. Moreover, there was no evidence of an inacceptable number of outliers for any IAA group.

Table 22

Comparison of serum and femoral whole blood mortuary admission specimens of *trans*-IAA, *cis*-IAA and TIAA concentrations.

	***trans*-IAA (mg/L)**	***cis*-IAA (area ratio)**	**TIAA (mg/L)**
Pairs	17	19	11
Blood median [min - max]	0.006 [0.001 – 0.043]	0.087 [0.006 – 0.486]	0.010 [0.001 – 0.074]
Serum median [min - max]	0.018 [0.002 – 0.124]	0.268 [0.034 – 2.840]	0.031 [0.002 – 0.191]
Ratio (Serum/Blood)	3.0	3.1	3.0
p value	< 0.001*	< 0.001*	0.001*

*significant difference ($p < 0.05$) utilizing a Wilcoxon two-tailed matched-pairs test

Note: There was an insufficient amount of RIAA and HIAA detected to perform analysis.

5.3.4 Discussion

This current study examines key forensic toxicology parameters that provide a greater understanding when interpreting postmortem IAA results to confirm beer consumption. In 2014, 78% of Australians believe the country has an alcohol problem with over 36% of drinkers claiming they "drink to get drunk"**,** costing more than $15 billion annually [4, 236, 247]. A previous study has demonstrated the high prevalence of beer consumption in forensic casework with over half of all alcohol positive cases confirming beer intake prior to death [245]. This research provides additional information when interpreting such results.

The postmortem redistribution processes during the postmortem interval can lead to variations in drug concentrations which can affect the way in which case results are interpreted [234, 248-252]. Comparison of mortuary admission and autopsy blood IAA concentrations can assist in determining the PMR phenomena [253]. Generally, this study showed that the natural IAA groups were subject to significant changes in concentration when femoral blood was collected on admission to the mortuary and compared to blood taken at autopsy, approximately 4 days later. Such fluctuations in blood drug concentrations have been shown when bodies are stored for long periods of time, hence the timely collection of specimens is preferable [254, 255]. Furthermore, it has been shown that PMR may occur in the early hours following death and therefore the extent to which PMR may have already taken place prior to the mortuary admission specimen is unknown [253]. Nonetheless, specimens collected on admission to the mortuary that are closer to the time of death may help to minimize these changes [256, 257]. The comparison of the pre-autopsy interval and change in IAA concentration for each case reflected this. It appeared that there were smaller fluctuations closer to the time of death. However this comparison of individual cases also

demonstrated the non-uniform trend for IAA concentrations to increase, and also decrease, throughout the PMI.

Although the body of the deceased cases were refrigerated immediately upon admission to the mortuary, studies investigating the stability of drugs in a decomposing body to simulate the influence of PMI have shown that many can quickly degrade following death [60, 255]. Long-term stability studies demonstrated that IAA and reduced IAA at low concentrations in blood underwent minimal degradation under refrigerated storage temperatures [231]**.** While this stability study provides information on the changes which can occur to IAA stability in a controlled environment; they cannot mimic the considerable influence that bacteria, fungi, body and trauma, and other factors, are known to influence analyte concentrations following death [60, 258-260]. Even with the possibility of losses, the natural IAA concentrations were shown to generally increase during body storage and exhibit PMR with AUT/ADM ratios 1.6 to 1.7.

Fat tissue and skeletal muscle, are possible body compartments where substance accumulation is allowed and source for the redistribution of IAA into the blood after death [234, 250, 261]. As this is most significant in central blood, the effects of PMR can possibly be minimalized with the collection of peripheral (e.g. femoral) blood [251, 262, 263]. This exaggerated increase in PMR from centrally located blood was demonstrated in one case where heart blood natural IAA concentrations were significantly higher than the peripheral specimens. These matched blood specimens demonstrated the time and site collection dependence of PMR.

Although there is no technique to measure the degree of redistribution, it is largely accepted that PMR is magnified for substances that collect in high concentrations in body compartments, which are commonly lipophilic, have an appropriate pK_a, or have high

volumes of distribution (Vd), commonly greater than 3 L/kg [251, 253, 263]. A quantitative structure-activity relationship (QSAR) model predicted a relatively small Vd range of 0.56-0.58 L/kg for the IAA groups [264]. However the distribution-coefficient (logD) for IAA groups was estimated to range from ~2.5 – 4 at pH 7.4 using a QSAR model [265] (with equal weighting [266, 267]). These logD properties may demonstrate the ability for IAA to collect in body compartments. Furthermore, the IAA groups have been shown to diffuse across lipophilic bilayers dependent on partition-coefficients (logP), pK_a, and molecular size [215]. The acidic IAA groups possess a pK_a of ~3-4 and have logP properties ranging from ~2.5 - 4.5 demonstrating good lipophilicity (log P) capable of transport across cell membranes [172, 268].

Such properties of the IAA groups demonstrate the lipophilicity of these compounds, sufficient to explain the elevation of the natural IAA concentrations in the heart blood of the case study following a prolonged PMI. Further investigation that provides *in vivo* data may further assist in the interpretation of IAA PMR in casework.

This paper also compared the serum-to-blood distribution (S/B ratio) of *trans*-IAA, *cis*-IAA and TIAA in postmortem specimens. Whole blood and serum (or plasma) are commonly obtained and analyzed in forensic investigations [269]. It is well known that drugs are unevenly distributed between the fluid and cellular phases of blood [270]. Although whole blood has recently been shown suitable for the analysis of postmortem and pharmacokinetic controlled studies [226, 239, 245], detection off IAA in serum was also found to be potentially more useful with higher concentrations of IAA in serum compared to blood [245]. Acidic and neutral compounds will primarily bind to albumin that if saturated, may bind to lipoprotein [270]. Serum concentrations for IAA are also dependent on the hematocrit value [271]. As it will not be possible to measure hematocrit in some of the circumstances in which

whole-blood samples have been analyzed, the comparison between serum and whole blood presented here can be used to compare results obtained in different blood specimens.

A consistent S/B ratio of ~3 was shown that did not show any obvious outliers throughout the concentration range. Serum (and plasma) to blood ratios are commonly measured in antemortem specimens, however the hemolysis of the erythrocytes in postmortem whole blood and therefore the liberation of intracellular water commonly [271]. This may explain the considerably high IAA and reduced IAA S/B values. Nonetheless, such a difference in serum and blood concentrations in postmortem casework demonstrates that specimen matched calibration and quality control matrices should be used when analyzing natural and reduced IAA.

5.3.5 Conclusion

This study showed that the IAA and reduced IAA groups detected in postmortem blood and serum are subject to postmortem phenomena such as redistribution. These studies confirm that although postmortem concentrations cannot be easily compared to clinical concentrations, the presented findings do provide a greater understanding to assist interpretation of forensic casework where the confirmation of beer is needed.

Key Points

1. IAA are subject to considerable postmortem redistribution
2. There is an association between the postmortem interval and the extent of postmortem redistribution
3. There is evidence that different sites of blood collection can influence IAA concentrations
4. The serum to whole blood ratio of IAA concentration was ~3:1 in postmortem blood specimens

CHAPTER 6.

BEER PROFILES

Blood IAA results can confirm beer consumption, however the analysis of IAA levels in the beer itself can allow for further conclusions to be drawn. **Chapter 6** examines the presence and concentrations of natural and reduced IAA groups in a range of popular beers in order to provide a reference catalogue. This is achieved by altering parts of the method from **Chapter 2**. The profiles of brown, green and clear bottled beer can allow for the comparisons of blood results, such as those in **Chapter 4 and 5**, to be made.

CHAPTER 6.1

IAA IN BEER METHODOLOGY

Based in part from:

Rodda, L.N., D. Gerostamoulos, and O.H. Drummer. *The Quantification of Hop-Derived Iso-α-acid Compounds in Beer using UHPLC-MS/MS*. in Forensic and Clinical Toxicology Association Inc. Conference. 2013. Sydney, Australia. **(Appendix 1.8)**

6.1.1 Introduction

Recently, methodology to determine beer consumption by the detection of iso-α-acids (IAA) in blood and other specimens was established [190, 226, 239, 245]. Such results are assisted with the confirmation that the beer in question actually contains the particular IAA group detected in blood. A quantitative IAA value that could distinguish between high- and low-hopped beers would also be favorable to help interpret high or low blood IAA concentrations. Specifically, the pharmacokinetic studies in **Chapter 4** required qualitative and quantitative results in order to assist in the interpretation of blood and urine results [226, 239].

International bitterness units (IBU) are used by the alcohol industry as a measurement of the bitterness profile of beer with one unit being approximately equal to one mg/L of total IAA [173]. The IBU scale provides a measure of the bitterness of beer based on a spectrophotometry, measuring the UV absorption of an iso-octane extract of acidified beer at 275 nm for bitter type compounds (IAA included) [272]. However, this method is rather unspecific as other beer congeners that do not provide bitterness are also measured [173, 187]. There is also the European Bitterness Units scale (EBU), which measures the *perceived* bitterness of beer by the tasting of beer itself. Theoretically, the IBU and EBU numerical values should be the same however, as these scales are determined slightly differently, this is not always true. Furthermore, these techniques do not generally determine reduced IAA. For these reasons, the accurate analytical measurement of all IAA groups is desirable.

Due to IAA are prone to becoming light-struck in the presence of sunlight, brown glass has historically being used to bottle beer [174]. However, the development of range of "lightproof" reduced IAA derived from the natural IAA, allowed manufactures to use a marketable clearer glass for bottling [242]. By chance, the reduced IAA can not only provide altered intensities of bitterness, but also enhance beer foam appearance and stability. When

used to improve foam and not particularly bitterness, they may be added at relatively lower strengths and, if desired, may safely be used in conjunction with traditional natural hopping regimes [178, 179]. Reduced IAA hop-products such as Redihop® (RIAA), Tetrahop Gold® (TIAA) and Hexahop® 95 (HIAA) [223-225] are just some examples of the commercially available reduced IAA products that are available as potassium salt preparations ready to be added directly to the finished beer (post-fermentation) [165, 179]. The detection of reduced iso-α-acids in blood may provide information on the type of beer consumed for example if clear bottled has been consumed.

The aim of this study was to develop an altered version of the current validated IAA blood method for the quantification of these beer markers in beer itself. This could determine the prevalence of IAA, RIAA, TIAA and HIAA in a large number of beers and form a reference catalogue for interpretation with blood IAA results.

6.1.2 Methodology

6.1.2.1 Authentic Beers

A number of local and domestic, popular and craft, beers were bought at local stores, kept at room temperature, and freshly opened prior to analysis.

6.1.2.2 Preparation of Blank Beer Matrix

For calibration and quality control standards, an un-hopped beer matrix was prepared in-house based on a pale-ale styled beer. One liter of this blank beer was prepared using ingredients and sterilizing chemicals purchased from Grain and Grape (Melbourne,

Australia). 100 g of light malt powder was combined with 5 g sugar and 1 g salt with enough 30 °C water to provide a 1 L total volume. This wort was then heated to boiling and boiled for 20 min. The mixture was then rapidly cooled with an immersion wort chiller and transferred to a sanitized fermenter where ale yeast powder was added and the wort allowed to ferment for 3 weeks between 20-22 °C. To ensure the beer was not contaminated by bacteria, particularly in un-hopped beer, brewing sterilization techniques were employed throughout the process.

6.1.2.3 Sample Preparation and Apparatus

To prepare the sample for the analytical system, a 10 mL beer sample was transferred into a disposable salinized glass bottle and degassed by sonication for 7.5 minutes immersed in cold water (~4 °C). The sample then underwent a final degassing and filtration by a 0.22 µm filter (Phenomenex, Melbourne, Australia). Finally, 20 µL of degassed and filtered beer was directly injected into the UHPLC-MS/MS system. The analytical conditions were identical to the previous IAA blood validated method [190].

6.1.2.4 Preparation of Calibration Model and Control Samples

The calibration standards were prepared using the blank beer matrix spiked with the working solutions to obtain the final concentrations of 0.1, 1, 10 and 50 mg/L for each IAA group. The quality control samples (QC) were prepared using the blank beer matrix spiked with the working solutions to obtain the final concentrations of 5 mg/L (low) and 25 mg/L (high), and were stored at -60 °C before analysis.

6.1.3 Results and Discussion

The described UHPLC and C_{18} solid-core column technology under gradient elution resolved the isobaric analogs and isomers within 10 min, compared to current techniques in the beer industry that resolve within 20-50 min [153, 176, 194, 216]. Similarly, beer samples are often filtered with 0.2-0.5 µm filters and degassed by sonication where direct injection is commonly employed. Recently, mobile LC systems have enabled the on-site measurement in a mobile laboratory with the direct injection of beer [273].

Following the isomerisation of α-acids to iso-α-acids, minor analogs post-, pre- and adpre-AA are also converted into their respective isomers. Although they are generally regarded as insignificant due to their relatively low concentrations, co-elution may be problematic particularly with ultraviolet detection [153, 177]. Many of these previous HPLC methods generally use DAD detection [173, 194, 216]. The use of mass spectrometry potentially solves this issue due to the differences of selection based on molecular weights and detected ions. Additionally, the increased specificity allows for the accuracy required when analyzing IAA content in beer for medico-legal casework.

The altered sample preparation proved to be sufficient for the detection of all IAA groups above 0.1 mg/L. The calibration fit showed a coefficient of determination (r^2) of >0.99 for all groups. It is known that the *trans*-IAA are present in the foam in greater proportion than the *cis*-IAA due to their lower water solubility [174]. For this reason, care was taken to include a representative 10 mL sample of the beer when freshly opened, as such to mimic authentic consumption.

Table 23 shows the IAA results of the variety of analysed beers of different styles, colored bottles and parts of the world.

Table 23

A reference catalogue of the detected IAA and Reduced IAA concentrations in beers

Vessel	Beer	Hop Content (mg/L)			
		Natural	Reduced		
		trans-IAA	RIAA	TIAA	HIAA
Can	Guinness Draught	7			
Brown Bottle	Asahi Super Dry	5			
	Bitburger	9			
	Coopers Pale Ale	7			
	Little Creatures Pale Ale	9			
	Sierra Nevada Pale Ale	14			
	Tiger	8			
	Victoria Bitter	6			
	West Coast Indian Pale Ale	20			
	XXXX Gold	6			
Green Bottle	Becks	13			
	Carlsberg	7	0.3	3	Trace
	Cascade Premium Light	4		5	Trace
	Hahn Premium Pilsner	4		2	
	Heineken	7			
	James Boag's Premium Light	4		5	
	Pure Blonde	5			
	Stella Artois	4		Trace	
	Tsingtao	5			
Clear Bottle	Amstel	Trace	5	1	
	Coopers Clear	Trace	19		0.3
	Corona	1			
	Fosters Light Ice	Trace		18	0.1
	Hahn Premium Light			6	
	Miller Genuine Draft			6	
	Sol Mexican	Trace	20	4	Trace
	Spitfire Kentish Ale	10			
	Tooheys Extra Dry	Trace		11	0.1
	XXXX Summer Bright	Trace		7	Trace

Trace = < 0.1 mg /L detected (signal:noise > 3:1)

Beers historically have ranged from 20-60 IBU however currently, they range from ~6-30 IBU, with the exception of some craft beers [165, 274]. Considering that IBU measures *trans* and *cis* isomers, and there is generally a higher proportion of *cis* isomers, our brown bottle *trans*-IAA results show comparable results. As expected, no reduced IAA groups were observed in brown bottled beer.

The bittering effect is less noticeable in beers with a high quantity of malt, so the use of more hops is needed in heavier beers to balance the flavor. This is seen with the high IAA content of the Sierra Nervada Pale Ale and West Coast Indian Coast Pale Ale beers that are generally considerably heavier styled beers.

Interestingly, although the natural IAA group degrades when exposed to light [166], brewers have used natural hop products in green, and more surprisingly, in clear bottled beers. Indeed a mixture of natural and reduced IAA are shown to be used in many green bottled beers allowing for bitterness to be retained even if the natural IAA degrade. The lower amount of natural IAA present in green bottled beer in comparison to brown is compensated with the addition of the light-proof reduced IAA products.

All clear bottles contained reduced IAA, excluding the Corona beer which contained solely naturally IAA. This was surprising and demonstrates the need for the analysis of the beer in question itself. Coopers Clear and Sol Mexican beers containing RIAA show a relatively high concentrations, possibly in order to achieve the desired bitterness due to the lower bitterness properties of RIAA [172].

Due to this synthesis of reduced IAA, residual natural IAA may be present in beer hopped with reduced IAA products. This explains the trace levels of natural IAA observed in over half of the clear bottled beers. As these concentrations are generally below the threshold

where noticeable 3-MBT is produced, the presence of trace natural IAA does not influence the quality of the beer [192].

Among other non-natural additives, the non-natural, reduced forms of IAA are not allowed in beers from the German market due to the "Reinheitsgebot" law stating that only natural hop products, water, malt and yeast, may be used in the brewing process [180]. This is confirmed in this study where the German beers, Bitburger and Becks, contained only natural IAA.

The IAA isomers are naturally found in fresh beer at ratios ~1:2 (*trans*:*cis*) [174]. However, Corona exhibited a ~1:27 ratio (data not shown). The *trans* isomer has a half-life of ~9 months, compared to *cis* with a half-life ~5 years [175]. As the Corona beer was produced in Mexico, the long transport and storage periods, may be the cause for the loss of *trans*-IAA [166]. Further degradation may also be possible if exposed to light for periods of a time due to the clear glass of the bottle.

In summary, the interpretation of the detected beer-specific ingredient congeners in blood and urine that demonstrate the consumption of beer in medico-legal casework, is assisted with profiles of the IAA and reduced IAA in commonly consumed beers, as presented here.

CHAPTER 7.

CONCLUSIONS

The outcomes of this research are discussed in **Chapter 7** and conclusions drawn from the results of previous chapters including their implications for alcohol congener analysis and more broadly in forensic toxicology. The use of detecting iso-α-acids in biological specimens as beer-specific markers is emphasised. The strengths, impact and limitations of this research are examined, whilst also summarising the developed novel technique. Future perspectives are suggested that will complement and continue to develop the use of alcohol congener analysis in forensic toxicology.

7.1 Introduction

Before looking at the research findings in their wider context, it is beneficial to recapitulate the structure of this research and the development of the thesis. **In Chapter 1**, the research problem was presented and the justification for undertaking the research was outlined. In addition, **Chapter 1** described the terminology used in this thesis and the alcohol congener analysis niche. The centrality of the research problem to the field of forensic toxicology was identified and the literature that provided the historical research was reviewed. This review of the literature exposed widespread limitations and interpretation inconsistencies when alcohol congener analysis uses the fermentation by-products in alcoholic beverages. The feasibility of identifying the exact source of alcohol by this technique was deemed unlikely.

A hypothesis emerged that indeed redirected the research that would be undertaken during this PhD onto a different path compared to traditional ACA investigations. A possible alternative approach that may determine the consumed alcoholic beverage was identified by the use of beverage-specific congeners as ingredient biomarkers. Iso-α-acids were identified as potential markers for beer consumption. Naturally, several questions were identified that would require answering in order to provide a conclusive novel approach to the field. Of the most pivotal question, even if a method to detect IAA in blood could be developed, would they even be bioavailable in a sufficient concentration for practical use as a forensic toxicology service? Additionally, other fundamental knowledge gaps such as the stabilities, pharmacokinetics, and prevalence and postmortem behaviour, of the IAA required attention.

With the finding that a blood IAA method could be developed and validated for use in a forensic setting, and IAA could indeed be detected in a volunteer post-consumption of beer in a pilot study, **Chapter 2**; ethics applications (**Appendix 2**) and project plans were assembled to target these later research questions examined in **Chapters 3 – 6**.

7.2 Review of Findings

Fermentation by-product alcohol congener analysis is a technique used in Germany to investigate after-drinking defense claims by examining the feasibility of the claimed alcoholic beverage consumption. The review in **Chapter 1** examined the forensic application of ACA in determining the source of alcohol. It demonstrated concerns surrounding the current ACA approach due to the limitations, uncertainties, the difficult nature of carrying out the evaluation, varying methodologies used by experts and the limited knowledge of the variation in pharmacokinetics of these congeners in humans. For example, blood sampling must occur within 1–3 h of cessation of drinking and a significant BAC of 0.08 must be present. Additionally, many of the fermentative by-product congeners can either be produced endogenously, by bacterial putrefaction during storage, or obtained from sources other than consumption of the claimed alcohol beverage. The Individual pharmacokinetic variability and the consumption of vastly different alcoholic beverage types can alter the expected congener concentrations. Furthermore, there is a reliance on information from the defense such as body weight, length of drinking times and case circumstances.

Even though the references in the review were translated to English, the high proportion of literature in German makes its transference into the international community a challenging task. It was difficult to obtain a universally accepted evaluation method for ACA. However the examined examples in the literature required careful interpolation that allows for potentially wide uncertainty ranges.

Nonetheless, this method of ACA has shown to be a prominent tool to examine after-drink type cases in Germany. However this technique does exactly determine the alcoholic beverage source for an elevated alcohol concentration and therefore its use in other forensic scenarios was fundamentally limited.

Ingredient biomarkers were hypothesized to have the potential for application in these other case scenarios by determining the specific source of alcohol. It also offered an alternative and potentially more defendable approach to examine after-drinking claims. The review in **Chapter 1** highlighted a considerable opportunity for investigations into the development of methodology to detect ingredient biomarker congeners, specific to a range of alcoholic beverages in biological specimens. Subsequent research was then based on particular alcohol beverages, of which beer was selected first. Investigation into brewing techniques through the literature, in addition to discussions and visits to a multinational brewery along with other local breweries, identified that the uniqueness and abundance of IAA would be a good candidate for the confirmation of beer.

These IAA compounds, derived from the hop plant, indeed could be detected in the blood and urine of beer drinkers subsequently confirming beer ingestion. To achieve this verification, a protein precipitation extraction and ESI-UHPLC-MS/MS method was developed, **Chapter 2**. LCMS technology was initially determined to be the most appropriate, considering the chemical properties of the IAA groups. Extracts were injected onto a C_{18} solid-core column, and after tedious modifications, a final gradient elution achieved the separation of isobaric analogs and isomers within a 10-min run time. This separation was quite important as although the brewing industry developed approximate IAA concentrations in beer, the analysis in blood for forensic application required a more selective detection of individual analytes. Certainly the quick rum time assists in the turnaround time of results.

Electrospray ionization in negative multiple reaction monitoring mode was used to monitor three transitions for each of the analytes that were ultimately grouped together to form a calibration curve for quantification of each of the four IAA groups.

This enabled detection of IAA analytes in both antemortem and postmortem blood. However for use in the forensic community, the method was fully validated according to international guidelines that included extraction efficiency, matrix effects, process efficiency, ion suppression/enhancement of co-eluting analytes, selectivity, crosstalk, accuracy and precision, stabilities, and lower limits of quantification. To confirm the applicability of such work, the *trans*-IAA were quantified in the blood of a volunteer post-consumption of beer, demonstrating the validity to progress onto pharmacokinetic studies.

The above guidelines for the forensic toxicology community describe the required parameters for validating developed methods, including the processed sample, freeze thaw and bench-top stabilities. However, the examination of any analyte degradation in stored specimens also required examination in order to determine the accuracy of IAA concentrations when specimens are subjected to prolonged, and variable, storage conditions.

Chapter 3 focused on the the long-term stability of IAA in whole blood. Pools of blank blood spiked with the four IAA groups at two low and high concentration levels were stored at 20 °C, 4 °C and -20 °C; and extracted in duplicate in weeks 1, 3, 5 and 8. In addition to the IAA group concentrations, the area ratios of the individual analytes within the four IAA groups were also measured to determine which particular isomers or analogs were subject to greater degradation and therefore responsible for the overall group instability. All four IAA groups showed losses greater than 30% after 8 weeks of storage under room temperature conditions. In particular, the natural IAA group observed major losses of 96% and 85% losses for low and high concentrations, respectively. Some degradation in all IAA groups were seen at 4 °C specimens predominantly due to the ‘n’ analogs of the group that showed an increased instability in blood. The -20 °C storage conditions resulted in minimal changes in concentrations of all analytes.

Even though IAA and reduced IAA compounds appear to be stable over lengthy storage times in beer, instability was demonstrated in blood over 8 weeks of storage at warmer than frozen temperatures. The sufficient stability of IAA in frozen storage conditions was confirmed in the re-analysis of authentic blood from a volunteer post-beer consumption. After 12 months, *trans*-IAA were more or less the same as when analysed immediately after collection.

The detection of blood IAA that confirmed the consumption of beer in a single volunteer showed to some degree, bioavailability. However to better understand how the IAA are handled by the body, pharmacokinetic studies must be performed. Volunteer drinking studies investigated the pharmacokinetics of IAA, described in **Chapter 4**. Concentrations of IAA were determined in the blood and urine of five volunteers over 6 h following the consumption of small volumes of beer containing relatively high or low concentrations of natural IAA. In similar studies, the pharmacokinetics of reduced IAA were determined using clear bottled beer consumption. The natural and reduced IAA were found to show small inter-variable differences in concentration-time profile, and possess pharmacokinetic data such as quick absorption rates and half-lives ranging between ~30-46 minutes.

Comparison of 0.5 and 2 h blood specimens demonstrated that the *trans* isomers were eliminated faster than the *cis* counterparts for the natural IAA group. Similarly, comparison of RIAA analyte ratios within their group indicates that some analytes eliminate relatively faster than others and there was also observations of metabolite products.

Preliminary urine analysis showed only unmodified ‘co’ analytes detectable throughout the 6 h for natural IAA. Whilst reduced IAA urine analysis showed only unmodified RIAA analytes were detectable throughout 6 h. It was suggested that natural IAA, TIAA and HIAA analytes are extensively metabolised by phase I pathways.

In the natural IAA studies the qualitative monitoring showed that the *cis*-IAA isomers were detected at all time-points and indeed provides a valuable marker due to its higher abundance naturally in beer.

The studies in **Chapter 4** also provide additional knowledge and literature for toxicologists that may assist in medico-legal investigations for judicial processes. The amount of beer consumed was calculated to give about 0.05% at the peak BAC. This demonstrates the sensitivity of the developed method as commonly, higher BAC are observed in forensic related casework. Moreover, such experiments confirms that the discriminate confirmation of either natural IAA or reduced IAA from brown or transparent bottled beers, respectively, is feasible

To conclusively investigate the application of the analytical method and the beer ingredient biomarker approach in general, IAA were examined in postmortem specimens, **Chapter 5**. The increased functionality of the death investigation process in Victoria where mortuary admission specimens are collected assisted the studies. The prevalence of IAA, and therefore the prevalence of beer consumption, in authentic casework were determined. In the assessment of 130 authentic coronial cases, ~57% of positive BAC cases showed beer consumption prior to death, and an even higher prevalence (87%) in casework where "beer" was mentioned in the case circumstances. Vitreous humour and urine specimens contained very low concentrations and prevalence of IAA groups. The correlations between IAA and BAC were also assessed with a weak association observed providing little evidence for back-calculation type investigations.

Additionally the analysis of a range of specimen types with different blood collection sites and times demonstrated postmortem redistribution phenomena. Although PMR was only weakly associated with the length of time from death to the collection of specimens. There were insignificant blood site collection and gender differences however, a serum/blood ratio

of ~3 was calculated. This assessment of fluctuations in compound concentrations from body tissue to tissue is important to understand in order to interpret postmortem toxicological results.

Lastly, the sample preparation of the analytical method was altered and used to assess the IAA profiles of ~30 different brown, green and clear bottled, local and international beers in **Chapter 6**. This reference catalogue assists forensic toxicologists in comparing and interpreting the results of authentic and unknown casework. Results were as expected for brown bottled beer and more or less for green bottled beer also. However the observation of only natural IAA in arguably the most popular clear bottled beer, demonstrated the need for the beer in question to be assessed. Otherwise a clear difference in beer profiles is observed for clear bottles, containing reduced IAA with only residual traces of natural IAA.

7.3 Values of Research

During this project, a number of issues arose which must be taken into consideration when interpreting the results of this research.

The developed analytical method's matrix effects in whole blood were accessed during validation with significant ion enhancements observed. The matrix effects were generally an enhancement of IAA ions, not uncommon with electrospray ionisation. These matrix effects were observed to be less apparent on closer inspection in serum and almost unnoticeable in un-hopped beer, vitreous humor and urine matrices. The employment of suitable full calibration models and quality controls is recommended and the validation of newly established methodology required.

Although the isolation of individual IAA analytes is feasible with the use of semi-preparative HPLC reference standard, a commercial reference standard may be more desired. Such analysis of individual analytes may provide more detail for toxicologists however may not necessarily influence the outcome. It is also noteworthy that this would be more costly whilst also requiring additional efforts when making calibration and quality control standards.

Indeed the consumption of gruit beer, beer bittered and flavoured with a herb mixture instead of hops, would not be detected [275]. This holds true for similar beers such as spruce or mugwort beers where hops are not utilised [276]. Although many types of beers have historically attempted to be bittered by herbs, such alcoholic beverages are today uncommon and only popular before the extensive use of hops centuries ago in isolated areas of the world.

As described in **Chapter 4**, reduced IAA, specifically RIAA and TIAA have recently been used in supplementary medicines for their suggested effectiveness in osteoarthritis, rheumatoid arthritis and fibromyalgia patients. However as mentioned, these are relatively rare products and generally in 10-100 times higher concentrations than found in beer which would result in exaggerated blood values.

The detection of IAA does not determine that the observed BAC is solely due to beer consumption. The co-consumption of other alcoholic beverages that also contributes to elevate BAC cannot be ruled out. Importantly, the detected of IAA indicates the relatively recent ingestion of beer.

During the end of this candidature, colleagues from Dusseldorf, Germany, investigated the possibility of using the malt-derived compound hordenine, as a possible marker for beer [277]. It seems they have opted to choose this as a marker for beer as it is produced from germinated barely, which is used in brewing. Indeed, early research into this approach demonstrated elevated levels of this marker in serum [278]. However, it may be less specific

to beer as it found in barley, also used for other food products, and more recently isolated as a nutritional supplement for bodybuilding [279]. In addition, it is unable to distinguish between brown and clear bottled consumption. For these reasons, the analysis of IAA for the determination of beer consumption is still an appropriate approach. However, this technique may be useful to assist in the herb bittered beers (gruit and spruce) previously mentioned, or to confirm beer consumption when supplement medicines containing reduced IAA are claimed to be consumed.

Likewise, there is a strong impact of the findings of this work.

The culmination of this research has been the development, for the first time, of the specific detection of beer consumption, the most widely consumed alcoholic beverage. In addition to alcohol abuse being a leading factor in many crimes and accidents, the results of this thesis are therefore an important result for forensic science. The dissemination of the chapters as papers in peer-reviewed publications provides for the use of these findings by forensic toxicologist in authentic casework internationally. It also allows for the technique to be critiqued and others to build on current work.

On attendance to the International Association of Forensic Toxicologists (TIAFT) meeting in Portugal 2013, this research was presented in front of several leading forensic alcohol experts, of which one was chairing the session. Feedback from this and the review paper was positive with the acknowledgment of a novel and applicable method being developed. Additionally, during the candidature I visited a German Forensic Institute who routinely undertakes alcohol congener analysis where beer consumption is commonly at question. On presentation of this methodology, it was indicated that this technique would be useful for their casework and will be employed. Finally, correspondence with an leader in the hop and beer analysis for the brewing industry, stated this work, particularly **Chapters 2 and 4**, to be

indeed useful for the wider beer industry. This demonstrates the potential for the usefulness of such work in disciplines outside of the forensic field.

7.4 Future Aspects

The human metabolism of IAA and reduced IAA can be further extrapolated with the investigation of either modification or conjugates metabolite products. Such metabolism studies can commonly utilise full scan MS to observe products in blood or urine from volunteers post-consumption of beer. Likewise high resolution MS has indeed assisted in similar studies in order to determine the likely structure of unknown compounds. Other metabolite identifying techniques that use fungi, bacteria, liver microsomes or *in silico* techniques may also be employed.

A likely step is the identification of other alcoholic beverage ingredient biomarkers. With wine being the obvious next target, the possibility of markers being obtained by the phenolic compounds found in the skin, flesh or stem of grapes is feasible. To distinguish from consumption of simply grape juice, the detection of compounds from the oak barrel may strengthen the conclusion of wine consumption. Furthermore, once a range of beverage-specific markers have been shown to be bioavailable and suitable targets for the identification of a wide-variety of alcoholic beverages; if feasible, the development of a single LCMS method would be ideal for forensic institutes and quite possibly make such analysis used more routinely when a BAC is observed. This identification of other alcohol beverage-specific ingredient markers would give more completion to the alcohol congener analysis discipline.

7.5 Conclusion

An initial review on the status of alcohol congener analysis described the limitations of performing the traditional approach routinely in wider medico-legal scenarios. The subsequent research in this thesis provides the comprehensive examination of detecting iso-α-acids in biological specimens to determine beer consumption and provide information on the source of alcohol, without the requirement of circumstantial evidence. The presented research may be used in isolation or in conjunction with traditional ACA as the required information necessitates. For this reason, the application of the presented research would be on a case-by-case basis that may be used more consistently in after-drinking type cases than any other medico-legal scenarios. Furthermore such analysis would not be financially feasible or efficacious to perform routinely on case received in a forensic laboratory. Nonetheless where the requirement of information on beer ingested is valuable, this developed approach can comprehensibly provide such results.

The aims of the project were met and the results provided in peer-reviewed publications and conferences proceedings in order to disseminate throughout the wider forensic community. The relevant findings required to perform, and defend results obtained from authentic casework in a forensic setting, are presented in this thesis. Thesis by publication is the primary structure of this PhD thesis with a total of seven peer-reviewed publications (three in review) resulting from this project that comprehensively describes the novel confirmation of beer ingestion in medico-legal casework and the applicability for this technique in forensic institutes locally and internationally.

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APPENDICES

APPENDIX 1

PUBLICATIONS

APPENDIX 1.1:

CHAPTER 1.1 PUBLICATION

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REVIEW

Alcohol congener analysis and the source of alcohol: a review

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Abstract For many decades traditional alcohol congener analysis has provided the concentrations of fermentation by-product congeners found in blood, to ascertain if the claims of an individual regarding the alcoholic beverage(s) they have consumed were feasible, assisting in cases where after-drinking is involved. However, this technique does not provide information on the exact alcoholic beverage(s) consumed. More recently, ingredient biomarker congeners specific to certain alcoholic beverages have been detected in blood, making it possible to identify the particular alcoholic beverage consumed and therefore the source of alcohol (albeit only for a limited number of beverages). This novel approach may reduce current limitations that exist with traditional methods of detecting fermentation by-product congeners, which restrict the use of alcohol congener analysis internationally and for other medico-legal scenarios. This review examines the forensic application of alcohol congener analysis in determining the source of alcohol and other techniques.

Keywords Alcohol congener analysis · After-drinking · Congener · Fermentation by-products · Ingredient biomarkers · Review

Introduction

The oldest evidence of alcohol production dates back to 10,000 B.C. [1]. Alcohol remains the most widely consumed drug in the world, with a worldwide annual average consumption of 6.5 L (of pure ethanol) per person [2]. Alcohol is commonly abused, presenting a global health issue which contributes to over 2.5 million deaths annually, in addition to a significant financial burden on society resulting from alcohol-related crimes and health care costs [2–4]. For many years, alcohol has been the most commonly detected substance in routine forensic analysis, particularly in cases involving violence, sexual assault, and motor vehicle accidents [2, 5]. This has provided extensive information for the interpretation of blood alcohol concentrations (BAC) [6, 7]. Widmark published the first detection method [8] and accompanying pharmacokinetics [9] of ethanol in the early 1920s and 1930s. This field of research was advanced by Elbel [10] when a forensic focus on blood alcohol research within the German speaking countries was established [11–13]. In the same decade, Machata developed the first gas chromatography with flame ionization detection (GC-FID) method for analysis of simple volatiles in blood by direct [14] and headspace (HS-GC-FID) injections [15]. This subsequently led to a method for the quantitative analysis of ethanol, which remains fundamentally similar to the methods used today [16].

Machata devised the initial workup and concept of investigating the source of alcohol by correlating detected congeners in blood with concentrations found in the beverage consumed [17], termed alcohol congener analysis (ACA). Congeners are all other compounds in an alcoholic beverage, other than water and ethanol, [1] that assist in the distinctive aroma, flavor and appearance of the beverage [18]. By the late 1970s, concentrations of a set target of fermentation by-product congeners were determined in a vast range of spirit [19], wine [20] and beer [21] type alcoholic beverages [22]. Throughout the 1980s and until 2000, Bonte advanced the technique of fermentation by-product

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congener detection by designing [20, 21] and then advancing a HS-GC-FID technique using the purge and trap technology [23–25]. The understanding of alcohol congener analysis (ACA) in blood was developed with the use of numerous drinking experiments and case studies [26–30]. In Germany, ACA has been useful to verify claims of after-drinking where determining the feasibility of the claimed consumption of alcohol can play a pivotal role in the case [28, 31]. However, this approach cannot determine the exact origin of the consumed ethanol. To date, ACA has not been applied in other medico-legal cases and is only used very minimally in post-mortem cases [32, 33].

In the last decade, Lachenmeier and Schulz targeted some ingredient biomarker congeners to detect congeners specific to a beverage type [34–38]. This approach identified the type of alcoholic beverage consumed, however this was limited to herbal flavored spirits.

This review aims to understand the significance of ACA in determining the source of alcohol, the analytical techniques used for quantitative detection of congeners and how the concentrations of these compounds can be applied to forensic cases.

Methodology

Papers were selected and reviewed based on a comprehensive PubMed search for articles about ACA, in all languages, published to date. Specific keywords were used to identify articles relating to congeners found in alcoholic beverages and biological specimens, analytic methodology, interpretation and medico-legal applications.

Application of ACA to forensic specimens

ACA has provided valuable information in certain types of forensic cases where understanding the source of alcohol is useful. Effective and more unique biomarkers of specific beverages may have wider applications in forensic science than the existing ACA methodology, which is limited only for use in after-drinking cases.

After-drinking

After-drinking (also known as the hip flask defense) refers to the situation where, after a motor vehicle accident, the driver flees from police and is later apprehended, returning a positive blood alcohol concentration (BAC) which s/he claims is a result of alcohol consumption after the incident [28, 39, 40]. Drivers invariably claim to have been sober prior to the incident, maintaining the drink consumed post-offense (most commonly a strong spirit) was used to calm their nerves. This type of defense has been the top-ranked defense claim in Germany and in many parts of Europe, forming approximately 90 % of all drink-driving defense claims [30]. Although this type of defense has also been used outside these countries [41], no data exists on the frequency of this claim outside of central Europe [39]. In Hampshire, England, there are procedures utilized by the local authorities to deal with drivers who have initiated a claim using the after-drink defense [42]. Local legislation can render these defense tactics unusable, such as in Norway where it is illegal to consume alcohol within 6 h after driving if there is reason to believe that the police may wish to investigate the driver [30]. This type of defense tactic may be used by a driver in countries such as Germany where the police can submit an individual to a BAC assessment after driving has ceased for a period of time. For example, in Victoria, Australia, under the Road Safety Act [43], police can submit a driver to an alcohol test within 3 h of driving. Furthermore, if heavier penalties described in the Crimes Act are suspected, such as manslaughter, police may perform an alcohol analysis of the driver after the 3 h period [44]. This scenario may also occur more frequently when the obligation lies with the prosecution to prove that the offense occurred, which is the case in Germany and many other countries.

Previous approaches and techniques to assist in the prosecution of these defenses exist. Back-calculation of the BAC [45, 46] is generally acceptable [47]. However, this will result in wide ranges [12] and likely over- or under-estimations [48]. The technique of double blood sampling where the magnitude and direction of the ethanol concentration in the second blood sample is compared to the first (and similarly for blood to urine comparisons) has been utilized in the past [27]. However, it has been concluded that these methodologies have little advantage over traditional back-calculation from a single sample to support such evidence of after-drinking claims [26, 27] and generally require significant demand both from the police and the toxicologist. For this reason, ACA of fermentation by-products has been used consistently in Germany to examine such claims, supported by extensive literature and the ease of taking only one specimen [27, 28, 49]. The development of ingredient biomarker ACA techniques may provide supporting evidence for such claims.

Drug facilitated assaults

Ethanol is the most common drug detected, and more often than not; the only drug detected, in claims of drug facilitated sexual assault or “drink spiking” [50–52]. This has been demonstrated in countries including Australia [53, 54] and the UK [55, 56]. With data showing very few cases actually contain sedative or other stupefying drugs it is clear that high alcohol consumption is an underlying factor

for these cases [57, 58]. Evidence in a case indicating what beverages were consumed may assist in determining if a higher strength spirit was added to a lower strength beverage, unsuspectingly increasing the ethanol intake of the victim. With a mean time of 5.9 h of sampling after an alleged spiking from one UK study [55], current ACA practices would be unable to provide assistance due to the 3 h limit of the technique. Furthermore, drink spiking is regarded as the second most used defense against drink-driving in Germany and Sweden [30, 59]. Detection of alcohol sources other than what is expected may assist in prosecuting defendants in drug facilitated assault and drink spiking cases.

Post-mortem ethanol confirmation and reporting

It is possible to differentiate between ingested ante-mortem ethanol and post-mortem production [60]. This is feasible through ethyl glucuronide detection in blood, urine and hair as a marker of ante-mortem ethanol ingestion [61–63], although it has been found to be unstable under some post-mortem putrefactive conditions [64]. 1-Butanol is a marker for putrefaction [60] particularly when detected in concentrations above 0.03 mg/L [65]. However, it has been detected consistently in fruit spirits and in wine at low concentrations [22, 66] limiting the application of current ACA practices in decomposed cases. Detection of ingredient biomarkers may be more useful to determine the source of ethanol in decomposed cases. Additionally, it may offer insight into a case otherwise not possible where there are coronial or legal outcomes relating to the type of alcoholic beverage consumed.

Fermentation by-product congener ACA

Although fermentation by-product congeners are found in nearly all alcoholic beverages, different quantities of each congener detected are dependent on the amount and type of the beverage consumed. In order to determine a likely source, or to refute a claim made by the defendant, scientists compare the actual and theoretical blood congener concentrations under a specified set of circumstances [20, 21, 34].

Congener content of alcoholic beverage

Beer commonly contains approximately 800 congeners [67], wine: 600 [68] and spirits: 800 [69] at generally very low concentrations (~1:1,000) compared with the corresponding ethanol content [1, 35]. Analysis has been undertaken on different types of alcoholic beverages [19–21, 70–76]. Table 1 describes the concentrations of the common fermentation by-product type congeners found in a range of alcoholic beverages. However, the claimed consumed beverage may contain slightly different amounts of congeners depending on the batch and storage conditions. This suggests that analysis of the beverage itself may be necessary, albeit not always possible. The production techniques described below aim to alter the ethanol, water and congener content to that of the desired final product, including the concentrations of fermentation by-products that are targeted by ACA.

Fermentation

Under anaerobic conditions, sugars are converted into ethanol and carbon dioxide by particular strains of yeasts. However, other volatile substances are also produced by this fermentation process. These targeted fermentation by-products typically consist of methanol and the "fusel alcohols" (a German term for bad liquor) 1-propanol, isobutanol, 1-butanol, 2-methyl-1-butanol or 3-methyl-1-butanol, among others. The amount of these congeners produced is largely subject to availability of amino acids during production, known as the Ehrlich mechanism [77]. Yeast replaces the amino moiety with a hydroxyl group to form the alcohol [78], except in the case of methanol, where pectins are the source for its production [79]. For example, amino acids such as threonine, leucine, isoleucine and valine have been shown to produce 2-methyl-1-butanol, 3-methyl-1-butanol, 1-propanol and isobutanol [18, 80, 81]. Other factors affecting the production of alcohol congeners are the presence of other carbon sources such as carbohydrates [1]. The source of these ingredients is contained in the plant materials used to produce the range of fermented alcoholic beverages available, i.e., beer (cereal grains), wine (grapes), sake (rice) and cider (apple or pear); resulting in varying congener content for each. In addition, different strains of yeast ferment at variable rates, consequently producing different congener profiles [82].

Distillation

Distillation is not only capable of increasing the alcohol by volume percentage (ABV) [83], but can also alter the relative congener concentrations produced during fermentation. Distillation separates ethanol and volatile congeners based on their boiling points, with congeners of similar or lower boiling points to ethanol also undergoing distillation (particularly methanol). This concentration effect is demonstrated by fermented beverages generally containing lower concentrations of total congeners per volume when compared with distilled beverages [22]. However, when congener concentrations are compared to the concentration of ethanol (i.e., congener/ABV), the relative difference in congener concentration between fermented and distilled

Table 1 Published common fermentation by-product congeners and ethanol concentrations in alcoholic beverages in mg/L (top row) compared to milligrams of congener per standard drink (using the ABV—bottom row), demonstrating the difference of congener content between beverages becomes less distinct when ABV is taken into account

Alcoholic beverage	*n*	Ethanol (ABV)[b]	Concentration	Methanol	1-Propanol	1-Butanol	2-Butanol	Isobutanol	2-Methyl-1-butanol	3-Methyl-1-butanol	References
Beer	653	5	mg/L	1–27	10–124	0–6	0	9–109	37–124	10–248	[31, 70]
			mg/std drink[a]	3–7	3–6	0–2	0	2–28	10–32	3–64	
Wine	813	14	mg/L	8–151	15–63	0–9	0–1	24–109	34–314	4–183	[31, 70]
			mg/std drink[a]	1–14	1–6	0–<1	0–<1	2–10	3–30	<1–17	
Fortified Wine	4	20	mg/L	125–329	54–63	4–15	N/A	60–95	N/A	115–166	[70]
			mg/std drink[a]	8–21	3–4	<1–1	N/A	4–6	N/A	<1–11	
Brandy	120	40	mg/L	176–4,766	79–3,445	0–359	0–1,088	58–441	678–961	28–158	[31, 70]
			mg/std drink[a]	60–153	3–110	0–12	0–35	2–14	22–31	1–5	
Whiskey	113	40	mg/L	6–328	22–205	0–5	0	20–487	102–1,247	26–1,200	[31, 70, 75]
			mg/std drink[a]	<1–11	<1–70	0–<1	0	<1–16	33–40	1–39	
Rum	28	40	mg/L	6–131	34–3,633	0–1	0–126	8–455	0–788	0–219	[31, 70]
			mg/std drink[a]	<1–4	1–116	0	0–4	<1–15	0–25	0–7	
Vodka	31	40	mg/L	0–170	0–102	<1	0	<1–164	0	0–90	[31, 70, 75]
			mg/std drink[a]	0–5	0–3	<1	0	<1–5	0	0–3	

[a] 10 grams of ethanol (Australian standard drink) [90]

[b] Typical ABV for beverage class [70]

beverages is reduced (Table 1). For example, wine and the spirit produced from distilling wine, brandy, contain ~24–109 and ~58–441 mg/L of isobutanol, respectively, demonstrating a significant difference between the two beverages. However, when the ABV is taken into account, these become ~2–10 and ~2–14 mg of isobutanol per standard drink (10 g of ethanol) for wine and brandy, respectively. This indicates a more similar ingestion of isobutanol content in order to consume the same amount of ethanol, providing less differentiation of isobutanol ingestion between the two beverages to distinguish between them for ACA purposes.

Further production techniques

Later production practices have been designed to alter the congener content further in order to produce the final drinking product. Maturation can also remove, change or add congeners, due to interactions with the barrel material, chemical reactions in the liquid and/or evaporation [1]. Secondary fermentation may take place as yeast acts on the remaining sugars in the liquid, further modifying the fermentation by-product congener content of the beverage [1]. Variable conditions such as oxygen levels, temperature and duration of production can also vary the composition of congeners [18, 84, 85]. Additionally, the blending of beverages can create new products, such as blended whiskeys or fortified wines (wine and brandy) providing new congener profiles.

Manufacturers of alcoholic beverages

Large variations in congener content may exist within a type of beverage. It is common for the same type of beer to be produced by breweries in several countries under license. Hence, results from a batch produced in another country may not be representative of a beverage produced elsewhere, particularly when production techniques have changed [86]. Additionally, individuals producing alcoholic products using simple grains, fruits, potatoes and/or rice [87], have less control of fermentation by-product congener concentrations. This can cause differences in congener concentration of up to ten-fold higher when compared with their commercial counterparts [88, 89].

Other sources of congeners

Fermentation by-product congeners may also be sourced from intentional or accidental consumption of products of industry, medicines or the home; albeit generally at relatively low levels. Absorption of ethanol through the skin from antiseptics or hand sanitizers has not been shown to raise ethanol levels sufficiently to affect BAC implications

[91, 92] with the products regarded as safe for everyday use [93, 94]. However, congener absorption from the use of hand sanitizers has been demonstrated with concentrations of 0.2 and 2 mg/L of 1-propanol and isopropanol detected in blood, respectively [95]. Another study revealed isopropanol concentrations of up to 12 mg/L in serum after using presurgical skin disinfection [96]. In a "worst-case model" of repeated applications with a commercially available hand sanitizer, concentrations of 18.0 and 10.0 mg/L of 1-propanol and isopropanol, respectively, were detected [97]. Other occupational absorption of congeners has been shown to occur in workers exposed to 1-butanol in their workplace, where concentrations of up 0.2 mg/L of free 1-butanol were detected in urine [98]. Fruit juices have also been shown to contain methanol derived from some fruits, nectars and syrups, ranging from low concentrations to those in excess of 100 mg/L [99]. Furthermore, these refreshments have also been shown to raise the concentrations of methanol in blood due to the pectin within the cells of the fruit undergoing metabolism to methanol in the colon [100]. Drinking experiments with fruit juices have shown methanol concentrations of up to ~0.5 mg/L in blood [101]. Similarly, a large variety of chocolates containing predominantly alcoholic fruit brandies and liqueurs showed considerable concentrations of ethanol and congeners [102]. Although experiments involving ingestion of the chocolates have not been performed, it would be unlikely that sufficient blood concentrations of congeners would occur with reasonable consumption of the chocolates. After-drinking claims involving circumstances such as these described above have not been documented. Nonetheless, absorption or ingestion of congeners through sources other than alcoholic beverages requires consideration as a potential misrepresentation to the sensitive nature of ACA interpretation.

Pharmacokinetics of fermentation by-product congeners

Considerable investigation has been undertaken surrounding the pharmacokinetics of the targeted congeners, in order to determine the theoretical congener concentrations in blood. Although similar to ethanol, the pharmacokinetics of low and higher alcohols do not completely fit the Widmark model, designed originally for ethanol. Drinking experiments with isolated congeners [103–105] and whole beverages [106–108] helped develop blood congener concentration curves with the use of previously established data on ethanol. This provided a better understanding of the pharmacokinetics of congeners, when combined with data on their physicochemical properties (Table 2). However, caution is still necessary when interpreting the fermentation by-product congener results.

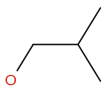
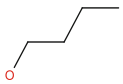
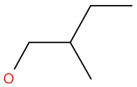
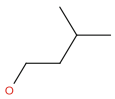
Absorption

Compared with ethanol, alcohol congeners have a slower absorption rate [70]. Differences in beverage absorption occur largely due to Fick's Law, where higher concentrated beverages such as spirits diffuse across membranes faster than lower concentration beverages, such as beer. Food can delay the absorption, while an empty stomach and beverages containing high amounts of carbon dioxide (carbonated beverages), accelerate absorption [109].

Distribution

The reduction factor (r_A) described for each congener in ACA literature is comparable to the volume of distribution and is used for extrapolating the theoretical blood concentration using the back calculation in a revised Widmark formula (Eq. 1). Like ethanol, methanol, 1-propanol, isopropanol and 2-butanol are water soluble and are readily distributed into the body water. The higher alcohols of 1-butanol and isobutanol are less hydrophilic, while 2- and 3-methyl-1-butanol are approximately 50 % soluble in body water, have a higher r_A (Table 2) and consequently penetrate more rapidly into tissues. The proportion of the body available for distribution of the congeners varies considerably throughout the population, affecting the r_A for each congener. Anthropometric parameters (body fat, body mass, age and gender) have led to much discussion regarding the potential to increase the accuracy of BAC detection [110]. However, ACA research is not as comprehensive. There have been improvements for ethanol using updated anthropometric measurements that take into account body mass index (BMI) or total body water (TBW) [111]. For example, overweight and obese individuals may not fit the r_A regressions currently set for ACA. People with a BMI over 30 (clinically obese) result in abnormally low r_A. and those with a BMI over 40 (grossly obese) would have unattainable r_A values [47]. As demonstrated by Watson for ethanol [112], the TBW must also be taken into account. This is achieved by using updated population data for both men and women, utilizing modern anthropometric measurements. This formula provides a more accurate r_A when incorporated into the Widmark formula for ethanol [113]. There have been many updated versions since, with the most recent account presenting a mathematical model of five previously published updated versions of the Widmark model [114, 115]. Here, Posey [110] recognized that having several different populations with more variety in anthropometric measures, better represents the general population for ethanol back-calculations. However, the fermentation by-product ACA methodology utilizes mean values from drinking experiments without incorporating any additional parameters, except for weight.

Table 2 Physicochemical properties of congeners and associated Widmark constants [126]

Compound CAS #	Structural formula	Formula	Molecular weight	Boiling point (°C)	Density (g/mL at 25 °C)	Vapor pressure (kPa at 20 °C)	Water solubility (g/L at 20 °C)	Volume of distribution [31] (r_A)	Congener concentration [31] (C_{time})
Ethanol 64-17-5		C_2H_6O	46.068(2)	78.3	0.785	5.90	Soluble	N/A	N/A
Methanol 67-56-1		CH_4O	32.0419(9)	64.7	0.787	12.8	Soluble	$r_{A\ min} = 0.6$ $r_{A\ med} = 0.7$ $r_{A\ max} = 0.8$	$C_{30} = 0.79 \times C_0 + 0.01 \pm 0.58$ $C_{90} = 0.89 \times C_0 + 0.08 \pm 0.44$ $C_{150} = 0.95 \times C_0 + 0.16 \pm 0.28$
Isopropanol 67-63-0		C_3H_8O	60.095(2)	82.5	0.781	4.4	Soluble	N/A	N/A
1-Propanol 71-23-8		C_3H_8O	60.095(2)	97.2	0.800	1.90	Soluble	$r_{A\ min} = 0.6$ $r_{A\ med} = 0.7$ $r_{A\ max} = 0.8$	$C_{30} = 0.72 \times C_0 \pm 0.05$ $C_{90} = 0.59 \times C_0 + 0.01 \pm 0.07$ $C_{150} = 0.48 \times C_0 + 0.01 \pm 0.12$
2-Butanol 78-92-2		$C_4H_{10}O$	74.122(3)	99.5	0.802	1.65	260 [127]	N/A	N/A
Isobutanol 78-83-1		$C_4H_{10}O$	74.122(3)	107.9	0.798	1.06	80	$r_{A\ min} = 1.1$ $r_{A\ med} = 1.3$ $r_{A\ max} = 1.5$	$C_{30} = 0.56 \times C_0 + 0.03 \pm 0.11$ $C_{90} = 0.40 \times C_0 + 0.03 \pm 0.09$ $C_{150} = 0.30 \times C_0 \pm 0.04$
1-Butanol 71-36-3		$C_4H_{10}O$	74.122(3)	117.7	0.806	0.67	79	N/A	N/A
2-Methyl-1-butanol 137-32-6		$C_5H_{12}O$	88.148(4)	128.0	0.815	0.43	36	N/A	N/A
3-Methyl-1-butanol 123-51-3		$C_5H_{12}O$	88.148(4)	131.1	0.805	0.31	25	$r_{A\ min} = 1.6$ $r_{A\ med} = 2.0$ $r_{A\ max} = 2.4$	$C_{30} = 0.32 \times C_0 \pm 0.05$ $C_{90} = 0.15 \times C_0 \pm 0.04$ $C_{150} = 0.07 \times C_0 \pm 0.06$

Some use three levels to allow for variation (minimum, medium and maximum) [31], while others suggest using a variation of ±30 % [28]. This may be due to the complexities of having multiple compounds of interest that increase the drinking experiment size and also the relatively low amount of ACA performed when compared to ethanol back-calculations internationally. Nonetheless, this presents potentially significant inaccuracies when performing ACA in the general population and particularly in individuals who do not accurately fit the r_A regression patterns.

Metabolism

The congener alcohols can remain unchanged, or undergo phase I (oxidation) or phase II (conjugation) metabolism [116]. The lower alcohol congeners predominantly favor phase I metabolism in the liver where competition at the alcohol dehydrogenase (ADH) enzyme binding sites occurs with the highly abundant ethanol. To explain the influence that relatively high concentrations of ethanol have on the metabolism of the congeners, as well as competition between congeners themselves, animal [117] and human [23, 118] trials were conducted, with and without ethanol. Further drinking trials comprising of congener(s) vs congener(s) of methanol [119], 1-propanol [104, 119], isopropanol [119] and isobutanol [105], were performed, with and without concurrent administration of ethanol to demonstrate how the pharmacokinetics of each congener were altered. Phase II conjugation involved reactions with glucuronic acid alongside the carbon chain length, as seen with higher alcohols [120], to the extent that 2- and 3-methyl-1-butanol were almost completely conjugated [31].

The phenomenon of continual methanol build up has been heavily investigated, as methanol metabolism is inhibited by ADH when ethanol levels are above approximately 0.2 g/kg [119, 121]. This is regularly seen in alcoholics allowing methanol concentrations to build up to potentially toxic levels, well above the endogenous levels of 0.86 ± 0.76 mg/kg that are found to be in the blood of non-alcoholic drinkers [119]. For this reason, measurement of blood methanol has been shown to be useful as a diagnostic tool to distinguish acute from chronic alcoholism [122–124]. However, this methanol accumulation then also requires consideration when evaluating ACA as it has the potential to alter the expected methanol concentration.

Excretion

With the exception of methanol, the elimination of alcohol congeners generally follows first-order kinetics when ethanol levels are high, due to their lower blood concentrations and other pathways of metabolism [31, 121]. Excretion of the unchanged congener alcohols through urine and expired air and has been suggested to be from 1 to 2 % [28] and up to 5-10 % [125]. In general, the higher alcohol congeners are excreted faster, due to the increasing affinity of ADH to longer carbon-chained alcohols. It has also been suggested that 2- and 3-methyl-1-butanol have less importance than lower alcohol congeners as their metabolism and excretion occur relatively rapidly [28]. If the ethanol concentration is low enough and does not alter hepatic metabolism, excretion for all alcohol congeners is increased [31].

Analytical methodology for fermentation by-product ACA

The current analytical technique to determine the fermentation by-product congeners in beverages and blood is by HS-GC-FID and can include trapping and/or cryofocusing techniques to increase the sensitivity. Often ~0.1–0.3 mL of blood is used in order to achieve the required limits of detection (LOD) of ~0.1 mg/L for methanol and ~0.01 mg/L for the remaining alcohol congeners [28, 31]. Calibration curves are not linear for all congener alcohols and are matrix dependent [28] emphasizing the need for internal standards. T-butanol (*tert*-butanol) is the most commonly used internal standard as it is not found in beverages, nor is it produced endogenously or from bacterial putrefaction [15, 128–130]. Matrix effects have been demonstrated for the HS-GC-FID method; these may be reduced with dilution when analyzing alcoholic beverages [35], however, due to the required sensitivity, dilutions of biological matrices is limited. The addition of perchloric acid in order to precipitate proteins and minimize matrix effects has been trialed, but is problematic as it also dilutes the congener concentrations [28, 131]. Other pretreatment techniques available to increase the sensitivity of the analysis include employing solid-phase microextraction (SPME) or in-tube extraction (ITEX) that enhances the recovery of certain analytes. However, competitive binding may be problematic as the high ethanol content decreases the recovery of other analytes of lower concentrations [132]. Competitive binding was not detected using trapping techniques supporting it as the preferred current technique [35]. Chromatographically, large glass columns packed with absorbent material (Carbowax 20M/Carbopack B) [123, 133] were traditionally used but more recently, laboratories have achieved better chromatography with capillary columns (CP-Sil-19, CP-Wax-52, Rtx-BAC1, Rtx-BAC2 etc.) [31, 130]. However, care is required to minimize the water content entering the capillary column, as degradation of chromatography can occur even at low levels in the headspace [134]. Problems exist where co-elution of peaks occurs, particularly when a small peak of a congener is on the tail of the highly abundant ethanol peak [28].

Stability of congeners

The stability of congeners in blood has also been investigated and, like ethanol, they have shown to alter with inappropriate storage conditions. Long term (12 month) storage of blood at room temperature show considerable changes in congener concentrations [65] and even some bacterial production of congeners [135]. When refrigerated at 4 °C, up to 10 % loss of congeners has been demonstrated, compared with no change to congener concentrations when stored frozen at −27 °C for the same 12 month period [65]. However, refrigeration is generally accepted as a suitable storage temperature for after-drinking claims where freezing is not possible. Even with appropriate storage conditions, correct antiseptic techniques are also required, as congener production by bacteria is possible if specimens are not handled aseptically. Correct handling of the specimen, sufficient preservative (e.g., sodium fluoride) and refrigeration, is adequate to inhibit ethanol and congener bacterial artifact production, while minimizing losses in blood concentrations [65, 135]. Bacterial ethanol production can be prevented by either refrigeration and/or addition of sodium fluoride, with a concentration of at least 1 % (w/v), as a preservative in urine [136] and blood [137]. However, one study showed that even with similar preservative concentrations, contamination, most likely from blood diluters, resulted in ethanol degradation by bacteria growth when left at room temperature [138]. This suggests that a preservative (such as sodium fluoride) of at least 1 % (w/v) under refrigerated temperatures is required for sufficient inhibition of bacteria and to increase the stability of congeners.

Evaluation of ACA

The evaluation process involves the back-calculation of each targeted congener to provide the theoretical concentrations of the congeners in blood using the claimed circumstances surrounding consumption, in conjunction with the alcoholic beverage congener content, anthropometric measurements, and known pharmacokinetic parameters of the congeners. The following brief ACA workflow for fermentation by-product congeners has been extrapolated from the review by Bonte where the congeners primarily targeted were: methanol; 1-propanol; isobutanol; 2-butanol; 1-butanol; and 3-methyl-1-butanol [31]. This is achieved by determining the congener concentrations in the beverage(s) that were claimed to be consumed either from published literature, or analysis of the beverage itself. The amount of each congener consumed is determined by multiplying the volume of beverage consumed with the concentration of that congener in the beverage. As the pharmacokinetics of each congener differ and with competition between ethanol occurring at the ADH binding site, each congener must be considered individually when performing the interpretation. The final theoretical concentration of the congener in the blood at a certain time of drinking cessation (C_0) is attained using body weight of the individual and the r_A placed in the Widmark formula (Eq. 1). Only for the methanol, isobutanol, 1-propanol and 3-methyl-1-butanol congeners are the revised version of the Widmark formula provided, described as:

$$C_0 = \frac{\text{amount of the congener consumed (mg)}}{\text{body weight (kg)} \times r_A} \quad (1)$$

Drinking experiments have determined three parameters of the confidence for the r_A of each congener based on pharmacokinetic variability (i.e., minimum, medium and maximum). The use of the appropriate r_A is important as variation occurs between the ranges. However, details on how to select an appropriate r_A are not described, suggesting that it is left to the judgment of the evaluating analyst.

Obtaining the C_{time} is achieved by subtracting the cessation of drinking time from the time of sampling, in minutes. This is required to provide the appropriate formula for each specific congener, as shown in Table 2. Using the C_0 calculated using the above Widmark formula, a congener concentration can then be calculated for each congener at the time of drinking cessation. Interpolation can then assist for times in-between these time points by estimating the concentration of the congener. Extrapolation is required if blood was taken outside of the 1–3 h time points. However naturally, this increases the uncertainty and creates limitations for use of the analysis. Relatively wide standard deviations provide confidence ranges for the expected congener(s) blood concentrations which the actual blood congener(s) concentration is compared with [28]. If the actual congener concentrations fall within the theoretical congener ranges, the claimed alcoholic beverage consumption is feasible and an opinion regarding the case can be made.

It is suggested that this methodology is most useful for 1-propanol and isobutanol and refers to these congeners as the most important of all the congeners in the evaluation of ACA. Due mainly to the very low concentrations in blood after alcohol consumption, 2- and 3-methyl-1-butanol are considered to be of minor importance in ACA and accordingly, methodology on how to evaluate the concentrations of these congeners has not been provided.

There are anomalies with fermentation by-product ACA in regards to the congener content in specific beverage types. For example, specifying that a certain beverage was consumed is usually not possible; only if the claim is feasible or not. However, as fruit brandies contain relatively high amounts of 1-butanol (Table 1), it may indicate that this type of beverage was consumed should the

congener be present. In contrast, beverages containing an extremely low congener content, such as vodka [22], gin and clear rum [1], will provide little or no detectable congeners in the blood, consequently complicating evaluation. Additionally, isopropanol is not found in alcoholic beverages unless intentionally added as an adulterant, as seen in some designer drinks [109]. However, detectable isopropanol levels may be due to endogenous production associated with acetone formation in diabetic ketoacidosis, vigorous exercise or prolonged alcohol abuse [119, 139–141], potentially making confirmation of an isopropanol laced drink difficult. Consumption of multiple different beverage types throughout a drinking session can cause issues with evaluating each congener accurately. Also, ACA is of little use if the same drink is allegedly consumed before and after an incident. Finally, in order to obtain sufficient congener concentrations to meet sensitivity requirements and accurate evaluations, a BAC of 0.08 and above must be present [31]. This may not be suitable in Australia and most other countries where the legal limit is 0.05 % or lower.

Additional evaluating techniques

There are different approaches in the calculation of likely congener content. Iffland and Jones [28] and Krause [11] use exponential elimination of the congeners (i.e., half-life) to determine time dependent changes [105]. The drinking cessation (C_0) is calculated as for Bonte and then used in the formula below (Eq. 2). However, the mean r_A is used for each congener, referenced by Bonte as r_A medium. This differs by the approach used by Bonte where specific formulae (r_A = minimum, medium or maximum) and a standard deviation with each drinking cessation time (Table 2) for each congener were given. Instead, a 30 % variation in distribution provides a confidence range for each congener.

$$C_t = C_0 \times e^{-k \times t} \quad (2)$$

The elimination constant (k) is given by substituting the half-life ($t_{½}$) of the congener as such.

$$k = \frac{0.693}{t_{\frac{1}{2}}} \quad (3)$$

Interpretations and opinions are handed to the courts referring to the claims of the after-drink defense equating from "almost certainly excluded" to "irrefutable" conclusions. In 2000, a German institute reported issuing up to a hundred expert opinions for ACA annually [31]. These opinions contained conclusions that confirmed only around 1 % of the defense's claims, rejected 75 %, ruled 14 % as improbable and were unable to determine in 15 % of cases.

ACA quality assurance

In Germany, a working group called "Alcohol consumption and after-drinking" was founded in 2001 by the German Society of Toxicological and Forensic Chemistry (GTFCh) [142]. In 2006 the group showed the methodology for satisfactory proficiency testing of 16 participating German laboratories performing ACA on fermentation by-products [143]. Prior to this, Iffland and Jones suggested that due to some unsatisfactory external proficiency results, additional investigation was required to standardize the analysis and interpretation throughout laboratories conducting ACA [28]. Nonetheless, Bonte had high confidence in his approach which appeared to be accepted by the courts, at least in the German jurisdiction [31]. This is supported with a recent retrospective study that indicates that German courts strongly refer to the ACA expert opinions in 80.6 % of after-drinking type offense cases, concluding that this technique is accepted in forensic practice [144].

Ingredient biomarker congener ACA

Other than from by-products of fermentation, congeners also exist in beverages as a result of the ingredients and materials used during production. These other substances includes aldehydes, esters, histamines, additives, coloring agents, tannins, phenols and other organic and inorganic compounds [1, 70] and are often beverage-specific. To date, the detection of ingredient biomarker congeners that are uniquely present in certain alcoholic beverage types has only been explored in some herbal spirits for the detection of eugenol [36], anethole [37] and menthone [38]. The detection of these ingredient biomarkers may indicate the consumption of herb, aniseed and peppermint-type liqueurs, since they are not present in other alcoholic beverages. There are no other ingredient biomarker congeners identified specifically for other alcoholic beverages including beer and wine thus far. The identification of such substances may prove useful in the future to determine if beer, wine or some other beverage was consumed.

Unique substances can potentially be identified from manufacturers that determine certain congeners as part of their legislative responsibility [66, 145, 146] or for research purposes in the pursuit of a better product [76, 147–149]. Additionally, profiling of alcoholic beverages has been performed to assist in age markers [150] and authentication claims [76], with some achieving this by principal component analysis [150, 151]. The knowledge obtained by the alcohol industry will assist in discovering potential targets as ingredient biomarkers for a specific alcoholic beverage.

Whatever substance(s) is targeted as a potential unique marker, it is mandatory that research studies assess how

unique this marker(s) may be and of course the bioavailability and pharmacokinetics in humans under various simulated conditions [1]. The targeted congener must also be detectable using conventional techniques.

Analytical methodology for ingredient biomarker ACA

Analytical techniques for the detection of ingredient biomarker congeners are capable of utilizing wider techniques than current ACA practices of HS-GC-FID since they possess larger molecular weights. There has been recent use of MS in the detection of higher molecular weight congeners other than fermentation by-products (such as aromatic congeners) [152], and with SPME pretreatment to detect congeners sourced from herbs [36–38]. The use of high performance liquid chromatography coupled with tandem mass spectrometry (LC–MS) has only been used for flavonoids in beer [153, 154]. Ultimately MS detection is necessary to gain the sensitivity and selectivity required for medico-legal purposes.

Conclusion

Fermentation by-product ACA is a technique used in Germany to investigate after-drinking defense claims by examining the feasibility of the claimed alcoholic beverage consumption. This review expresses concerns surrounding the current ACA approach due to the limitations, uncertainties, the difficult nature of carrying out the evaluation, varying methodologies used by experts and the limited knowledge of the variation in pharmacokinetics of these congeners in humans. For example, many of the fermentative by-product congeners can either be produced endogenously, by bacterial putrefaction during storage, or obtained from sources other than consumption of the claimed alcohol beverage. Additionally, blood sampling must occur within 1–3 h of cessation of drinking and a significant BAC of 0.08 must be present. Furthermore, there is a reliance on information from the defense such as body weight, length of drinking times and case circumstances. Individual pharmacokinetic variability and the consumption of vastly different alcoholic beverage types alter the expected congener concentrations. It is difficult to obtain a universally accepted evaluation method for ACA, which also requires careful interpolation that allows for potentially wide uncertainty ranges. Although these factors exist, it has shown to be a prominent tool used for after-drink type cases in Germany; although it is inadequate for determining the alcohol source in other scenarios.

Although minimally researched to date, ingredient biomarkers offer an alternative and potentially more defendable approach to examine after-drink claims, having the potential for application in other cases by determining the specific source of alcohol. This review highlights a considerable opportunity for investigations into the area of developing methodology for the detection of ingredient biomarker congeners for a range of commonly consumed alcoholic beverages in biological specimens.

Key Points

1. Besides ethanol and water, alcoholic beverages contain a number of compounds called congeners that can be used to determine the type(s) of beverage consumed.
2. Providing proof that a particular alcoholic beverage is the source of alcohol may assist in cases where the type of beverage consumed is at question.
3. Fermentation by-product ACA may assist in determining the feasibility of claimed alcoholic beverage(s) consumption however, it does not indicate the source of alcohol.
4. Detection of ingredient biomarkers from alcoholic beverages may provide a means to identify the source of alcohol consumed.

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APPENDIX 1.2:

CHAPTER 2.1 PUBLICATION

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RESEARCH PAPER

The rapid identification and quantification of iso-α-acids and reduced iso-α-acids in blood using UHPLC-MS/MS: validation of a novel marker for beer consumption

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Abstract A method for the detection of iso-α-acid (IAA) type ingredient congeners that are derived from the hop plant (*Humulus lupulus* L.) was developed to detect recent consumption of beer in blood. Three structurally similar but chemically altered IAA, also used as beer-specific ingredients, are known as "reduced IAA", consisting of the rho-, tetrahydro-, and hexahydro-IAA were also targeted. The use of a simple protein precipitation extraction and ultrahigh-performance liquid chromatography system coupled with a tandem mass spectrometer system enabled detection of these analytes in both antemortem and postmortem blood. Extracts were injected onto a C_{18} solid-core column under gradient elution to achieve separation of isobaric analogs and isomers within a 10-min run time. Electrospray ionization in negative multiple reaction monitoring mode was used to monitor three transitions for each of the analytes that were ultimately grouped together to form a calibration curve for quantification of each of the four IAA groups. The method was fully validated according to international guidelines that included extraction efficiency, matrix effects, process efficiency, ion suppression/enhancement of co-eluting analytes, selectivity, crosstalk, accuracy and precision, stabilities, and lower limits of quantification. Finally, applicability of the method described was demonstrated by the detection of IAA ingredient congeners in the blood of a volunteer following the consumption of a relatively small amount of beer in a pilot study.

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Keywords Alcohol congener analysis · Beer · Ingredient congener · Hop-derived iso-α-acids · UHPLC-MS/MS · Validation

Introduction

Compounds other than ethanol and water that are present in alcoholic beverages are termed congeners and may be detected in blood and urine following alcoholic beverage consumption. The detection and interpretation of these congeners is termed alcohol congener analysis (ACA) and can provide information for after-drinking (or hip flask) defense cases regarding the feasibility of claimed alcohol consumption prior and/or subsequent to a motor vehicle incident [1–3]. Traditionally, ACA has been used to detect levels of fermentation by-product congeners (typically alcohols) that are found in nearly all alcoholic beverages [1, 4, 5]. However, the exact origin of the consumed ethanol cannot be determined and consequently, its use in a postmortem setting has been minimal [6–8].

Congeners may also exist in a beverage as a result of the ingredients used during beverage production, in the form of different classes of compound (i.e., not only alcohols) [9]. Ingredient congeners may often be beverage-specific and present as targets for detection in biological fluid to determine consumption of specific alcoholic beverages [8]. To date, the detection of ingredient congeners present in alcoholic beverage has only been explored in some herbal spirits (e.g., eugenol, anethole, and menthone) [10–12]. Furthermore, ACA that targets ingredient congeners may have fewer limitations to that of the fermentation by-products and can potentially be utilized in a broader range of medicolegal cases where determining the source of consumed alcohol is in dispute. There are no beer ingredient congeners thus far identified as targets for

ingredient congener ACA, indicating the potential to identify targets in this common beverage.

Beer is the oldest and most widely consumed alcoholic beverage; just short of two billion hectoliters were produced in 2012 worldwide [13]. Beer traditionally utilizes the four ingredients—water, yeast, malt, and hops—and may contain other ingredients for preservation or specific flavoring [14, 15]. These ingredients provide up to 800 congeners consisting of not only alcohols but also aldehydes, esters, histamines, additives, tannins, phenols, and other organic and inorganic compounds [2, 14, 16, 17]. Of the range of congeners in beer, those identified as specific to beer and potential targets as ingredient congeners for ACA are those derived from the hop plant.

Traditionally in the boiling phase of beer brewing, the female cones (strobiles) from the hop plant (*Humulus lupulus* L.) are added to the wort (a liquid containing malt-derived sugars). Initially, hops were used to impart the desired bitterness and aromatic *hoppy* properties to the beer. It was later established that hops also stabilizes the beer, including the foam or head [14, 18, 19] and protects the beer principally against gram-positive bacteria and/or microbial damage [20, 21]. Dried hops contain approximately 2–17 % of alpha acids (AA), also known as α-acids or humulones, that consist of three major analogs (defined as n-, co-, and ad-AA) which are almost bitterless [16]. However, after the wort containing the hops is boiled for a prolonged time, the AA molecules are converted into the intensely bitter isomerised-α-acids (IAA) with *cis* and *trans* configurations (Fig. 1) [22–24]. The wort is then cooled and yeast is added; following fermentation, the beer is filtered and packaged into the final product.

Whole extract of the hop cone is also used as a natural herbal remedy commonly in combination with Valerian (*Valeriana officinalis*) for similar complementary properties as a mild sedative in humans for anxiety, insomnia, nervousness, memory, and mood disorders [25–27]. However, as this is a whole extract and does not undergo a boiling (isomerization) process, IAA are not present. Other than for beer, there are no other known uses for hops that undergo isomerisation and develop IAA, presenting a specific ingredient congener for beer.

Six stereoisomers of IAA are produced from the three predominant AA analogs that isomerise into diasterisomers [28]. The compounds are structurally very similar, only differing in the nature of the saturated acyl side chain and the absolute configuration of one of the chiral centers (Fig. 1). The IAA are water soluble, tensioactive, have low pK_a values of ~3.5 and are responsible for the bitter character of beer and foam stabilization [16]. International bitterness units (IBU) are used by the alcohol industry as a measurement of the

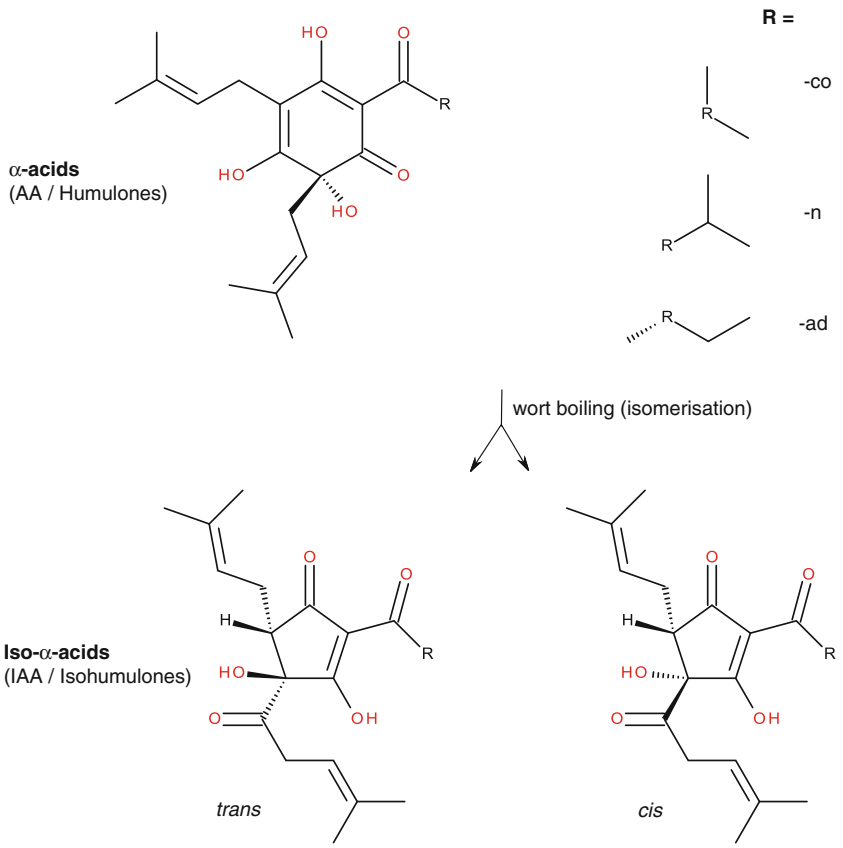

Fig. 1 Isomerisation of α-acid to iso-α-acids in diasteroisomeric *cis* and *trans* forms after boiling of the wort, including the three major analogs

bitterness profile of beer with one unit being approximately equal to 1 mg/L of total IAA [29]. Beers historically ranged from 20 to 60 IBU; however, currently, they range from ~6 to 30 IBU with the exception of some craft beers [21]. It is known that the *cis*/*trans* ratios in beer are 68:32 [30]; however, the *trans*-IAA are present in the foam in greater proportion than the *cis*-IAA due to their lower water solubility. This hydrophobic property also explains the lower half-life of the *trans*-IAA (~1 year) compared to the *cis*-IAA (>5 years) [31] of which a variety of degradation products are formed [32]. Following the isomerization, minor analogs post-, pre-, and adpre-AA are also converted into their respective isomers; however, they are generally regarded as insignificant due to their relatively low concentrations [33]. Although co-elution may be problematic particularly with ultraviolet detection [34], the use of mass spectrometry potentially solves this issue due to the differences of selection based on molecular weights and detected ions.

The IAA are prone to becoming light-struck in the presence of sunlight (near ultraviolet, blue light) and oxygen resulting in familiar and undesirable "skunky" aroma producing compounds, of which 3-methyl-2-butene-thiol (3-MBT) being the most offensive [30]. This historically led to beer being bottled in brown or green, lightproof glass. In recent years, a range of so-called light-stable "reduced IAA" were developed from the naturally available IAA, namely rho-IAA (RIAA), tetrahydro-IAA (TIAA), and, more recently, hexahydro-IAA (HIAA). By reducing the relatively weak double bonds or carbonyl group in the side chains of IAA to stronger single bonds as seen with the reduced IAA, photolytic cleavage is unable to occur on that side chain and the production of 3-MBT is prevented from developing (Fig. 2).

This reduction process provides altered intensities of bitterness [21]. Coincidently, due to increasing hydrophobicity of the compounds (in order of RIAA to HIAA to TIAA), enhanced beer foam stability, appearance, and "cling" are

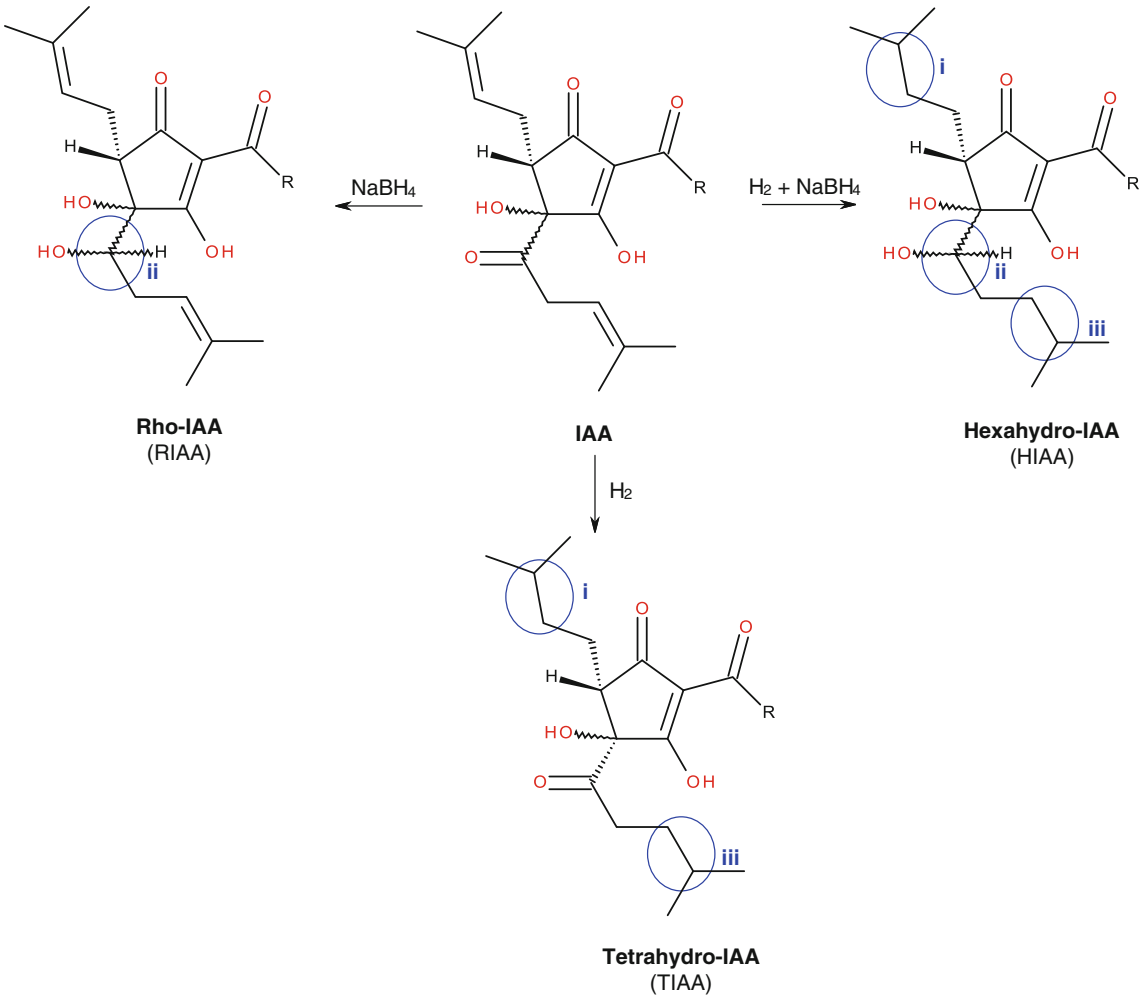

Fig. 2 The synthesis of reduced-IAA and properties of increased hydrophobicity (*i*, *iii*) and photolytic cleavage prevention (*ii*, *iii*). The carbonyl group in the side chain of IAA is reduced with sodium borohydride to produce rho-IAA. Reduction by hydrogenation of both side chains double carbon bonds within the IAA produce the tetrahydro-IAA derivative. Execution of both the sodium borohydride and hydrogenation processes yields the hexahydro-IAA derivative of IAA

observed. As 2.4 and 4.2 ppm (for TIAA and HIAA, respectively) have been sufficient to show foam stabilization to that of the natural IAA; reduced IAA used for the purpose of foam improvement are added in lower concentrations [35].

The reduced IAA products are available as potassium salts preparations ready to be added directly to the finished beer (postfermentation) [21, 36]. Among other non-natural additives, the non-natural reduced forms of IAA are not allowed in beers for the German market due to the "Reinheitsgebot" law stating that only natural hop products, water, malt, and yeast may be used in the brewing process [37].

The aim of this study was to develop a fully validated method for the detection of IAA and reduced IAA in human blood as potential specific ingredient congeners for the confirmation of beer consumption using an ultrahigh-performance liquid chromatography system coupled with a tandem mass spectrometer (UHPLC-MS/MS). This may also alleviate some issues and limitations of current methodologies that detect only fermentation by-product congeners. The detection of reduced IAA in blood may also provide information on the type of beer consumed, for example, beer within a clear glass bottle.

Experimental

Chemicals and reagents

Reference standards for DCHA-Iso, ICS-I3 (containing 62.3 % *w*/*w* of *trans*-IAA); DCHA-Rho, ICS-R2 (containing 65.3 % *w*/*w* of *cis*-RIAA); Tetra, ICS-T2 (containing 99.4 % *w*/*w* of TIAA); DCHA-Hexa, ICS-H1 (containing 65.7 % *w*/*w* of *cis*-HIAA); and ICE-3 (containing 44.64 % *w*/*w* of AA) were obtained from Labor Veritas (Zurich, Switzerland). The isotope-labeled internal standard (IS) nimodipine-d_7 was purchased from PM Separations (Brisbane, Australia). Acetonitrile (ACN), methanol, and formic acid were purchased from Merck (Melbourne, Australia). Ammonium formate was purchased from Sigma Aldrich (Sydney, Australia). All chemicals were of analytical grade or better and water was purified using a Milli-Q Ultrapure Water System from Waters (Sydney, Australia).

Specimens

Preserved blank blood 10 mL samples (containing 200 mg sodium fluoride and 30 mg potassium oxalate) for calibration purposes and validation experiments were obtained from a local blood bank (Melbourne, Australia). Blood of the volunteer in the application to authenticity study was collected in sterile 5 mL Venosafe blood tubes containing 9 mg sodium fluoride and 9 mg potassium oxalate purchased from Hazpak (Melbourne, Australia). Concentrations of the preservatives are therefore at least 2.25 % of each and blood samples were stored at −20 °C, sufficient to inhibit bacterial alteration of ethanol and congeners [38, 39].

Apparatus

The UHPLC-MS/MS system comprised of a Shimadzu MS 8030 quadrupole mass spectrometer (Melbourne, Australia) operated in the electrospray ionization (ESI) in negative mode and a Shimadzu Nexera UHPLC system (Melbourne, Australia) that consisted of a degasser, two eluent pumps, a column oven and a chilled autosampler. For MS data evaluation, Shimadzu Postrun and Shimadzu Browser Analysis (Melbourne, Australia) softwares were used. GraphPad Prism 5.04 from GraphPad Software (San Diego, USA) was used for statistical analysis.

UHPLC conditions

Gradient elution was performed on a Kinetex C_{18} (3.0× 150 mm, 2.6 μm) column coupled with a SecureGuard C_{18} Ultra guard column (3.0×10 mm, 2.6 μm), both purchased from Phenomenex (Melbourne, Australia). The mobile phases consisted of 50 mmol/L aqueous/ACN (90:10) ammonium formate pH 2.8 (eluent A) and ACN containing 0.1 % formic acid (eluent B). The flow rate of the mobile phase was 0.5 mL/min and was degassed by the integrated Shimadzu Nexera degasser during use. The gradient was programmed as follows: 0–0.5 min hold at 50 % eluent B; 0.5–6.0 min eluent B increasing to 60 %; 6.0–9.5 min eluent B increasing to 75 %; and 9.5–10 min eluent B hold at 75 %. Before the start of batch analysis and before each injection, the UHPLC system was flushed for 2 min (90 % eluent B) and equilibrated at starting conditions (50 % eluent B) for 3 min. The column oven was maintained at 30 °C, the autosampler was operated at 4 °C and the autosampler needle was rinsed before and after aspiration of the sample using methanol.

MS/MS conditions

The MS data were acquired with the following ESI inlet conditions: nebulizing gas and drying gas were nitrogen at a flow rate of 3.0 and 12.5 L/min, respectively; the interface voltage was set to 4.8 kV, desolvation line temperature was 190 °C, and the heat block temperature was 500 °C. The mass spectrometer was operated in negative multiple reaction monitoring mode (MRM) with argon as the collision-induced dissociation gas at a pressure of 230 kPa; the detector voltage was set to 1.72 kV.

Specific MRM conditions for nimodipine-d_7 were auto-optimized by direct flow injection of 1 μL of a 1 mg/mL solution in acidic methanol. However, as the IAA and reduced IAA compounds are only available as a combined mixture of analogs, each analyte was manually optimized by injecting 10 μL of a 1 mg/mL solution of the respective IAA group in

acidic methanol onto the column. With the system in Q1 scan mode, the retention time and a precursor mass (Q1 mass) were obtained for each analytes. Using product ion scan mode in a series of repeated injections over a range collision energies (CE) from 10 to 50 V at increments of 2 V, the three most abundant product ions and optimal CE for each individual analyte were obtained. The most abundant product ion was selected as the quantifier ion and subsequent two ions as suitable qualifiers. The results of the auto and manual optimizations are summarized in Table 1. All analyte dwell times were set at 25 ms.

Preparation of stock solutions, control samples and calibration standards

Individual stock solutions of each IAA group and nimodipine-d_7 were prepared at a concentration of 1 mg/mL using acidic methanol (0.05 % formic acid) and methanol, respectively. The working solutions of IAA were prepared by pooling IAA stock solutions to make a single 0.1 mg/mL working solution with subsequent dilutions at the following concentrations: 0.01, 0.001, and 0.0001 mg/mL; using acidic methanol. Stock and working solutions were stored at −20 °C for a maximum time frame of 6 and 1 months, respectively.

The calibration standards were prepared using pooled blank blood spiked with the working solutions to obtain the final concentrations of 0.001, 0.01, 0.05, 0.15, 0.06, 2, 5, and 10 mg/L. These calibrations provided the levels required for varying calibration models for each IAA group (Table 2).

The quality control (QC) samples were prepared using pooled blank blood spiked with the working solutions to obtain the final concentrations of 0.1 mg/L (low), 0.8 mg/L (med), and 6 mg/L (high; Table 2), and were stored at −60 °C before analysis.

Sample preparation

Briefly, 100 μL of blood underwent protein precipitation in a 2 mL Eppendorf tube from Eppendorf (Sydney, Australia) using 200 μL of cold (−20 °C) ACN containing the IS nimodipine-d_7 (0.5 mg/L) for 5 min on a shaker at 2,500 rpm. After a 10-min rest and subsequent centrifugation at 15,000 rpm for 10 min, the supernatant was transferred to an autosampler vial and evaporated to dryness under nitrogen using a Ratek dry block heater DBH10 (Melbourne, Australia) operated at room temperature. The residue was reconstituted in 50 μL of a mixture of eluent A and eluent B (60:40, *v*/*v*). Twenty microliters of the final extract was injected into the UHPLC-MS/MS system.

Validation

Full validation was performed as per international guidelines [40, 41]. Although results are reported as the concentration of the groups (rather than individual analytes), individual analytes were validated for such parameters as selectivity, the stabilities and matrix effects, as they each have their own chemical properties. Accuracy and precision was based on the calibration model of total area of the analytes consisting in that group.

Selectivity and crosstalk

Selectivity experiments were carried out using 10 antemortem and 10 postmortem blood samples obtained by the authors' laboratory for routine toxicological requirements. In total, the 20 different blood samples were analyzed to demonstrate no interference with endogenous peaks and targeted ions, including potential crosstalk of ions within the MS. An additional two zero samples (blank sample+IS) were analyzed to check for the absence of analyte ions in the respective peaks of the IS. Furthermore, pooled blank blood was spiked with either *trans*-IAA, *cis*-RIAA, TIAA, *cis*-HIAA, or AA in duplicates at concentrations of the upper limit of calibration curve 10 mg/L using individual stock solutions to check for the absence of interference with IS ions. This also ensured there was no interference between IAA groups as the IAA/AA family of compounds are structurally similar and include similar fragmented product ions. Pooled blank blood was also spiked with a mix of ~350 common therapeutic drugs ranging from levels 1–10 mg/L to determine any interference with individuals taking medications concurrent to beer consumption.

Extraction efficiencies, matrix effects, and process efficiencies

The extraction efficiencies, matrix effects, and process efficiencies were estimated with a set of three different samples at two concentrations (low and high QC levels) with five samples each according to the simplified approach described by Matuszewski et al. [42]. The IS was estimated concurrently at a concentration used in the described method. Sample set 1 represented the neat standard, sample set 2 represented blank matrix spiked after extraction, and sample set 3 consisted of blank matrix spiked before extraction. Extraction efficiencies were estimated by comparison of the peak area of the samples of set 2 to those of set 3. For the matrix effects, the peak area of the samples of set 2 was compared to those of set 1. For process efficiencies, the peak area of the samples of set 3 was compared to set 1. All values are reported in percentage. General acceptability levels of 50 % extraction efficiency or more were applied. Values over 100 % for matrix effects indicate ion enhancement, while values below 100 % indicate ion suppression.

Table 1 Groups and analytes, retention times (RT) [min], multiple reaction monitoring (MRM) transitions Q1 and Q3 masses [Da], Q1 pre-bias [V], collision cell energy (CE) [V], and Q3 pre-bias [V], used in LC-ESI-MS/MS

Group	Analyte	(RT [min])	Ions	Q1 mass [Da]	Q3 mass [Da]	Q1 pre-bias [V]	CE [V]	Q3 pre-bias [V]
IAA	**I1** *trans*-isocohumulone[a]	(2.70)	Quant	347.30	251.05	12	12	20
	I2 *cis*-isocohumulone	(2.95)	Qual		182.05		16	14
			Qual		329.10		18	26
	I3 *trans*-isohumulone	(3.20)	Quant	360.90	264.95	16	14	22
	I4 cis-isohumulone[a]	(3.45)	Qual		195.95		16	24
	I5 *trans*-isoadhumulone	(3.75)						
	I6 *cis*-isoadhumulone[a]	(4.05)	Qual		291.95		14	22
RIAA	**R1** *cis*-rho-isocohumulone 1	(4.25)	Quant	348.90	251.10	16	16	20
	R2 *cis*-rho-isocohumulone 2	(4.90)	Qual		181.95		16	24
			Qual		233.00		20	10
	R3 *cis*-rho-isohumulone 1	(5.55)	Quant	362.90	265.25	16	16	20
	R4 *cis*-rho-isohumulone 2	(5.70)	Qual		196.20		16	24
	R5 *cis*-rho-isoadhumulone 1	(6.30)						
	R6 *cis*-rho-isoadhumulone 2	(6.55)	Qual		247.00		18	20
TIAA	**T1** *trans*-tetrahydro-isocohumulone	(4.55)	Quant	350.90	239.05	16	16	14
	T2 *cis*-tetrahydro-isocohumulone	(4.90)	Qual		253.00		14	20
			Qual		235.10		30	18
	T3 *trans*-tetrahydro-isohumulone	(5.10)	Quant	364.90	267.10	16	14	22
	T4 *cis*-tetrahydro-isohumulone	(5.75)	Qual		321.10		16	18
	T5+6 *trans*+*cis*-tetrahydro-isoadhumulone	(6.50)	Qual		125.00		42	40
HIAA	**H1** *cis*-hexahydro-isocohumulone 1	(6.90)	Quant	353.00	253.00	16	16	20
	H2 *cis*-hexahydro-isocohumulone 2	(7.45)	Qual		235.00		18	18
			Qual		209.10		16	16
	H3 *cis*-hexahydro-isohumulone 1	(7.80)	Quant	367.00	267.10		16	22
	H4 *cis*-hexahydro-isohumulone 2	(8.45)	Qual		249.00		32	30
	H5+6 *cis*-hexahydro-isoadhumulone 1+2	(9.10)	Qual		223.05		14	12
	IS nimodipine-d_7	(4.55)	Quant	424.20	122.10	10	22	14
			Qual		92.30		33	12
			Qual		301.20		21	23

[a]Residual analytes from TIAA standard, not quantified

Table 2 Concentrations of calibration standards and quality control samples of all studied grouped analytes in milligrams per liter

Group	Weighting[a]	Calibration standards								Quality controls		
		1	2	3	4	5	6	7	8	Low	Med	High
trans-IAA	$1/x$	0.001	0.01	0.05	0.15	0.6	2	5	10	0.1	0.8	6
cis-RIAA	$1/x$	n/a	n/a	0.05	0.15	0.6	2	5	10	0.1	0.8	6
TIAA	$1/x^2$	n/a	n/a	0.05	0.15	0.6	2	5	10	0.1	0.8	6
cis-HIAA	$1/x^2$	n/a	0.01	0.05	0.15	0.6	2	5	10	0.1	0.8	6

[a] Weighting was used for analysis using calibration standards 1–6 for quantification of samples below 2 mg/L. Otherwise no weighting was applied when analysis using full curve to quantify higher concentrations from 2 to 10 mg/L

Linearity

The calibration model was based on the total area of the analytes consisting in that group as ratio to the IS area. Blank blood aliquots were spiked at concentrations given in Table 2 and extracted as described previously to obtain calibration standards. Replicates ($n=6$) at each of the eight concentration levels were analyzed. As the expected concentrations of IAA in blood are unknown, the calibration model was tiered into two calibration levels based on the QC to be analyzed. Quality controls low and medium were analyzed using calibration standards 1–6 while the high QC was analyzed using the full (1–8) calibration curve. All groups were visually checked for a linear or quadratic fit and weighting (none, $1/x$ or $1/x^2$). Daily calibration curves using the same concentrations (single measurements per level) were prepared with each batch of validation and authentic samples.

Lower limit of quantification

The lower limit of quantification (LLOQ) was defined as the lowest point of the calibration curve of the method (Table 2) and fulfilled the requirement of LLOQ, signal-to-noise ratio ≥10:1 for quantifier and qualifier of the lowest abundant peak in the respective group. Furthermore, it was tested whether the quantifier/qualifier ratio of the MRM signals was within the acceptable limits and if there were at least 12 data points available at this concentration for each analyte of the group. Limit of detection values were not systematically evaluated due to the multiple analytes present in each group, a signal-to-noise ratio ≥3:1 of at least one analyte is sufficient to confirm detection of the respected group [40, 41].

Processed sample stability

The stability of the processed samples during batch analysis under the conditions of the described method were estimated. Quality control samples at low and high concentrations ($n=9$ of each) were extracted as described previously and resulting extracts pooled. Aliquots of these pooled extracts at each concentration level were transferred to autosampler vials and injected into the UHPLC-MS/MS system and analyzed under the described method. The time intervals between the analyses of the QC samples were extended to 3.1 h by the injection of 11 blank samples repeated over a 24.8 h period. Stability of the extracted analytes was tested by regression analysis plotting absolute peak areas of each analyte at each concentration versus injection time. The instability of the processed samples was indicated by a negative slope, significantly different from zero ($P \leq 0.05$) [41, 43].

Freeze/thaw and bench-top stability

Combined freeze/thaw and bench-top stability were evaluated by analysis of low and high QC samples ($n=6$ of each) before (control samples) and after four and eight freeze/thaw cycles (stability samples) for evaluation of freeze/thaw stability. Samples completed in total eight freeze/thaw cycles by undergoing 22 h freezing period (−60 °C) and a thawing period of 2 h at room temperature to incorporate bench-top stability. The experiments were carried out together with the accuracy and precision experiments and the concentrations of the control and stability samples were calculated via daily calibration curves. For stability, there are two criteria which have to be fulfilled: the ratio of means (stability/control) has to be within 90–110 % and the 90 % confidence interval has to be within 80–120 % from the control sample [40].

Accuracy and precision

Low-, medium-, and high-quality control samples were prepared at the previously described concentrations and in duplicate each QC concentration was analyzed over a period of eight consecutive days using the described method using daily calibration curves. Accuracy was calculated for each analyte and bias determined by calculating the percent deviation of the mean of all calculated concentration values at a specific level from the respective nominal concentration. Repeatability (within-day precision) and time-different intermediate precision were calculated as relative standard deviation (RSD) [44]

using one-way analysis of variance (ANOVA) with the grouping variable "day". For low and medium concentrations, the acceptance limit was set to 20 % CV and RSD, respectively, and 15 % CV and RSD, respectively, for high concentrations.

Application to authentic samples

A normal healthy male volunteer was administered ~570 mL of West Coast IPA from Green Flash Brewery (San Diego, USA) described as 95 IBU and therefore assumed that ~55 mg of total IAA was consumed. The volunteer consumed this quantity of beer at 7.3 % alcohol by volume in order to obtain ~0.05 blood alcohol concentration, the legal limit in Australia and many other countries. Blood was sampled prior (zero), 0.5, 2, and 6 h postconsumption where the zero sample acted as the control and blank blood of the participant.

Results and discussion

Separation and detection

Sample preparation and methods of detection

Historically, the bitter content of beer was estimated by a broad photometric analysis to provide an IBU rating [45] and has shown to suffice for the brewing industry to attribute a general bitterness profile for beers. Currently, the technique predominately used in routine beer and hop product analysis ensures consistent testing using a standardized HPLC-UV method by the European Brewery Convention (EBC method 7.8) [46]. Other analytical techniques utilizing capillary electrophoresis [47] and HPLC-UV [23, 34] showed improvement; however, accurate and specific determination of IAA and reduced IAA content was not possible until the emergence of LC-MS(MS) [33, 34, 48]. These LC-MS(MS) methods are highly selective and sufficiently sensitive for the analysis of beer that contain a relatively high concentration of IAA. Our method describes an extraction with sufficient recovery to allow for the analysis of IAA compounds in human blood after consumption of beer. A typical sample chromatogram acquired in ESI-negative mode shows the separation of the analytes (Fig. 3). Liquid–liquid extraction was found to be inappropriate when in combination with the highly acidic buffers required to improve extraction of the acidic IAA compounds. The combination would lyse the red blood cells and cause intracellular matrix to fall into the extracted sample and interfere with analysis. Protein precipitation was demonstrated as a sufficient clean-up step that did not degrade the biological matrix or result in a loss of recovery of the analytes. The extraction was found to be further optimized with the use of cold (−20 °C) ACN and the supernatant allowed to rest for 10 min after centrifugation.

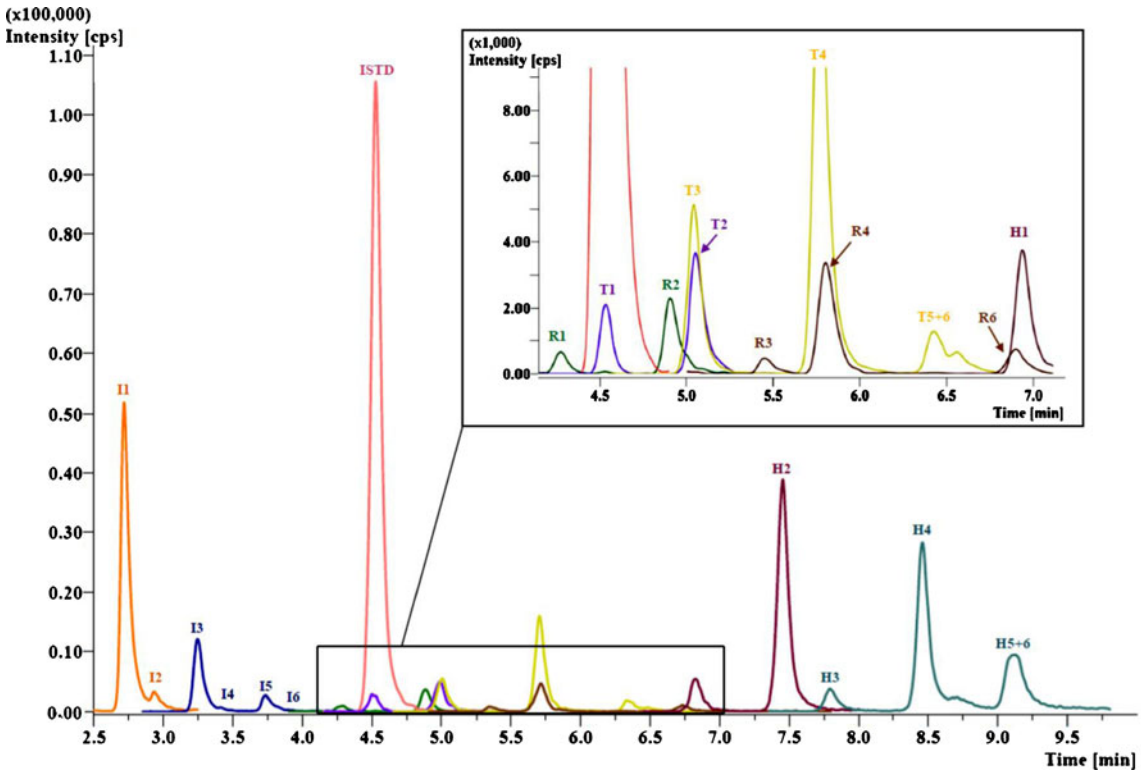

Fig. 3 Chromatograph of individual analytes at spiked blood concentrations of 1 mg/L for the *trans*-IAA, *cis*-RIAA, TIAA, and *cis*-HIAA groups (0.1 mg/L for IS) using the validated LC-MS/MS method presented. Residual *cis*-IAA (mainly analyte I2, but also I4 and I6 in higher concentrations) remain in the *trans*-IAA reference standard and as carry-over during production

Only in recent years have methods (using typically C_{18} columns) shown near complete separation and selectivity of individual IAA [33, 34] and reduced IAA analytes [29], requiring a run time of 20–50 min. After extraction from blood, our method separated the analytes using a gradient elution on a core–shell C_{18} column within 10 min. Preliminary experiments showed increased chromatographic selectivity and sensitivity using this column with considerably lower flow rates under high pressure (data not shown). The decrease of flow rate over the run improved the separation and peak shape of late eluting compounds. This chromatographic system was further optimized by applying a lower pH to eluent A (preferably at least 1 pH unit below the ~3.5 pKa of IAA) in order to obtain improved ionization of acidic compounds. However, phosphate buffers are not compatible with MS detection and ammonium acetate buffer systems allowing for relatively low pH ranges were tested with poor chromatography results. Although the lowest capacity of the chosen ammonium formate buffer system is pH 2.8, this pH demonstrated the best separation of nearly all analytes. The class of IAA compounds contains two analogs (n- and ad-IAA) with the same molecular weight and there are structural isomers within these analogs (*cis* and *trans*; see Figs. 1 and 2). It is therefore expected that separation difficulties with compounds of related chemistries and selectivity issues may be encountered with isobaric compounds with similar fragmentation. To avoid misidentifications, chromatographic separation of these isobaric compounds needed to be achieved. The determination of retention times within a batch of analysis compared to the IS in positive samples was necessary to avoid misidentifications of isobaric compounds. Some isobaric structural isomers (T5 and T6, as well as H5 and H6) were not baseline separated. However, as they are present in their respective standards and in the reduced hop products used, the total area of the combined peaks was sufficient to achieve reasonably accurate quantification under the described conditions.

Stability

Information from the IAA manufacture Labor Veritas and EBC indicate that IAA standards are unstable after a day in methanol but the use of phosphoric acid in methanol for stock and working solutions was sufficient to stabilize most of the compounds. However, degradation of *trans*-IAA in the DCHA-Iso, ICS-I1 standard has still been demonstrated [34]. Due to the known deterioration with MS metallic parts following the continual use of the nonvolatile phosphoric acid, formic acid was substituted. A 0.05 % concentration of formic acid in methanol was found to stabilize the IAA sufficient for stock and working solutions to be stored at −20 °C over a 6- and 1-month period, respectively. As reduced on-column stability of the analytes was observed with increases in temperatures (data not shown), the column oven was maintained at a minimal temperature above ambient (30 °C).

Grouped standards

Due to the unavailability of individual analyte standards and as each analyte has variable abundance within the respective group, the LLOQ of the group was restricted to the analyte with the lowest abundance with a signal-to-noise ratio of 10:1. However, detection of individual analytes with a signal-to-noise ratio of 3:1 [40] was sufficient to report beer consumption as detected. This also proves problematic for the determination of MRM transitions which resulted in the precursor and product ions meticulously selected manually by repeated injections of standards at different conditions described in Table 1. This review process ensured that all fragment ions were explainable as possible fragments of the respective chemical structure. To improve overall sensitivity of IAA compounds, a thorough source optimization was undertaken improving all groups of analytes with the IAA group recording the greatest gains. This reflects the relatively lower LLOQ for this group specifically and results in better sensitivity for the majority of beer consumption. The dwell times were optimized depending on the signal response of each individual analyte by repeated injection; all analytes obtained >30 points across the peaks. Although there are more theoretical reduced IAA isomers present in brewing products and reference standards (i.e., the *trans* isomers of the RIAA and HIAA groups), the extremely low relative abundance of these analytes did not provide sufficient detection limits. These analytes were also unable to be detected at high (10 mg/L) concentrations and would therefore not interfere with quantification even with large beer consumption. Although the manufacturer of the IAA standards claim that residual pre-, post-, and adpost-IAA may be present from the production process [46], the difference in precursor ions to that of the scheduled MRMs allows for accurate selectively of targeted IAA using MS.

Calibration model

As a deuterated derivative of IAA is currently unavailable, the chemical and structural similarities of various compounds were examined. Nimodipine-d_7 was selected as a suitable IS for the calibration model. Other techniques have used an *ECHO* technique consisting of a shortly followed second injection containing the IAA group as the IS [33]. However, a closely followed injection of IS does not compensate for injection volume variability and is not wholly appropriate when gradient elution chromatography is undertaken. The relatively low abundance of R5 only produced a signal-to-noise ratio of 10:1 at total *cis*-RAA concentrations of 2 mg/L. Therefore, the R5 analyte does not contain a peak in Fig. 3 and validation data using QCs at lower concentrations were also

unable to be performed. For this reason, it was not used for calculation of the concentration curve or for any samples. As mentioned earlier, the calibration models involved two curves for analysis. The same calibrations standards were used for quantification, the two curves only differed on whether points 7 and 8 were included when quantifying high-quality controls. All IAA groups were spiked into all standards, irrespective of whether the particular group would be included in the calibration level (i.e., groups *cis*-RIAA, TIAA, and *cis*-HIAA were not examined at calibration level 1 due to their LLOQ, see Table 2; however, the groups were still present in the extracted samples). Antemortem blood was chosen as the matrix for the calibration standards, matrix effects and quality controls rather than postmortem blood. Excess blank postmortem blood from deceased persons is difficult to obtain ethically for assay calibration purposes and matrix effects studies, whereas antemortem blood was readily available through blood donor banks.

Validation

The described procedure was validated according to internationally accepted recommendations [40, 41]. For the detection of the IAA and reduced IAA, three MRM transitions were used for each analyte; their use and their respective peak area ratios enabled unambiguous identification of all IAA and reduced IAA compounds included in the assay. The selectivity studies showed no interference or crosstalk in 20 different clinical and postmortem samples.

Table 3 shows mean values of extraction efficiencies and matrix effects of the corresponding variation over five different blood samples. Datasets in which the variation (minimum and maximum values in percentage) is greater than 20 % difference of the mean value are marked in bold type. Overall, the method showed satisfactory extraction efficiencies for most analytes; however, variation occurred for many analytes at low concentrations. Table 3 also shows the mean values of matrix effects and the corresponding variation over five different blood samples with strong ion enhancement demonstrated. The described extraction procedure showed no significant matrix effects over five different blank blood samples in high concentrations. However, nearly half of the analytes demonstrated variation in matrix effects at low concentrations. Matrix effect studies of IS nimodipine-d_7 were also performed at the target concentration with no significant matrix effects observed over five different blank blood samples with

Table 3 Matrix effects and recoveries in percent [range] of all targets and IS

Analyte		Matrix effects		Extraction efficiency	
		QC low	QC high	QC low	QC high
I1	*trans*-isocohumulone	139 [133–150]	77 [70–85]	75 [63–90]	68 [60–77]
I3	*trans*-isohumulone	195 [179–207]	84 [79–88]	97 [87–110]	69 [59–78]
I5	*trans*-isoadhumulone	287 [266–310]	107 [101–114]	**78 [47–101]**	76 [65–89]
R1	*cis*-rho-isocohumulone 1	272 [257–297]	143 [133–155]	**92 [52–109]**	72 [61–85]
R2	*cis*-rho-isocohumulone 2	313 [254–345]	123 [118–130]	**66 [30–97]**	71 [61–82]
R3	*cis*-rho-isohumulone 1	214 [200–226]	98 [95–102]	71 [65–80]	65 [58–73]
R4	*cis*-rho-isohumulone 2	537 [525–550]	135 [126–143]	**86 [57–102]**	67 [59–78]
R6	*cis*-rho-isoadhumulone 2	–[b]	187 [171–217]	–[b]	68 [53–82]
T1	*trans*-tetrahydro-isocohumulone	**174 [95–232]**	128 [122–134]	**139 [98–165]**	86 [74–96]
T2	*cis*-tetrahydro-isocohumulone	265 [238–284]	82 [78–89]	100 [83–117]	79 [71–89]
T3	*trans*-tetrahydro-isohumulone	–[b]	90 [85–95]	–[b]	81 [71–91]
T4	*cis*-tetrahydro-isohumulone	209 [199–223]	87 [80–94]	**121 [75–147]**	**73 [54–84]**
T5+6	*tran*+*cis*-tetrahydro-isoadhumulone	–[b]	86 [72–93]	–[b]	83 [72–93]
H1	*cis*-hexahydro-isocohumulone 1	**208 [147–240]**	87 [77–95]	**117 [98–141]**	71 [63–78]
H2	*cis*-hexahydro-isocohumulone 2	**238 [148–341]**	102 [92–111]	83 [51–97]	59 [50–69]
H3	*cis*-hexahydro-isohumulone 1	–[b]	95 [86–106]	–[b]	63 [53–74]
H4	*cis*-hexahydro-isohumulone 2	**257 [146–277]**	105 [96–115]	**73 [63–94]**	60 [51–71]
H5+6	*cis*-hexahydro-isoadhumulone 1+2	**209 [158–315]**	104 [96–115]	116 [97–135]	66 [56–79]
IS	nimodipine-d_7[a]	83 [79–90]		68 [58–76]	

Datasets with variations (minimum and maximum value in percent) greater than 20 % difference of the mean value (not acceptable) are marked in bold type, $n=5$

[a] The IS nimodipine-d_7 was analyzed at a concentration of 0.1 mg/L during the sample preparation of the described method

[b] Abundance of analyte relatively low in quality control, subsequent area unattainable

Table 4 Accuracy [in percent], intermediate (time-dependent intermediate precision) [in percent RSD] and repeatability (within-day precision) [in percent RSD] of the UHP LC-MS/MS assay for IAA type groups in blood

Group	Quality control	QC LOW (0.1 mg/L)	QC MED (0.8 mg/L)	QC HIGH (6 mg/L)
trans-IAA	Repeatability	7.0	5.8	7.7
	Precision	**31.1**	17.8	**17.2**
	Accuracy	19.0	5.4	9.2
cis-RIAA	Repeatability	7.4	12.7	9.3
	Precision	**23.0**	12.7	**15.4**
	Accuracy	18.1	0.9	5.6
TIAA	Repeatability	5.0	8.0	8.0
	Precision	15.9	16.5	**17.4**
	Accuracy	**34.7**	12.0	11.4
cis-HIAA	Repeatability	9.6	16.5	6.1
	Precision	**24.3**	**27.3**	**18.7**
	Accuracy	3.3	10.6	5.7

Datasets outside required limits are marked in bold type, $n=2$ (over 8 consecutive days)

satisfactory results. The described method demonstrated satisfactory process efficiency of 50 % or more for all analytes.

Linear regression was applied to all studied groups and no weighting applied when using the full calibration curve for high QC analysis. As a result of heteroscedasticity that is commonly encountered for calibrations ranges spanning more than 1 order of magnitude [40], a weighted model ($1/x$ for IAA and *cis*-RIAA, $1/x^2$ for TIAA, and *cis*-HIAA; Table 2) was used for the calibration curve using standards 1–6 for lower and medium QC analysis. The calibration fit showed a coefficient of determination of $r^2>0.95$ for all groups and calibration ranges.

In the freeze/thaw and long-term stability experiments, the ratio of means (stability versus control samples) was within

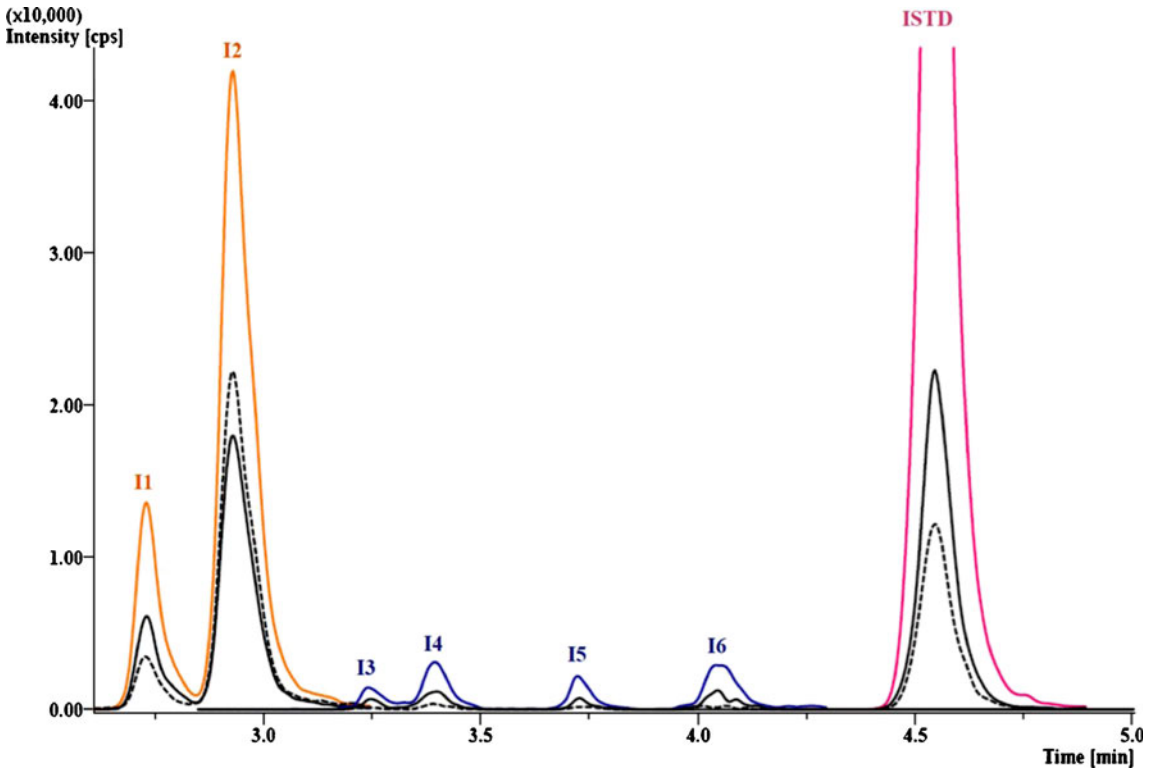

Fig. 4 Chromatograph of blood taken 30 min after consumption of ~570 mL beer containing ~55 g of IAA (*trans* and *cis*) from a volunteer. The concentration of the *trans*-IAA (analytes I1, I3, and I5) was ~0.2 mg/L. The *cis*-IAA group (analytes I2, I4, and I6) are unable to be quantified due to the unavailability of a reference standard; however, they do provide evidence for the consumption of beer. All IAA analytes demonstrate baseline separation as not to interfere with quantification results. *Colored lines* demonstrate quantification ions; *black full* and *dashed lines* indicate the two other MRMs

90–110 %, whereas the 90 % CIs for stability samples were within 80–120 % of the respective control means. Stability issues have demonstrated degradation of the t*rans*-IAA standard when stored at 20 °C for 24 h *without* light exposure [34], potentially problematic for processed sample stability. However, in the processed sample extracts, the acidic eluent conditions that the samples were reconstituted with assisted in sufficiently stabilizing the analytes for over 24 h analytes at low and high concentrations. Stability was potentially assisted as the described method utilizes a chilled autosampler for storage of processed samples at 4 °C.

Accuracy data was within the acceptance interval of ±15 % (±20 % for low and medium) of the nominal values for all IAA groups excluding TIAA at low QC concentrations. Within-day (repeatability) and intermediate precision required limits of ±15 % RSD (±20 % for low and medium) of which all repeatability data was within. However, the results summarized in Table 4 demonstrate variability in intermediate precision for low and high concentrations in most groups when criteria above are applied. The calibration model incorporating total peak areas as a single group may induce greater variation in accuracy and precession results. The synthesis of a deuterated IAA may help alleviate some of the problems. Overall, the grouped calibration using the described IS provides an approximate quantification for *trans*-IAA, *cis*-RIAA, TIAA, and *cis*-HIAA and is a good representative value for the amount of IAA, RIAA, TIAA, and HIAA, respectively, in blood postconsumption of beer.

Application to authentic samples

Detection of all IAA analytes were present in the 0.5 and 2.0 h duplicate bloods at *trans*-IAA concentrations of ~0.2 (Fig. 4) and ~0.02 mg/L, respectively, of the volunteer. Blood taken 6 h postconsumption detected the I1 analyte only and therefore accurate quantification was not possible. This is most probably due to the increased sensitivity of this presented method for that particular analyte, compared to analogs I3 and I5 at the similar concentrations. However, detection of solely I1 at 6 h demonstrates how detection of individual analytes is sufficient to show beer consumption. Similarly, detection of the I2, I4, and I6 analytes of the *cis*-IAA group that were not quantified due to unavailability of a reference standard was also able to confirm beer consumption. Furthermore, should *trans* isomers undergo a more rapid metabolism and/or have since degraded at the time of sampling, the *cis* isomers have a much longer half-life and may still be used to indicate beer consumption over a greater detection window. The detection of beer ingredient congeners in the participant of the pilot study at a relatively low amount of beer consumption than what might be typical, demonstrates high sensitivity and consequently, applicability of the method.

Conclusion

The UHPLC-MS/MS assay presented is a suitable procedure for the separation, detection, and quantification of IAA, RIAA, TIAA, and HIAA compounds as beer-specific ingredient congeners in blood samples to confirm beer consumption. Validation has proven to be selective, linear, accurate, and precise for the range of beer ingredient congeners at concentrations expected to be found in blood. The *trans*-IAA were quantified in a volunteer postconsumption of beer and demonstrates possible applicability for clinical and forensic toxicological casework.

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Conflict of interest The authors declare that there is no conflict of interest.

Ethics Approval for the human consumption of beer and subsequent specimen retrieval and analysis was obtained from the Human Research Ethics Committee at the Victorian institute of Forensic Medicine.

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APPENDIX 1.3:

CHAPTER 3.1 PUBLICATION

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The stability of iso-α-acids and reduced iso-α-acids in stored blood specimens

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ABSTRACT

The long-term stability of the iso-α-acids, and three structurally similar but chemically altered iso-α-acids (known as 'reduced iso-α-acids' and consisting of the rho-, tetrahydro- and hexahydro-iso-α-acid groups) were investigated in whole blood. Pools of blank blood spiked with the four beer-specific ingredient congener groups at two different concentration levels were stored at 20 °C, 4 °C and −20 °C; and extracted in duplicate in weeks 1, 3, 5 and 8, using a previously published method. A loss of 15% of the initial concentration was considered to indicate possible instability and losses greater than 30% demonstrated significant losses. The individual analytes within the four iso-α-acid groups were also measured to determine which iso-α-acids were subject to greater degradation and were responsible for the overall group instability. All four iso-α-acid groups showed significant losses after 8 weeks of storage under room temperature conditions in particularly the natural iso-α-acid group where major losses were observed (96% and 85% losses for low and high concentrations, respectively). Some degradation in all iso-α-acid groups were seen at 4 °C samples predominantly due to the 'n' analogs of the groups showing an increased instability in blood. The −20 °C storage conditions resulted in minimal changes in concentrations of all analytes. Higher than frozen storage temperatures can result in substantial changes on the stability of the iso-α-acid type groups in blood. The aim of this study was to highlight the stabilities of the IAA analytes in order to assist in the interpretation of IAA in stored blood specimens.
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1. Introduction

Methodology for novel alcohol congener analysis that allows detection of ingredient congeners specific to beer has recently been developed in blood [1]. Detection of these compounds in blood allows for the confirmation of beer ingestion which is potentially useful in cases where an after-drinking (hip-flask) defense is being used [2,3], but also in other medico-legal casework where the source of alcohol is sought [4]. These beer-specific ingredient congeners consist of the hop-derived iso-α-acids (IAA) type compounds that are formed following the isomerization of α-acid (AA) contained within the hops that are added during the boiling process of brewing beer (Fig. 1). Furthermore, derivatives of the natural IAA group exist and are termed 'reduced IAA', consisting of the rho-, tetrahydro-, and hexhydro-IAA (RIAA, TIAA and HIAA, respectively). The three reduced IAA groups are used for their light protective and foam stabilizing properties (Fig. 1), can also be detected post-consumption of particular beers containing these types of compounds.

During the boiling phase of brewing beer, the three predominant AA analogs (co, n and ad) isomerize into diasteromers to produce six IAA stereoisomers [5], differing only in the absolute configuration of one of the chiral centers and saturated acyl side-chain (Fig. 1). The tensioactive, water soluble and low p*Ka* (~3.5) properties of the IAA compounds lead to the intense bitterness of beer and also provide foam stabilization [5,6]. The IAA are subject to radical-assisted oxidation [7] and furthermore, in the presence of sunlight and oxygen, the undesirable "skunky" smelling 3-methyl-2-butene-thiol (3-MBT) is produced when beer is not protected by lightproof brown-bottled glass (or less-so green) [8,9]. However in recent years, the development of a range of light-stable reduced IAA allows for clear glass bottles to be used. By reducing the susceptible carbonyl group in the side chains or double bonds of IAA to less susceptible single bonds (i.e. reduced IAA), the production of 3-MBT by photolytic cleavage is prevented (Fig. 1).

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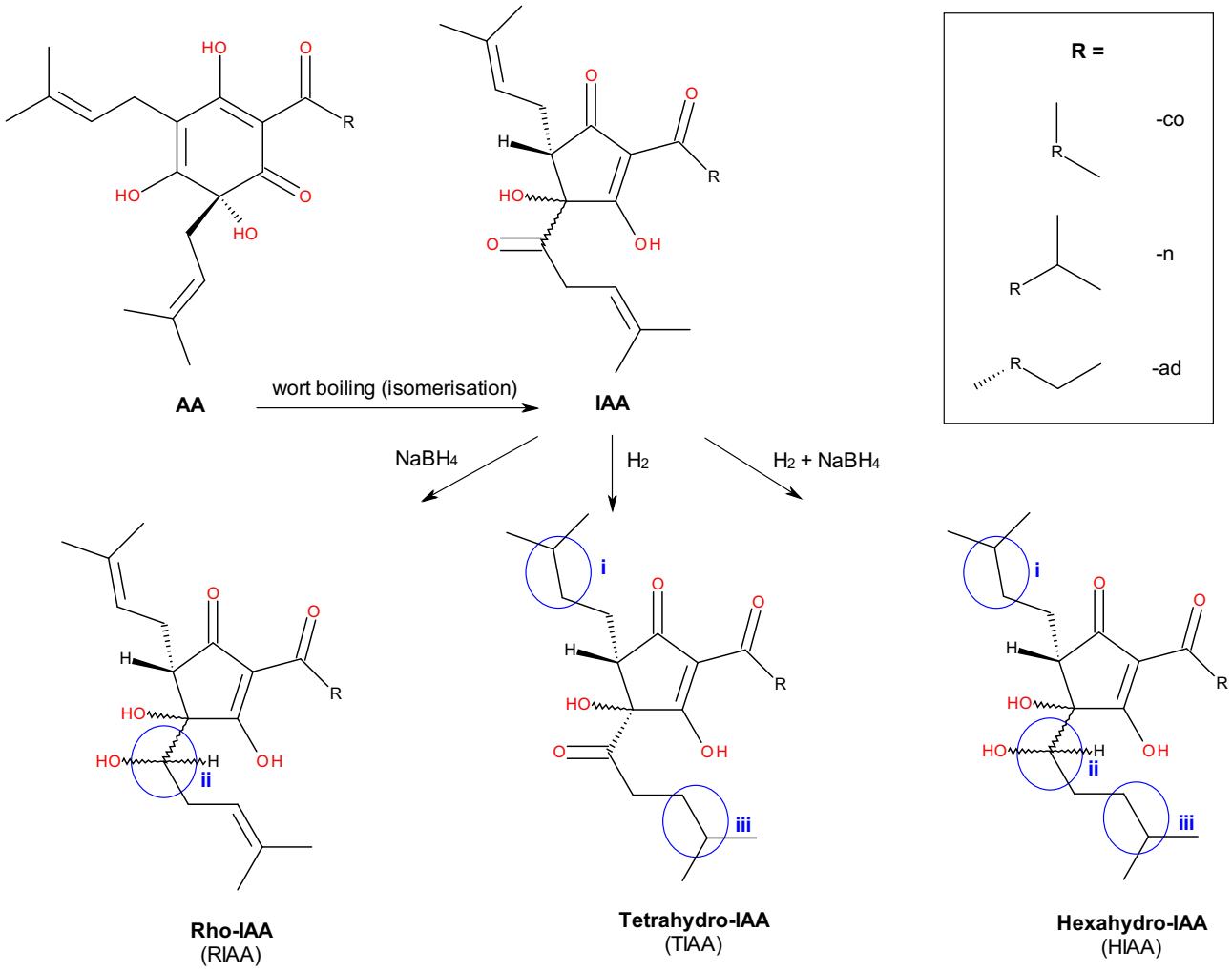

Fig. 1. The isomerization of the α-acid (AA) structure including the three major analogs (co, n and ad) into the iso-α-acid (IAA) diasteromers and subsequent synthesis of the three reduced IAA groups. Sites of increased hydrophobicity (i and iii) and photolytic cleavage prevention (ii and iii) properties are highlighted in the reduced IAA. The reduced carbonyl group produces rho-IAA. Reduction of both side-chains double carbon bonds within the IAA produces the tetrahydro-IAA derivative. Execution of processes yields the hexahydro-IAA derivative of IAA.
Modified from Figs. 1 and 2 from Rodda et al. [1].

Assessment of analyte stability in bioanalytical methods is regularly achieved during method validation using experiments: freeze/thaw stability, bench-top stability, stability in the processed samples, and long-term stability in the sample matrix [10]. All but the long term-stability was performed for the method validation of an UHPLC–MS/MS method for the determination of these compounds in blood [1]. Therefore the stability of the analytes in blood during common storage conditions (e.g. ambient temperature, refrigeration and frozen) should be evaluated over a time period that includes at least the length of typical sample collection, sample handling and analytical preparation for authentic samples [11,12].

Degradation experiments targeting IAA have only been carried out in aqueous solutions, beer wort or in finished beer [7–9,13–20]. This is generally performed to demonstrate how to most efficiently isomerize the AA to IAA during brewing and to measure the loss of beer quality during storage, a known issue with the light sensitive natural IAA compounds. The varying stabilities of these compounds has been shown where certain analogs and isomers, in particular the *trans*-IAA, have been shown to degrade more so than others, into the non-bitter final degradation product, humulinic acid [21].

The aim of this study is to determine the pattern of stability of the four groups of IAA in stored blood samples at different temperatures at a number of time intervals over an eight-week period and to investigate if particular analogs and/or isomers are subject to degradation more so than others.

2. Materials and methods

2.1. Chemicals and reagents

Reference standards for: DCHA-Iso, ICS-I3 (containing 62.3%, w/w of *trans*-IAA); DCHA-Rho, ICS-R2 (containing 65.3%, w/w of *cis*-RIAA); Tetra, ICS-T2 (containing 99.4%, w/w of TIAA), and; DCHA-Hexa, ICS-H1 (containing 65.7%, w/w of *cis*-HIAA); were obtained from Labor Veritas (Zurich, Switzerland). The monitored analogs and isomers of the four IAA groups were: **trans-IAA** (I1 = *trans*-isocohumulone; I2 = *cis*-isocohumulone; I3 = *trans*-isohumulone; I4 = *cis*-isohumulone; I5 = *trans*-isoadhumulone; I6 = *cis*-isoadhumulone); **cis-RIAA** (R1 = *cis*-rho-isocohumulone 1; R2 = *cis*-rho-isocohumulone 2; R3 = *cis*-rho-isohumulone 1; R4 = *cis*-rho-isohumulone 2; R6 = *cis*-rho-isoadhumulone 2); **TIAA** (T1 = *trans*-tetrahydro-isocohumulone; T2 = *cis*-tetrahydro-isocohumulone; T3 = *trans*-tetrahydro-isohumulone; T4 = *cis*-tetrahydro-isohumulone; T5 + 6 = *trans* + *cis*-tetrahydro-isoadhumulone); and **cis-HIAA** (H1 = *cis*-hexahydro-isocohumulone 1; H2 = *cis*-hexahydro-isocohumulone 2; H3 = *cis*-hexahydro-isohumulone 1; H4 = *cis*-hexahydro-isohumulone 2; H5 + 6 = *cis*-hexahydro-isoadhumulone 1 + 2).

The isotope labeled internal standard (IS) nimodipine-d_7 was purchased from PM Separations (Brisbane, Australia). Acetonitrile (ACN), methanol and formic acid were purchased from Merck (Melbourne, Australia). Ammonium formate was purchased from Sigma–Aldrich (Sydney, Australia). All chemicals were of analytical grade or better and water was purified using a Milli-Q Ultrapure Water System from Waters (Sydney, Australia).

2.2. Specimens

Whole blood for stability experiments was obtained from the local Red Cross Blood Bank (Melbourne, Australia) in lithium heparin-coated plastic bags (500 mL). The blood was aliquoted into 10 mL polypropylene tubes containing 200 mg sodium fluoride and 30 mg potassium oxalate. Blood was analyzed using a previously published method [1] and was found to be free of IAA. Preserved blood samples were stored at −20 °C prior to analysis, preservative was sufficient to inhibit bacterial alteration of ethanol and fermentation by-product congeners [22,23].

2.3. Apparatus and analytical conditions

The UHPLC–MS/MS system comprised of a Shimadzu MS 8030 quadrupole mass spectrometer (Melbourne, Australia) operated in the electrospray ionization (ESI) in negative mode and a Shimadzu Nexera UHPLC system (Melbourne, Australia) that consisted of a degasser, two eluent pumps, a column oven (30 °C) with a Kinetex C_{18} (3.0 mm × 150 mm, 2.6 μm) column, and a chilled autosampler. For MS data evaluation, Shimadzu Postrun and Shimadzu Browser Analysis (Melbourne, Australia) softwares were used. GraphPad Prism 5.04 from GraphPad Software (San Diego, USA) was used for statistical analysis. The mobile phases consisted of 50 mmol/L aqueous:ACN (90:10) ammonium formate pH 2.8 (eluent A) and ACN containing 0.1% formic acid (eluent B). The apparatus and analytical conditions were as described in the previously validated method [1].

2.4. Preparation of stock solutions and extraction

Preparation of stock solutions, calibration standards, quality controls, stability samples and extraction procedures were performed as published previously [1]. The extraction consisted of a protein precipitation of the whole blood using ACN with the resulting supernatant dried under nitrogen and the residue reconstituted with 50 μL of a mixture of eluent A and eluent B (60:40, v/v).

2.5. Long-term group stability

'Low' (0.1 mg/L) and 'High' (6 mg/L) concentrations of the IAA groups were prepared in 50 mL volumetric flasks using pooled blank blood and inverted on a rotary wheel for 1 h at room temperature to ensure homogeneity, 500 μL aliquots was transferred into 2 mL Sarsteadt tubes (Sarsteadt, Adelaide, Australia). The batch of low and high aliquots were then divided into 4 groups of different storage temperatures: 20 °C, 4 °C and −20 °C (stability samples); and −60 °C (reference samples). All specimens were housed in light-proof boxes during storage. Furthermore, previous bench-top stability experiments showed the IAA analytes to be stable for up to 2 h [1], therefore degradation of analytes during the process of developing stability samples does not affect experimental results. These samples of were evaluated in duplicates at weeks 1, 3, 5 and 8.

The concentrations of the IAA groups were calculated using the daily calibration curves included in each assay as per previously described methodology [1]. Importantly, a relative concentration calculated as the ratio of the means (stability samples vs. reference samples), in percent was used to compare stabilities. Stability of the IAA groups was measured as 'acceptable' for <15% variation, 'some losses' determined by ≥15 to <30% degradation and 'significant losses' with degradation of ≥30%.

2.6. Individual analyte stability

The specific stabilities of the individual analytes of the four IAA groups were determined in order to demonstrate if certain analytes were particularly responsible for the loss in total group stability. The analyte peak areas of the high concentration stability samples were examined in the final week of the long-term stability experiment (week 8) for the 20 °C, 4 °C and −20 °C storage temperatures. The relative response ratio for each analyte was calculated as the ratio of the peak area response (analyte/internal standard) of the stability samples duplicates vs. the mean area of the reference samples, in percent.

Multiple unpaired *t*-tests using the Holm–Sidak method assessed the storage temperatures of 4 °C and 20 °C against −20 °C to demonstrate if a significant degradation occurred ($p < 0.05$) with each analyte.

2.7. Ethics

This research was approved by the Victorian institute of Forensic Medicine's Human Research Ethics Committee (E04/12).

3. Results

3.1. Long-term group stability

The relative degradation patterns of the four IAA groups in the long-term stability experiment are shown in Fig. 2. The mean of the duplicate results were plotted and an exponential one-phase decay regression model applied that best profiles natural chemical and biological processes. Dotted and dashed lines outline 15% and 30% variations, respectively, and demonstrate that acceptable losses of <15% were seen for all groups at −20 °C temperatures in both low an high concentrations. Although generally showing a slightly less stable profile compared to −20 °C, most IAA groups at 4 °C also showed acceptable losses at conclusion of the study. However, some losses > 15% were seen with *cis*-RIAA (29%) at low concentrations and high concentrations of TIAA (26%) at 4 °C storage conditions. Furthermore, the low levels of TIAA resulted in significant losses of 35% at the same temperature, after 8 weeks of storage.

At 20 °C, the four IAA groups showed further losses below the 30% range at both low and high concentrations. The reduced IAA groups of *cis*-RIAA, TIAA and *cis*-HIAA, showed similar degradation profiles; after initial losses at ~30% variation, the concentrations then plateaued surprisingly at ~1–3 weeks of storage. However, the *trans*-IAA group continued to degrade throughout the eight weeks with only 4% and 15% of the initial concentration remaining at the conclusion of the stability experiment at both concentrations.

3.2. Individual analyte stability

The individual analyte stabilities of the four IAA groups that are comprised of analogs and isomers are presented in Fig. 3. The *cis* isomers of the IAA group (I2, I4 and I6) are residual by-products of the *trans*-IAA production and their concentrations are unable to be calculated due to the lack of a '*cis*' reference standard. However; the sufficient abundance of I2 and I4 allowed for the peak areas to be monitored in order to demonstrate degradation. Notably, these

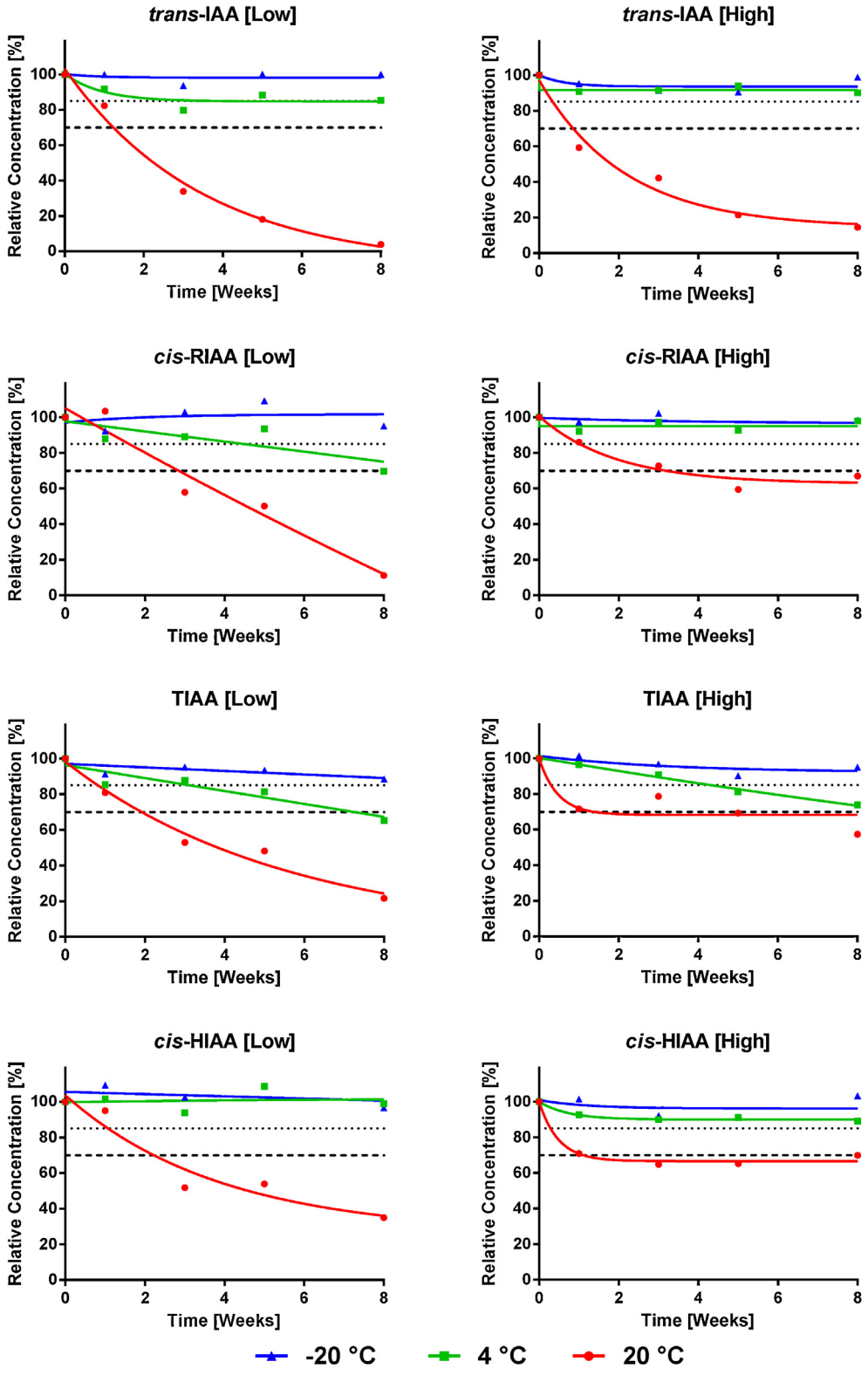

Fig. 2. The relative concentration (%), as a ratio compared to the reference sample (−60 °C), of 'Low' (0.1 mg/L) and 'High' (6 mg/L) levels of the four IAA groups in blood in long-term stability samples stored for 8 weeks at 20 °C, 4 °C and −20 °C. Symbols represent the mean of the duplicates with an exponential one phase decay regression model applied. Dotted and dashed lines outline 15% and 30% variations, respectively.

cis-IAA do not contribute to the *trans*-IAA group instability as demonstrated in Fig. 2.

At −20 °C storage little degradation was observed for all analytes (Fig. 3). At 4 °C a significant loss was seen for I3 and a visible loss also for the equivalent *cis* isomer, I4. Remarkably, all the 'n' analogs (i.e. I3, I4, R3, R4, T3, T4, H3 and H4; see Fig. 1 for analog definition) degraded to a larger extent than the corresponding 'co' and 'ad' analogs. There was a major and significant loss at 20 °C for these analogs, with some losses also seen at 4 °C in all but the RIAA 'n' analogs (R3 and R4). It was also evident that all the *cis*- and

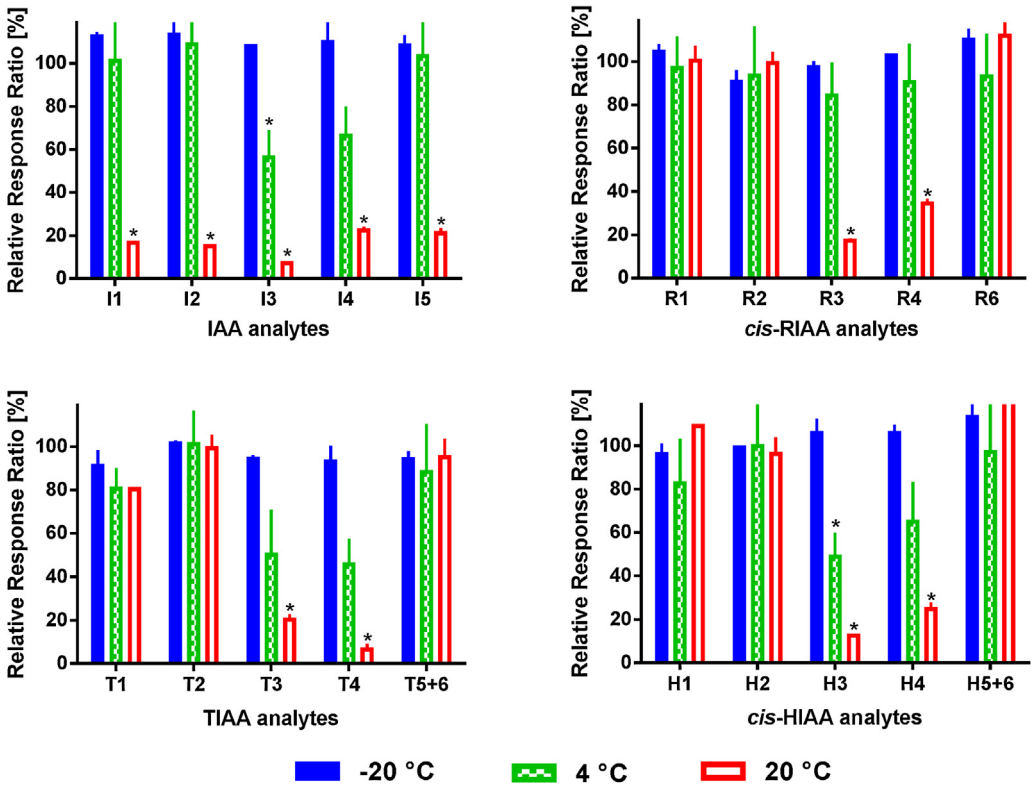

Fig. 3. The peak area response (analyte/internal standard) as a ratio compared to the reference sample (%) in the four IAA groups stability samples in 'High' concentrations in duplicate after 8 weeks of storage at 20 °C, 4 °C and −20 °C. I6 (*cis*-isoadhumulone) and R5 (*cis*-rho-isoadhumulone 1) are not included due to extremely low abundance in the commercial reference standards. I2 (*cis*-isocohumulone) and I4 (*cis*-isohumulone) are not included in the stability of the total *trans*-IAA group presented in Fig. 2. Error bars demonstrate the standard deviations. Multiple unpaired *t*-tests using the Holm–Sidak method assessed the storage temperatures of 4 °C and 20 °C against −20 °C to demonstrate significant degradation (*), $p < 0.05$, with each analyte.

trans-IAA analogs resulted in major significant losses when subject to 20 °C storage conditions, as verified in both low and high concentrations in Fig. 2.

4. Discussion

The recent development and validation of methodology to detect these compounds in blood now enables their detection in biological specimens as well as in beer. Freeze/thaw and bench-top stabilities of the compounds were accessed after 8 freeze/thaw cycles of 22 h freezing periods (−60 °C), followed by thawing periods of 2 h at ambient temperatures [1]. However, their long-term stabilities in whole blood required investigation. The storage temperatures were chosen as these are regular temperatures that toxicological specimens are exposed to.

The assessment and definition of 'stability' varies throughout the literature as demonstrated by Shah et al. who defined stability as the "the chemical stability of an analyte in a given matrix under specific conditions for given time intervals" [11]; whilst Hartmann et al. gave the definition "Absence of an influence of time on the concentration of the analyte in a sample" [24]. Although there are varied definitions and parameters to assess the long-term stability of a compound in matrices [11,12,24–26], they are generally similar in design and outcomes. These experimental setups commonly recommend analyzing 'reference samples' at time zero and a set of 'stability samples' after just one certain time-point of storage, in replicates of ∼5 in both low and high therapeutic concentrations [24,26]. Suggestion of stability issues is determined by statistical tests that assess if drug concentrations degrade significantly between reference and stability samples. However, as the IAA compounds have not been assessed previously in a biological matrix for stability, a degradation profile with fewer replicates and more time points is advantageous. In order to determine instabilities of the targeted groups, the autosampler stability approach first used by Wieling et al. [27] and subsequently modified to demonstrate when the losses occurred [28], was used in these experiments.

The individual analyte stability was also investigated at high concentrations that provided a better indication of losses than at lower concentrations. The exacerbated losses in IAA groups shown at low concentrations may be more likely due to the lower limit of quantification of the experimental methodology, rather than true instability. The individual analyte stability was also assessed using −20 °C samples as a control (as it showed almost no degradation for all analytes).

The possibility of analytes degrading into related compounds within the group seems unlikely as it has been shown that during prolonged beer storage, the isomers do not epimerize into another isomer [29]. Literature suggests these compounds degrade into other products (e.g. humulinic acid; 3-MBT, and; tri- and tetracyclic *trans*-IAA degradation products) [8,13,14,20,21,29].

The *cis*-IAA isomers I2 and I4 were shown to degrade at a similar rate as the corresponding *trans* isomers. This differs in brewing chemistry literature that shows that the *trans*-IAA are more hydrophobic which consequently results in the lower half-life in beer of the *trans*-IAA (∼9–12 months) compared to the *cis*-IAA (>5 years) [14,29,30], leading to range of degradation products to be formed in beer [29,31]. A possible explanation for this may be the acidic nature of beer (pH ∼ 4), compared to that of blood (pH ∼ 7.4), better stabilizes the *cis* isomer, where the near neutral blood does not support *cis* stability. Interestingly, data showed that the degradation in blood was analog based, rather than by isomer and furthermore that both *cis*- and *trans*-IAA analytes are subject to major losses at room temperature, irrelevant of isomeric or analog configuration.

The thermodynamic stability of IAA has also been investigated in order to determine kinetic models and efficient utilization of hop products [13] and demonstrated although boiling temperatures are required for the isomerization of AA to IAA, the prolonged exposure to such temperatures degrades IAA rapidly [19]. It has been shown that handling, storage and aging of beer affects *trans*-IAA to degrade by ~70% after 8 months of storage at 28 °C in a glass bottle [31]. Huang et al. demonstrated that temperature has the most profound influence in IAA degradation where a recovery of ~85% of IAA was seen at temperatures of 90 °C after 6 h, whilst all IAA were degraded at 130 °C for the same time period [13]. Not surprisingly, this correlates with results from this study where a decrease in temperature showed a consistent increase in the stabilities of the IAA groups.

Iso-α-acid degradation studies investigating the effect pH of the aqueous matrices demonstrated that increasing basic matrices was detrimental to IAA stability [13]. At 100 °C and over 6 h, an increase of pH from 4.5 to 5.5 resulted in losses of IAA from 36% to ~45%; however, an increase to pH 6.5 increase the rate of degradation giving a loss of ~70% IAA. As the pH of blood is ~7.4, it can be expected that the near neutral nature of this biological matrix leads to a similar degradation rate of the IAA groups. Furthermore, blood extract samples containing IAA that were reconstituted in acidic eluent conditions stabilized the IAA analytes, at least for 24 h [1].

In summary, even though IAA and reduced IAA compounds appear to be stable over lengthy storage times in beer, instability was demonstrated in blood over 8 weeks of storage at warmer than frozen temperatures. Although the overall stability of the IAA and reduced IAA groups were acceptable during refrigerated storage (4 °C), increased degradation of the 'n' analogs was observed. Significant losses were seen at ambient storage temperatures for all groups, particularly with the natural IAA group.

Conflict of interest

The authors declare no conflict of interest.

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APPENDIX 1.4:

CHAPTER 4.1 PUBLICATION

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Pharmacokinetics of Iso-α-Acids in Volunteers Following the Consumption of Beer

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Hop-derived iso-α-acid (IAA) ingredient congeners are specific to beer. Concentrations of IAAs were determined in blood of five volunteers over 6 h following the consumption of small volumes of beer containing relatively high (Pale Ale beer) or low (wheat beer) concentrations of IAAs. IAAs were quickly absorbed with peak *trans*-IAA concentrations at 0.5 h followed by a drop of generally 10-fold at 2 h and low or not detectable *trans*-IAA levels at 6 h. However, the qualitative monitoring showed that the *cis*-IAAs were detected at all time-points. Preliminary pharmacokinetics of these compounds in humans shows relatively small interindividual differences and an estimated short half-life of ∼30 min. Comparison of 0.5 and 2 h blood specimens demonstrated that the *trans* isomers were eliminated faster than the *cis* counterparts. Preliminary urine analysis showed only unmodified 'co' analytes detectable throughout the 6 h. In authentic forensic casework where typically large amounts of conventionally hopped beer are consumed, this approach may provide a novel method to target ingredient congeners consistent with beer ingestion.

Introduction

Iso-α-acids (IAAs) are derived from the hop plant (*Humulus lupulus* L.) and are present in beer following the isomerization of α-acids (AAs) during the boiling phase of the brewing process. Six stereoisomers of IAAs are produced from the three predominant AA analogs (co, n and ad) that each isomerize into diastereoisomers (Figure 1). The compounds are structurally very similar, only differing in the nature of the saturated acyl side-chain and the absolute configuration of one of the chiral centers (1).

Beers commonly contain ∼6–30 International Bitterness Units (IBUs, one unit being approximately equal to 1 mg/L of total IAA); however, certain styles of 'highly hopped' craft beers often contain much higher amounts (2, 3). The *cis*: *trans* ratios in beer are usually ∼68 : 32 (4). However, the *trans*-IAAs are present in the foam in greater proportion than the *cis*-IAAs due to their lower water solubility, which also explains the lower half-life of the *trans*-IAAs (<1 year) compared with the *cis*-IAAs (∼5 years) during beer storage (5). The long-term stabilities of the IAA in stored blood recently concluded that the IAA 'n' analogs were most susceptible to degradation; however, refrigeration and freezing provided acceptable stability (6).

It has been proposed that due to the IAA content, beer may also have medicinal properties, including weight loss, antidiabetic, anticarcinogenic and anti-inflammatory (7–14); animal models show that diets enriched with IAAs and administered orally to mice (7–9) and rats (10), led to physiological changes; suggesting that these compounds may be bioavailable. Furthermore, IAAs were dosed to New Zealand white rabbits where it was determined that the total bioavailability of IAA was 13.0%, of which diastereomerization showed no influence; however, the more lipophilic 'n' analogs showed greater bioavailability compared with other analogs (12). More recently using human *in vitro* models, IAAs were shown to be highly permeable across Caco-2 intestinal cells (15), while also shown to promote gastric acid secretion in human gastric cancer cells (16). The AA compounds, of which IAAs originate from and are structural similar, were shown to permeate epithelial membranes and demonstrate absorption also in human *in vitro* models (15).

Recently, these IAAs have been detected in blood of one volunteer following the consumption of a high-hopped beer using an ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS-MS) procedure (17). This confirmation of beer consumption method specifically targets and quantifies the *trans* stereoisomers of the IAA group, whereas the *cis* counterparts are qualitatively monitored (Figure 1). Detection of all IAA analytes were present at 0.5 and 2 h post-dose with *trans*-IAA concentrations of ∼0.2 and ∼0.02 mg/L, respectively. This pilot study demonstrated that these compounds were bioavailable and suggested the possible applicability for clinical and forensic toxicological casework.

This may represent another method of performing alcohol congener analysis to confirm the consumption of beer in routine forensic casework and in after-drinking (or hip-flask) defense cases where the feasibility of claimed alcohol consumption prior and/or subsequent to a motor vehicle incident is in question (18–21).

The aim of this study was to detect and determine the pharmacokinetics of IAAs in the blood and urine of human volunteers given controlled doses of two types of beer containing different amounts of IAAs.

Experimental

Drinking study design

Five healthy male volunteers were administered specific volumes of a relatively high-hopped beer (Little Creatures Pale Ale from Perth, Australia) and a relatively low-hopped beer (Erdinger Weissbier from Erding, Germany; Table I). Studies were performed separately over a day each with a wash-out period of at least 1 week between studies, and no alcohol was permitted for 48 h prior to each of the study days.

To estimate blood alcohol concentration (BAC), a revised version of the Widmark formulae by Posey *et al.* was employed that mathematically combines recent variations by other authors into a single formula to provide an accurate 'Widmark factor' for each individual and subsequently the volume of beer required to produce a peak BAC of 0.05 g/100 mL, the legal limit in Australia (21–23). The anthropometric measurements (i.e., height, weight and age) of each participant are described in Table II.

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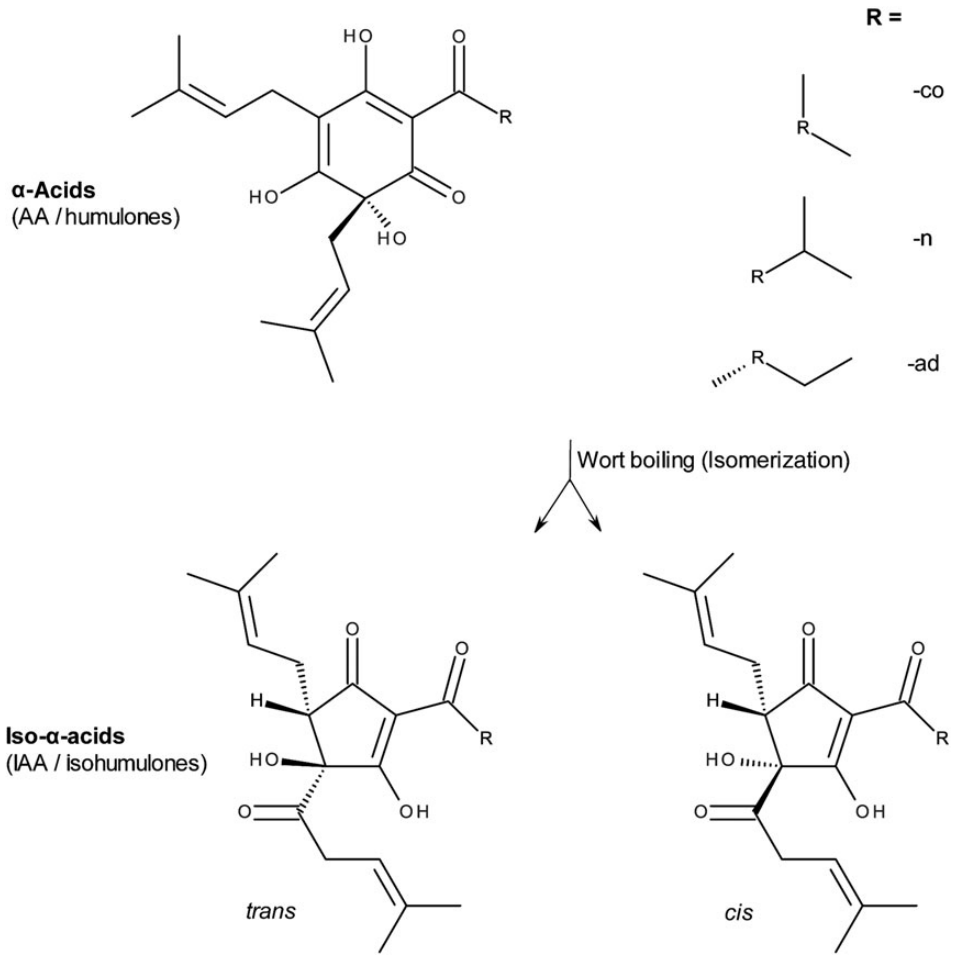

Figure 1. Isomerization of α-acid to iso-α-acids in diasteroisomeric *cis* and *trans* forms after boiling of the wort, including the three major analogs.

Table I

Details of the Beers Consumed during the Two Drinking Studies of Relatively High- and Low-Hopped Beers, the Class and Style of that Beer, Location of the Brewery, the Batch Number of the Beers, Alcohol Content, the Described IBUs and Approximate Total Iso-α-Acid Content

Beer consumed	Class	Style	Brewery	Location	Batch (expiry)	Ethanol (ABV%)	IBU/IAA (mg/L)
High-hopped	Craft	Pale Ale	Little Creatures	Perth, Australia	10174/1 (Exp 3/2014)	5.2	∼40
Low-hopped	Popular	Weissbier	Erdinger	Erding, Germany	L221311 (Exp 11/2013)	5.3	∼10

ABV%, alcohol by volume (volume/volume %); IBU, ∼1 mg/L of total IAA.

Table II

Anthropometric Measurements (Height, Weight and Age) for the Volunteers and Volumes of High-Hopped (Little Creatures Pale Ale) and Low-Hopped (Erdinger Weissbier) Beers Consumed

Volunteer	Gender	Height (cm)	Weight (kg)	Age (years)	Widmark factor (22)	High-hopped beer consumption (mL)	Low-hopped beer consumption (mL)
A	Male	179	105	37	0.594	802	787
B	Male	186	80	32	0.675	694	681
C	Male	175	72	25	0.675	624	613
D	Male	178	98	44	0.611	770	755
E	Male	182	90	39	0.641	741	727

Widmark factor, an estimate of the volume of distribution calculated using the anthropometric measurements of the volunteers.

Blood was obtained by a registered phlebotomist and urine by the volunteers themselves at prior (zero), 0.5, 2 and 6 h post-consumption time-points. The 0-h specimens acted as the control and blank blood and urine of the respective participant. In order to minimalize absorption variables, all participants fasted from food on the morning of the study and the administered beer

was required to be consumed within 10 min. No drink and food were permitted until 2 h post-consumption.

Specimens

Preserved blank blood (10 mL of samples containing 200 mg sodium fluoride and 30 mg potassium oxalate) for instrument calibration purposes were obtained from a local blood bank (Melbourne, Australia). Blood of the participants in the drinking studies were collected in sterile 5-mL Venosafe blood tubes containing 9 mg sodium fluoride and 9 mg potassium oxalate purchased from Hazpak (Melbourne, Australia).

Blank urine for instrument calibration purposes was obtained from the authors after abstinence from beer and other alcoholic beverages for 1 week. Blank and volunteer urine specimens were contained in 50 mL of urine pots and stored at −20°C until analysed.

Chemicals and reagents

The *trans*-IAA reference standards were DCHA-Iso, ICS-I3 (containing 62.3% w/w of *trans*-IAA), obtained from Labor Veritas (Zurich, Switzerland) and contain primarily *trans*-isocohumulone, *trans*-isohumulone and *trans*-isoadhumulone that were grouped and quantified together (*trans*-IAA). However, during commercial production, residual *cis*-IAAs remain and were utilized to qualitatively monitor *cis*-isocohumulone, *cis*-isohumulone and *cis*-isoadhumulone (Figure 1).

The isotope-labeled internal standard nimodipine-d_7 was purchased from PM Separations (Brisbane, Australia). Acetonitrile (ACN), methanol and formic acid were purchased from Merck (Melbourne, Australia). Ammonium formate was purchased from Sigma-Aldrich (Sydney, Australia). All chemicals were of analytical grade or better, and water was purified using a Milli-Q Ultrapure Water System from Waters (Sydney, Australia).

IAA analytical method

IAA determination was performed using a previously published UHPLC–MS-MS method that was validated for blood analysis (17). Briefly, the extraction consisted of a protein precipitation of 200 μL of whole blood using −20°C ACN with the resulting supernatant dried under nitrogen and the residue reconstituted with 50 μL of a mixture of eluent A and eluent B (60 : 40, v : v). Urine analysis was performed using this method, replacing blood for urine in the calibration model.

The UHPLC–MS-MS system comprised of a Shimadzu MS 8030 quadrupole mass spectrometer (Melbourne, Australia) operated in the electrospray ionization in negative mode and a Shimadzu Nexera UHPLC system (Melbourne, Australia) that consisted of a degasser, two eluent pumps, a column oven (30°C) with a Kinetex C_{18} column (3.0 × 150 mm, 2.6 μm) and a chilled autosampler. For MS data evaluation, Shimadzu Postrun and Shimadzu Browser Analysis (Melbourne, Australia) softwares were used. GraphPad Prism 5.04 from GraphPad Software (San Diego, CA, USA) was used for statistical analysis. The mobile phases consisted of 50 mmol/L of aqueous : ACN (90 : 10) ammonium formate pH 2.8 (eluent A) and ACN containing 0.1% formic acid (eluent B).

Preparation of stock solutions, calibration standards, quality controls, stability samples and extraction procedures were performed as published previously (17).

Blood alcohol analysis

Blood alcohol analysis was undertaken in conjunction with routine blood alcohol analysis within the Toxicology Department at the Victorian Institute of Forensic Medicine. Twenty-five microliters of blood were diluted with 1,000 μL of deionized water and directly injected into a gas chromatograph coupled with flame ionization detection and a packed glass column (0.2% carbowax 1500 on carbopack C 80/100 mesh, Sigma-Aldrich) for separation and quantification. This method has been in use for over two decades, utilizes daily calibrations and ensures all quality controls are within range.

Ethics

Approval for the human consumption of beer and subsequent specimen retrieval and analysis was obtained from the Ethics Committee of the Victorian Institute of Forensic Medicine (EC 04/2012).

Results

The IAA content of the two beers and their sources are shown in Table I. Neither alcohol nor IAA analytes were detected in any of the pre-dose blood or urine specimens.

Following the consumption of ~600–800 mL of relatively high-hopped beer, the *trans*-IAAs were detected in all post-dose blood specimens (Figure 2). IAA concentrations peaked at 0.5 h in all volunteers. Results were similar between participants with the *trans*-IAA reaching ~0.1 mg/L at 0.5 h and dropping to ~0.01–0.02 mg/L by 2 h with only one participant having *trans*-IAA detected at the final 6-h time-point.

Consumption of the low-hopped beer showed *trans*-IAA levels also peaking at 0.5 h, although at a lower ~0.02 mg/L concentration compared with a higher hopped beer. At 2 h, these concentrations had dropped to ~0.002 mg/L, just above the lower limit of quantification. In both studies, the *trans*-IAA levels fell ~10-fold from 0.5 to 2 h. This rate of decrease in both studies after 90 min suggests a *trans*-IAA half-life ($t½$) of ~30 min.

cis-IAAs were detected in the 0.5-, 2- and 6-h blood specimens of all participants in both high- and low-hopped studies, of which the 'co' analog was consistently most abundant (data not shown).

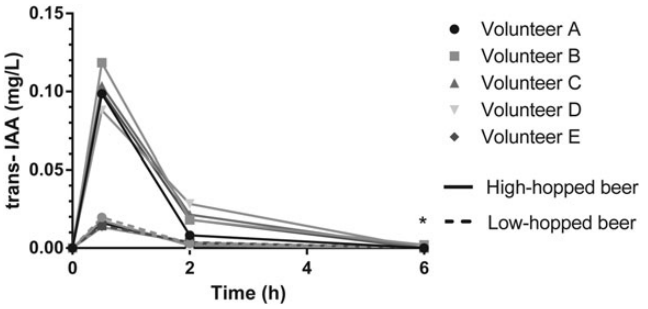

Figure 2. The *trans*-IAA (mg/L) blood concentration–time profiles of the five volunteers at 0, 0.5, 2 and 6 h post-consumption of Little Creatures Pale Ale ('high-hopped' beer) and the Erdinger Weissbier ('low-hopped' beer). *cis*-IAAs were detected in all volunteers at the 0.5-, 2- and 6-h time-points. Asterisk denotes that *trans*-IAA was only detected in volunteer B at the 6-h time-point of the high-hopped study.

Since the 'co' analogs were the most abundant ions monitored and were therefore the best analogs to demonstrate variations in elimination between stereoisomers. Figure 3 shows the two 'co' analytes within the IAA group (*trans*-isocohumulone and *cis*-isocohumulone) and compares the areas of these analytes at the 0.5- and 2-h time-points. Results show that levels of the *trans* isomer fell ~5–10% more in the 90-min timespan in both studies, suggesting that the *trans* isomers may be eliminated more rapidly. Notable differences in the ratios between studies of ~10% were also observed in the low-hopped study.

The mean and standard deviation of *trans*-IAA, BAC and the comparison of *trans* and *cis* metabolism results for the five participants in both studies are shown in Table III.

Analysis of urine for *trans*-IAA showed only the 'co' analogs detected in volunteers at all time-points in the high-hopped study. Although quantification is not possible due to the detection of only one analog, there was a noticeable variation in the area responses between volunteers. Only one volunteer in the low-hopped beer study had a detectable *trans* 'co' analog (at 0.5 h). No 'n' or 'ad' analogs were detected in any volunteer at any time-point. However, the *cis*-IAA 'co' analog was detected in all volunteers in both studies at all time-points post-consumption.

Discussion

Although one volunteer had given detectable concentrations of IAAs after consumption of a high-hopped beer in a pilot study (17), the pharmacokinetics of IAAs in the blood of humans have not yet been established. This current study provided some basic pharmacokinetic information on IAAs, largely in agreement with previous animal models that demonstrated that IAA was rapidly absorbed, had a $t^{1}/_{2}$ of 32 ± 1.8 min and only trace amounts of unmodified IAAs were excreted in the urine (12).

Interindividual differences in pharmacokinetics were small as *trans*-IAA concentration over time profiles between participants demonstrated no obvious outliers. The low- and high-hopped beers were described as ~10 and ~40 IBU, respectively; therefore, although similar volumes of the beer were consumed, participants in the low-hopped beer study consumed ~4-fold less IAA. This correlated well as the *trans*-IAA levels between the beer studies showed a similar profile with differences in *trans*-IAA concentrations of approximately four times. Furthermore, the IBU of the Indian Pale Ale which was consumed in the pilot study was approximately double that of the Little Creatures Pale Ale used in this study (17). This compared well with the *trans*-IAA concentrations at 0.5 and 2 h from this current study being approximately half to that of the pilot study.

Although differences between individuals were shown to be small, these data only provide a limited understanding of the pharmacokinetics of these compounds and does not currently permit correlations of *trans*-IAA and BAC levels to be made or estimate when alcohol consumption occurred. Furthermore, future studies should account for potential gender differences with the inclusion of female volunteers.

As the absorption of ethanol is commonly accepted to provide peak BAC at ~1 h post-consumption (24), the 0.5- and 2-h time-points most likely do not represent the peak BAC and explain the lower than BAC 0.05 g/100 mL target levels observed.

Since the low-hopped wheat beer was described as only ~10 IBU and beers naturally contain less *trans*-IAA than *cis*, it is expected that the concentrations of *trans*-IAA in blood to be only a few mg/L. With participants consuming between ~600 and 800 mL of Erdinger Weissbier, only ~1–3 mg of *trans*-IAA was estimated to have been consumed, thus making blood IAA detection more difficult. However, confirmation of beer ingestion almost throughout the 6 h demonstrates the sensitivity of the method and possible applicability for the detection of different styles of beers.

Notable differences in the overall *trans*: *cis* ratios between studies of ~10% less in the low-hopped (Erdinger Weissbier) were observed. This is most likely due to the *trans* isomer being less stable in beer long term and a slight loss occurring during the transportation from Germany to Australia, rather than any pharmacokinetic variation between beer studies taking place.

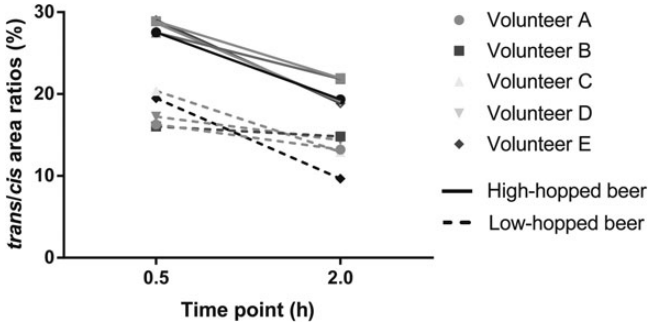

Figure 3. The comparison of *trans*-isocohumulone/*cis*-isocohumulone area ratios in the blood of the five volunteers at 0.5 and 2 h post-consumption of beer in the high- and low-hopped beer drinking studies in order to demonstrate metabolism differences between *trans* and *cis* isomers.

Table III

The Mean and Standard Deviations of the BAC (g/100 mL), Blood *trans*-IAA (mg/L) and *trans*-IAA/*cis*-IAA Area Ratios of the Five Volunteers in the High- and Low-Hopped Drinking Studies at Three Time-Points Post-Drinking

Analysis	*n*	High-hopped			Low-hopped		
		Time-point (h)			Time-point (h)		
		0.5	2	6	0.5	2	6
BAC (g/100 mL)	5	0.034 ± 0.005	0.028 ± 0.004	<0.01	0.032 ± 0.008	0.024 ± 0.005	<0.01
trans-IAA (mg/L)	5	0.102 ± 0.011	0.019 ± 0.007	0.004 ± 0.001	0.016 ± 0.003	0.003 ± 0.001	<0.001
trans-/*cis*-IAA	5	28.332 ± 0.739	20.212 ± 1.542	n/a	17.890 ± 1.941	12.998 ± 2.016	n/a

n/a, not performed at 6 h time-point due to area response below the limit of detection (signal : noise <3).

Although no significant differences were detected between stereoisomers during long-term storage in blood (6), this study demonstrated that the *trans* isomers may be more subject to metabolism *in vivo*. Furthermore, the *cis* isomers are also ~1.5 times more abundant in beer to that of the *trans* counterpart (4) and as previously mentioned, are stable for longer in beer during storage. Finally, the detection of *cis*-isocohumulone at 6 h post-consumption was possible where *trans* isomers were not detected. Therefore, it is noteworthy that although the *cis* isomers were unable to be quantified, they may present as possibly important qualitative markers when greater detection windows of beer consumption are required.

Additional pharmacokinetic information can be obtained with use of the *in silico* metabolism pathway prediction modeling software such as 'MetaPred', which suggested that the cytochrome P450 (CYP) 2C9 was primarily responsible for the oxidation of the IAA compounds (25). Furthermore, 'SmartCyp', a CYP-mediated metabolism prediction tool, suggested that the most probable site of metabolism would occur at the end of both side-chains on the four methyl groups (26). Similarly, Cattoor *et al.* (12) demonstrated that IAA conjugates were not significantly present in rabbits post-ingestion of large amounts of IAAs and also proposed that Phase I metabolism was the primary pathway for the elimination of IAA.

Preliminary urine analysis showed a significant difference in the elimination of unmodified IAA analogs with the 'co' being the only detectable analog. Although *cis*-IAA 'co' analytes were able to be abundantly detected, the development of an analytical method for metabolites or conjugates of IAA may allow for longer windows of detection while also providing further information on the metabolism pathways of IAAs.

Reduced IAAs are chemically synthesized from IAAs and are commonly used in either green or clear glass bottles for their photolytic protective properties (2–4, 12, 13). They may be used in isolation or in conjunction with traditional IAA hopping techniques. Detection of reduced IAAs in blood and urine following the consumption of these beers may allow for further discrimination between the ingestion of different beers. Additionally, determination of accurate IAA concentrations in a range of popular, craft and homemade beers would benefit forensic toxicologists in correlating blood IAA results with the beer(s) suspected to have been consumed.

In conclusion, although outside the scope of this study, more IAA pharmacokinetic data may be achieved with the oral and intravenous dosing of pure IAA formulations and with specimens collected more often. While these studies are limited to five volunteers and two different types of beer, the detection of IAA in blood and perhaps in other specimens may be possible in forensic casework, particularly where typically large amounts of conventionally hopped beer are consumed and the confirmation of beer consumption is valuable.

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Conflict of interest

None declared.

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APPENDIX 1.5:

CHAPTER 4.2 PUBLICATION

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Title: Pharmacokinetics of Reduced Iso-α-acids in Volunteers following Clear Bottled Beer Consumption

Article Type: Original Research Article

Keywords: Alcohol congener analysis; reduced iso-α-acids; beer; pharmacokinetics; blood; urine

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Abstract: Reduced iso-α-acids (reduced IAA) consisting of the rho-, tetrahydro- and hexahydro-IAA groups are ingredient congeners specific to beer generally found in clear and also occasionally green bottled beer. Concentrations of reduced IAA were determined in the blood and urine of five volunteers over 6 hours following consumption of small volumes of beer containing each of the reduced IAA. The reduced IAA were quickly absorbed with peak concentrations at 0.5 h followed by a drop of about fivefold by 2 h. Preliminary pharmacokinetics of these compounds in humans shows relatively small inter-individual differences and an estimated short half-life varying between ~38-46 for the three groups. Comparison of RIAA analyte ratios within the group indicate that some analytes were eliminated relatively faster than others. Preliminary urine analysis showed only unmodified RIAA analytes were detectable throughout 6 h and suggests extensive phase I metabolism of TIAA and HIAA analytes. In authentic forensic casework where clear or green bottled beers are consumed, the identification of reduced IAA groups may provide a novel method to target ingredient congeners consistent with beer ingestion and suggest the type of beer consumed.

Suggested Reviewers: Kristof E Maudens
Pharmacist, Department of Pharmaceutical Sciences , University of Antwerp
[redacted]
Has recently published alcohol related articles (The influence of the body mass index (bmi) on the volume of distribution of ethanol. FSI 2014)

Fredrik Kugelberg
Toxicology, Linkoping Forensic Institute
[redacted]
Has published alcohol related articles and understands the alcohol congener analysis field.

Re: Cover letter for the Research Paper "Pharmacokinetics of Reduced Iso-α-acids in Volunteers following Clear Bottled Beer Consumption"

Dear the Editorial Office of Forensic Science International,

The authors ask that you please consider the above original research paper for publication in Forensic Science International.

This research examines the detection of iso-alpha-acids derived from hops in blood and urine of volunteers given controlled does of beer from clear or green-bottles.

This study builds that has shown that reduced iso- alpha -acids can be used as unique markets for beer consumption and may have application in forensic cases when the source of alcohol needs to be established particularly in after-drinking (hip flask) defences. These are light-stable compounds that are found specifically in clear (and to some extent green) bottled beer. The detection of these compounds in blood and urine demonstrates evidence of recent consumption of beer.

Such data provides forensic laboratories an ability to interpret the blood concentrations of these beer markers in forensic casework.

The research was approved by the ethics committee of the Victorian Institute of Forensic Medicine.

This manuscript is not under consideration for publication in any other journal.

All authors have materially participated in the research and in the article preparation. LR conducted the experimental analyses and evaluation of data and organised the volunteer study as a PhD student of Monash University; DG was a co-supervisor and was involved in the design of the research and the wider PhD dissertation and in the preparation of the paper; and OHD was the principal supervisor and was also actively involved in the design of all aspects of the research including preparation of the manuscript.

All authors have approved the final article and agree for it to be reviewed and if deemed satisfactory published in FSI.

Signed

Luke Rodda PhD student

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May 25, 2014

TITLE

Pharmacokinetics of Reduced Iso-α-acids in Volunteers following Clear Bottled Beer Consumption

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Highlights

- Reduced iso-α-acids (IAA) are ingredient congeners found in beer.
- We examine their presence in volunteers over 6 hours given controlled doses of beer.
- Beer contained in clear or green bottles gave a blood alcohol concentration of near 0.05 %.
- Reduced IAA were detected in all volunteers.
- These unmodified forms may be useful markers for detection of beer consumption.

*Manuscript (without author details)
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ABSTRACT

Reduced iso-α-acids (reduced IAA) consisting of the rho-, tetrahydro- and hexahydro-IAA groups are ingredient congeners specific to beer generally found in clear and also occasionally green bottled beer. Concentrations of reduced IAA were determined in the blood and urine of five volunteers over 6 hours following consumption of small volumes of beer containing each of the reduced IAA. The reduced IAA were quickly absorbed with peak concentrations at 0.5 h followed by a drop of generally fivefold by 2 h. Preliminary pharmacokinetics of these compounds in humans shows relatively small inter-individual differences and an estimated short half-life varying between ~38-46 for the three groups. Comparison of RIAA analyte ratios within the group indicate that some analytes eliminate relatively faster than others and the observation of metabolite products. Preliminary urine analysis showed only unmodified RIAA analytes were detectable throughout 6 h and suggests extensive phase I metabolism of TIAA and HIAA analytes. In authentic forensic casework where clear or green bottled beers are consumed, the identification of reduced IAA groups may provide a novel method to target ingredient congeners consistent with beer ingestion and suggest the type of beer consumed.

KEYWORDS

Alcohol congener analysis; Reduced iso-α-acids; beer; pharmacokinetics; blood; urine

1. INTRODUCTION

Traditionally, iso-α-acids (IAA) that have been derived from the hop plant (*Humulus lupulus* L.) have been used in beer for their bitterness, bacteriostatic and hydrophobic properties [1-3]. However IAA are prone to becoming light-struck in the presence of sunlight (near ultraviolet, blue light) and oxygen resulting in familiar and undesirable "skunky" aroma producing compounds, of which 3-methyl-2-butene-thiol (3-MBT) being the most offensive [4]. This historically led to beer being packaged in lightproof glass such as brown, and to some extent green, bottles.

Over the last five decades, a series of "light-stable" derivatives called reduced IAA have been chemically synthesised from the naturally available IAA; namely rho-IAA (RIAA), tetrahydro-IAA (TIAA) and more recently, hexahydro-IAA (HIAA) [1, 5, 6]. By reducing the relatively weak double bonds or carbonyl group in the side chains of IAA to stronger single bonds, the photolytic cleavage that produces 3-MBT is unable to occur [2], see **Fig. 1**. These photolytic protective properties allows for the use of these products to be commonly used in isolation in unprotected clear glass bottles [2, 4, 7, 8]. However in order to stablise the bitterness of the beer, they may be used also in conjunction with traditional IAA hopping techniques in green bottles that are subject to low levels of light where some photolytic degradation will still occur to the natural IAA [9].

This reduction process provides altered intensities of bitterness and sensory properties [1, 10]. Additionally, due to increasing hydrophobicity of the compounds (in order of RIAA to HIAA to TIAA), enhanced beer foam stability, appearance and "cling" are observed (**Table 1**). Concentrations of 2.4 and 4.2 ppm have been sufficient to show foam stabilisation for TIAA and HIAA, respectively, demonstrating that reduced IAA used for the purpose of foam improvement only are added in lower concentrations [11].

Reduced IAA hop-products such as Redihop® (RIAA), Tetrahop Gold® (TIAA) and Hexahop® 95 (HIAA) [12-14] are just some examples of the commercially available reduced IAA products that are available as potassium salt preparations ready to be added directly to the finished beer (post-fermentation) [2, 15]. Amongst other non-natural additives, the reduced forms of IAA are prohibited in beers for the German market due to the "Reinheitsgebot" law that states only natural hops, water, malt and yeast, may be used in the brewing process [9]. A range of lager beers have shown to contain ~3-6 mg/L and ~5-28 mg/L of TIAA and RIAA, respectively [9]. Following the manufacture process, residual levels of up to 0.1 – 0.2% of the parent IAA have been found in reduced IAA products [16], this may lead to trace amounts of IAA in some beers.

Recently, natural IAA have been detected in the blood and urine of volunteers for up to six hours following the consumption of natural hopped brown bottled beers using a newly developed ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) procedure [17, 18]. These early IAA pharmacokinetic studies demonstrated that IAA compounds can be detected and suggests that the structurally similar reduced IAA may also be able to be targeted. Furthermore, there have been toxicity [19] and pharmacokinetic studies investigating reduced IAA, particularly RIAA and TIAA, due to their possible medicinal properties such as weight loss, anti-diabetic, anti-carcinogenic and anti-inflammatory [7, 8, 20-23]. Of these, two studies detected RIAA and TIAA in New Zealand white rabbits [7], and one measured RIAA in humans [8], confirming those reduced IAA groups examined were indeed bioavailable. The long-term stabilities of the reduced IAA groups in stored blood recently concluded that they were less susceptible to degradation than the natural IAA and refrigeration or freezing conditions provided acceptable stability [24].

This suggests that reduced IAA would be able to be detected in blood post consumption and therefore present as beer-specific ingredient congeners that if detected, can confirm beer ingestion. This may represent another method of performing alcohol congener analysis (ACA) to assist investigations into the source of alcohol in routine forensic casework and in after-drinking (or hip-flask) defence cases where the feasibility of claimed alcohol consumption prior and/or subsequent to a motor vehicle incident is in question [25-28].

The aim of this study was to detect and determine the pharmacokinetics of reduced IAA in the blood and urine of human volunteers given controlled volumes of three different types of beer containing different amounts of RIAA, TIAA and HIAA.

2. EXPERIMENTAL

2.1. Drinking study design

Five healthy volunteers were administered specific volumes of beers (**Table 2**) targeting RIAA (Amstel), TIAA (Hahn Premium Light) and HIAA (Coopers Clear). Beers were selected due to the levels of reduced IAA detected in preliminary investigations [29]. Coopers Clear was found to contain the highest levels of HIAA, however as it also contained relatively high levels of RIAA, this reduced IAA group was also examined in detail.

Studies were performed separately over a day each with a wash-out period of at least one week between studies. No alcohol was permitted for 48 h prior to each of the study days. In order to minimalise absorption variables, all volunteers fasted from food on the morning of the study and the administered beer was required to be consumed within 10 min. No drink and food was permitted until two hours post consumption.

To estimate blood alcohol concentration (BAC), a revised version of the Widmark formulae was employed that mathematically combines recent variations by other authors into a single formulae to provide an accurate 'Widmark factor' for each individual and subsequently the volume of beer required to produce a peak BAC of 0.05 g/100mL [30, 31], the legal limit in Australia. The anthropometric measurements (i.e. height, weight and age) and subsequent required volume of beer consumed for each volunteer were described (**Table 3**). Due to volunteer unavailability, volunteer A1 was replaced by A2 for the last two drinking studies.

Blood was obtained by a registered phlebotomist and urine by the volunteers themselves at prior (zero), 0.5, 2, 4 and 6 h post-consumption time-points. Due to delays in the phlebotomy, the 0.5 h time point bloods were collected between 0.5-1.25 h and at 0.75 h for the Amstel and Hahn Premium Light drinking studies, respectively. The zero hour specimens acted as the control and blank blood and urine of the respective volunteer.

2.2. Specimens

Preserved blank blood (10 mL samples containing 200 mg sodium fluoride and 30 mg potassium oxalate) for instrument calibration purposes were obtained from a local blood bank (Melbourne, Australia). Blood from the volunteers in the drinking studies were collected in sterile 5 mL Venosafe blood tubes containing 9 mg sodium fluoride and 9 mg potassium oxalate purchased from Hazpak (Melbourne, Australia). Volunteer blood specimens were analysed on the day of study and blank bloods were stored at -20 °C until analysed.

Blank urine for instrument calibration purposes was obtained from the authors after abstinence from beer and other alcoholic beverages for one week. Blank and volunteer urine specimens were contained in 50 mL urine pots and stored at -20 °C until analysis.

2.3. Chemicals and reagents

Reference standards for: DCHA-Rho, ICS-R2 (containing 65.3 % w/w of *cis*-RIAA); Tetra, ICS-T2 (containing 99.4 % w/w of TIAA); DCHA-Hexa, ICS-H1 (containing 65.7 % w/w of *cis*-HIAA), and; DCHA-Iso, ICS-I3 (containing 62.3 % w/w of *trans*-IAA); were obtained from Labor Veritas (Zurich, Switzerland).

The monitored analogs and isomers of the three reduced IAA groups were: **RIAA** (R1 = *cis*-rho-isocohumulone 1; R2 = *cis*-rho-isocohumulone 2; R3 = *cis*-rho-isohumulone 1; R4 = *cis*-rho-isohumulone 2; R5 = *cis*-rho-isoadhumulone 1; R6 = *cis*-rho-isoadhumulone 2); **TIAA** (T1 = *trans*-tetrahydro-isocohumulone; T2 = *cis*-tetrahydro-isocohumulone; T3 = *trans*-tetrahydro-isohumulone; T4 = *cis*-tetrahydro-isohumulone; T5+6 = *trans*+*cis*-tetrahydro-isoadhumulone), and; **HIAA** (H1 = *cis*-hexahydro-isocohumulone 1; H2 = *cis*-hexahydro-isocohumulone 2; H3 = *cis*-hexahydro-isohumulone 1; H4 = *cis*-hexahydro-isohumulone 2; H5+6 = *cis*-hexahydro-isoadhumulone 1+2).

The monitored analogs and isomers of the natural **IAA** group were: I1 = *trans*-isocohumulone; I2 = *cis*-isocohumulone; I3 = *trans*-isohumulone; I4 = *cis*-isohumulone; I5 = *trans*-isoadhumulone; I6=*cis*-isoadhumulone.

The isotope labeled internal standard nimodipine-d_7 was purchased from PM Separations (Brisbane, Australia). Acetonitrile (ACN), methanol and formic acid were purchased from Merck (Melbourne, Australia). Ammonium formate was purchased from Sigma Aldrich (Sydney, Australia). All chemicals were of analytical grade or better and water was purified using a Milli-Q Ultrapure Water System from Waters (Sydney, Australia).

2.4. Reduced IAA Analytical Methodology

Reduced IAA determination was performed using a previously published UHPLC-MS/MS method that was validated for blood IAA and reduced IAA analysis [18]. Briefly, the extraction consisted of a protein precipitation of 200 µL of whole blood using -20 °C ACN with the resulting supernatant dried under nitrogen and the residue reconstituted with 50 µL of a mixture of eluent A and eluent B (60:40, v:v). Urine analysis was performed using this method, replacing blood for urine in the calibration and quality control models.

The UHPLC-MS/MS system comprised of a Shimadzu MS 8030 quadrupole mass spectrometer (Melbourne, Australia) operated in electrospray ionisation negative mode. A Shimadzu Nexera UHPLC system (Melbourne, Australia) consisted of a degasser, two eluent pumps, a column oven (30 °C) with a Kinetex C_{18} column (3.0 × 150 mm, 2.6 µm from Phenomenx, Melbourne, Australia), and a chilled autosampler. For MS data evaluation, Shimadzu Postrun and Shimadzu Browser Analysis (Melbourne, Australia) softwares were used. GraphPad Prism 5.04 from GraphPad Software (San Diego, USA) was used for statistical analysis. The mobile phases consisted of 50 mmol/L aqueous:ACN (90:10) ammonium formate pH 2.8 (eluent A) and ACN containing 0.1 % formic acid (eluent B).

Preparation of stock solutions, calibration standards, quality controls, stability samples and extraction procedures were performed as published previously [18].

2.5. Blood alcohol analysis

Blood alcohol analysis was undertaken in conjunction with routine blood alcohol analysis within the toxicology department at the Victorian Institute of Forensic Medicine. Twenty-five microliters of blood was diluted with 1000 µL of deionised water and directly injected into a gas chromatograph coupled with flame ionisation detection and a packed glass column (0.2 % carbowax 1500 on carbopack C 80/100 mesh, Sigma–Aldrich, Sydney, Australia) for separation and quantification. This method has been in use for over two decades and utilises daily calibrations and ensures all quality controls are within range.

2.6. Ethics

Ethics was obtained from the Human Research Ethics Committee at the Victorian Institute of Forensic Medicine (E06/2013).

3. RESULTS

The IAA and reduced IAA content of the three beers and their sources are shown in **Table 2**. **Table 4** shows the mean and standard deviation BAC for the studies, lower than 0.05 g/100mL results suggest the peak BAC was not in correlation with the studies' time points. Neither alcohol nor IAA and reduced IAA analytes were detected in any of the pre-dose blood or urine specimens. Metabolism and/or modification of reduced IAA into other reduced IAA or IAA groups was not observed.

3.1. Blood reduced IAA concentration-time profiles

The reduced IAA blood concentration-time profiles of the three beer studies are shown in **Fig. 2**. **Table 4** shows the blood quantifiable mean and standard deviations values of the IAA and reduced IAA levels, or the detected result if only some of the IAA or reduced IAA analyte(s) of the group could be detected. All profiles showed reduced IAA concentrations peaking at maximum absorption times (T_{max}) of 0.5 h and decreased rapidly by 2 h with further elimination continuing throughout the remaining time points. Due to the high concentration of RIAA in the Coopers Clear beer, this group is also shown in additional to the originally targeted HIAA group in F**ig 2**.

Following the consumption of ~470-770 mL of Amstel beer, RIAA peak blood concentrations (C_{max}) of ~0.17 and ~0.05 mg/L were observed in 0.5 and 2 h bloods, respectively. However by the forth hour, the R2 analyte was the only detected analyte. In comparison and even though similar beer volumes were consumed, all RIAA analytes were able to be detected throughout the Coopers Clear study do to the higher content of RIAA in that beer. The half-life (t½) of RIAA remained constant between studies at ~46 minutes reflected by the similar four- to five-fold reduction in concentrations from the 0.5 to 2 h time points. With the exception of volunteer D in the Amstel drinking study due to phlebotomy delays, the RIAA profiles were similar between all volunteers in both studies.

All TIAA analytes were seen in the 0.5 h blood after ~850-1500 mL of Hahn Premium Light beer was consumed. Although a larger volume of beer was consumed due to the low alcohol content, elimination of analytes T3 to T6 only allowed for analytes T1 and T2 to be quantified at the later 2, 4 and 6 h time points. Examination of the 0.75 and 2 h time points showed a t½ of ~ 45 min for the TIAA group. As Amstel contained

~1 mg/L of TIAA, that drinking study showed detection of the T2 analyte in all 0.5 h bloods also. In **Fig 2**., male (A and B) and female (C, D and E) volunteers showed different TIAA profiles to each other with males generally showing higher concentrations, this is also reflected by the larger group standard deviation in **Table 4**.

After ~500-850 mL of Coopers Clear beer consumed, very low levels of blood HIAA were detected at only 0.5 and 2 h bloods of which a t½ of ~ 38 min can be estimated. No HIAA analytes were detected at 4 and 6 h time points. Although Hahn Premium Light contains trace levels of HIAA, no HIAA analytes were detected in the bloods from this study.

All 0.5 h bloods were able to detect traces of just I2 and all the IAA analytes in the Amstel and Coopers Clear beer studies, respectively.

3.2. RIAA analyte metabolism profile

Since RIAA in the Coopers Clear beer study was the only group where analytes were quantified throughout the six hours in blood (excluding R5 and R6 at 4 and 6 h), the relative metabolism rates of the analytes within the RIAA group were further examined to demonstrate variations in elimination (**Fig. 4**). Producing the same monitored ions to that of R3, R4, R5 and R6 (362.90>265.25; 362.90>196.20, and; 362.90>247.00 m/z), unknown peaks at 5.75 and 6.00 min (U/K 1 and U/K 2, respectively) were observed at the first 0.5 h time point and monitored thereafter. These demonstrate as metabolites of one, or more, of the RIAA analytes as they are not present in the control bloods, reference standard or the beer. The U/K1 peak initially is the more abundant of the two, however it gradually was also eliminated at a relatively quicker rate and eventually the U/K2 metabolite was more prominent by 2-4 h and onwards. The increasing relative abundance of the R3 analyte suggests it may be eliminated at a relatively slower rate. Similarly, the relative decreases in the R1, R4, R5 and R6 analytes may imply that they are subject to more extensive elimination. The R2 showed no obvious overall change in direction inferring an average type elimination profile relative to that of the collective RIAA group.

3.1. Urine reduced IAA concentration-time profiles

Table 5 shows the mean and standard deviations values of the reduced IAA levels and the detected IAA analytes in the urine of the drinking studies. Notably RIAA was the only reduced IAA group to be detected in urine post consumption. Urine RIAA concentrations in both Amstel and Coopers Clear studies fluctuated through the 6 hours and was moderately varied between volunteers as demonstrated by the relatively wide standard deviations in **Table 5**.

Similar to the blood results, trace levels of the *cis*- isomers of the IAA group could be detected in the urine specimens of the Amstel and Coopers Clear beers. Only I2 could be detected in the 0.5 and 4 h urines in the Amstel study, whilst I2 was detected throughout the six hours in the Cooper Clear study in addition to I4 at 0.5 and 2 h time points.

4. DISCUSSION

Previous studies have shown some pharmacokinetics of natural IAA in human volunteers [17, 18], RIAA and TIAA in animal models [7], and in humans dosed with large amounts of RIAA [8]. However the pharmacokinetics of all the reduced IAA in the blood and urine of humans following consumption of beer have not yet been established. This current study provided some basic pharmacokinetic information on reduced IAA, largely in agreement with previous studies [7, 8], in addition to providing HIAA pharmacokinetic data for the first time.

4.1. Pharmacokinetics

Cattoor *et al*. showed that RIAA and TIAA was 28% and 23% bioavailable, respectively, in New Zealand white rabbits [7]. This study also showed varied T_{max} of 4-12 h (RIAA) and 0.5-6 h (TIAA) following oral consumption, in comparison to this current study that demonstrated rapid absorption. The calculated human equivalent dose (HED) of oral administration of RIAA and TIAA in that study equated to ~500 mg for a 60-70 kg human and provided plasma C_{max} of ~6-8 mg/L [7]. Furthermore in another study by Hall *et al.*, two individual RIAA analytes each reached Cmax of ~1-3 mg/L 4 h after oral dosing of 700 mg of total RIAA to 2 healthy human volunteers [8]. This report also showed RIAA levels were ~70% less in plasma to that of whole

blood and goes further to suggest that total RIAA levels in whole blood would be higher than the reported plasma values. Our study had volunteers consume ~10-16 mg of RIAA in the Coopers Clear study and produced Cmax values of ~0.4 mg/L. The relatively large dose of 700 mg of RIAA orally in combination with food *ad libitum* may have delayed the Tmax and reduced the Cmax in comparison to our current study where absorption was potentially aided due to volunteers were fasted and lower doses given.

In general, the reduced IAA exhibit longer t½ to that of the natural IAA where it was determined that *trans*-IAA had a ~30 min ½ life [7, 17]. Our study showed reduced IAA groups had similar elimination slopes and comparable t½ (~45-46 min) to those found when rabbits were dosed (t½ = ~43 and ~41 min for RIAA and TIAA, respectively) [7].

Urine RIAA and TIAA was excreted 12% and 1%, respectively, unmodified following the rabbit model [7]. Comparably, our study also showed significant unchanged urine RIAA concentrations in both the Amstel and Coopers Clear studies, however our study did not detect TIAA in any urine samples. Enzymatic hydrolysis of plasma samples revealed that the presence of TIAA conjugates could not be significantly demonstrated indicating phase I metabolism the most likely limiting factor of bioavailability. However, it was estimated that 50% (plasma) and 22% (urine) of RIAA was either a sulphate or a glucuronide conjugate undergoing phase II metabolism [7].

Hall e*t al*. examined the inhibition of cytochrome P450 (CYP) isoenzymes by RIAA with CYP2C9 found to be the most strongly inhibited by RIAA followed by moderately inhibition of CYP2C19 [8]. Furthermore, Cattoor *et al*. examines the probable sights of metabolism for the RIAA and TIAA in detail and suggests phase I (IAA and TIAA) and phase II (RIAA) processes [7]. Additional pharmacokinetic information can be obtained with use of the *in silico* metabolism pathway prediction modeling software which suggested that the CYP2C9 was primarily responsible for the oxidation of the reduced IAA compounds [32]. Furthermore, a CYP-mediated metabolism prediction tool proposed the most probable sites of metabolism occurred at the end of both side chains on the four methyl groups for reduced IAA groups [33]. Further human *in vitro* and *in vivo* modelled studies are required to confirm such predictions.

Similar to previous IAA pharmacokinetic studies suggesting that *trans* isomers are metabolised at a quicker rate that the *cis* counterparts [17], altered ratios between analytes in the RIAA group were observed also, however no isomer or analog trend was obvious. As the RIAA concentrations peaked rapidly at 0.5 h, it is implied that the

252 differences in the ratio profiles of analytes over the time points is most probably due to metabolism and
253 excretion differences rather than variable absorption rates. Further studies may include larger doses of TIAA
254 and HIAA in order to provide elimination profiles of the individual analytes contained within those groups also.

255 Although outside the scope of this study, more reduced IAA pharmacokinetic data may be achieved with the
256 oral and intravenous dosing of pure reduced IAA formulations to humans with additional time points.

257

258 **4.2. Applicability**

259 Importantly, metabolism and modification of reduced IAA into other reduced IAA or IAA groups was not
260 observed, indicating the specificity of the reduced IAA hop-derived beer marker model suitable. Besides
261 possibly TIAA where males showed slightly higher levels, no obvious or significant differences in
262 concentration-time profiles were observed between genders in these preliminary drinking studies.

263 The RIAA study using LC-MS methodology to analyse the plasma of humans post-dosing with 700 mg of
264 RIAA has not progressed onto other IAA or reduced IAA analytes and does not achieve the sensitivity required
265 for application in forensic casework [8]. This current analytical technique has shown suitable sensitivity for the
266 confirmation of relatively low levels of beer ingestion up to six hours post consumption of certain reduced IAA
267 containing beers. The small number of beers containing HIAA and low concentrations of HIAA in those beers
268 suggests that RIAA and TIAA are of the more important reduced IAA groups to monitor.

269 The development of an analytical method for the metabolites and/or conjugates of reduced IAA may allow for
270 TIAA and HIAA determination when similar beer consumption volumes take place. Additionally, longer
271 windows of detection and further information on the metabolism pathways of reduced IAA in humans may also
272 be achieved. Urine methodology was not creatinine corrected, quantified results are approximate and as RIAA
273 concentrations fluctuated throughout the six hours, caution should be applied when reporting urine results.

274 Presumably due to residual deposits following reduced IAA manufacture and consequently trace IAA levels in
275 certain beers [16], some IAA analytes were detected in the Amstel and Coopers Clear beer studies in trace
276 amounts. Following consumption of natural hopped beer it was shown that *trans*-IAA concentrations well above
277 the lower limit of quantification were possible [17], this distinction may assist in determining if beer consumed
278 is from brown or clear bottles.

As a result of a study performed where osteoarthritis, rheumatoid arthritis and fibromyalgia patients were prescribed a combination of reduced-IAA and other anti-inflammatory substances [20, 23], there have been some supplementary medicines produced that contain RIAA and TIAA [34]. The particular doses of each are not described only that up to 225 – 370 mg of total reduced IAA content are in each tablet. To the best of the authors' knowledge these are not commonly used supplements and is the only use of reduced IAA outside of brewing. For these reasons, the use of these medicines as a defence tactic to mask authentic beer consumption seems unlikely.

Reduced IAA may be used in isolation or in conjunction with traditional IAA hopping techniques. Detection of reduced IAA in blood and urine may allow for discrimination between the ingestion of different bottled beers. Furthermore, determination of accurate IAA and reduced IAA concentrations in a range of popular, craft and homemade beers additional to current preliminary studies [29], would benefit forensic toxicologists in correlating blood IAA and reduced IAA results with the beer(s) suspected to have been consumed.

5. Conclusion

This study showed that the reduced IAA groups detected in the blood and urine correlated with the profiles of the beers consumed. The analytical methodology demonstrated the suitable sensitivity to confirm beer ingestion several hours post consumption of a variety of clear bottled beers. Whilst these studies are limited to five volunteers and three different types of beer, the detection of RIAA, TIAA and HIAA in blood and perhaps in other specimens is possible in forensic casework where typically large amounts of beer are consumed and the confirmation of beer and type of beer consumption is valuable.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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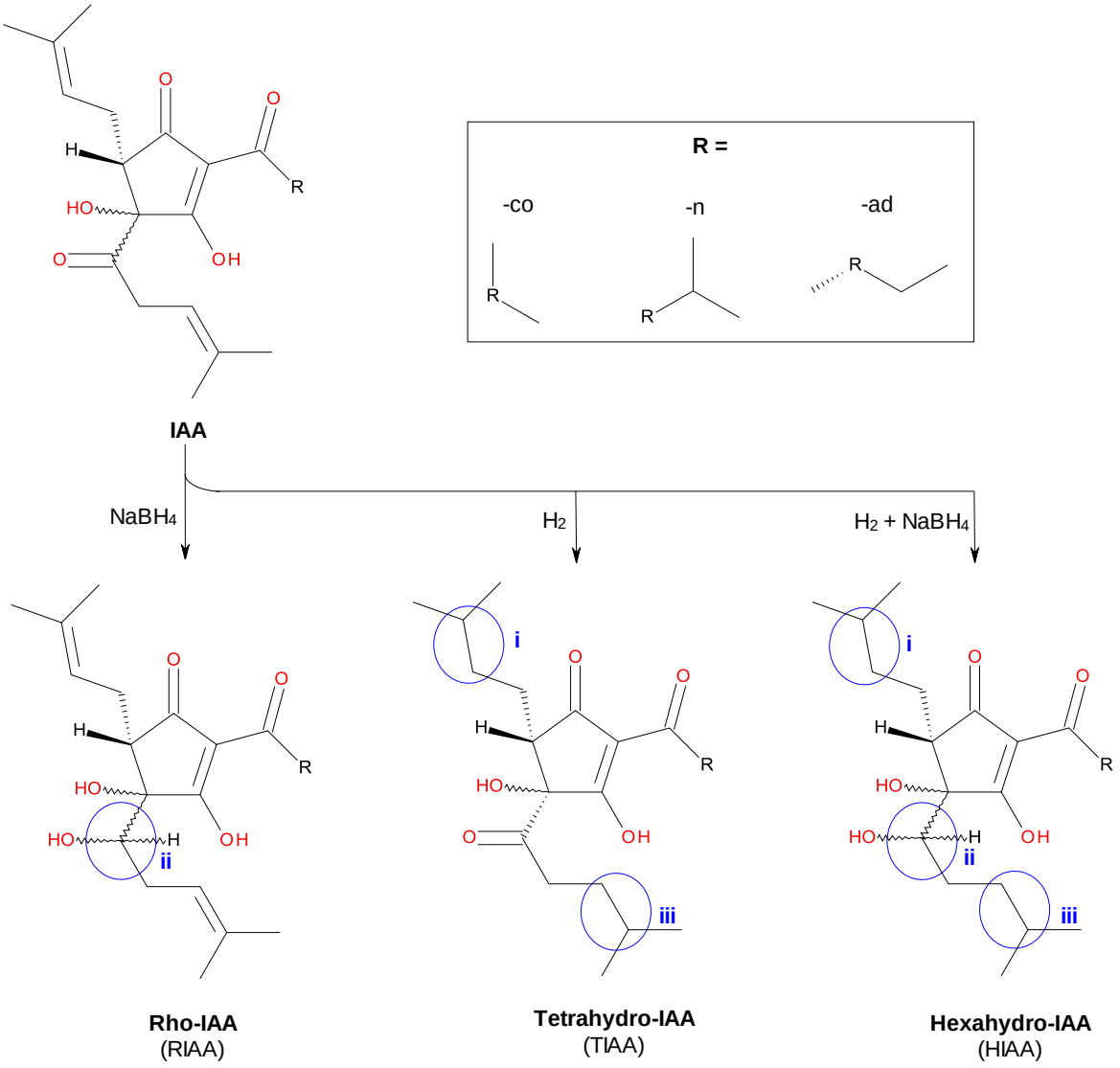
384 **FIGURES**



385

386 **Fig. 1**

387 The iso-α-acid (IAA) structure including the three major analogs (co, n and ad) and subsequent synthesis of the
388 three reduced IAA groups (RIAA, TIAA and HIAA). Sites of increased hydrophobicity (i, iii) and photolytic
389 cleavage prevention (ii, iii) properties are highlighted in the reduced IAA. The reduced carbonyl group produces
390 rho-IAA. Reduction of both side-chains double carbon bonds within the IAA produce the tetrahydro-IAA
391 derivative. Execution of both processes yields the hexahydro-IAA derivative of IAA.

392 **Fig. 2**

393 The reduced IAA groups blood concentration-time profiles (mg/L) of the five volunteers at 0, 0.5, 2, 4 and 6 h
394 post consumption of the Amstel (RIAA), Hahn Premium Light (TIAA) and Coopers Clear (RIAA and HIAA)
395 drinking studies. Due to delays in the phlebotomy, the 0.5 h time point bloods were collected between 0.5-1.25 h
396 and at 0.75 h for the Amstel and Hahn Premium Light drinking studies, respectively.

397 ^ = Only R2 was detected in all 4 h bloods.

398 # = volunteers only had T1 and T2 detected at 2, 4 and 6 h time points.

399 **Fig. 3**

400 Values represent the mean RIAA analyte/internal standard area ratios, as a percentage of the sum of the total
401 RIAA group of analytes, at each 0.5, 2, 4, and 6 h time points post consumption of Coopers Clear. Error bars
402 represent the standard deviation. U/K 1 and U/K 2 represent unknown metabolite peaks observed at 5.75 and
403 6.00 min, respectively. Values represent elimination (metabolism an excretion) rates that are proportionate to
404 other analytes within the RIAA group.

TABLES

Table 1

Summary of the natural and reduced IAA products used in brewing for their desriable bittering [10, 12-14, 35], foam stabilising [11], light protective [35] and sensory [10] properties.

Reduced Iso Product	Bittering Power (%)	Foam Stability	Light Stable[#]	Sensory Analysis
Iso-α-acids (IAA)	100	++	No	astringent, chalky, less fruity and vegetative
Rho- iso-α-acids (RIAA)	60-80	+	Very Good	medicinal, metallic, sharp, astringent
Tetrahydro- iso-α-acids (TIAA)	110-180	++++	Good	less medicinal, more vegetative and fruity, flat, dull
Hexahydro- iso-α-acids (HIAA)	100-170	+++	Very Good	medicinal, metallic, aspirin, vegetative, green

= the prevention of only the degradation product 3-methyl-2-butene-1-thiol (3-MBT) occurring after photolytic cleavage of side chains [36] (see **Fig. 1**)

410 **Table 2**

411 Details of the clear-bottled lager beers consumed during the three drinking studies targeting beers containing each of the three reduced-IAAs (RIAA, TIAA and HIAA)

412 including the variety, brewer, location of brewery, batch or expiry, alcohol content, and the approximate IAA and reduced IAA content of the beers [29].

Name	**Variety**	**Brewery**	**Location**	**Batch or Expiry**	**Ethanol (ABV%)**	**IAA (mg/L)**	**RIAA (mg/L)**	**TIAA (mg/L)**	**HIAA (mg/L)**
Amstel	Low Carb	Amstel Brouwerij B.V.	Amsterdam, Holland	April 14 3119528R1826	4.7	Trace < 0.1	~5	~1	-
Hahn Premium Light	Low Alchol	Hahn	Sydney, Australia	19 March 14 44:57 142	2.6	-	-	~6	-
Cooper Clear	Low-Carb	Coopers	Adelaide, Australia	05 Nov 14 13:41	4.5	Trace < 0.1	~19		~0.3

413 ABV% = alcohol by volume (volume / volume %)

414 **Table 3**

415 Anthropometric measurements (height, weight and age) that provide a Widmark factor required to estimate the volumes of the three beers that were consumed in the drinking

416 studies.

Volunteer	Gender	Height (cm)	Weight (kg)	Age (y)	Widmark factor [30]	Amstel beer consumption (mL)	Hahn Premium Light beer consumption (mL)	Coopers Clear beer consumption (mL)
A1	Male	186	80	32	0.675	768	n/a	n/a
A2	Male	182	90	39	0.641	n/a	1483	857
B	Male	175	72	25	0.675	691	1249	722
C	Female	173	73	36	0.510	530	958	554
D	Female	168	68	28	0.514	497	899	519
E	Female	166	64	33	0.521	475	858	496

417 Widmark factor: an estimate of volume of distribution calculated using the anthropometric measurements of the volunteers.

418 NB: Volunteer A1 was replaced by volunteer A2 in the two final drinking studies.

Table 4

The mean and stardard devations of the blood alcohol concentration (BAC g/100mL), and blood IAA, RIAA, TIAA and HIAA (mg/L) results of the five volunteers in the Amstel, Hahn Premium Light and Coopers Clear drinking studies at four time points post-consumption. If analytes were only detected then the indiviual analytes are indicated.

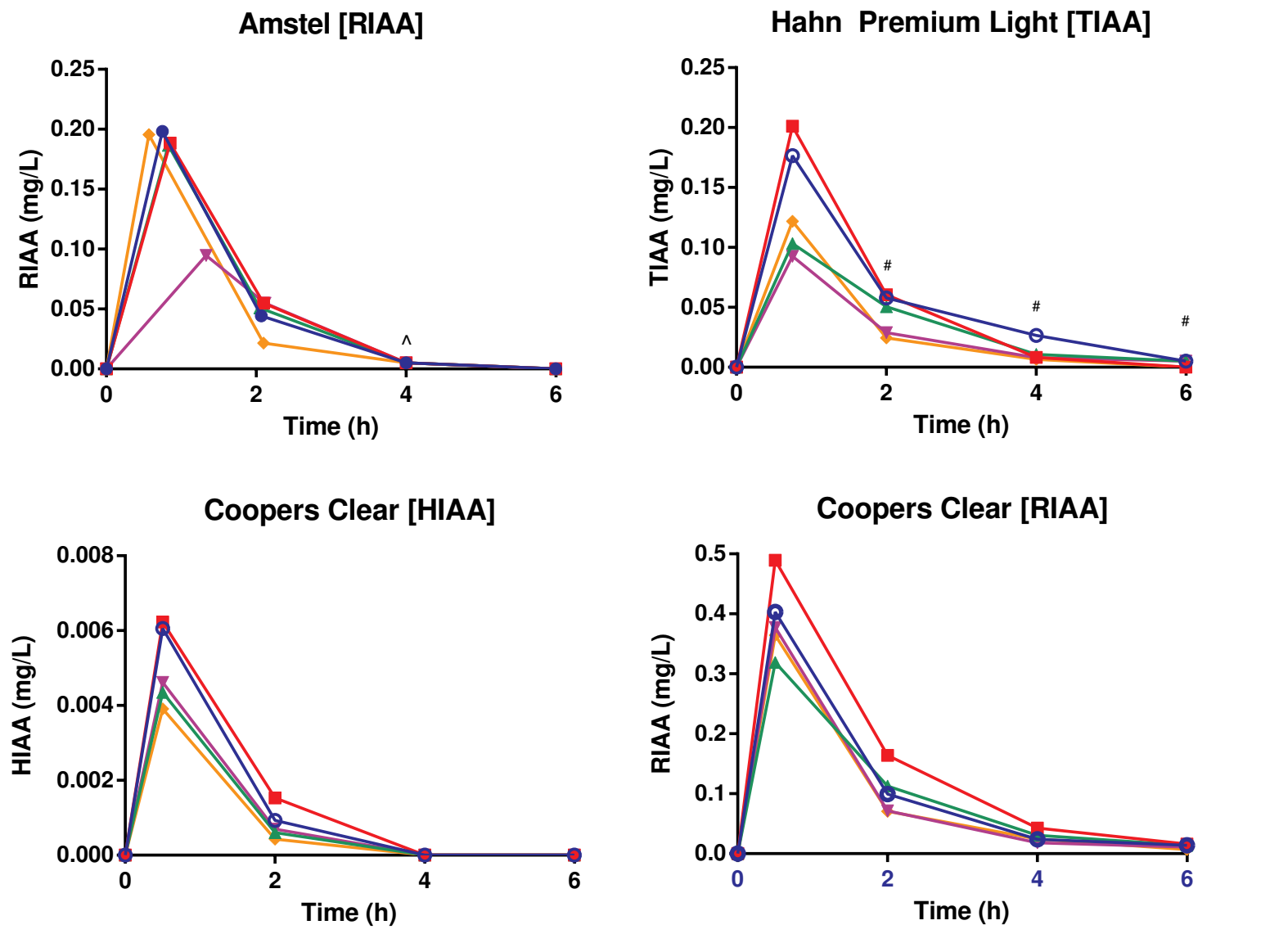
Analysis	n	Amstel				Hahn Premium Light				Coopers Clear			
		Time-point (h)				Time-point (h)				Time-point (h)			
		0.5-1.25	**2**	**4**	**6**	**0.75**	**2**	**4**	**6**	**0.5**	**2**	**4**	**6**
BAC	5	0.033 ±0.006	0.016 ±0.006	-	-	0.030 ±0.013	0.015 ±0.008	-	-	0.030 ±0.006	0.018 ±0.005	-	-
IAA	5	I2	-	-	-	-	-	-	-	I2, I4, I6	-	-	-
RIAA	5	0.173 ±0.044	0.045 ±0.014	R2	-	-	-	-	-	0.391 ±0.063	0.103 ±0.038	0.028 ±0.009	0.012 ±0.004
TIAA	5	T2	-	-	-	0.139 ±0.047	0.044 ±0.017	0.012 ±0.008	T1, T2	-	-	-	-
HIAA	5	-	-	-	-	-	-	-	-	0.005 ±0.001	0.001 ±0.001	-	-

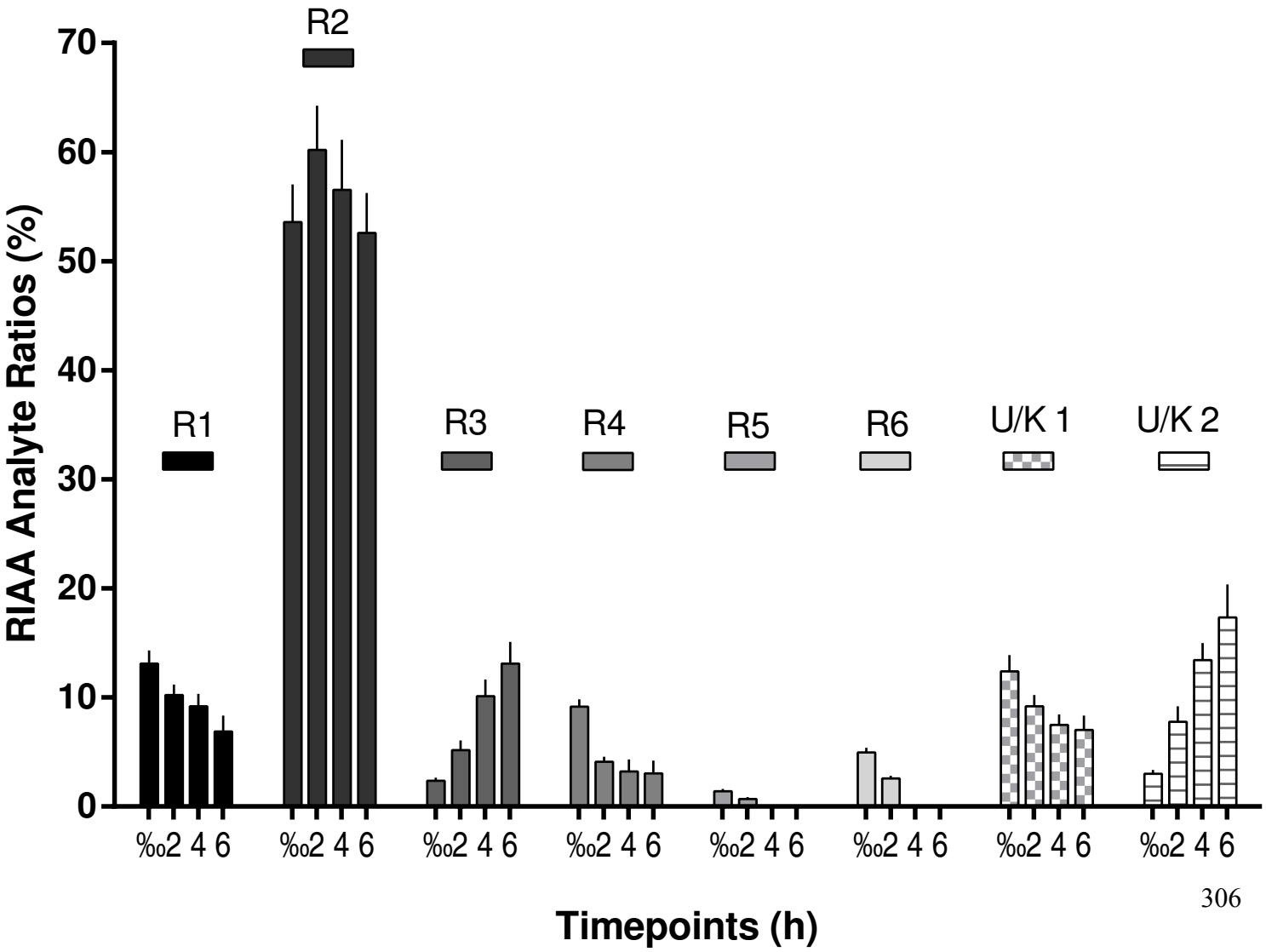
Table 5

The mean and stardard devations of the urine IAA, RIAA, TIAA and HIAA (mg/L) results of the five volunteers in the Amstel, Hahn Premium Light and Coopers Clear drinking studies at four time points post-consumption. If analytes were only detected then the indiviual analytes are indicated.

Analysis	n	Amstel				Hahn Premium Light				Coopers Clear			
		Time-point (h)				Time-point (h)				Time-point (h)			
		0.5-1.25	**2**	**4**	**6**	**0.75**	**2**	**4**	**6**	**0.5**	**2**	**4**	**6**
IAA	5	I2	-	I2*	-	-	-	-	-	I2, I4	I2, I4	I2	I2
RIAA	5	0.018 ±0.010	0.012 ±0.007	0.041 ±0.041	-	-	-	-	-	0.054 ±0.052	0.043 ±0.016	0.151 ±0.163	0.122 ±0.128
TIAA	5	-	-	-	-	-	-	-	-	-	-	-	-
HIAA	5	-	-	-	-	-	-	-	-	-	-	-	-

* = detected in only volunteers B and C


Amstel [RIAA]
RIAA (mg/L)
0.25
0.20
0.15
0.10
0.05
0.00
0
2
4
6
Time (h)
^
Hahn Premium Light [TIAA]
TIAA (mg/L)
#
Coopers Clear [HIAA]
HIAA (mg/L)
0.008
0.006
0.004
0.002
0.000
Coopers Clear [RIAA]
0.5
0.4
0.3
0.2
0.1
0.0
Volunteer A1
Volunteer B
Volunteer C
Volunteer D
Volunteer E
Volunteer A2


R1
R2
R3
R4
R5
R6
U/K 1
U/K 2
RIAA Analyte Ratios (%)
0
10
20
30
40
50
60
70
‰2 4 6
Timepoints (h)

APPENDIX 1.6:

CHAPTER 5.2 PUBLICATION

Drug Testing and Analysis

Detection of Iso-α-Acids to Confirm Beer Consumption in Postmortem Specimens

Journal:	*Drug Testing and Analysis*
Manuscript ID:	Draft
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Rodda, Luke; Monash University, Department of Forensic Medicine; Victorian Institute of Forensic Medicine, Toxicology Gerostamoulos, Dimitri; Monash University, Department of Forensic Medicine; Victorian Institute of Forensic Medicine, Toxicology Drummer, Olaf; Monash University, Department of Forensic Medicine; Victorian Institute of Forensic Medicine, Toxicology
Keywords:	beer, ingredient congener, iso-α-acids, postmortem, blood, urine, vitreous humor, serum
Abstract:	Iso-α-acids (IAA) can be used as markers for the consumption of beer. Postmortem specimens from a range of coronial cases were analyzed for IAA in order to determine the prevalence of beer consumption and any correlation to blood alcohol concentrations (BAC). A total of 130 cases were included in this study including those where beer was mentioned in the case circumstances, cases where beer was not mentioned specifically but alcohol was detected, and cases where neither beer was mentioned nor a positive BAC was present. Available blood, serum, vitreous humor and urine specimens were analyzed. Of the 50 cases where beer was mentioned, 87% had one or IAA detected. In cases that only had a positive BAC (n = 60), 57% of these cases also showed the presence of these beer markers. Iso-α-acids were detected in specimens obtained from traumatized, burnt and decomposed cases with a mention of beer consumption or where BAC was positive in blood. No IAAs were detected in cases where BAC was negative. There was little or no correlation between blood IAA concentrations and BAC. This study demonstrates the possible detection of IAA as a marker for beer consumption.

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TITLE PAGE

Detection of Iso-α-Acids to Confirm Beer Consumption in Postmortem Specimens

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ABSTRACT

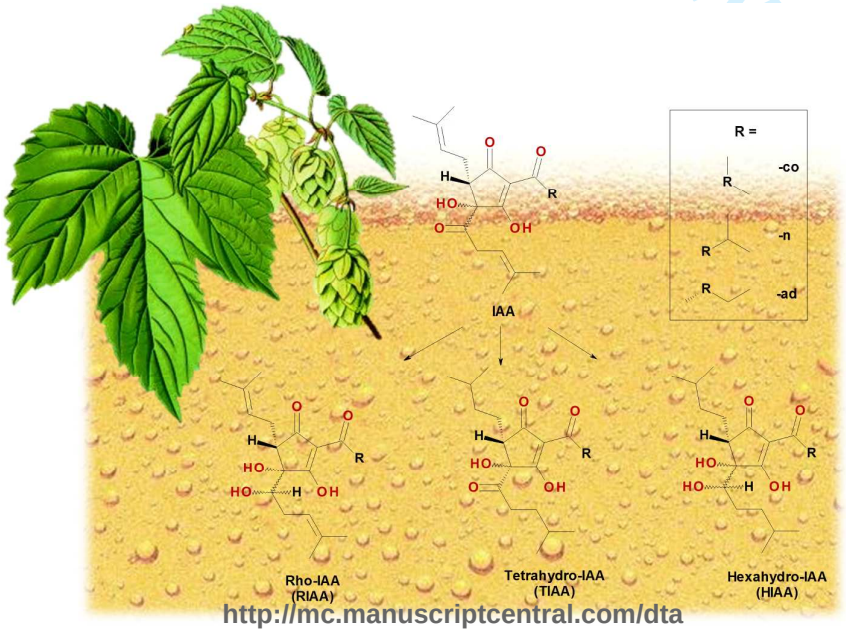
Iso-α-acids (IAA) can be used as markers for the consumption of beer. Postmortem specimens from a range of coronial cases were analyzed for IAA in order to determine the prevalence of beer consumption and any correlation to blood alcohol concentrations (BAC). A total of 130 cases were included in this study including those where beer was mentioned in the case circumstances, cases where beer was not mentioned specifically but alcohol was detected, and cases where neither beer was mentioned nor a positive BAC was present. Available blood, serum, vitreous humor and urine specimens were analyzed. Of the 50 cases where beer was mentioned, 87% had one or IAA detected. In cases that only had a positive BAC (n = 60), 57% of these cases also showed the presence of these beer markers. Iso-α-acids were detected in specimens obtained from traumatized, burnt and decomposed cases with a mention of beer consumption or where BAC was positive in blood. No IAAs were detected in cases where BAC was negative. There was little or no correlation between blood IAA concentrations and BAC. This study demonstrates the possible detection of IAA as a marker for beer consumption.

KEYWORDS

beer; ingredient congener; iso-α-acids; postmortem blood, urine, vitreous humor

DTA GRAPHICAL ABSTRACT

Iso-α-acids (IAA) can be used as markers for the consumption of beer. Postmortem specimens from a range of coronial cases were analyzed for IAA in order to determine the prevalence of beer consumption and any correlation to blood alcohol concentrations (BAC). 57% of cases that had a positive BAC showed the presence of one or more of these beer markers.



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1. INTRODUCTION

Alcohol is the most widely consumed drug in the world with approximately two billion hectoliters produced worldwide in 2012 [1, 2]. Alcohol abuse is at hazardous and harmful consumption levels in many countries resulting in a global health issue and is one of the most common analytes in forensic case work including motor vehicle crashes and assaults [3-6]. In some situations, the identification of which alcoholic beverage was consumed can assist the investigation and associated court case [7].

Compounds other than ethanol and water that are present in alcoholic beverages are termed congeners and may be detected in blood and urine following alcohol consumption, i.e. alcohol congener analysis (ACA). For many decades traditional ACA has determined the fermentation by-product congeners in blood to ascertain if the claims of alcoholic beverage(s) consumption by an individual was feasible, assisting in cases where after-drinking (or hip flask) defenses are involved [8, 9]. However this does not always determine the origin of the consumed ethanol and requires circumstantial evidence, generally provided by the offender [7, 10-12]. In death investigations this approach is limited to those compounds not being produced postmortem [13].

Recently, the authors developed a ultra high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) technique that was validated for the detection and quantification of several hop-derived iso-α-acids (IAA) and reduced IAA compounds [14]. These compounds are used in brewing for their bittering, bacteriostatic and foam stabilizing properties [15, 16]. However, they can also be used as beer-specific ingredient congeners that could confirm beer consumption. These substances have been detected in blood and urine of volunteers following controlled beer drinking studies [17, 18]. Depending on the type of IAA detected the type of beer consumed can also be identified [19].

This methodology has not yet been applied to authentic casework where such an approach may have advantages over, or compliment, traditional ACA techniques. Investigation into a variety of case scenarios that forensic toxicology laboratories are commonly presented (e.g. indication of beer consumption and positive BAC) is necessary in order to indicate IAA prevalence in different body tissues. Additional information such as any association between blood alcohol concentrations (BAC) and IAA concentrations is also required.

This study examines the presence of several IAA compounds in blood and other specimens taken from a selection of postmortem cases.

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2. EXPERIMENTAL

2.1. Case and specimen selection

The Victorian Institute of Forensic Medicine performs medico-legal investigations of deceased cases reported to the Coroners Court of Victoria. The toxicology laboratory within the Institute receives specimens on admission of the body to the mortuary (generally femoral whole blood and serum) and also following an autopsy when it is conducted with a larger range of body specimens including blood, serum, urine and vitreous humor. Coronial, autopsy and toxicological data (e.g. demographics, cause of death, drug exposure, BAC etc.) was obtained using the Institute's case management system.

There were a total of 130 cases of varying cause of death that were selected and placed into one of three groups based on the following criteria:

A. beer mentioned in case circumstances (n = 50);
B. positive BAC (ethanol > 0.01 g/100mL) and beer not mentioned (n = 60), and;
C. cases where neither beer was mentioned nor alcohol was detected (n = 20).

The mortuary admission blood of the cases was analyzed first (or autopsy blood if mortuary admission was not available). If any IAA analytes were detected, subsequent testing of all other blood (mortuary admission and autopsy), serum (mortuary admission and autopsy), vitreous humor and urine specimens were conducted.

2.2. Ethical Approvals

Permission for this research was obtained by the Research Advisory Committee (RAC 013/13) and Human Research Ethics Committee (EC 07/2013) of the Victorian Institute of Forensic Medicine.

2.3. Specimens

For mortuary admission specimens, blood (and often serum) was collected as soon as practicable after a body was admitted to the mortuary. At autopsy, additional blood and serum specimens from the same deceased person were collected, along with vitreous humor and urine specimens. Mortuary admission blood was refrigerated (~4 °C) prior to analysis, all other specimens were stored frozen (-20 °C). All blood specimens were collected in 10 mL

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polypropylene tubes containing 1 % sodium fluoride/potassium oxalate. Serum was obtained by centrifugation for 10 min at 2400 × g. Unless otherwise stated all blood specimens were collected from the femoral region.

Antemortem specimens were obtained by the laboratory if collected when the deceased was hospitalized prior to death.

Drug-free specimens were used for instrument calibration and quality control purposes. Preserved blank blood (10 mL samples containing 200 mg sodium fluoride and 30 mg potassium oxalate) was obtained from a local blood bank (Melbourne, Australia). Blank urine was obtained from the authors after abstinence from beer and other alcoholic beverages for one week. A blank postmortem serum and vitreous humor was obtained from a case previously analysed that showed no IAA analytes. All blank specimens underwent additional screening to ensure there were no IAA analytes or other interferences. All blank specimens were collected in polypropylene tubes (or containers for urine) and immediately frozen (-20 °C).

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2.4. Chemicals and reagents

Reference standards for the natural IAA and reduced IAA were obtained from Labor Veritas (Zurich, Switzerland): IAA DCHA-Iso, ICS-I3 (containing 62.3 % w/w of *trans*-IAA); rho-IAA (RIAA), DCHA-Rho, ICS-R2 (containing 65.3 % w/w of *cis*-RIAA); tetrahydro-IAA (TIAA), Tetra, ICS-T2 (containing 99.4 % w/w of TIAA), and; hexahydro-IAA (HIAA), DCHA-Hexa, ICS-H1 (containing 65.7 % w/w of *cis*-HIAA).

The isotope labeled internal standard nimodipine-d_7 was purchased from PM Separations (Brisbane, Australia). Acetonitrile (ACN), methanol and formic acid were purchased from Merck (Melbourne, Australia). Ammonium formate was purchased from Sigma Aldrich (Sydney, Australia). All chemicals were of analytical grade or better and water was purified using a Milli-Q Ultrapure Water System from Waters (Sydney, Australia).

2.5. IAA and Reduced IAA Analytical Methodology

Natural IAA (*trans*-IAA and *cis*-IAA) and reduced IAA (RIAA, TIAA and HIAA) determination was performed using a previously published validated UHPLC-MS/MS

method that was validated for blood analysis [14]. Briefly, the extraction consisted of a protein precipitation of 200 µL of whole blood using -20 °C ACN with the resulting supernatant dried under nitrogen and the residue reconstituted with 50 µL of a mixture of eluent A and eluent B (60:40, v:v). Preparation of stock solutions, calibration standards, quality controls, stability samples and extraction procedures were performed as published previously [14]. For the analysis of serum, vitreous humor and urine, blood was replaced by the respective blank matrix in the calibration and quality control models. The availability of IAA reference standards allowed for the quantification of *trans*-IAA, RIAA, TIAA and HIAA groups. Residual *cis*-IAA in the *trans*-IAA reference standard was used to allow for qualitative *cis*-IAA results.

The UHPLC-MS/MS system comprised of a Shimadzu MS 8030 quadrupole mass spectrometer (Melbourne, Australia) operated in electrospray ionization negative mode. A Shimadzu Nexera UHPLC system (Melbourne, Australia) consisted of a degasser, two eluent pumps, a column oven (30 °C) with a Kinetex C_{18} column (3.0 × 150 mm, 2.6 µm from Phenomenx, Melbourne, Australia), and a chilled autosampler. For MS data evaluation, Shimadzu Postrun and Shimadzu Browser Analysis (Melbourne, Australia) softwares were used. The mobile phases consisted of 50 mmol/L aqueous:ACN (90:10) ammonium formate pH 2.8 (eluent A) and ACN containing 0.1 % formic acid (eluent B).

The *cis*-IAA group was unable to be quantified due to lack of specific reference standard. However to allow for comparisons to be made, the internal standard/area ratios of each *cis*-IAA analyte were summed to provide a total are ratio for the *cis*-IAA group and given the following qualitative ratings (area ratio: < 0.01 = +; 0.01-0.09 = ++; 0.10-0.49 = +++; > 0.5 = ++++).

2.6 BAC:IAA Correlation

The association between BAC and IAA concentrations was determined using a two-tailed Pearson test. All positive BAC cases from both Group A and B that contained a matching pair with either *trans*-IAA, *cis*-IAA, RIAA, TIAA and/or HIAA in non-decomposed whole blood femoral specimens were considered. Additionally, correlation data were visually shown in a scatter plot to show if any obvious correlation existed between BAC and IAA results in these matching pairs.

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2.7 Descriptive and Statistical analysis

All statistical analysis was performed using GraphPad Prism 5.04 from GraphPad Software (San Diego, USA). Descriptive statistics were performed on all specimens and cases in all cohorts, and summarized.

3. RESULTS

Based on the described criteria there were 50, 60 and 20 cases analyzed for Groups A, B and C, respectively.

3.1. Prevalence

3.1.1. Specimens

The twenty specimens analyzed that had neither mention of beer consumption nor a positive BAC result (Group C) were all negative for natural IAA and reduced IAA analytes.

Table 1 shows the prevalence and mean concentrations of natural and reduced IAA by Groups. The concentration ranges for the IAA groups in all types of specimens ranged from trace levels to ~0.08 mg/L.

In all 125 instances when *trans*-IAA was detected in any blood specimen (both Group A and B), *cis*-IAA was also present. However, *cis*-IAA was detected in an additional 35 cases. This held true for all specimens; if *trans*-IAA was detected, *cis*-IAA was also present. The mean *trans*-IAA blood concentrations in Group A and B were not dissimilar, 0.011 and 0.013 mg/L, respectively ($P > 0.05$). Similarly, the average blood *cis*-IAA area ratio (+++) was qualitatively similar across the two groups of cases.

Of the cases where reduced IAA was detected, TIAA was shown to be the most prevalent followed by RIAA, with only one case in each cohort containing detectable HIAA. Only in one case of any group was reduced IAA detected without the presence of natural IAA; this case only contained a mortuary admission and autopsy blood specimen. On all other occasions at least *cis*-IAA were detected when RIAA, TIAA and/or HIAA were present.

The concentrations in serum were always higher than the corresponding blood specimen. All serum specimens analyzed contained natural IAA and had positive BAC. All bloods that were positive for a reduced IAA, also contained the appropriate reduced IAA in the serum

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specimen. However, two additional RIAA and an additional HIAA were detected during the serum screening.

There was a marked reduction in the prevalence and concentration of *trans*-IAA and more so for the reduced IAA groups in the vitreous humor specimens. Although the ability to detect *cis*-IAA in the vitreous humor specimens remained high (90%), the qualitative mean also decreased to trace levels (+). Similar prevalence and concentrations were seen in the urine specimens for the natural IAA. However, there was no reduced IAA detected in any of the urine specimens.

Overall, comparisons between the individual IAA groups were relatively similar to each other throughout the specimens in both groups (i.e. in order of prevalence *cis*-IAA > *trans*-IAA > TIAA > RIAA > HIAA). The prevalence and concentrations of all IAA groups between specimens decreased in order of serum > blood > vitreous humor > urine.

3.1.2. Cases

In Group A, natural IAA and reduced IAA were present in 86% and 52% of cases, respectively. In Group B, 53% and 28% of cases contained natural IAA and reduced IAA, respectively, and suggested that beer was consumed in 57% of these cases.

Table 2 summarizes the prevalence of natural and reduced IAA by age, gender, intent and condition of body in the two positive groups.

Both groups of cases tended to have the highest prevalence of beer consumption from below 30 years olds to the 40 – 49 age strata where 100% and 90% of cases were positive for beer consumption in Groups A and B, respectively. The consumption of beer decreased with age above 49 years old, although there were only a small number of cases in the over 70 age range. Natural IAA followed a similar trend however reduced IAA was more prevalent in younger ages (>30 – 39 years), close to similar levels to that of natural IAA.

A quarter of the BAC positive cohort were females (n = 14) however only 14% (n = 2) were positive for IAA. In contrast, 70% of the 48 males showed beer consumption through detection of IAA.

There was no apparent difference in prevalence of IAA by intent (natural, accident, suicide or undetermined).

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IAA analytes were also detected in antemortem (clinical) specimens and in cases that were traumatized or burnt as well as in cases that showed significant decomposition (although there was a marked difference of IAA prevalence between the groups in the decomposed cases).

3.1.3. Group A Case Details

Table 3 details all of the individual results such as BAC and IAA content by age, sex, cause of death and a brief account of the relevant circumstances in all 50 Group A cases. Forty three (86%) of these cases detected natural and/or reduced IAA beer markers with circumstances indicating a range of bottled and canned beers.

The seven cases not positive for beer consumption had circumstances that described beer being located only in the vicinity of the deceased and may simply be associated with the case but not necessarily directly consumed prior to death. Of these seven cases, 4 had negative BAC and 3 contained positive BAC (cases 6, 7 & 49). Cases 7 and 49 were decomposed and with BAC of 0.01 and 0.04 g/100mL, respectively, where postmortem bacterial ethanol production was a likely reason for the BAC.

There were 16 cases that had a negative BAC, 13 of these were found to have beer consumed with *cis*-IAA detected in all of those cases. However, only 8 of these detected *trans*-IAA with a relatively low mean concentration of 0.002 mg/L. TIAA was detected in two cases at low concentrations. The highest of these concentrations was Case 46 having a *trans*-IAA of just 0.005 mg/L.

Case 13 was a male with brown bottled glass present around a chest injury that occurred during a motor vehicle collision. This may possibly indicate contamination of bodily tissue with beer contained within the bottle. The femoral blood was both positive for BAC and natural IAA beer markers. Toxicology testing had confirmed a similar BAC level (to that of the blood) in the vitreous humor confirming alcohol consumption prior to death. Furthermore, TIAA was also present, a reduced IAA used for green or clear bottled consumption. In conjunction with the positive natural IAA determination, this suggests consumption of at least colored bottled beer prior to death. Unfortunately, the vitreous humor was unavailable for IAA analysis.

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In Cases 34 and 43,antemortem specimens obtained prior to death (in hospital). showed trace levels of natural IAA. The circumstances associated with these two cases described heavy drinking the night before (Case 34) and just one beer consumed ~6 h prior to death (Case 43).

The highest *trans*-IAA concentration was 0.056 mg/L for case 11 where the deceased was said to have consumed ~12 long neck beers (a 750 mL bottle) a day. It was also described that of the day of death a brown bottled beer that utilizes traditional natural IAA hopping techniques was consumed. As such, additional beer consumption from beer containing reduced IAA was likely to have also been consumed as 0.003 mg/L of TIAA was also detected.

Eight cases also mentioned other types of alcoholic beverages possibly indicating co-consumption with beer, specifically: wine (Cases 36, 45 and 48), whiskeys including "scotch" and "bourbon" (Cases 20, 21, 33, 37), and rum (case 23). This may demonstrate the ability to detect IAA even if co-consumption of other beverages has taken place.

3.2. Correlation of IAA:BAC

Fig. 1 shows a plot of individual BAC and matched *trans*-IAA, *cis*-IAA, RIAA and TIAA results for all cases analyzed in non-decomposed femoral whole blood specimens. These data showed essentially no correlation between IAA groups, although there was a weak correlation for TIAA (r^2 0.169, p = 0.02).

There were no substantial *trans*-IAA and TIAA concentrations (> ~0.01 mg/L) when BAC was ~0.05 g/100mL or less. In contrast, it is possible to have low IAA concentrations even if BAC are high. This is demonstrated with the clustering of points along the x-axis.

4. DISCUSSION

This validated methodology that uses natural IAA and reduced IAA groups as ingredient congeners to confirm beer consumption, has analyzed two controlled beer pharmacokinetic studies, and stability investigations [14, 17, 18, 20]. This technique can be important in forensic science when a confirmation of circumstances is required or even to complement existing congener analysis to determine what beverage may have been consumed after an incident. The detection of *trans*-IAA, *cis*-IAA, or both, demonstrates natural IAA presence. Similarly, the detection of RIAA, TIAA and/or HIAA determines the presence of reduced

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IAA. If a case had natural IAA, reduced IAA, or both, in one or more specimens, it has been shown that beer had been consumed at some time prior to death (Beer Positive). This approach also may be used in broader applications. The routine reporting of beer consumption in clinical and forensic toxicology may assist in the circumstances, and outcomes, of many types of medico-legal situations.

Besides ACA, other related drink driving scenarios such as claims to have one's drink laced (i.e. drink spiking), is another highly used defence in Germany and Sweden to explain an elevated BAC [21, 22]. Similarly, in drug facilitated sexual assaults where drink spiking has been claimed, ethanol is the most common drug detected, and more often than not the only drug detected [23-25]. It is not uncommon for additional alcohol to be added to the beverage of an unsuspecting victim and the excess alcohol consumption to be the underlying factor for assisting in the assault [26-30]. Should a considerable amount of IAA be shown to be present, this may verify a high rate of beer consumption and assist in such cases.

It is currently possible to differentiate antemortem ethanol obtained by ingested of alcoholic beverages to that of post-mortem bacterial production through ethyl glucuronide detection [12]. However this marker of antemortem ethanol ingestion has been found to be unstable under post-mortem putrefaction conditions [31]. This study shows the ability to confirm the presence of IAA in decomposed specimens and may provide an additional tool to confirm if the ethanol is genuine or a postmortem artifact.

The circumstances provided in this study do not explicitly distinguish between acute beer intake and beer consumption for possibly days prior to death. It could be feasible that a higher than 86% agreement rate for beer consumption in Group A could be achieved using only cases when acute beer ingestion was confirmed. Nonetheless these results show the ability to detect the beer consumption when beer is indeed mentioned in the case circumstances and suggests applicability in authentic settings.

Our study showed that over half of the cases (57%) that were recruited only on the basis of having a positive BAC showed markers of beer consumption. This is comparable to the current trend of apparent alcohol beverage consumption that suggests that ~41% of all alcoholic beverages consumed in Australia are beer products which reflects beer being the most popular alcoholic beverage, ranked fourth internationally for beer consumption (~110 L per capita annually) [2, 32].

The qualitative analysis of the *cis*-IAA group is shown here to be advantageous as they were detected in ~10% of all beer positive cases, when no other IAA was detected. However, due

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to the sensitivity for *cis*-IAA detection, the prolonged window of detection for this group may therefore not definitively demonstrate acute beer consumption, particularly if at trace levels.

A high number of cases that had a positive BAC and aged between 40 and 49 years were also shown to have consumed beer (90%), considerable higher than the other age groups. Reduced IAA were more common in younger years, a possible direct reflection of how clear bottled beer is marketed to these generations [33].

This study shows that free and unchanged IAA are also found in high concentrations in serum and low concentrations in vitreous humor and urine specimens, which therefore can be used to compliment any blood-derived results in postmortem cases.

Reduced IAA are commonly used in clear, and in in conjunction with natural hopping techniques in green bottles [19]. Their presence in the demonstrated casework suggests either clear and/or green bottled consumption. The single case where only reduced IAA was detected suggests it was unlikely that brown bottled beer was consumed, at least in significant amounts.

Trace levels of natural IAA can also be found in beers using only reduced IAA bittering products due to residual amounts remaining following the synthesis of reduced IAA [19]. It was recently shown that volunteers who consumed only clear bottled beer that contained trace amounts of natural IAA, resulted in trace levels of natural IAA (mainly *cis*-IAA) in their blood in the following hours [18]. Additionally, many green bottled beers contain significant levels of both natural and reduced IAA [19] which may explain the high rate of natural IAA detection when reduced IAA was also detected in these studies, particularly for very low natural IAA levels. Although the co-consumption of brown and clear (or green) bottled beer cannot be ruled out if both natural and reduced IAA are detected, it at least confirms the consumption of clear or green bottled beer.

Of the reduced IAA groups, monitoring of TIAA seems to be of most importance followed by RIAA, with only one case in each cohort detecting HIAA analytes. These profiles are a reflection by the prevalence of reduced IAA in a range of common beers, of which TIAA and RIAA are the most commonly used reduced hop products [19].

RIAA and TIAA have been used to treat patients suffering rheumatic disorders [34, 35]. A supplementary medicine has been produced that containing a combined total of 225-370 mg of RIAA and TIAA [36]. This is not a commonly used supplement and is the only apparent use of reduced IAA outside of brewing.

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Previous natural IAA and reduced IAA pharmacokinetic studies described a relatively similar concentration-time profiles to that of alcohol [17, 18]. These controlled drinking studies involved measured quantities of beer and showed good correlations between BAC and IAA concentrations when measured over several hours post consumption. Not surprisingly on a population scale where such controls are not possible there are only (at best) weak associations between blood alcohol and IAA concentrations. This will be caused by factors such as concurrent use of other alcoholic beverages, variable ethanol and IAA congener profiles between beers, and varying pharmacokinetics (e.g. adsorption, half-life) between ethanol and IAA [17, 18]. Additionally, the possible alcohol intake from multiple sources (that do not contain IAA), may explain the tendencies of the correlations to trend towards the x-axis where higher BAC but low IAA concentrations lie. Such variables will not allow back-calculations to be performed. Additionally, there may be other changes in a postmortem context such as postmortem redistribution that may affect tissue concentrations. At least in this cohort, it was shown that IAA concentrations higher that ~0.01mg/L contained significant BAC levels.

5. Conclusion

The analytical methodology demonstrated the suitable sensitivity and selectivity to detect IAA in postmortem specimens and help to confirm beer ingestion. Using this novel approach, the high prevalence of beer consumption in society was demonstrated with over half of all alcohol positive cases confirming beer intake sometime prior to death. Such examination of cases provides a greater understanding of the typical detection rates of IAA type compounds in authentic casework. Although serum showed higher concentrations, the analysis of whole blood also proved suitable. As is common in many aspects of forensic toxicology, postmortem phenomena demand further investigations and make back-calculations of drugs, or alcohol, a challenging task.

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For Peer Review

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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For Peer Review

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Detection of Iso-α-Acids to Confirm Beer Consumption in Postmortem Specimens

Table 1.

A summary of the mean concentration and prevalence of the natural and reduced IAA groups in the whole blood, serum, vitreous humor and urine of cases in Groups A and B.

	Beer Mentioned (Group A)			**BAC Positive and Beer not Mentioned (Group B)**							
	Blood[1]		**Case**	**Blood[1]**		**Serum[1]**		**Vitreous Humor**		**Urine**		**Case**
	mean	Prevalence	Prevalence	mean	Prevalence	mean	Prevalence	mean	Prevalence	mean	Prevalence	Prevalence
trans-IAA (mg/L)	0.011	41 (72%)	38 **(76%)**	0.013	47 (58%)	0.027	20 (100%)	0.002	7 (35%)	0.002	10 (59%)	26 **(43%)**
cis-IAA (area ratio)	+++	50 (88%)	43 **(86%)**	+++	53 (65%)	+++	20 (100%)	+	18 (90%)	++	16 (94%)	32 **(53%)**
Natural IAA		50 (88%)	43 **(86%)**		53 (65%)		20 (100%)		18 (90%)		16 (94%)	32 **(53%)**
RIAA (mg/L)	0.005	7 (21%)	7 **(14%)**	0.006	5 (7%)	0.008	7 (35%)		0 (0%)		0 (0%)	7 **(12%)**
TIAA (mg/L)	0.010	21 (37%)	20 **(40%)**	0.019	26 (32%)	0.033	15 (75%)	Det[2]	1 (5%)		0 (0%)	18 **(30%)**
HIAA (mg/L)	0.020	1 (2%)	1 **(2%)**		0 (0%)	Det[2]	1 (5%)		0 (0%)		0 (0%)	1 **(25%)**
Reduced IAA		24 (48%)	23 **(52%)**		27 (33%)		16 (80%)		1 (5%)		0 (0%)	17 **(28%)**
Beer Positive[3]	50 (88%)		43 **(86%)**	54 (67%)		20 (100%)		19 (95%)		16 (94%)		34 **(57%)**
Total Tested	57		**50**	81		20		20		17		**60**

[1] mortuary admission and/or autopsy.

[2] Detected = Det

[3] Beer Positive = the detection of one or more of the natural and/or reduced IAA groups in any specimen provides the prevalence of beer in that case.

Note: Available additional blood, serum, vitreous humor and urine specimens were only analyzed if the initial blood (generally the mortuary admission) for that case was positive for natural IAA and/or reduced IAA.

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Appendix 1.6

Detection of Iso-α-Acids to Confirm Beer Consumption in Postmortem Specimens

Table 2.

Prevalence of the natural and reduced IAA groups by age, gender, intent and condition of body in Groups A and B.

Characteristics		**Group A** (n = 50)				**Group B** (n = 60)				**Total** (n = 110)			
		n	Natural IAA (%)	Reduced IAA (%)	Beer Positive[1] (%)	n	Natural IAA (%)	Reduced IAA (%)	Beer Positive[1] (%)	n	Natural IAA (%)	Reduced IAA (%)	Beer Positive[1] (%)
Age (years)	<30	5	80	60	**80**	7	57	57	**57**	12	67	58	**67**
	30-39	5	80	20	**80**	12	67	50	**67**	17	71	41	**71**
	40-49	11	100	64	**100**	10	80	40	**90**	21	90	52	**95**
	50-59	13	92	54	**92**	8	38	0	**38**	21	71	33	**71**
	60-69	12	83	33	**83**	14	50	14	**50**	26	65	23	**65**
	≥70	4	50	25	**50**	9	33	11	**33**	13	38	15	**38**
Gender	Male	46	85	43	**85**	48	65	39	**70**	92	75	41	**77**
	Female	4	100	75	**100**	14	14	7	**14**	18	33	22	**33**
Intent	Natural	23	83	30	**83**	29	50	11	**50**	41	68	22	**68**
	Accident	15	100	60	**100**	18	67	50	**67**	33	82	55	**82**
	Suicide	5	60	20	**60**	9	44	56	**56**	14	50	43	**57**
	Undetermined	7	86	71	**86**	15	47	20	**47**	22	59	36	**59**
Body Condition	Normal	38	87	45	**87**	37	68	38	**73**	75	77	41	**80**
	Decomp	8	75	25	**75**	15	7	0	**7**	23	30	9	**30**
	Trauma	3	100	100	**100**	7	86	86	**86**	10	90	90	**90**
	Burnt	1	100	100	**100**	0				1	100	100	**100**
	Antemortem	3	67	0	**67**	1	0	0	**0**	4	50	0	**50**

[1] Beer Positive = the detection of one or more of the natural and/or reduced IAA groups in any specimen provides the prevalence of beer in that case.

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Appendix 1.6

Detection of Iso-α-Acids to Confirm Beer Consumption in Postmortem Specimens

Table 3.
Details of 50 cases where beer was mentioned in the case circumstances (Group A).

ID	Age/sex	Intent	Cause of Death	Quality	Femoral Blood	BAC (g/100mL)	Natural IAA		Reduced IAA			Beer Positive[2]	Circumstances[3]
							trans-	*cis-*	RIAA	TIAA	HIAA		
1	F /61	Acc	Alcohol & drug toxicity	N	Admin	0.02	**0.010**	+++				✓	Alcoholic. 1 x brown bottled beer located
2	M /62	Nat	Heart disease	N	Admin	0.03	**0.008**	+++		**0.008**		✓	Consumed 3 heavy beers night before death
3	M /53	Nat	Heart disease	N	Admin	0.02	**0.007**	+++		**0.001**		✓	Alcoholic. Consumed ½ to 1 cartons of beer per day
4	M /51	Nat	Heart disease	N	Admin	ND	**0.002**	++				✓	Consumes 24 beers per day
5	M /58	Unk	Heart disease	N	Admin	0.08	**0.026**	+++ +	**0.001**	**0.001**		✓	Alcoholic. 3 slabs of brown bottled beer[3] per week
6	M /28	Sui	Hanging	N	Admin	0.08							Beer cans located
7	M /37	Sui	CO poisoning	D	Admin	0.01							Half empty beer bottle located
8	M /60	Unk	Heart disease	B	Autopsy Heart	0.01	**0.006**	++	**0.004**	**0.014**		✓	1 x empty brown bottled mid-strength beer[3] located
9	M /48	Sui	CO poisoning	D	Admin	0.10	**0.015**	+++	**0.011**	**0.001**		✓	3 x empty 750 mL bottles of brown bottled beer[3] located
10	F /52	Nat	Alcohol & drug toxicity	N	Admin	0.19	**0.014**	+++		**0.001**		✓	Alcoholic. Consumed several stubbies and perhaps other type of alcohol
11	M /71	Unk	Heart disease	N	Admin	0.11	**0.056**	+++ +		**0.003**		✓	Alcoholic. Consumes ~12 long neck beers a day. Day of death = brown bottled beer
12	M /65	Nat	Heart disease	N	Admin	0.09	**0.021**	+++		**0.031**		✓	Consumed 7 x mid-strength beers
13	M /42	Acc	Motor vehicle collision	T	Admin	0.19 (0.17)[#]	**0.025**	+++ +		**0.003**		✓	Brown beer bottle glass present around chest injury
14	M /25	Unk	Drug toxicity	N	Admin	0.02	**0.003**	+++		**0.003**		✓	Regularly consumes ~8 beers a day
15	M /47	Sui	Hanging	N	Admin	ND		(+)				✓	Beer cans & bottles located
16	M /45	Unk	Alcohol & drug toxicity	D	Admin	0.23	**0.005**	++	**0.001**			✓	Consumes a minimum of 6 beers per day
17	M /42	Acc	Haemoperitoneum & liver cirrhosis	N	Admin	0.11	**0.003**	++				✓	Alcoholic. ~20 empty brown bottled beer[3] cans & empty wine cask located
18	F /19	Acc	Drug toxicity	N	Admin	0.08	**0.001**	++		**0.001**		✓	Empty beer bottle located
19	M /57	Nat	Liver cirrhosis	D	Autopsy Cavity	0.07	**0.002**	++				✓	Alcoholic. Beer cans located near deceased
20	M /58	Nat	Heart disease	N	Admin	0.27	**0.009**	+++				✓	Consumed 4 beers & unknown quantity of scotch during the 6 h prior to death
21	M /77	Nat	Aortic aneurysm	N	Admin	ND							Known to drink beer & bourbon
22	M /60	Nat	Heart disease	N	Admin	0.02	**0.008**	++				✓	Can of beer on side table
23	M /77	Nat	Heart disease	N	Admin	ND		(+)				✓	Empty beer stubby & ¾ full bottle of rum located

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Appendix 1.6

Detection of Iso-α-Acids to Confirm Beer Consumption in Postmortem Specimens

ID	Age/sex	Intent	Cause of Death	Quality	Femoral Blood	BAC (g/100mL)	Natural IAA		Reduced IAA			Beer Positive[2]	Circumstances[3]
							trans-	*cis-*	RIAA	TIAA	HIAA		
24	M /49	Nat	Heart disease	N	Admin	ND	**0.001**	++		**0.002**		✓	Consumes 2 beers each day
					Autopsy			(++)		**0.001**		✓	
25	M /60	Nat	Heart disease	N	Admin	0.04	**0.005**	++		**0.004**		✓	Consumes 20-30 pots of beer daily
26	M /45	Nat	Acute pancreatitis	N	Admin	ND	**0.003**	++				✓	Consumed 10 x 375 mL brown bottled beer[3] cans a day prior to death
27	M /40	Acc	Drug toxicity	N	Admin	0.07	**0.021**	+++ +	**Det[1]**			✓	Consumed beer
28	M /51	Acc	Alcohol & drug toxicity	N	Admin	0.13	**0.009**	+++		**0.020**	**0.020**	✓	Stubby of beer located
29	M /60	Nat	Chronic alcoholism	N	Admin	ND	**0.001**	+				✓	Alcoholic. Empty beer cans located
					Autopsy		**0.001**	+				✓	
30	M /69	Nat	Abdominal aortic aneurysm	D	Admin	0.05		(+)				✓	Beer cans located
					Autopsy			(++)				✓	
31	M /52	Sui	Hanging	N	Admin	0.18	**0.017**	+++ +	**0.010**			✓	Beer cans located
32	M /55	Acc	Drug toxicity	N	Admin	0.07	**0.013**	+++ +	**Det[1]**	**0.030**		✓	30 empty beer cans located
33	M /85	Nat	Spine operation complications	N	Ante-mortem	ND							2 beers & 5 whiskeys 48 h prior to death due to fall & subsequent surgery
34	M /53	Acc	Head injury	N	Ante-mortem	0.22	**Det[1]**	++				✓	Consumed 1 slab of beer within day, seizing at 16:30, hospitalised at 18:30, died next day
35	M /35	Acc	Aviation accident	T	Autopsy	0.06	**0.008**	+++		**0.010**		✓	Beer consumed for some time prior to piloting plane
36	M /49	Acc	Drug toxicity	N	Admin	0.08	**0.002**	++		**Det[1]**		✓	Consumed ~6 beers & 2 L of cask wine
37	M /29	Acc	Train incident	T	Admin	0.26	**0.043**	+++ +		**0.040**		✓	Consumed a large quantity of beer & whiskey 5 h prior to death
38	M /28	Acc	Drug toxicity	N	Admin	ND	**Det[1]**	++				✓	Consumed ~11 stubbies of beer over a 12 h period prior to bed, died ~14 h later
39	M /64	Nat	Heart disease	N	Admin	ND							Light drinker. Consumed beer once a week
40	M /55	Nat	Heart disease	N	Admin	ND	**0.002**	++		**0.002**		✓	½ glass of beer ~8 hours prior to death
41	F /48	Acc	Alcohol & drug toxicity	N	Admin	0.17	**0.004**	+++		**0.010**		✓	Consumed at least ½ a slab of beer during day prior to falling over & hitting head
42	M /58	Nat	Heart disease	N	Admin	0.03		(++)				✓	Alcoholic. 1 empty slab & 3 unopened slabs located
					Autopsy		**0.001**	++				✓	
43	M /33	Unk	Unascertained	N	Ante-mortem	ND		(+)				✓	Consumed 1 heavy beer 355 mL bottle with meal ~6 h prior to death
					Admin	ND		(+)				✓	

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Appendix 1.6

Detection of Iso-α-Acids to Confirm Beer Consumption in Postmortem Specimens

ID	Age/sex	Intent	Cause of Death	Quality	Femoral Blood	BAC (g/100mL)	Natural IAA		Reduced IAA			Beer Positive[2]	Circumstances[3]
							trans-	*cis-*	RIAA	TIAA	HIAA		
44	M /57	Nat	Heart disease	N	Autopsy	ND							Consumed 1 light beer with meal ~2 h prior to death
45	M /48	Nat	Pulmonary embolism	N	Autopsy	ND	**0.001**	++				✓	Consumed beer & wine night prior to death
46	M /67	Nat	Heart disease	D	Admin	ND	**0.005**	++				✓	Alcoholic. Many empty beer bottles located
47	M /68	Nat	Heart disease	D	Admin	0.05	**0.004**	+++				✓	Beer can & bag of empty cans located
					Autopsy		**0.015**	+++				✓	
48	M /33	Acc	Drug toxicity & anabolic steroid use	N	Admin	ND		(+)				✓	Beer & wine bottles located
49	M /64	Nat	Heart disease	D	Autopsy	0.04							Empty beer bottles located
50	M /33	Acc	MVA pass	N	Admin	0.13	**0.039**	+++ +				✓	~3-4 beers claim to have been consumed
					Autopsy		**0.030**	+++ +				✓	

[1] Detected = Det

[2] Beer Positive = the detection of one or more of the natural and/or reduced IAA groups in any specimen provides the prevalence of beer in that case.

[3] Specific brands of beer have been de-identified

Intent (Nat = natural, Acc = accident; Unk = undetermined);

Cause of Death (Carbon monoxide = CO; Motor vehicle accident unrestrained passenger = MVA pass);

Quality = Quality of body/specimens (N = normal; D = decomposed; T = traumatized; B = burnt);

Femoral Blood (Admin = collection on admission to mortuary);

BAC = blood alcohol concentration (ND = ethanol not detected < 0.01 g/100mL; # = Vitreous Humor result);

parenthesis = *cis*-IAA only detected

"Stubby" is colloquially known in Australia as a small 375 mL bottle of beer; "long neck" = 750 mL bottle; Carton/slab = pack of 24 beers;

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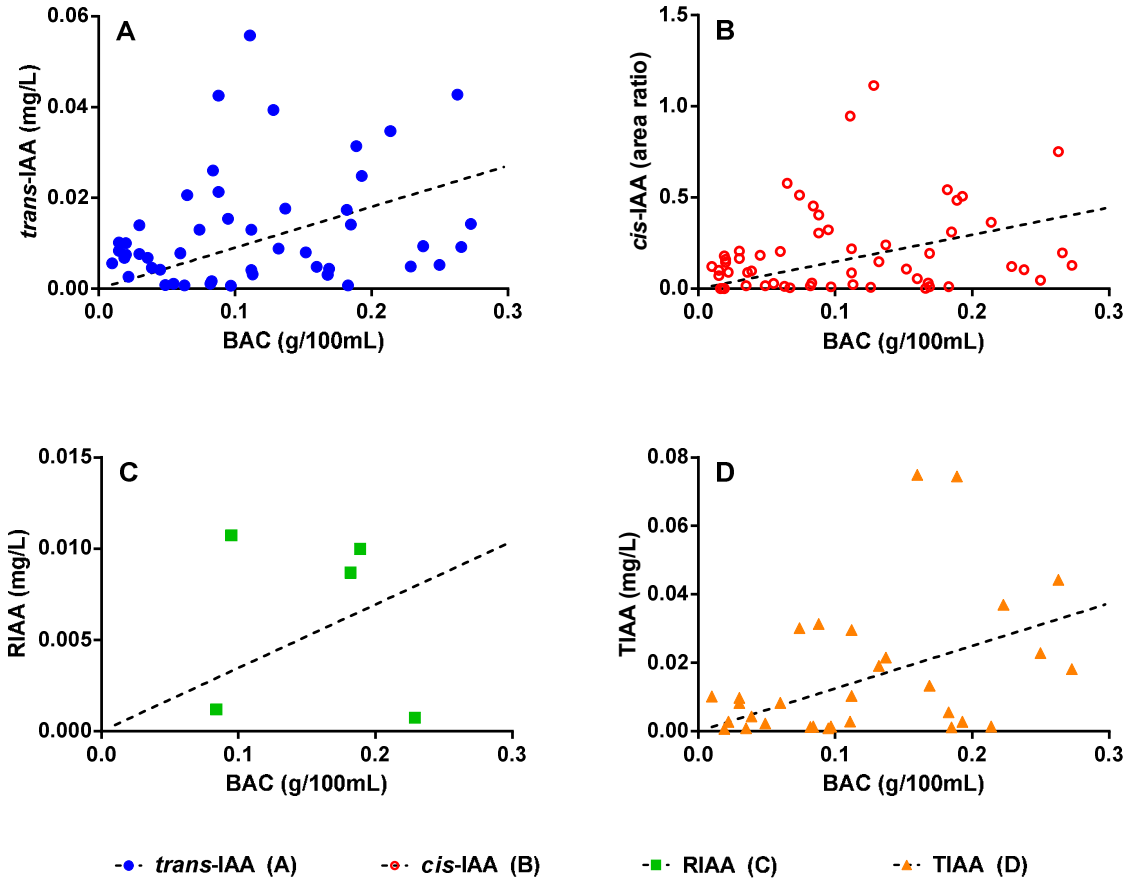
Fig. 1.

Scatter plot and correlation analysis[1] of BAC with presence of either *trans*-IAA, *cis*-IAA, RIAA and/or TIAA in non-decomposed femoral whole blood specimens for all cases. A weak positive association is shown between increasing BAC and IAA results.



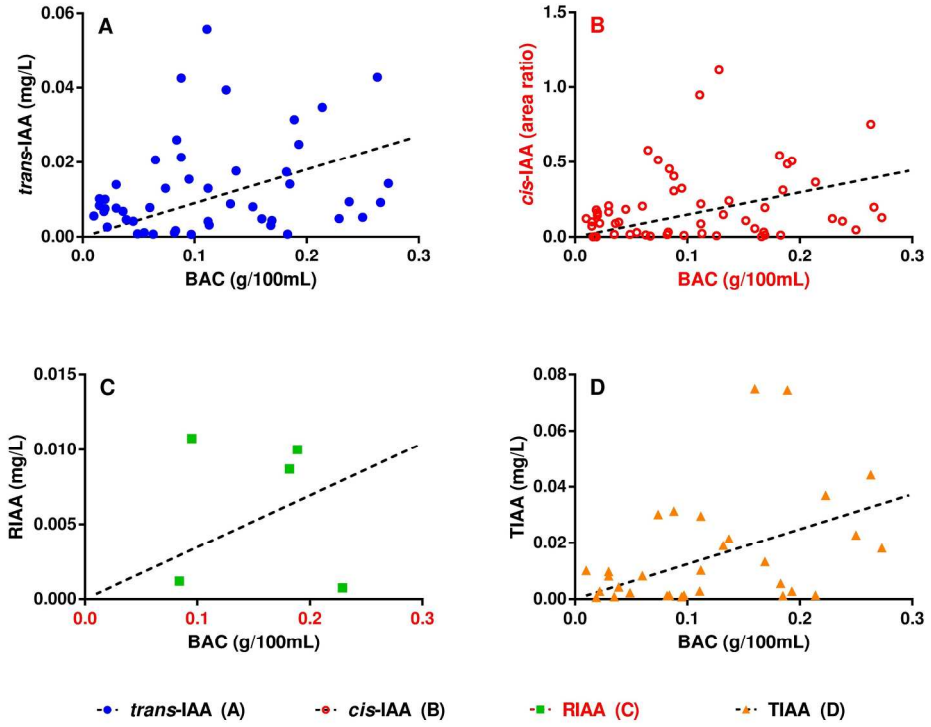
	BAC vs. *trans*-IAA	BAC vs. *cis*-IAA	BAC vs. RIAA	BAC vs. TIAA
Pairs	48	56	5	31
R^2	0.064	0.051	0.010	0.169
p value[1]	0.082	0.096	0.873	0.022*

R^2 = coefficient of determinations

[1]two-tailed Pearson matched pairs correlation test. *$p < 0.05$

Note: HIAA had too few pairs (n = 1) to perform correlation analysis.

Appendix 1.6



201x154mm (300 x 300 DPI)

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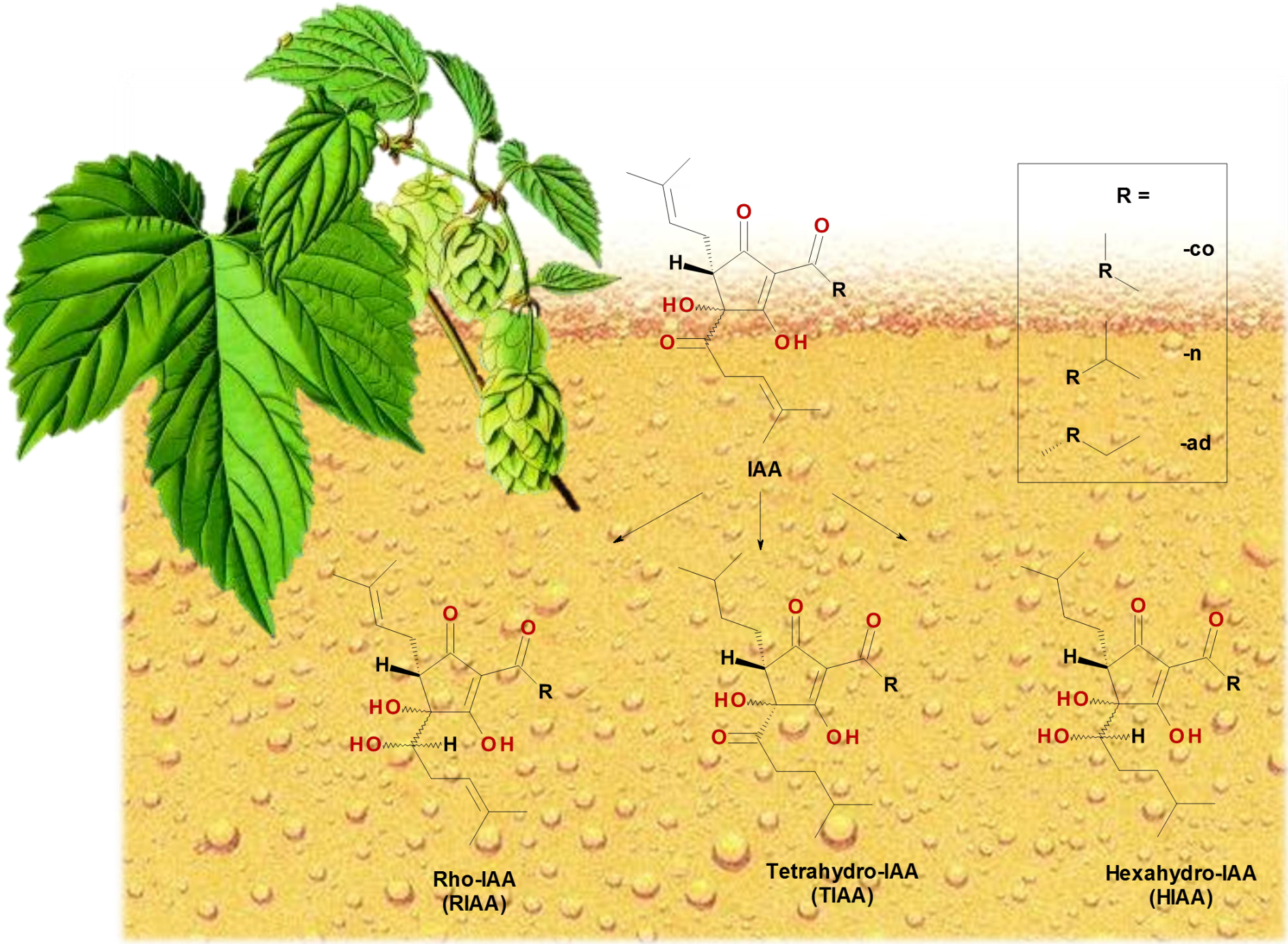
Appendix 1.6


R =
-co
-n
-ad
IAA
Rho-IAA
(RIAA)
Tetrahydro-IAA
(TIAA)
Hexahydro-IAA
(HIAA)

APPENDIX 1.7:

CHAPTER 5.3 PUBLICATION

Forensic Science, Medicine and Pathology

The Postmortem Redistribution of Iso-α-acids in Postmortem Specimens

--Manuscript Draft--

Manuscript Number:	
Full Title:	The Postmortem Redistribution of Iso-α-acids in Postmortem Specimens
Article Type:	Original Article
Keywords:	beer; ingredient congener; iso-α-acids; postmortem; postmortem redistribution; blood, serum.
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Abstract:	Iso-α-acids (IAA) and reduced IAA can be used as beer-specific ingredient congeners to confirm beer consumption when detected in blood and other specimens using a UHPLC-MS/MS method. Recent analysis of postmortem casework demonstrated a high prevalence of beer consumption and the possibility of providing the source of alcohol in forensic casework. Research outlined in this manuscript has examined the degree to which the interval after death and quality of blood affects the concentration of IAA in postmortem cases. Postmortem whole blood and serum were analysed in cases where natural or reduced IAA groups were detected. The trans-IAA, cis-IAA and TIAA groups were subject to postmortem redistribution (PMR), although only weakly associated with the length of time from death to collection of specimens. Serum had 3-fold higher concentrations than blood for trans-IAA, cis-IAA and TIAA. These studies confirm that although postmortem concentrations cannot be easily compared to concentrations found in living persons but the presented findings do provide some understanding to assist the interpretation where the confirmation of beer consumption is required in forensic casework.
Suggested Reviewers:	Peter Stockham Toxicologist, Forensic Science Centre [redacted] Peter has published in the past and has the relevant knowledge to assess postmortem redistribution effectively.
	Michael Robertson, PhD Consultant toxicologist, Independent Forensic Consulting [redacted] Michael has also published in the past and has the relevant knowledge to assess postmortem redistribution effectively.

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TITLE PAGE

The Postmortem Redistribution of Iso-α-acids in Postmortem Specimens

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Manuscript (Do not include any personal info i.e. name, affiliation, etc.)
Click here to view linked References

ABSTRACT

Iso-α-acids (IAA) and reduced IAA can be used as beer-specific ingredient congeners to confirm beer consumption when detected in blood and other specimens using a UHPLC-MS/MS method. Recent analysis of postmortem casework demonstrated a high prevalence of beer consumption and the possibility of providing the source of alcohol in forensic casework. Research outlined in this manuscript has examined the degree to which the interval after death and quality of blood affects the concentration of IAA in postmortem cases. Postmortem whole blood and serum were analysed in cases where natural or reduced IAA groups were detected. The *trans*-IAA, *cis*-IAA and TIAA groups were subject to postmortem redistribution (PMR), although only weakly associated with the length of time from death to collection of specimens. Serum had 3-fold higher concentrations than blood for *trans*-IAA, *cis*-IAA and TIAA. These studies confirm that although postmortem concentrations cannot be easily compared to concentrations found in living persons but the presented findings do provide some understanding to assist the interpretation where the confirmation of beer consumption is required in forensic casework.

KEYWORDS

beer; ingredient congener; iso-α-acids; postmortem; postmortem redistribution; blood, serum

1. INTRODUCTION

Alcohol consumption results in a significant increase in deaths, hospitalizations and alcohol-related crimes and is reflected domestically with the recent findings that spotlight the dangers of alcohol-fuelled violence [1, 2]. Responsible for ~4.7 L of the pure alcohol ingestion per capita annually, beer is the most commonly consumed alcoholic beverage and third most popular overall beverage following water and tea [3, 4].

Iso-α-acids (IAA) and three structurally similar but chemically-altered IAA known as reduced IAA provide the bitter properties in beer. However they can also be used as beer-specific ingredient congeners to confirm beer consumption. In the authors' laboratory, a protein precipitation extraction and ESI-UHPLC-MS/MS method was developed and validated for the detection of these compounds in blood [5]. The long-term stabilities of these analytes in stored blood specimens was assessed over 8 weeks with freezing (-20 °C) and refrigeration (4 °C) conditions determined as acceptable [6]. The analysis of the blood and urine from volunteers consuming a range of brown and clear bottled beers in small amounts in controlled drinking studies demonstrated successful bioavailability of these compounds [7, 8]. Additional pharmacokinetic data demonstrated small inter-variable differences in concentration-time profile, quick absorption rates and half-lives ranging between ~30-46 minutes.

Recently this methodology was applied to the human tissue from forensic postmortem cases to determine the ability to detect natural IAA and reduced IAA groups and ultimately to demonstrate the prevalence of beer consumption [9]. Nearly 90% of all cases that had "beer" mentioned in the circumstances or autopsy report of the cases, contained an IAA beer marker. It was further shown in a separate cohort 57% of cases that had no mention of beer ingestion but contained a positive alcohol concentration, demonstrated beer consumption prior to death. Such data demonstrates the high prevalence of beer consumption in Australia.

Further investigation of postmortem casework is necessary in order to obtain information such as postmortem redistribution (PMR) [10] and the comparison between serum and whole blood concentrations. This study aims to examine such postmortem phenomena to provide the relevant toxicology data required in order to interpret and confirm beer consumption when IAA type compounds are detected in casework.

2. EXPERIMENTAL

2.1. Case and Specimen Selection

The Victorian Institute of Forensic Medicine performs medico-legal investigations of deceased cases reported to the Coroners Court of Victoria. The toxicology laboratory within the Institute receives specimens on admission of the body to the mortuary (generally femoral whole blood and serum) and also following an autopsy when it is conducted with a larger range of body specimens including another whole blood and serum. Coronial, autopsy and toxicological data (e.g. demographics, cause of death, drug exposure, BAC etc.) were obtained using the Institute's case management system. No decomposed cases were considered for analysis.

2.2. Ethical Approvals

Permission for this research was obtained by the Research Advisory Committee (RAC 013/13) and Human Research Ethics Committee (EC 07/2013) of the Victorian Institute of Forensic Medicine.

2.3. Specimens

For mortuary admission specimens, blood (and often serum) was collected as soon as practicable after a body was admitted to the mortuary. At autopsy, additional blood and serum specimens from the same deceased person were collected. Mortuary admission blood was refrigerated (~4 °C) prior to analysis, all other specimens were stored frozen (-20 °C). All specimens were collected in polypropylene tubes with all whole blood specimens containing 1% sodium fluoride/potassium oxalate preservative. Serum was obtained by centrifugation for 10 min at 2400 × g. Blood was collected from the femoral region unless otherwise indicated.

Drug-free specimens were collected for instrument calibration and quality control purposes. Preserved blank blood (10 mL samples containing 200 mg sodium fluoride and 30 mg potassium oxalate) was obtained from a local blood bank (Melbourne, Australia). A blank postmortem serum was obtained from a case previously analyzed that showed no IAA analytes. All blank specimens underwent additional screening to ensure there were no IAA analytes or other interferences. All blank specimens were collected in polypropylene tubes and immediately frozen (-20 °C).

2.4. Chemicals and reagents

The details of the reference standards for the natural IAA and reduced IAA obtained from Labor Veritas (Zurich, Switzerland) are: IAA DCHA-Iso, ICS-I3 (containing 62.3 % w/w of *trans*-IAA); rho-IAA (RIAA), DCHA-Rho, ICS-R2 (containing 65.3 % w/w of *cis*-RIAA); tetrahydro-IAA (TIAA), Tetra, ICS-T2 (containing 99.4 % w/w of TIAA), and; hexahydro-IAA (HIAA), DCHA-Hexa, ICS-H1 (containing 65.7 % w/w of *cis*-HIAA).

The isotope labeled internal standard nimodipine-d_7 was purchased from PM Separations (Brisbane, Australia). Acetonitrile (ACN), methanol and formic acid were purchased from Merck (Melbourne, Australia). Ammonium formate was purchased from Sigma Aldrich (Sydney, Australia). All chemicals were of analytical grade or better and water was purified using a Milli-Q Ultrapure Water System from Waters (Sydney, Australia).

2.5. IAA and reduced IAA analytical methodology

Natural IAA (*trans*-IAA and *cis*-IAA) and reduced IAA (RIAA, TIAA and HIAA) determination was performed using a previously published UHPLC-MS/MS method that was validated for blood analysis [5]. Briefly, the extraction consisted of a protein precipitation of 200 µL of whole blood using -20 °C ACN with the resulting supernatant dried under nitrogen and the residue reconstituted with 50 µL of a mixture of eluent A and eluent B (60:40, v:v). Preparation of stock solutions, calibration standards, quality controls and extraction procedures were performed as published previously [5]. Blank serum replaced whole blood in the calibration and quality control models for the analysis of serum casework. The availability of IAA reference standards allowed for the quantification of *trans*-IAA, RIAA, TIAA and HIAA groups. Residual *cis*-IAA in the *trans*-IAA reference standard was used to allow for qualitative *cis*-IAA results.

The *cis*-IAA group was unable to be quantified due to lack of specific reference standard. However to allow for comparisons to be made, the internal standard/area ratios of each *cis*-IAA analyte were summed to provide a total are ratio for the *cis*-IAA group.

The UHPLC-MS/MS system comprised of a Shimadzu MS 8030 quadrupole mass spectrometer (Melbourne, Australia) operated in electrospray ionisation negative mode. A Shimadzu Nexera UHPLC system (Melbourne, Australia) consisted of a degasser, two eluent pumps, a column oven (30 °C) with a Kinetex C_{18} column (3.0 × 150 mm, 2.6 µm from Phenomenex, Melbourne, Australia), and a chilled autosampler. For MS data evaluation, Shimadzu Postrun and Shimadzu Browser Analysis (Melbourne, Australia) softwares were used. The mobile phases consisted of 50 mmol/L aqueous:ACN (90:10) ammonium formate pH 2.8 (eluent A) and ACN containing 0.1 % formic acid (eluent B).

2.6 Statistical analysis

All statistical analysis was performed using GraphPad Prism 5.04 from GraphPad Software (San Diego, USA).. The median, range and population size (n) was reported and $p < 0.05$ was considered statistically significant for all analyses

2.6.1. Postmortem redistribution

To demonstrate PMR any changes in natural and reduced IAA median concentrations between mortuary admission (ADM) and autopsy (AUT) bloods was determined. The median postmortem interval (time of death – time of collection, PMI), median concentrations and their ranges, and the AUT/ADM ratio were examined. The Wilcoxon matched-pairs signed rank test ($p < 0.05$) was performed on the concentrations of natural and reduced IAA in femoral whole bloods from cases that had five or more paired mortuary admission and autopsy specimens.

Additionally any correlation between the pre-autopsy interval (collection time difference between mortuary admission and autopsy specimens) and changes in IAA concentrations for the same cases were plotted to demonstrate if PMR was influenced by a prolonged or delayed specimen collection. The following formula was used to evaluate the change in concentration [%] between ADM and AUT specimens:

$$\frac{\mathrm{Conc(AUT)} - \mathrm{Conc(ADM)}}{\mathrm{Conc(ADM)}} \times 100 = \Delta\mathrm{Conc}\ [\%]$$

If ΔConc [%] > 0 an increase in concentration was observed between ADM and AUT blood specimens, if ΔConc [%] < 0 a decrease in concentration was observed between ADM and AUT blood specimens.

Finally, any influence of different blood collection sites on the concentrations of natural and reduced IAA were accessed. Femoral and non-femoral (heart, cavity and subclavian) specimens were compared in paired specimens with positive IAA groups.

2.6.3. Serum/blood ratios

The serum to blood ratios (S/B) for the natural and reduced IAA groups were estimated. Comparison of whole blood and serum specimens collected only from the femoral region at admission to the mortuary admission were considered in order to determine if a significant difference exists in the IAA median concentrations by utilizing a Wilcoxon two-tailed matched-pairs test.

3. RESULTS

The analysis of a large cohort of authentic casework provided for the statistical examination for *trans*-IAA, *cis*-IAA and TIAA in most test parameters. Unfortunately, the low prevalence of the RIAA and HIAA ($n < 5$) did not allow these IAAs to be considered. However, these three common IAA groups were largely similar in results between each of the test parameters.

Table 1 shows the *trans*-IAA, *cis*-IAA and TIAA median concentrations for ADM and AUT bloods. There was a significant difference between the specimens for *trans*- and *cis*-IAA which had AUT/ADM ratios of 1.6 and 1.7, respectively. The median PMI (time of specimen collection - time of death) for the natural IAA were similar for the ADM and AUT specimens at ~0.4 and 4.8 days, respectively. However, TIAA showed no difference in median concentrations and had a median AUT collection time approximately a day earlier than the natural IAA group.

The influence of the pre-autopsy interval was also examined by comparing against the difference in specimen collection time of a case with the change in IAA concentration in the same case, **Fig. 1**. There were 18, 22 and 9 cases for *trans*-IAA, *cis*-IAA and TIAA, respectively. The pre-autopsy interval ranged up to ~8.5 days with each case plotted in sequence from lowest to highest pre-autopsy interval. The largest ΔConc was ~1000%, whilst there were also some cases where concentrations were considerably lower than at mortuary admission. Although there appears to be a slight relationship for AUT concentrations to rise with prolonged collection times, there were also cases where all IAA concentrations decreased, particularly for cases collected between ~4-5 days difference.

There were insufficient non-femoral (heart, cavity and subclavian) whole blood specimens that were positive IAA groups to suitably compare to matched femoral blood concentrations. However, a single case study describes the difference in natural IAA concentrations from multiple bloods taken at mortuary admission and at autopsy, where three autopsy bloods were collected from different sites, **Table 2**. Redistribution was observed between bloods collected at the same time at autopsy with 0.010, 0.010 and 0.026 mg/L *trans*-IAA concentrations in the femoral, subclavian and cavity bloods, respectively. Similar redistribution of analytes was observed with the *cis*-IAA area ratios. This analysis demonstrates the potential fluctuations in IAA redistribution between specimens collected from femoral and non-femoral blood collection sites. Following a pre-autopsy interval length of ~8.5 days, there was also a slight increase in natural IAA in the autopsy femoral blood, when compared to the mortuary admission.

An assessment of the *trans*-IAA, *cis*-IAA and TIAA median concentrations, S/B ratios and p-values of cases with both serum and blood specimens are given in **Table 3**. A significant and marked difference was demonstrated between the

matched serum and whole bloods for the three IAA concentrations and resulted in an average S/B ratio of consistently ~3 for the analyzed IAA groups. In addition, linear regression analysis (serum vs. blood concentrations) was performed on the plotted S/B ratios (data not shown) that demonstrated a uniform trend between matched pairs. This demonstrated that the increase in concentrations did not show any changes in ratios over the concentration range for the IAA groups under investigation. Moreover, there was no evidence of an inacceptable number of outliers for any IAA group.

4. DISCUSSION

This current study examines key forensic toxicology parameters that provide a greater understanding when interpreting postmortem IAA results to confirm beer consumption. In 2014, 78% of Australians believe the country has an alcohol problem with over 36% of drinkers claiming they "drink to get drunk", costing more than $15 billion annually [11-13]. A previous study has demonstrated the high prevalence of beer consumption in forensic casework with over half of all alcohol positive cases confirming beer intake prior to death [9]. This research provides additional information when interpreting such results.

The postmortem redistribution processes during the postmortem interval can lead to variations in drug concentrations which can affect the way in which case results are interpreted [14-19]. Comparison of mortuary admission and autopsy blood IAA concentrations can assist in determining the PMR phenomena [20]. Generally, this study showed that the natural IAA groups were subject to significant changes in concentration when femoral blood was collected on admission to the mortuary and compared to blood taken at autopsy, approximately 4 days later. Such fluctuations in blood drug concentrations have been shown when bodies are stored for long periods of time, hence the timely collection of specimens is preferable [21, 22]. Furthermore, it has been shown that PMR may occur in the early hours following death and therefore the extent to which PMR may have already taken place prior to the mortuary admission specimen is unknown [20]. Nonetheless, specimens collected on admission to the mortuary that are closer to the time of death may help to minimize these changes [23, 24]. The comparison of the pre-autopsy interval and change in IAA concentration for each case reflected this. It appeared that there were smaller fluctuations closer to the time of death. However this comparison of individual cases also demonstrated the non-uniform trend for IAA concentrations to increase, and also decrease, throughout the PMI.

Although the body of the deceased cases were refrigerated immediately upon admission to the mortuary, studies investigating the stability of drugs in a decomposing body to simulate the influence of PMI have shown that many can quickly degrade following death [22, 25]. Long-term stability studies demonstrated that IAA and reduced IAA at low

concentrations in blood underwent minimal degradation under refrigerated storage temperatures [6]. While this stability study provides information on the changes which can occur to IAA stability in a controlled environment; they cannot mimic the considerable influence that bacteria, fungi, body and trauma, and other factors, are known to influence analyte concentrations following death [25-28]. Even with the possibility of losses, the natural IAA concentrations were shown to generally increase during body storage and exhibit PMR with AUT/ADM ratios 1.6 to 1.7.

Fat tissue and skeletal muscle, are possible body compartments where substance accumulation is allowed and source for the redistribution of IAA into the blood after death [16, 17, 29]. As this is most significant in central blood, the effects of PMR can possibly be minimalized with the collection of peripheral (e.g. femoral) blood [18, 30, 31]. This exaggerated increase in PMR from centrally located blood was demonstrated in one case where heart blood natural IAA concentrations were significantly higher than the peripheral specimens. These matched blood specimens demonstrated the time and site collection dependence of PMR.

Although there is no technique to measure the degree of redistribution, it is largely accepted that PMR is magnified for substances that collect in high concentrations in body compartments, which are commonly lipophilic, have an appropriate pKA, or have high volumes of distribution (Vd), commonly greater than 3 L/kg [18, 20, 31]. A quantitative structure-activity relationship (QSAR) model predicted a relatively small Vd range of 0.56-0.58 L/kg for the IAA groups [32]. However the distribution-coefficient (logD) for IAA groups was estimated to range from ~2.5 – 4 at pH 7.4 using a QSAR model [33] (with equal weighting [34, 35]). These logD properties may demonstrate the ability for IAA to collect in body compartments. Furthermore, the IAA groups have been shown to diffuse across lipophilic bilayers dependent on partition-coefficients (logP), pKa, and molecular size [36]. The acidic IAA groups possess a pKA of ~3-4 and have logP properties ranging from ~2.5 - 4.5 demonstrating good lipophilicity (log P) capable of transport across cell membranes [37, 38].

Such properties of the IAA groups demonstrate the lipophilicity of these compounds, sufficient to explain the elevation of the natural IAA concentrations in the heart blood of the case study following a prolonged PMI. Further investigation that provides *in vivo* data may further assist in the interpretation of IAA PMR in casework.

This paper also compared the serum-to-blood distribution (S/B ratio) of *trans*-IAA, *cis*-IAA and TIAA in postmortem specimens. Whole blood and serum (or plasma) are commonly obtained and analyzed in forensic investigations [39]. It is well known that drugs are unevenly distributed between the fluid and cellular phases of blood [40]. Although whole blood has recently been shown suitable for the analysis of postmortem and pharmacokinetic controlled studies [7-9], detection off IAA in serum was also found to be potentially more useful with higher concentrations of IAA in serum

compared to blood [9]. Acidic and neutral compounds will primarily bind to albumin that if saturated, may bind to lipoprotein [40]. Serum concentrations for IAA are also dependent on the hematocrit value [41]. As it will not be possible to measure hematocrit in some of the circumstances in which whole-blood samples have been analyzed, the comparison between serum and whole blood presented here can be used to compare results obtained in different blood specimens.

A consistent S/B ratio of ~3 was shown that did not show any obvious outliers throughout the concentration range. Serum (and plasma) to blood ratios are commonly measured in antemortem specimens, however the hemolysis of the erythrocytes in postmortem whole blood and therefore the liberation of intracellular water commonly [41]. This may explain the considerably high IAA and reduced IAA S/B values. Nonetheless, such a difference in serum and blood concentrations in postmortem casework demonstrates that specimen matched calibration and quality control matrices should be used when analyzing natural and reduced IAA.

In summary, this study showed that the IAA and reduced IAA groups detected in postmortem blood and serum are subject to postmortem phenomena such as redistribution. These studies confirm that although postmortem concentrations cannot be easily compared to clinical concentrations, the presented findings do provide a greater understanding to assist interpretation of forensic casework where the confirmation of beer is needed.

KEY POINTS

1. IAA are subject to considerable postmortem redistribution
2. There is an association between the postmortem interval and the extent of postmortem redistribution
3. There is evidence that different sites of blood collection can influence IAA concentrations
4. The serum to whole blood ratio of IAA concentration was ~3:1 in postmortem blood specimens

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Table 1.

Comparison of mortuary admission (ADM) and autopsy (AUT) whole blood femoral specimens for *trans*-IAA, *cis*-IAA and TIAA concentrations.

		***trans*-IAA (mg/L)**	***cis*-IAA (area ratio)**	**TIAA (mg/L)**
Mortuary Admission femoral blood (ADM)	**Median PMI (days)**	0.3	0.2	0.4
	Median concentration	0.008	0.059	0.010
	[min - max]	[0.001 – 0.043]	[0.010 – 1.114]	[0.001 – 0.030]
Autopsy femoral blood (AUT)	**Median PMI (days)**	4.9	4.7	3.7
	Median concentration	0.013	0.099	0.010
	[min - max]	[0.001 – 0.068]	[0.008 – 1.105]	[0.001 – 0.058]
Pairs		18	22	9
Ratio (AUT/ADM)		1.6	1.7	1.0
p value		0.008*	0.034*	0.844

PMI = Postmortem Interval

*significant difference ($p < 0.05$) utilizing a Wilcoxon two-tailed matched-pairs test.

Table 2.

The time and site collection dependent PMR in a 66 year old male who died of natural causes with untraumatized body specimens collected.

	Mortuary admission	**Autopsy**		
	Femoral	**Femoral**	**Subclavian**	**Cavity**
***trans*-IAA (mg/L)**	0.007	0.010	0.010	0.026
***cis*-IAA (area ratio)**	0.090	0.245	0.230	0.523
PMI (days)	1.1	9.6	9.6	9.6

Table3.

Comparison of serum and femoral whole blood mortuary admission specimens of *trans*-IAA, *cis*-IAA and TIAA concentrations.

	***trans*-IAA (mg/L)**	***cis*-IAA (area ratio)**	**TIAA (mg/L)**
Pairs	17	19	11
Blood median	0.006	0.087	0.010
[min - max]	[0.001 – 0.043]	[0.006 – 0.486]	[0.001 – 0.074]
Serum median	0.018	0.268	0.031
[min - max]	[0.002 – 0.124]	[0.034 – 2.840]	[0.002 – 0.191]
Ratio (Serum/Blood)	3.0	3.1	3.0
p value	< 0.001*	< 0.001*	0.001*

*significant difference ($p < 0.05$) utilizing a Wilcoxon two-tailed matched-pairs test

Note: There was an insufficient amount of RIAA and HIAA detected to perform analysis.

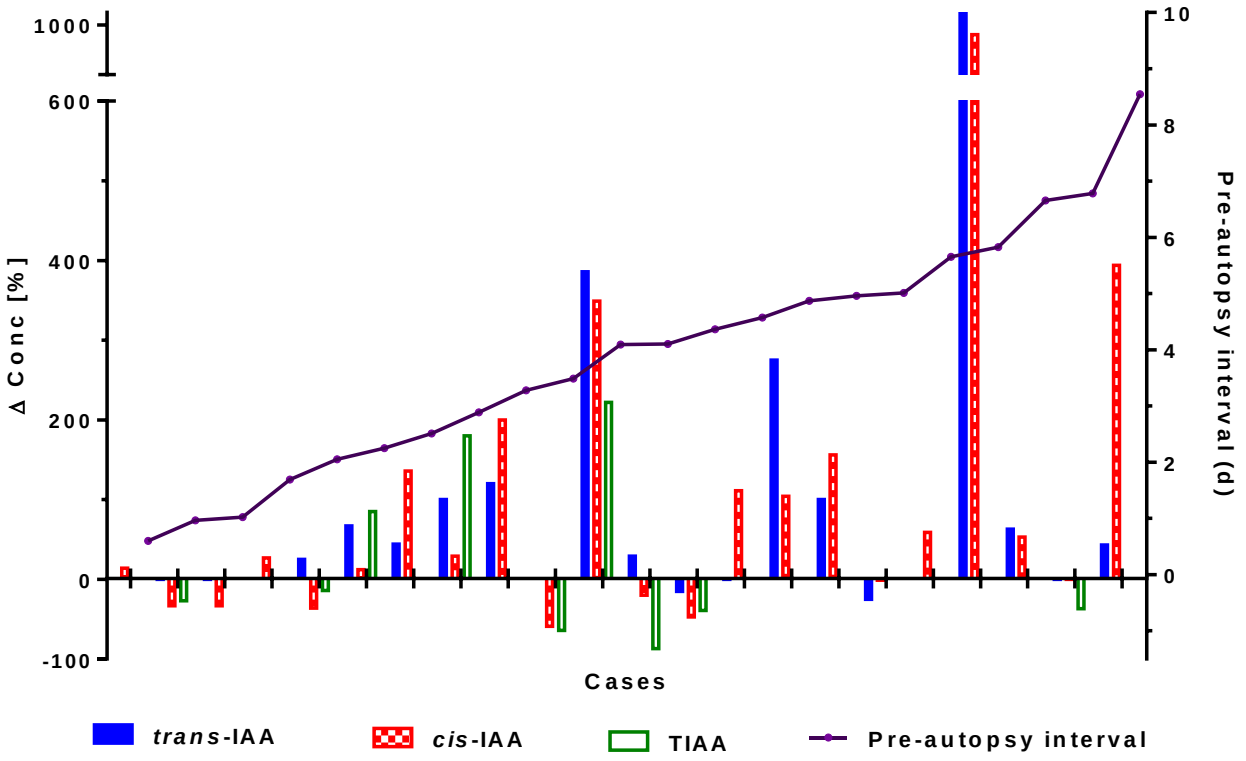
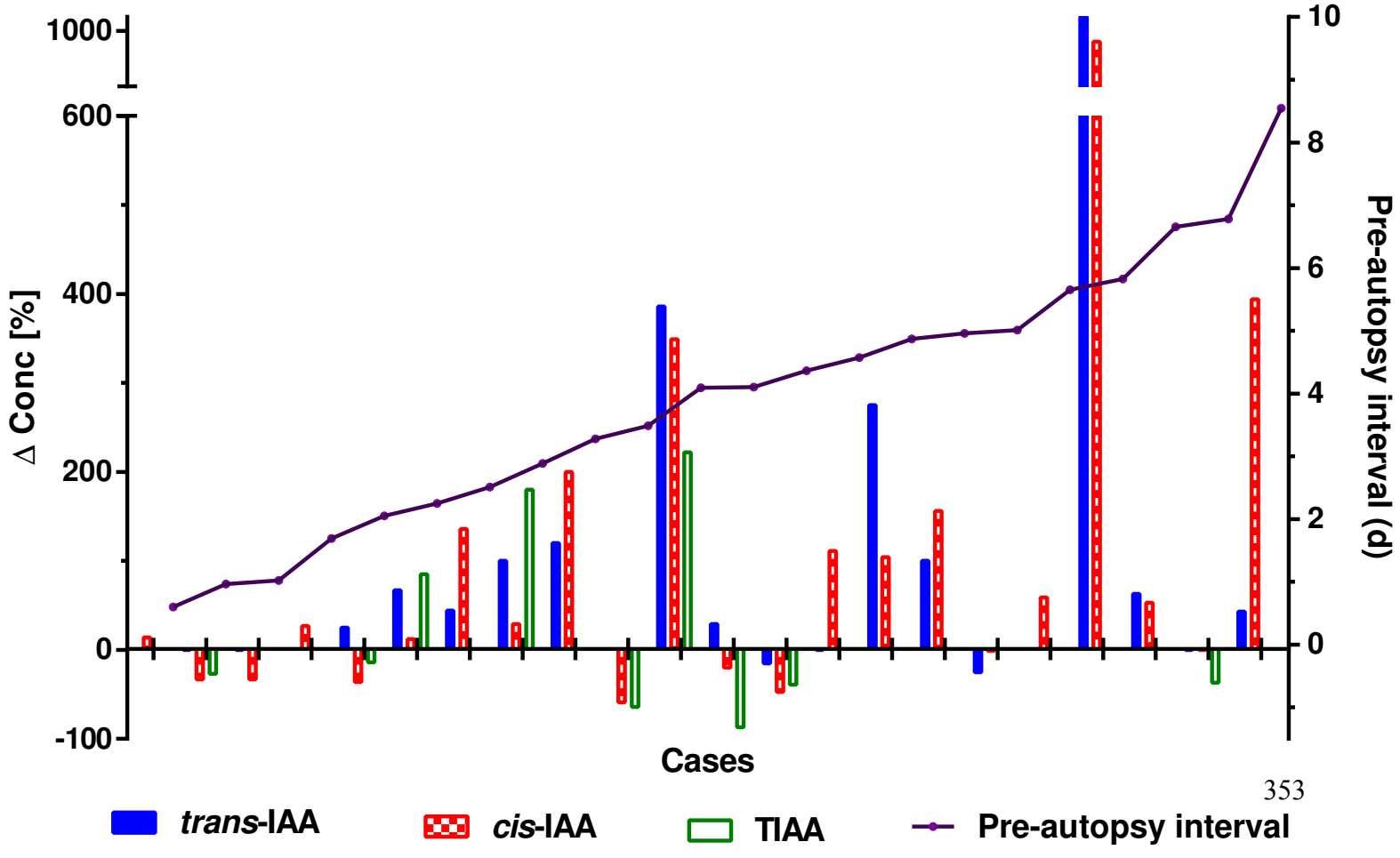


Fig. 1.

Changes in IAA concentrations were compared to the pre-autopsy interval (collection time difference between mortuary admission and autopsy) in femoral whole bloods from non-decomposed cases that had paired mortuary admission and autopsy samples that were positive for *trans*-IAA, *cis*-IAA and/or TIAA


1000
600
400
200
0
-100
Δ Conc [%]
10
8
6
4
2
0
Pre-autopsy interval (d)
Cases
trans-IAA
cis-IAA
TIAA
Pre-autopsy interval

APPENDIX 1.8:

CHAPTER 6.1 CONFERENCE POSTER

The Quantification of Hop-Derived Iso-α-acid type Compounds in Beer using uHPLC-MS/MS

L. N. RODDA[1,2], D. GEROSTAMOULOS[1,2], O. H. DRUMMER[1,2]

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luke.rodda@monash.edu

DEPARTMENT OF FORENSIC MEDICINE
School of Public Health and Preventive Medicine

Introduction:

A method for the quantification of iso-α-acid (IAA) type ingredient congeners that are derived from the hop plant (*Humulus lupulus* L.) in beer was developed to form a reference catalogue. Three structurally similar but chemically-altered IAA, also used as beer-specific ingredients known as "reduced IAA" (Fig. 1) were also targeted, namely rho-, tetrahydro- and hexhydro-IAA (RIAA, TIAA and HIAA, respectively).

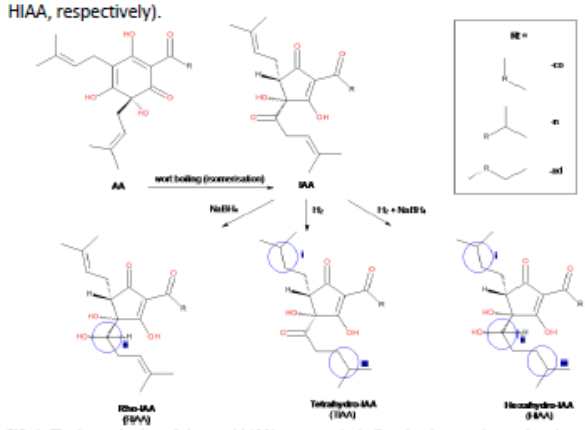

FIG. 1: The isomerization of the α-acid (AA) structure including the three major analogs (co, n and ad) into the IAA diasteromers and subsequent synthesis of the three *reduced* IAA groups. Sites of increased hydrophobicity (i, iii) and photolytic cleavage prevention (ii, iii) properties are highlighted in the reduced IAA that provide light-protective and foam stabilization properties.

Aims:

- To develop a method of quantification of the IAA type groups in Beer.
- To determine the prevalence of *trans*-IAA, *cis*-RIAA, TIAA and *cis*-HIAA in a large number of beers and form a reference catalogue for novel alcohol congener analysis used to determine whether beer may be the source of alcohol (FACTA Inc. 2013 Conference Oral #27).

Methods:

- Following degassing by sonication and 0.22 µm filtration, 20 µL of beer was directly injected into the uHPLC-MS/MS system.
- Separation of isobaric analogs and isomers within a 10 min run-time was achieved using a C_{18} solid-core column under gradient elution.
- Three transitions were monitored using electrospray ionisation in negative multiple reaction monitoring mode for each analyte.
- Analytes were ultimately grouped accordingly to form a calibration curve (0.1 – 50 mg/L, r^2>0.99) for quantification of the IAA and reduced IAA groups.
- A large number of local and domestic, popular and craft, beers were analysed.

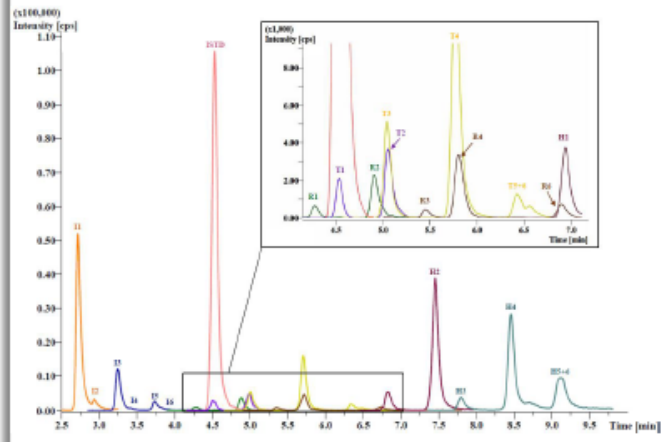

FIG. 2: Chromatograph of individual analytes at 1 mg/L concentrations for the *trans*-IAA, *cis*-RIAA, TIAA, and *cis*-HIAA groups. The description of individual analytes has previously been described [2].

TABLE 1. A reference catalogue of the detected IAA and Reduced IAA concentrations in beers

Vessel	Beer	Hop Content (mg/L)			
		Natural	Reduced		
		trans-IAA	cis-RIAA	TIAA	cis-HIAA
Can	Guinness Draught	7			
Brown Bottle	Asahi Super Dry	5			
	Bitburger	9			
	Coopers Pale Ale	7			
	Little Creatures Pale Ale	9			
	Sierra Nevada Pale Ale	14			
	Tiger	8			
	Victoria Bitter	6			
	West Coast Indian Pale Ale	20			
	XXXX Gold	6			
Green Bottle	Becks	13			
	Carlsberg	7	0.3	3	Trace
	Cascade Premium Light	4		5	Trace
	Hahn Premium Pilsner	4		2	
	Heineken	7			
	James Boag's Premium Light	4		5	
	Pure Blonde	5			
	Stella Artois	4		Trace	
	Tsingtao	5			
Clear Bottle	Amstel	Trace	5	1	
	Coopers Clear	Trace	19		0.3
	Corona	1			
	Fosters Light Ice	Trace		18	0.1
	Hahn Premium Light			6	
	Miller Genuine Draft			6	
	Sol Mexican	Trace	20	4	Trace
	Spitfire Kentish Ale	10			
	Tooheys Extra Dry	Trace		11	0.1
	XXXX Summer Bright	Trace		7	Trace

Results & Discussion:

- Sufficient separation was achieved that provided accurate determination of targeted beer-specific ingredient congeners in commonly consumed beers.
- Interestingly, Table 1 demonstrates that although the IAA group degrades when exposed to light [2], brewers have used natural hop products in green, and more surprisingly, in clear bottled beers.
- The lower amount of natural IAA present in green bottled beer in comparison to brown is substituted with addition of the *light-proof* reduced IAA products.
- Corona showed a low *trans*-IAA content, likely a result of being subject to light consequently causing major degradation of the susceptible isomer during improper storage [2].
- Trace levels of IAA are observed as a residual by-product following the reduced IAA production, as seen in several clear bottles.
- Beers containing RIAA required a relatively higher concentration to achieve the desired bitterness due to the lower bitterness properties of RIAA [2].

In summary, the interpretation of detected beer-specific ingredient congeners in blood and urine, where demonstrating the consumption of beer is of importance in medico-legal casework [3], is assisted with details of the IAA and reduced IAA content in commonly consumed beers as presented here.

References and Acknowledgements:

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WE ACKNOWLEDGE SHIMADZU FOR THE 2013 FACTA INC. CONFERENCE PHD FULL STUDENT SCHOLARSHIP AWARD

APPENDIX 2

ETHICS APPROVALS

Ethics Number ***(Appendix)***	**Human Research Ethics Committee & Site**	**Approval date**	**Aim & Outcome**
EC 04/2012 *(Appendix 2.1)*	Victorian Institute of Forensic Medicine, Australia	22.02.2013	Method development and validation. Pilot and secondary volunteer drinking studies for Natural IAA.
EC 04/2012 amendment *(Appendix 2.2)*	As Above	29.05.2013	Safer phlebotomy technique used to obtain blood in the above studies.
EC 06/2013 *(Appendix 2.3)*	As Above	09.10.2013	Secondary volunteer drinking studies for the Reduced IAA.
EC 07/2013 *(Appendix 2.4)*	As Above	07.08.2013	Recruitment and analysis of coronial postmortem blood and urine specimens for IAA.
EC 07/2013 amendment *(Appendix 2.5)*	As Above	21.03.2014	Approval to analyse 20 vitreous humor specimens from the above coronial cases for IAA.

Appendix 2.1: EC 04/2012

Victorian Institute of Forensic Medicine
57-83 Kavanagh Street Southbank Victoria, Australia 3006
MONASH University

22 February 2012

Dear Mr Rodda

RE: EC 4/2012: Alcohol congener analysis of beer components in revealing beer consumption in a forensic context

I am happy to inform you that the VIFM Ethics Committee considered your application for the above project on 16 February 2012 and granted full **approval** having determined that the project meets the requirements of the NHMRC National Statement on Ethical Conduct in Human Research.

Please note the following requirements:

- The Reference Number for your project is **EC 4/2012**
- The VIFM Ethics Committee is to be notified immediately of:
  - any matter that arises that may affect the conduct or continuation of the approved project or have serious or unexpected adverse effects for next of kin.
  - any unforeseen events that might affect continued ethical acceptance of the project
  - any proposed changes to the original protocol. Changes must be approved by the Committee before they are implemented
  - discontinuation of the protocol before its completion and reasons why
- You are required to provide an Annual Report every 12 months and to provide a Final Report at the end of the project.
- Approval is only granted for three years, after which time a Final Report is to be submitted and the project renewed with a new application.
- The VIFM would also appreciate receiving copies of any relevant publications, papers, theses, conferences presentations or audiovisual materials that result from this research. The VIFM should be acknowledged as a source of research data or tissue in any such publications.

All future correspondence regarding this project must be sent to [redacted] and include the EC Reference Number and the project title. Hard copies of signed documents or original correspondence are to be sent to:

Ms Fiona Leahy, Executive Officer
VIFM Ethics Committee
57-83 Kavanagh Street
Southbank Victoria 3006

TELEPHONE: +61 3 9684 4444 **FACSIMILE:** +61 3 9682 7353 **EMAIL:** forensic@vifm.org **WEB SITE:** www.vifm.org

If you have any queries regarding this application you are welcome to contact me on 9684 4358 or email: Fiona.Leahy@vifm.org.

Good luck with your research.

Yours sincerely,

[redacted]

Ms Fiona Leahy

Executive Officer
VIFM Ethics Committee

Appendix 2.2: EC 04/2012 Amendment

Victorian Institute of Forensic Medicine
57-83 Kavanagh Street Southbank Victoria, Australia 3006
MONASH University

29 May 2013

Mr Luke Rodda
Victorian Institute of Forensic Medicine

Dear Luke

RE: EC 4/2012: Alcohol congener analysis of beer components in revealing beer consumption in a forensic context

Thank you for your application to the VIFM Ethics Committee to amend the above project to amend the means of collecting blood from the voluntary participants.

The VIFM Ethics Committee has considered and approved this amendment.

If you wish to discuss this matter further, please contact me on [redacted] or at [redacted]

Good luck with your research.

Yours sincerely

[redacted]

FIONA LEAHY

Senior Medico-Legal Adviser
Executive Officer, VIFM Ethics Committee

TELEPHONE: [redacted] FACSIMILE: +61 3 9682 7353 EMAIL: [redacted] WEB SITE: www.vifm.org

Appendix 2.3: EC 06/2013

Victorian Institute of Forensic Medicine
57-83 Kavanagh Street Southbank Victoria, Australia 3006
MONASH University

9 October 2013

Mr Luke Rodda
Department of Forensic Medicine
VIFM

Dear Luke,

RE: Alcohol congener analysis of beer components in revealing beer consumption in a forensic context: Reduced-iso-α-acid study EC 6/2013

Thank you for providing a revised application for the above research project that:

- updated the registration details of the supervising medical practitioner and nurse
- broadened the inclusion criteria of the participants to allow the participation of women
- Amended the PICF to include a question to the effect of "is there any health or medical reason why you cannot participate in the study".

This information has been sent to the VIFM Ethics Committee members who have endorsed the approval of this project.

If you have any queries regarding this application you are welcome to contact me on (03) 9684 4358 or email: Fiona.Leahy@vifm.org.

Good luck with your research.

Yours sincerely,

[redacted]

Ms Fiona Leahy

Executive Officer
VIFM Ethics Committee

[redacted]

Appendix 2.4: EC 07/2013

Victorian Institute of Forensic Medicine
57-83 Kavanagh Street Southbank Victoria, Australia 3006
MONASH University

7 August 2013

Mr Luke Rodda
Department of Forensic Medicine
VIFM

Dear Luke,

RE: Alcohol congener analysis of beer components in revealing beer consumption in a forensic context: Application in authentic cases **EC 7/2013**

I am happy to inform you that the VIFM Ethics Committee considered your application for the above project on 1August 2013 and has granted **full approval** having determined that the project meets the requirements of the NHMRC National Statement on Ethical Conduct in Human Research.

Please note the following requirements:

- The Reference Number for your project is **EC 7/2013**
- The VIFM Ethics Committee is to be notified immediately of:
  - any matter that arises that may affect the conduct or continuation of the approved project or have serious or unexpected adverse effects for next of kin.
  - any unforeseen events that might affect continued ethical acceptance of the project
  - any proposed changes to the original protocol. Changes must be approved by the Committee before they are implemented
  - discontinuation of the protocol before its completion and reasons why
- You are required to provide an Annual Report every 12 months and to provide a Final Report at the end of the project.
- Approval is only granted for three years, after which time a Final Report is to be submitted and the project renewed with a new application.
- The VIFM would also appreciate receiving copies of any relevant publications, papers, theses, conferences presentations or audiovisual materials that result from this research. The VIFM should be acknowledged as a source of research data or tissue in any such publications.

All future correspondence regarding this project must be sent to [redacted] and include the EC Reference Number and the project title. Hard copies of signed documents or original correspondence are to be sent to:

[redacted]

Ms Fiona Leahy, Executive Officer
VIFM Ethics Committee
57-83 Kavanagh Street
Southbank Victoria 3006

If you have any queries regarding this application you are welcome to contact me on (03) [redacted]

Good luck with your research.

Yours sincerely,

[redacted]

Ms Fiona Leahy

Executive Officer
VIFM Ethics Committee

Appendix 2.5: EC 07/2013 Amendment

Victorian Institute of Forensic Medicine
57-83 Kavanagh Street Southbank Victoria, Australia 3006
MONASH University

21 March 2013

Mr Luke Rodda
Department of Forensic Medicine
VIFM

Dear Luke,

RE: Alcohol congener analysis of beer components in revealing beer consumption in a forensic context: Application in authentic cases EC 7/2013

Thank you for your application to the VIFM Ethics Committee to amend the above project to allow for the testing of vitreous humour for approximately 20 coronial cases.

The VIFM Ethics Committee has considered and approved this amendment.

If you wish to discuss this matter further, please contact me on [redacted] or at [redacted]

Good luck with your research.

Yours sincerely

Yours sincerely,

[redacted]

Ms Fiona Leahy

Executive Officer
VIFM Ethics Committee

[redacted]