Hassan, Huda (2011) *An evaluation of the stability and prevalence of alcohol and related biomarkers in biological matrices with applications to the interpretation of medico-legal cases.* PhD thesis.

http://theses.gla.ac.uk/3002/

Copyright and moral rights for this thesis are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This thesis cannot be reproduced or quoted extensively from without first obtaining permission in writing from the Author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the Author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Glasgow Theses Service
http://theses.gla.ac.uk/
theses@gla.ac.uk

An Evaluation of the Stability and Prevalence of Alcohol and Related Biomarkers in Biological Matrices with Application to the Interpretation of Medico-Legal Cases

Thesis Submitted in Accordance with the Requirements of the University of Glasgow for the Degree of Doctor of Philosophy

by

HUDA MUSTAFA A. HASSAN

Forensic Medicine and Science
(School of Medicine)

November 2011

© Huda Mustafa A.Hassan

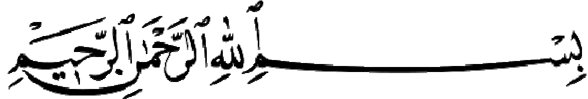
To my parents

Mustafa and Ameera

"وَقُل رَّبِّ ارْحَمْهُمَا كَمَا رَبَّيَانِي صَغِيرًا"

"And say: My Lord! Bestow on them Your Mercy as they did bring me up when I was small"

Acknowledgement



First I would like to thank God for all of my blessings, His infinite liberality will always exceed all our wishes and our thoughts. I would like to thank King Abdullah (Custodian of the Two Holy Mosques) for his kindness and generosity, the Saudi Government, Royal Embassy, Saudi Cultural Bureau and Ministry of Higher Education, as well as King Faisal Specialist Hospital and Research Center in Riyadh (Pathology laboratory, Academic Affairs and Hospital Administration) for my scholarship and supporting my study not only financially.

I would like to express my sincere gratitude to my supervisor Dr. Gail Cooper for her enthusiasm, her inspiration, and her great efforts that helped guide me throughout my research and the writing of my thesis. With her support she helped make this thesis possible. She always did so many thoughtful things that had her special touch, I have no other words other than, thank you, thank you, thank you!

I must also thank all of the Toxicologists, Dr. Robert Anderson for all his support and lots of good ideas and tips to support my research, Dr. Hazel Torrance for her encouragement, advice and support, and great tips especially during the LC-MS/MS project. My sincere thanks also go to Dr. Karen Scott for all her advice and support. She was the one who enlightened me at the start of my research by helping me to operate and set up methods on the GC/MS.

I would also like to take this opportunity to thank Dr. Fiona Wylie, and Dr. Eleanor Miller and to wish them all the best. Many thanks to Dr. John Clark for his kindness, I really liked when he would wish me a good morning or see you tomorrow in Arabic.

I would like to express my thanks to the Technicians who helped me get to grips with some of the equipment and for all their support during my research, and to all of the staff within Forensic Medicine and Science; the Secretaries, Administrators and IT staff for all their support. I must say that Forensic Medicine and Science was like a second home for me and made me feel so welcome. It is an excellent place to come and study with fantastic resources and great staff who encourage you.

I would also like to thank and recognize the University of Glasgow Staff especially in the School of Medicine, the Graduate School, the International Office, Library, IT help desk, courses tutors, and all other staff because with their support I have had a great environment to study and continue my research without obstacle. I feel very happy and honored to have had the opportunity of studying at such a distinguished institution and with such a distinguished team.

Warm thanks to Professor Maceij Bogusz who encouraged and supported me to apply for my PhD and for inviting me work as a co-author of a chapter in his book. For also supporting me during my work with him at the King Faisal Hospital and throughout my PhD, without which I could not have achieved so much!

To my late Dad, whose memories inspired me to persevere. I will not forget to thank my lovely mother who always prays for God to help me and to make my life easier. I also value the support of my eldest brother Abdulkader, all brothers and sisters in Saudia Arabia who always kept in touch with me and made me feel surrounded by a loving family.

I would also like to express my deepest gratitude to my late husband, Sameer (SAM - his favorite name) who always advised and encourage me before he passed away during my first year of my PhD to continue my study no matter what happened.

Also I cannot forget my four children, Mashael, Sarah, Mishaal and Ali who have been ever so patient when I had to leave them for long periods of time during the busy parts of my research and for helping me at home. I knew they were very proud of me, especially when I have won awards or had success during my studies. They give me strength in my life and have supported me during the difficult times. Thank you!

I would like to extend my thanks to all friends and colleagues specially Dr. Abdulkareem Alfazil who is the one whose understanding and virtue I can equally confide and whose opinion I can value for its wisdom and its sincerity.

I will not forget to extend my thanks to our family's friend Mr. Naveed for his great support since we arrived to Glasgow and during difficult time.

Many thanks for my neighbours Elina and family for their kindness and care.

Finally, this thesis could not have been possible without all the support I have had and I hope my work will benefit my country and other countries in some way.

Huda Hassan
October 2011

Table of Contents

ACKNOWLEDGEMENT III
LIST OF TABLES IX
LIST OF FIGURES XI
LIST OF APPENDICES XIII
AUTHOR'S DECLARATION XIV
ABSTRACT XV
LIST OF ABBREVIATIONS XVII
CHAPTER 1 - INTRODUCTION 1
1.1 History of Alcohol Use 1
1.1.1 Alcohol Standard Measure 2
1.2 Prevalence of Alcohol Use 3
1.3 Alcohol-Related Harm 4
1.3.1 Alcohol-Rehlated Social Harm 5
1.3.2 Alcohol-Related Deaths 6
1.3.3 Road Traffic Incidents Involving Alcohol 8
1.3.4 Foetal Alcohol Spectrum Disorders (FASD) 9
1.3.5 Alcohol-Related Harm Reduction Plan in Scotland 13
1.4 Interpretative Challenges Associated with Medico-legal Investigations Involving Ethanol 14
1.5 Pharmacokinetics of Ethanol 14
1.6 Other Volatiles of Interest 17
1.7 Sample Selection for the Analysis of Volatiles 18
1.8 Stability of Alcohol in Post-Mortem Blood 22
1.9 Analysis of Ethanol and Related Volatiles 23
1.9.1 Headspace Gas Chromatography-Flame Ionization Detection (HS-GC-FID) 24
1.9.2 Selection of a Suitable Internal Standard for Analysis of Volatiles 25
1.9.3 Analytical Considerations for Method Development (Volatiles) 28
1.10 Analytical Biomarkers of Alcohol Consumption 29
1.10.1 Introduction 29
1.10.2 Markers of Acute and Chronic Alcohol Consumption 30
1.11 β-Hydroxybutyrate (BHB) 34
1.11.1 Analysis of BHB in Biological Matrices 38
1.11.2 Gas Chromatography/Mass Spectrometry (GC/MS) 38
1.12 Fatty Acid Ethyl Esters (FAEEs) 41
1.12.1 Sample Selection for the Analysis of FAEEs 42
1.12.2 Analysis of FAEEs in Biological Matrices 44
1.12.3 Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS) 47
1.13 Aims and Objectives: 50
CHAPTER 2 - EVALUATION OF COMMON VOLATILES IN POST-MORTEM BLOOD BY HS-GC-FID 52
2.1 Introduction 52
2.2 Materials and Methods 52
2.2.1 Statistical methods 52
2.2.2 Chemicals and Reagents 54
2.2.3 Preparation of Blank (Volatile-Free) Blood 54
2.2.4 Preparation of Working Standards and Quality Controls 54
2.2.5 Post-Mortem Case Samples 56
2.2.6 Instrumentation 56
2.2.7 Optimisation of GC Conditions 57

2.2.8 Sample Preparation ... 57
2.2.9 Method Validation ... 57

2.3 Results and Discussion ... 59
2.3.1 Optimisation of GC Conditions ... 59
2.3.2 Method Validation ... 62
2.3.3 Prevalence of Volatiles in Post-Mortem Blood ... 65

2.4 Conclusion ... 70

CHAPTER 3 - APPLICATION OF A VALIDATED METHOD FOR THE ANALYSIS OF VOLATILES IN BIOLOGICAL MATRICES ... 72

3.1 Introduction ... 72

3.2 The Effect of Preservative and Antioxidant Reagents on the Stability of Volatiles in Blood ... 73
3.2.1 Introduction ... 73
3.2.2 Sample Preparation ... 73
3.2.3 Results and Discussion ... 74

3.3 Stability of Ethanol and Acetone in Paired Preserved and Unpreserved Post-Mortem Blood ... 78
3.3.1 Introduction ... 78
3.3.2 Methodology ... 78
3.3.3 Results and Discussion ... 79

3.4 Stability of Ethanol in Preserved Blood Following Long-Term Storage at Room Temperature ... 88
3.4.1 Introduction ... 88
3.4.2 Methodology ... 88
3.4.3 Results and Discussion ... 89

3.5 Evaluation of the Prevalence of Volatiles in Urine, Vitreous Humour and Bile and their Correlation with Femoral Blood. ... 91
3.5.1 Introduction ... 91
3.5.2 Methodology ... 91
3.5.3 Volatile Standards ... 92
3.5.4 Results ... 92
3.5.5 Discussion ... 98

3.6 Evaluation of the Immunalysis Ethyl Alcohol Enzymatic Assay as a Semi-Quantitative Test in Comparison to HS-GC-FID ... 100
3.6.1 Introduction ... 100
3.6.2 Chemicals and Reagents ... 101
3.6.3 Methodology ... 102
3.6.4 Method Validation ... 102
3.6.5 Results and Discussion ... 103

3.7 Conclusion ... 106

CHAPTER 4 - DETERMINATION OF β-HYDROXYBUTYRATE (BHB) IN POST-MORTEM BLOOD AND URINE USING GAS CHROMATOGRAPHY -MASS SPECTROMETRY (GC-MS) ... 109

4.1 Introduction ... 109

4.2 Method Development ... 109
4.2.1 Materials ... 109
4.2.2 Instrumentation ... 112
4.2.3 Method Evaluation ... 113
4.2.4 Results and Discussion ... 115

4.3 Method Validation ... 120
4.3.1 Linearity ... 120
4.3.2 Limit of Detection (LOD) and Limit of Quantification (LOQ) ... 121
4.3.3 Precision and Accuracy ... 121
4.3.4 Recovery and Matrix Effect ... 121
4.3.5 Stability ... 122
4.3.6 Case samples ... 122
4.3.7 Results and Discussion ... 123

4.4 Study Investigating the Relationship between β-hydroxybutyrate (BHB), Acetone and Ethanol in Post-Mortem Cases 129
4.4.1 Introduction 129
4.4.2 Methodology 129
4.4.3 Results and Discussion 132

4.5 Conclusion 138

CHAPTER 5 - APPLICATION OF THE METHOD VALIDATED FOR THE ANALYSIS OF β-HYDROXYBUTYRATE (BHB) TO THE ANALYSIS OF γ-HYDROXY-BUTYRATE (GHB) AND β-HYDROXY-β-METHYLBUTYRATE (HMB) 139

5.1 Introduction 139

5.2 Determination of GHB in Post-Mortem Blood and Urine using GC-MS 140
5.2.1 Introduction 140
5.2.2 Prevalence of GHB in Biological Matrices 141
5.2.3 Analysis of GHB 143
5.2.4 Materials 144
5.2.5 Methodology 144
5.2.6 Method Validation 145
5.2.7 Results and Discussion 145

5.3 Simultaneous Determination of HMB, BHB and GHB in Plasma and Urine using GC/MS 154
5.3.1 Introduction 154
5.3.2 Analysis of HMB 157
5.3.3 Materials 158
5.3.4 Methodology 159
5.3.5 Method Validation 159
5.3.6 Results and Discussion 159

5.4 Conclusion 169

CHAPTER 6 - FATTY ACID ETHYL ESTERS 171

6.1 Introduction 171

6.2 Materials and Methods 171
6.2.1 Chemicals and Reagents 171
6.2.2 Instrumentation 172
6.2.3 LC-MS-MS Operating Conditions 172
6.2.4 Meconium Samples 173
6.2.5 Preparation of Stock Standard Solutions 174
6.2.6 Preparation of Quality Control (QC) Material 175
6.2.7 Sample Extraction Procedure 176

6.3 Instrument Method Optimisation 177
6.3.1 Optimization of the Fragmentor Voltage and Collision Energy 177

6.4 Method Validation 177
6.4.1 Selectivity 177
6.4.2 Linearity 178
6.4.3 Limit of Detection (LOD) and Limit of Quantification (LOQ) 178
6.4.4 Accuracy and Precision 179
6.4.5 Recovery study 179
6.4.6 Assessment of Matrix Effects 179
6.4.7 Carryover 180

6.5 Results and Discussion 180
6.5.1 Optimization of the Fragmentor Voltage and Collision Energy 180
6.5.2 Specificity 183
6.5.3 Validation Results 185
6.5.4 Recovery and Matrix effect 186
6.5.5 Carryover 188
6.5.6 FAEEs in Meconium 189

6.6 Conclusion 194

CHAPTER 7 - CONCLUSION AND FUTURE WORK 195

7.1 **Conclusion** 195
7.2 **Future work** 197
CHAPTER 8 - REFERENCE LIST 199

List of Tables

Table 1-1: Legal Drink Drive Limit by Country for Ethanol in Whole Blood (19;20) 8
Table 1-2: Summary of Statistics on Prevalence of Foetal Alcohol Exposure by Country 12
Table 1-3: Ratio of Alcohol in Alternative Biological Specimens to BAC*. 21
Table 1-4: Published advantages and disadvantages of Different Internal Standards Used for the Analysis Volatiles by HC-GC-FID 27
Table 1-5: Alcohol and Other Volatile Levels in Blood. 28
Table 1-6: Acute and Chronic Alcohol Biomarkers. 33
Table 1-7: Main Differences Between AKA and DKA.(55;130) 36
Table 1-8: Levels of BHB Measured in post-mortem Blood, Urine and Vitreous Humour from a Range of Case Types. 37
Table 1-9: Summary of Published Methodologies for the Analysis of BHB in Biological Matrices 40
Table 1-10: The Difference of the Techniques Used for the Determination of FAEEs in Biological Samples 45
Table 1-11: Summary of Techniques for the Analysis of FAEEs in meconium. 46
Table 2-1: Preparation of Mixed Working Standard Solutions 55
Table 2-2: Preparation of Calibration Standards 55
Table 2-3: Preparation of QC Material 56
Table 2-4: Summary of GC Conditions Used to Optimise Separation of Volatiles 59
Table 2-5: Optimised GC Parameters used to Evaluate Methods 1 and 2 62
Table 2-6: LOD and LOQ for Validated Methods 63
Table 2-7: Intra-day and Inter-day Precision for Volatiles in Blood 64
Table 2-8: Recovery and Accuracy for Methods 1 and 2 in Blood 65
Table 2-9: Ethanol Concentrations Measured in Post-Mortem Blood by two Methods 66
Table 2-10: Acetone Concentrations Measured in Post-Mortem Blood two Methods 66
Table 2-11: Elevated Post-mortem Blood n-propanol Concentrations. 68
Table 3-1: Preservative and Antioxidant Concentrations by Group 73
Table 3-2: Ethanol Concentrations in Paired Post-Mortem Blood Samples. 80
Table 3-3: Acetone Concentrations in Paired Post-Mortem Blood Samples 81
Table 3-4: ummary of Correlation Data for Ethanol in Blood. 83
Table 3-5: Comparison of Positive and Negative Results for Ethanol in BloodVersus Vitreous Humour 93
Table 3-6: Summary of Mean Ethanol Ratio and Correlation for Blood in Comparison to Vitreous Humour (VH), Bile and Urine. 93
Table 3-7: Comparison of Positive and Negative Results for Ethanol in Blood Versus Bile 94
Table 3-8: Comparison of Positive and Negative Results for Ethanol in Blood Versus Urine 95
Table 3-9: Comparison of Positive and Negative Results of Acetone in Blood versus Vitreous Humour 96
Table 3-10: Comparison of Positive and Negative Results of Acetone in Blood Versus Bile 96
Table 3-11: Summary of Mean Acetone Ratio and correlation for Blood in Comparison to Vitreous Humour (VH), Bile and Urine. 97
Table 3-12: Comparison of Positive and Negative Results for Acetone in Blood Versus Urine 98
Table 3-13: Ethanol Results for HS-GC-FID versus Enzymatic Assay highlighted mismatched cases in yellow 104
Table 3-14: Ethanol in Blood (Enzymatic-v-HS-GC-FID) 105
Table 3-15: Mismatched Ethanol Results (Enzymatic versus HS-GC-FID) 106
Table 4-1: Preparation of BHB Calibration Standards 110
Table 4-2: Preparation of BHB Quality Control Samples 111
Table 4-3: Methods Evaluated for the Determination of BHB in Blood and Urine. 113
Table 4-4: R^2 and Linear Range of BHB for Different Methods 119
Table 4-5: Summary of Blood and Urine Extraction Methods for the Analysis of BHB 120
Table 4-6: BHB Intra day and Interday Precision in Blood and Urine 124
Table 4-7: BHB Recovery and Matrix Effect Data 124
Table 4-8: BHB Stability Data for Spiked Blood Stored at 2-5ºC for 15 Days 125
Table 4-9: BHB Summary of Case Results 126
Table 4-10: BHB Intraday, Interday Precision, Accuracy and Recovery Data 132
Table 4-11: Mortem Cases Classified by Cause of Death and Blood BHB Level 133

Table 4-12: Post-Mortem Cases with Blood BHB Concentrations Between 151-250 mg/L 135
Table 5-1: GHB Published Levels in the Living and Post-Mortem 143
Table 5-2: GHB Intra-day and Inter-day Precision for Blood and Urine 148
Table 5-3: Recovery, Efficiency and Accuracy of GHB 150
Table 5-4: Case Samples with High/Toxic Levels of GHB in Post-Mortem Blood and Urine 150
Table 5-5: Urinary Levels of HMB, BHB and Serum Leucine in Diabetic and Non-Diabetic Patients(206) 157
Table 5-6: Summary of the Published Methods for the Analysis of HMB. 157
Table 5-7: LOD, LOQ and Linearity for HMB, BHB and GHB 164
Table 5-8: HMB, BHB and GHB, Intraday and Interday Precision, Recovery and Accuracy 164
Table 5-9: HMB, GHB and BHB Concentrations Measured in Spiked Plasma and Urine [following (A) Initial tests and (B) after 12 months storage at -20°C]. 165
Table 5-10: HMB, GHB and BHB level in urine and plasma at baseline and after 0-4 hours(urine) and 2hours(plasma) following 3g dose of HMB 169
Table 6-1: Summary of LC and Ion Source Parameters 173
Table 6-2: Summary of FAEEs Stability Study by Moore et al.(153) 174
Table 6-3: Conversion of FAEEs Concentrations from nmol/g to ng/g 176
Table 6-4: FAEES and FAEEs-d5 Optimization of Product Ion and Collision Energy (CE) 182
Table 6-5: MRM (m/z) Transitions for Quantification of FAEEs 182
Table 6-6: Linearity, LOD, and LOQ of FAEEs in Meconium 185
Table 6-7: Intra-day and Inter-day Precision and Accuracy 186
Table 6-8: Recovery and Matrix Effects for FAEEs 187
Table 6-9: Case Samples With Results Exceeding ULOQ for most of FAEEs 190
Table 6-10: The Positive and Negative FAEEs Results and the 95% of Confidence Interval Level 190

List of Figures

Figure 1-1: Alcohol Prohibition Poster. 2
Figure 1-2: Consumption of Pure Alcohol in Litres Per Person (>15 years old) in 2005. 3
Figure 1-3: Regional Variations in Alcohol-Related Mortality in Great Britain. 7
Figure 1-4: William Hogarth 1697-1764 (Gin Lane). 9
Figure 1-5: Factors Affecting Blood Alcohol Concentrations. 15
Figure 1-6: Ethanol Metabolism. 15
Figure 1-7: Gas Chromatogram with Headspace Autosampler (GC-HS) 24
Figure 1-8: Formation of Ketone Bodies 34
Figure 1-9: BHB and BHB-TMS-Derivative 38
Figure 1-10: Electron impact ionization. 39
Figure 1-11: FAEES and D5-FAEES Structures 41
Figure 1-12: Illustration of Triple Quadrupole Mass Spectrometry. 48
Figure 1-13: Electrospray Ionization (ESI) (A) and (B). 49
Figure 2-1: Separation of Volatiles by HS-GC-FID 60
Figure 2-2: Volatile Calibration Curves 63
Figure 2-3: Correlation of Ethanol (A) and Acetone (B) concentrations in post-mortem blood using n-propanol (Method 1) versus t-butanol (Method 2) as an Internal Standard. 67
Figure 3-1: Stability Study Design and Spiked Sample Preparation Guide 74
Figure 3-2: Stability of Volatiles Spiked in Blood at Room Temperature 25 ±2°C) 76
Figure 3-3: Fitted Line Plots of Ethanol concentrations in Paired Blood Samples. 85
Figure 3-4: Fitted Line Plots of Acetone concentrations in Paired Blood Samples. 86
Figure 3-5: Correlation Between Road Traffic [Ethanol] following Long-Term Storage 89
Figure 3-6: Road Traffic Preserved Blood Percentage Recovery of Ethanol 90
Figure 3-7: Road Traffic Preserved Blood - Percentage Recovery of Ethanol following Long-Term Storage at Room Temperature. 90
Figure 3-8: Comparison of Ethanol Concentrations in Paired Blood and Vitreous Humour Samples 93
Figure 3-9: Comparison of Ethanol Concentrations in Paired Blood and Bile Samples 94
Figure 3-10: Comparison of Ethanol Concentrations in Paired Blood and Urine Samples 95
Figure 3-11: Comparison of Acetone Concentrations in Paired Blood and Urine 97
Figure 3-12: Enzymatic Alcohol Reaction 101
Figure 4-1: Chromatogram and Spectra for BHB and GHB-D6 from a Blood Control (50 mg/L) 115
Figure 4-2: Chromatogram and Spectra for BHB and GHB-D6 in Post-Mortem Urine (300 mg/L) 116
Figure 4-3: BHB and GHB-D6 Extracted Ions for Spiked Blood using LLE and derivatization Conditions (1) 117
Figure 4-4: BHB and GHB-D6 Extracted Ions for Spiked Blood using LLE Method and Derivatisation Conditions (3). 118
Figure 4-5: Calibration Curves for BHB in Blood (A) and Urine (B) 123
Figure 4-6: Post-mortem Case Samples with BHB in (A) Blood (343 mg/L) and (B) Urine (>500 mg/L) 128
Figure 4-7: BHB Calibration Curves for Blood (A) and Urine (B) using the Agilent GC-MS. 130
Figure 4-8: BHB Overlaid Chromatograms for Spiked Blood (A) and Spiked Urine (B) at Different Concentrations. 131
Figure 4-9: Prevalence of BHB at Different Concentrations in Post-Mortem Blood 133
Figure 4-10: Percentage of Cases with Varying Blood BHB Concentrations by Cause Death 134
Figure 4-11: Acetone Concentrations in Post-Mortem Blood Relative to BHB Levels 137
Figure 4-12: Ethanol Concentrations in Post-Mortem Blood Relative to BHB Levels 137
Figure 5-1: Chemical Structures of BHB, HMB, GHB and GHB-D_6 139
Figure 5-2: In vivo metabolism of GHB, GABA, GBL and 1,4BD 140
Figure 5-3: Chromatogram and Full-Scan Spectrum of GHB extracted from spiked blood (50 mg/L). 146
Figure 5-4: Chromatogram and Full-Scan Spectrum of GHB extracted from spiked urine (50 mg/L). 147
Figure 5-5: GHB Calibration Curves for Blood and Urine 148
Figure 5-6: GHB Extracted Ion Chromatograms for Blank Blood (A) and Urine (B) 149

Figure 5-7: Chromatograms (A) and (C) and Full-Scan Spectra (B) and (D) for GHB Extracted from Blood and Urine, respectively, from Case # 36 at Levels >500 mg/L. 151
Figure 5-8: GHB Levels in Post-Mortem Blood and Urine in Cases with no History of GHB and/or GBL Misuse. 152
Figure 5-9: Leucine Metabolism and Formation of β-hydroxy-β-methylbutyrate (HMB) 155
Figure 5-10: (A) HMB, (B) BHB, (C) GHB and (D) GHB-D6Chromatograms and Full-Scan Spectra for Unextracted Derivatized Standards (100 mg/L). 160
Figure 5-11: Chromatogram and Full-Scan Spectrum for HMB, BHB and GHB 161
Figure 5-12: Chromatogram and Full-Scan Spectrum for HMB, BHB and GHB 162
Figure 5-13: HMB, GHB and BHB Calibration Curves for Urine (A) and Plasma (B) 163
Figure 5-14: Chromatogram and Full-Scan Spectrum for HMB Extracted from a Plasma Case Sample (37 mg/L) Versus the Spiked Plasma Control at 50 mg/L and Blank Plasma. 166
Figure 5-15: Chromatogram and Full-Scan Spectrum for HMB extracted from a Urine Case Sample (500 mg/L) versus the Spiked Urine Control at 50 mg/L and Blank Urine. 166
Figure 5-16: (A) Chromatogram and (B) Full-Scan Spectrum for BHB 167
Figure 5-17: (A) Chromatogram and (B) Full-Fcan Spectrum of GHB 168
Figure 6-1: Fragmenter Ion Optimization of FAEEs 180
Figure 6-2: Fragmenter Ion Optimization of D5-FAEEs and Ethyl Heptadecanoate 181
Figure 6-3: Collision Energy Optimization of FAEEs and D_5-FAEEs 181
Figure 6-4: Exogenous Specificity Study Spiked Meconium with FAME Mix Standard alone 8000ng/g against Spiked FAEES 8000ng/g plus FAME 8000ng/g Compared with Unextracted FAEES and Unextracted FAME of 10 mg/L. [The arrow indicates no interference]. 183
Figure 6-5: Total Ion Chromatogram (TIC) of Extracted FAEEs 8000 ng/g in Meconium Versus Extracted Blank and FAME standard of 8000 ng/g with Internal Standard. 183
Figure 6-6: Extracted FAAEs With and Without Internal Standard. 184
Figure 6-7: Ion Suppression Experiment. Figure (A) Overlaid Chromatograms 187
Figure 6-8: Carryover study – Total Ion Chromatogram of Highest Calibrator 188
Figure 6-9: Carryover Study – (MRM) Chromatogram of Highest Calibrator 188
Figure 6-10: Comparison of Positive and Negative Findings for Individual FAEEs in Meconium 189
Figure 6-11: Cumulative concentrations of the FAEES in meconium 191
Figure 6-12: Cumulative Concentration of all FAEE Results (500–10,000ng/g) 192
Figure 6-13: Overlaid MRM chromatogram of High-Result FAEE Case samples 192
Figure 6-14: MRM Chromatogram of Negative FAEES Case Samples 193

List of Appendices

Appendix 1-1 Summary of published techniques for the Determination of Fatty acid ethyl esters (FAEEs) in biological matrices. 219
Appendix 2-1 Summary of Published Techniques for Volatile analysis 222
Appendix 3-1 Stability Study of volatiles using t-butanol as an Internal Standard 225
Appendix 3-2 Stability Study of volatile using n-propanol Internal standard 231
Appendix 4-1 BHB QC Charts 233
Appendix 4-2 BHB, Acetone and Ethanol Concentrations in Post-mortem blood and urine 234
Appendix 5-1 Summary table of techniques used for detection of GHB and related compounds 238
Appendix 5-2 GHB concentrations in blood and urine postmortem case samples 244
Appendix 5-3 GHB QC Charts 249
Appendix 5-4 HMB/BHB and GHB QC Charts 250
Appendix 6-1 Preparation and optimization of in house deuterated internal standard 251
Appendix 6-2 Endogenous interference Study 256
Appendix 6-3 FAEEs results in meconium 258
Appendix 7 Papers and Awards in Support of this thesis 261

Author's declaration

"I declare that, except where explicit reference is made to the contribution of others, that this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution"

Signature _______________________________

Printed name ____________________________

Abstract

Ethanol is a poison that adversely affects the health of individuals and is detrimental to society as a whole. Analysis of ethanol in biological matrices is the most frequent test carried out in forensic toxicology laboratories with application across a range of cases types including fatalities, road traffic accidents, criminal investigations and workplace drug testing.

The interpretation of ethanol concentrations in post mortem samples can be challenging in relation to medico-legal investigations. The source of ethanol can be as a result of the ingestion of an alcoholic beverage or it may have been formed after death. The stability of alcohol is also adversely affected by the presence of bacteria, high temperatures and unsuitable storage containers.

A robust and sensitive method was developed for the analysis of common volatiles such as ethanol, methanol, isopropanol, acetone and n-propanol using headspace gas chromatography coupled with a flame ionization detector (HS-GC-FID). The method was validated in accordance with ISO/IEC 17025, and was used to investigate the stability of volatiles in blood when stored under different conditions, and to investigate the prevalence of volatiles in different biological matrices collected post-mortem.

Storage of blood samples in the freezer within sample vials containing preservative and antioxidant improved the stability of all volatiles with the exception of methanol which remained stable under all conditions investigated. The identification of ethanol or acetone in vitreous humour was found to be a suitable alternative matrix in cases where femoral blood was unavailable or ethanol production was suspected. The concentration of ethanol in bile correlated well with femoral blood but to a lesser extent than vitreous humour. Urine was not a suitable matrix for estimating blood ethanol concentrations.

Alcohol biomarkers, β-Hydroxybutyrate (BHB) and fatty acid ethyl esters (FAEEs) have been reported as useful markers for, investigating the role of alcoholic ketoacidosis (AKA) in post-mortem cases, and foetal exposure to chronic maternal alcohol consumption, respectively. Methods were developed and

validated in accordance with ISO/IEC 17025 for BHB in blood and urine using gas chromatography - mass spectrometry (GC/MS), and for FAEEs in meconium by liquid chromatography - tandem mass spectrometry (LC/MS/MS).

Post-mortem case samples submitted to the Forensic Medicine and Science (FMS) toxicology laboratory for routine tests were analysed for BHB using the validated method. The findings highlighted the importance of measuring BHB in blood in all cases where the deceased has a history of alcohol misuse and where the cause of death cannot be determined following the post-mortem. The role of alcoholic ketoacidosis in the cases analysed in this study was significantly under-reported.

Meconium samples collected from infants born at the Princess Royal Maternity Hospital, Glasgow, were analysed for FAEEs, using the validated method to investigate the prevalence of each FAEE (ethyl laurate, ethyl myristate, ethyl palmitate, ethyl stearate, ethyl oleate, ethyl linoleate, ethyl linolenate, and ethyl arachidonate). The study found evidence of chronic maternal alcohol consumption in approximately one third of the cases tested, in contrast to self-reported use and highlights the importance of screening for the presence of FAEEs in meconium. The identification of suitable biomarkers of excessive maternal alcohol consumption during pregnancy, carried out as part of screening program, in addition to clinical evaluation would help to diagnose and support newborns with Foetal Alcohol Spectrum Disorder (FASD).

The method developed for the analysis of BHB in blood and urine was successfully adapted and validated for analysis of structurally similar drugs such as β-hydroxy-β-methylbutyrate (HMB), a legal dietary supplement, in plasma and urine collected from 8 subjects, pre- and post-administration of a 3g dose of HMB. A significant increase was observed in urine and plasma following administration of of HMB. The method was then applied to the analysis of post-mortem blood and urine to investigate the concentrations of exogenous and endogenous levels of γ-hydroxybutyrate (GHB).

List of Abbreviations

ABV	Alcohol by volume
ACN	Acetonitrile
AKA	Alcoholic ketoacidosis
ADH	Alcohol dehydrogenase
ALDH	Acetaldehyde dehydrogenase
ALT	Alanine aminotransferase
AMU (amu)	Atomic Mass Unit
ARBD	Alcohol-related birth defects
ARND	Alcohol-related neuro-developmental disorders
AST	Aspatate aminotransferase
BAC	Blood alcohol concentration
1,4BD	1,4-Butanediol
BHB	Beta-hydroxybutyrate
BSTFA + 1% TMCS	N,O-Bis (Trimethylsilyl) trifluoroacetamide + 1% trimethylchlorosilane
CDT	Carbohydrate deficient transferrin
CV%	Coeffecient of Variation percentage
CI	Confidence interval
R^2	Correlation Coefficient
CSF	Cerebral spinal fluid
D_5-FAEE	Deuterated fatty acid ethyl esters
DKA	Diabetic ketoacidosis
EI	Electron impact (or ionisation)
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionisation
EtOH	Ethanol
FAEE	Fatty acid ethyl esters
FAME	Fatty acid methyl esters
FAS	Foetal alcohol syndrome
FASD	Foetal alcohol spectrum disorder
FB	Femoral blood
FBM	Femur bone marrow
FID	Flame ionization detector (or detection)
FMS	Forensic Medicine and Science
FS	Full scan
EtG	Ethyl glucuronide
GABA	Gamma-Aminobutyric acid
GC	Gas chromatography
GC/MS	Gas chromatography-mass spectrometry
GBL	γ-Butyrolactone
GGT	γ-Glutamyl transferase
GHB	γ-hydroxybutyrate
GTOL	Glucuronidated 5-hydroxytryptophol
HMB	β-Hydroxy-beta-methylbutyrate
HPLC	High performance liquid chromatography
HS	Headspace
5-HIAA	5-hydroxyindole-3-acetic acid
5-HTOL	5-hydroxytryptophol

IEC	The International Electrochemical Commission
ISO	International Organisation for Standardisation
ISTD	Internal standard
L	Liter
LC	Liquid chromatography
LC/MS	Liquid chromatography-mass spectrometry
LC/MS/MS	Liquid chromatography-tandem mass spectrometry
LLE	Liquid liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LLOQ	Lower limit of quantification
MeOH	Methanol
mg	Milligrams
mL	Millilitre
m/z	Mass to charge ratio
NaF	Sodium fluoride
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide reduced form
NICE	National Institute for Health and Clinical Excellence
P	Preserved
PEth	Phosphatidylethanol
PFAS	Partial foetal alcohol spectrum
PP	Protein precipitation
QA	Quality assurance
QC	Quality control
RTA	Road traffic accident
SIM	Selective ion monitoring
SPE	Solid Phase extraction
SPME	Solid phase micro-extraction
SSALD	Succinic semialdehyde
SSA-Dehydrogenase	Succinic semialdehyde dehydrogenase
SSA-Reductase	Succinic semialdehyde reductase
St Dev.	Standard Deviation
SE	Standard Error of mean
TIC	Total ion chromatogram
UAC	Urine alcohol concentration
ULOQ	Upper limit of quantification
UP	Unpreserved
VAC	Viterous humour alcohol concentration
VH	Vitreous humour
v/v	Volume to volume
WHO	World Health Organisation
wt/v	Weight to volume
wt/wt	Weight to weight

Chapter 1 - Introduction

1.1 History of Alcohol Use

The exact date of the first use of ethanol (commonly known as alcohol) as a beverage is not known, but it is thought to have occurred as early as 10,000 BC.(1) Archaeologists have found evidence that barley had been used to make beer for centuries. The Egyptians made beer from corn, while in China an alcoholic drink “samshu” appeared around 7000 B.C. along with “sake” in Japan which is made from rice. Wine and beer became the daily thirst quenchers for many years until water suitable for human consumption became more widely available in the 19th century.(1)

Religious references to alcohol are both positive and negative. In the Bible an abundance of wine is used as an example of God's blessing, while a lack of wine is used as an example of God's punishment. Negative references include warnings against abusing alcohol and advocating abstinence if drinking will cause “a brother to stumble”. (2) In Islam, alcohol consumption is completely forbidden as it is believed that the harm associated with alcohol far outweighs its benefits. For the benefit and betterment of man, Islamic religion criminalizes the consumption, production, transportation, and sale of alcoholic beverages, although the strictness by which this prohibition was, and is, enforced varies between Islamic countries.(3;4)

The first half of the 20th century saw a period of alcohol prohibition in several countries, including some provinces in Canada (1907 -1948), the United States (1920 to 1933), and Russia and the Soviet Union (1914-1925).(4) After several years, prohibition was deemed a failure in North America and other countries as bootlegging (rum-running) became widespread and organized crime took control of the distribution of alcohol. Prohibition generally came to an end in the late 1920s or early 1930s.(4) Figure 1-1 is an example of the posters displayed at the time.

Figure 1-1: Alcohol Prohibition Poster.
" Reproduced with permission from druglibrary.org"(5)

In the United Kingdom, the sale or consumption of commercial alcohol has never been prohibited by law and is freely available to purchase in licensed premises if you are 18 years of age or older. However, for over 100 years the village of Bournville near Birmingham has not sold alcohol in any of their pubs, bars, or shops. Residents fought to maintain their alcohol-free zone, winning a court battle in March 2007 with Tesco (the largest supermarket chain in Britain) to prevent them from selling alcohol in its local outlet.(4)

1.1.1 Alcohol Standard Measure

Alcohol units are a measure of the volume of pure alcohol in alcoholic beverages. The standard drink is used in many countries to quantify alcohol intake. The standard drink contains one unit of alcohol and varies from one country to another according to how each country defines the unit of alcohol. The terms used for alcoholic drink measures include, units, alcohol by volume (%ABV), proof, pints, litres(L) and millilitres(mL).(6) One unit of alcohol in the United Kingdom is defined as 10 mL (8.0 grams of pure alcohol). This compares with 10 grams (12.7 mL) in Australia, while in the USA 1 unit of alcohol is equal to 17.7 mL and 1 pint equates to 473.176473 mL.(7)

The number of units of alcohol in a drink can be calculated by multiplying the volume of the drink in milliliters by the percentage alcohol content and then dividing by 1000. The alcohol content is expressed on the label as (%ABV).(7)

1.2 Prevalence of Alcohol Use

In 2005 (Figure 1-2), the total recorded worldwide consumption of alcohol by persons aged 15 years or older was 6.13 litres of pure alcohol per person. The highest consumption levels can be found in the developed world whilst the lowest consumption levels can be found in the countries of North Africa and sub-Saharan Africa, the Eastern Mediterranean region, and southern Asia and the Indian Ocean. These regions represent large populations of the Islamic faith, which have high rates of abstention.(8;9)

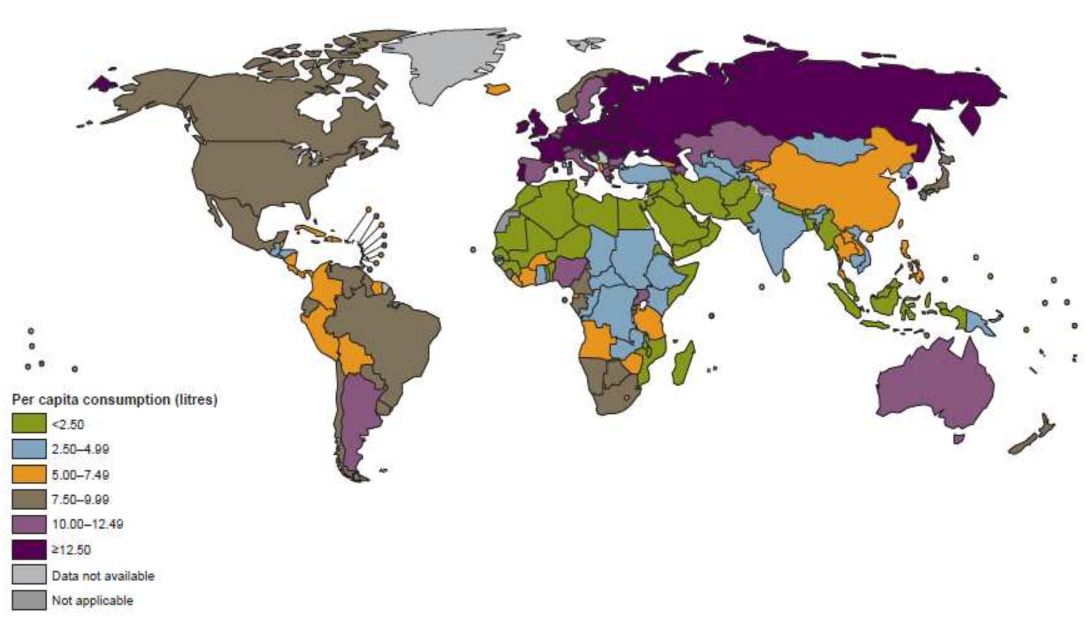


Figure 1-2: Consumption of Pure Alcohol in Litres Per Person (>15 years old) in 2005. " No permission is required if used for research private study or with noncommercial document with limited circulation such as an academic thesis or dissertation.(8)"

Scotland, a small northern European country with a population of approximately 5 million people has the eighth highest rate of ethanol consumption in the world. Estimated at 11.9 litres of pure alcohol per person based on sales figures, alcohol consumption in Scotland is significantly higher than that of England and Wales by 2.4 litres (9.6 litres).(10) In 2009, the population of men in Scotland

exceeding “sensible” drinking limits was more than half (53%) of the 25-34 age group, and 44% for women in the same age group. (10)

The UK Governments guidelines for sensible drinking recommends maximum daily drinking limits for men that should not exceed more than 3 - 4 alcohol units per day and for women a maximum of 2 - 3 units of alcohol per day. It is also advised that people should have at least two alcohol free days each week and as such weekly limits are set at no more than 21 units for men and 14 units for women.(11)

Although there is no official internationally agreed definition of “binge drinking” the Scottish Health Survey 2009(11) used the same definitions that were used in the Health Survey for England and the General Lifestyle Survey(12;13) to allow for comparability with other major alcohol consumption surveys in Britain. Binge drinking was defined as daily alcohol intake of more than 8 units for men and more than 6 units for women.(10;11) The prevalence of “binge drinking” was reported for 2009 with 26% of men and 17% of women drinking more than twice the recommended daily limit.

1.3 Alcohol-Related Harm

The World Health Organisation (WHO) objectives for public health policies and initiatives are to reduce the hazards caused by the harmful use of alcohol.(8;9) Alcohol is identified by the WHO as the third highest risk factor for ill health in developed countries, behind only tobacco and high blood pressure.(9)

Many health medical onditions are linked to alcohol. These conditions may be acute or chronic diseases or injuries. Two types of alcohol-related conditions were identified in the Scottish report of alcohol statistics for 2011.(10) The first were those wholly attributable to alcohol misuse, such as alcoholic liver disease, mental and behavioural disorders, alcoholic polyneuropathy, myopathy, cardiomyopathy, gastritis, degeneration of the nervous system, intentional and accidental poisoning by alcohol, toxic effects of alcohol, foetal alcohol syndrome and alcohol-induced chronic pancreatitis.

The second group are those partly attributable to alcohol, such as cancers of the lip, oral cavity and pharynx, oesophagus, liver, larynx, colorectum and breast, and injuries resulting from road traffic accidents, assaults, fire injuries, falls, and intentional self-harm. Other medical issues include coronary heart disease, stroke, hypertensive diseases and cardiac arrhythmias, gastrointestinal diseases including liver disease, oesophageal varices, chronic pancreatitis, psoriasis, spontaneous abortion, and epilepsy.(10)

Alcohol misuse is also associated with several infectious diseases including Human Immunodeficiency Virus (HIV)/Acquired Immunodeficiency Syndrome (AIDS), tuberculosis, and sexually transmitted infections. Alcohol consumption weakens the immune system and has a negative effect on patients' adherence to antiretroviral treatment.(9)

To investigate the extent of harm associated with alcohol use in Scotland, adults were classified into four different drinking categories based on the number of units consumed in one week.(10) These categories included "non-drinkers", "moderate drinkers", "hazardous drinkers" and "harmful drinkers".

The study concluded that while those classified as hazardous drinkers (men who consume between 21 – 50 alcohol units weekly and women between 14-35 units) may not have serious affects in the short-term, their excessive drinking could cause problems in the long-term.(10;11) The harmful drinking category included men who consumed over 50 units of alcohol weekly and women over 35 units, and were associated with causing physical, social, or psychological harm to themselves.

1.3.1 Alcohol-Rehlated Social Harm

Ninety-seven per cent of respondents to the Scottish Crime and Justice Survey considered alcohol abuse in Scotland to be a problem, with almost three quarters (74%) perceiving it as a "big problem". "Vomiting" and "having an argument" were the most common negative effects of alcohol to reported by children.(10) It is estimated that 65,000 children live with a parent with an alcohol problem. (14)

In 3 out of 5 incidents of violent crime (62%), victims believed their attacker to be under the influence of alcohol, while in almost a third of incidents of violent crime, victims were under the influence of alcohol themselves.(10)

1.3.2 Alcohol-Related Deaths

Alcohol-related deaths include acute intoxication, chronic alcoholism, trauma, hypothermia, accidents, suicides, and homicides.(6;14-16) Alcohol-related death rates in Scotland are twice as high as in England and Wales, and have doubled over the last 15 years.(14) One Scot dies of an alcohol-attributable cause every three hours.(14) and deaths related to alcohol in some areas in Scotland are 6 times higher than the UK average.(17) The alcohol-related mortality rate for males was more than twice that for females (30.0 per 100,000 population compared to 14.4 per 100,000 population, respectively).(10)

The dark purple colour in Figure 1-3 represents the highest mortality rate from alcohol misuse, corresponding to northern Scotland and the central belt cities of Glasgow, Dundee, and Edinburgh. This data does not include many other causes of death to which alcohol may have contributed, such as deaths due to cancer and liver disease.(18)

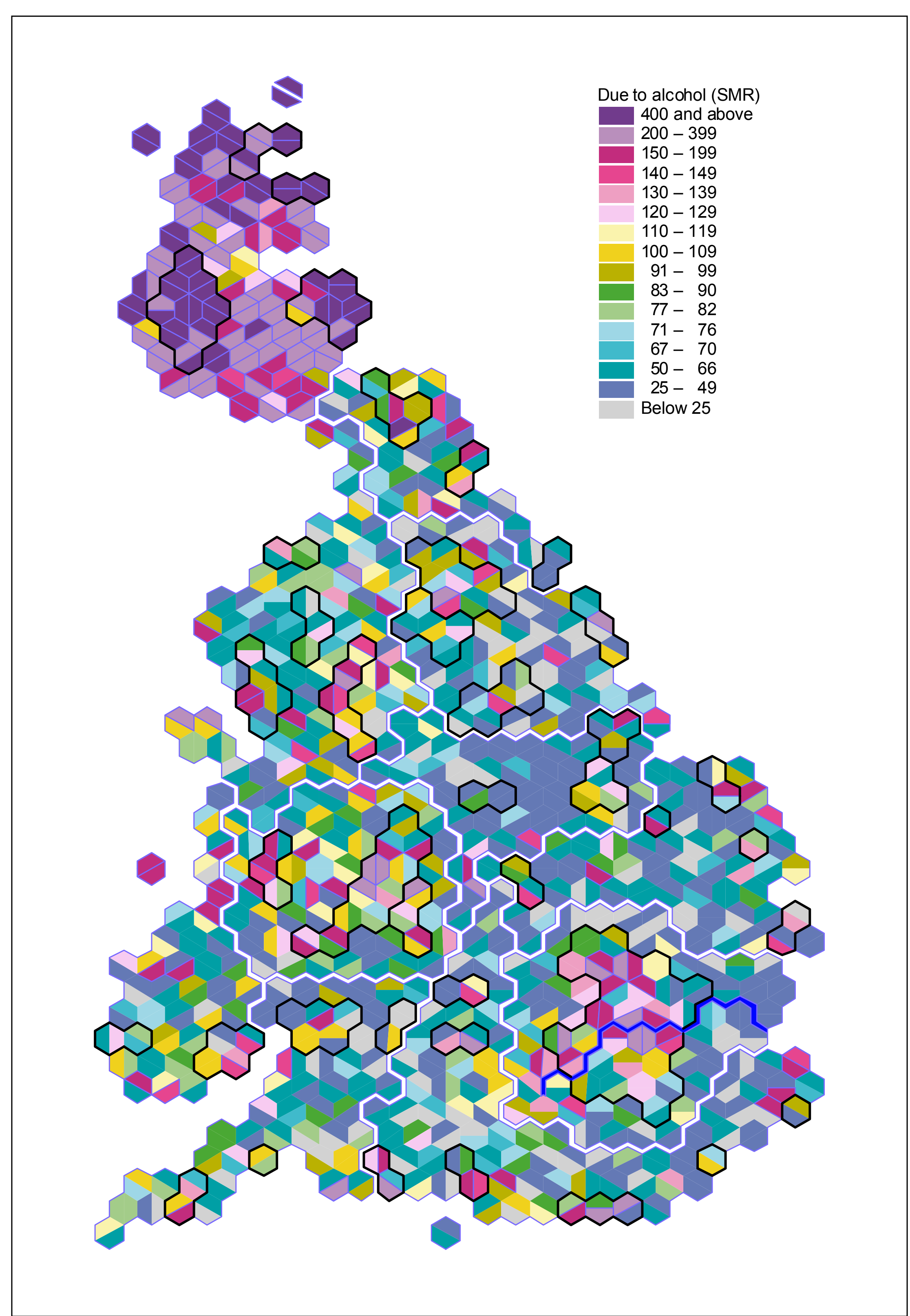


Figure 1-3: Regional Variations in Alcohol-Related Mortality in Great Britain. "Reproduced with permission from department of Geography University of Sheffield (18)"

1.3.3 Road Traffic Incidents Involving Alcohol

The offence of being drunk and incapable of some tasks, such as operating machinery, was first recognised in the 18th and 19th centuries.(6) In 1872, penalties were imposed for drunkenness of persons who were in charge of the safety of others, such as engine drivers, ship masters, taxi drivers, and service personnel.(6) The prescribed limits for alcohol in the current UK legislation (Road Traffic Act 1988)(6) are 80 mg/100 mL in blood, 107 mg/100 mL in urine and 35 µg/100 mL in breath. The ability of a driver to judge speed and distance while dealing with the unexpected is impaired when under the influence of alcohol with the risk of being involved in an vehicle accident increasing sharply if the blood alcohol concentration (BAC) of the driver is above the legal limit.(6) The transport legal limits for alcohol in blood vary considerably in different countries and two alcohol blood legal limits were reported for some countries such as india, the reason is not clearly stated but the highest limit is the one used as summarised in Table 1-1.

Table 1-1: Legal Drink Drive Limit by Country for Ethanol in Whole Blood (19;20)

Country	Legal limit (Blood)	Legal Limit (Blood) mg%
United States	0.08 g/100 mL	80
Great Britain	80 mg/100 mL	80
Netherlands	0.50 mg/mL	50
Sweden	0.20 mg/g	20
Norway	0.20 mg/g	20
Australia	0.05 %	50
Canada	0.08 %	80
Japan	0.03 %	30
India	0.03/0.015 %	30/15
Saudi Arabia	0.00 %	0

In Scotland, there were 8,504 offences of drunk driving in 2009-10(10) which was a decrease of 13% from 9,800 drunk driving offences in 2008-09. A total of

18,536 motorists were known to be involved in injury-producing road accidents in 2009. This figure does not include drivers involved with hit and run accidents who were not traced. Out Of the 18,536 motorists, 10,924 (59%) were requested to take a breath test. Of these only 429 (3.9%) cases gave a positive breath test including motorists who refused to take the test which equates to 2.3% of the total number of tests carried out (18,536).(10) In 2008 it was estimated that 40 people were killed in accidents involving motor vehicle drivers or riders with alcohol levels, above the current drink-drive limit of 80 mg of alcohol per 100mL of blood.(10)

1.3.4 Foetal Alcohol Spectrum Disorders (FASD)

People have the misguided belief that the effects of ethanol on the foetus have only recently been recognised. However, the link between ethanol and birth defects are noted throughout history. The Ancient Greek Philosopher, Aristotles (384-322 B.C.) noticed that the children of drunken women were often “morose and languid”(21) and in 1500 A.D., St. Francis warned women against consuming wine or strong drink during pregnancy to prevent the child from becoming “lunatic” (one who had imperfect memory).(21)

Figure 1-4: William Hogarth 1697-1764 (Gin Lane).
"This work has been identified as being free of known restrictions under copyright law"(22)

In 18th century Britain, the epidemic consumption of gin made people believe that it could lead to birth defects. When the price of gin decreased, the infant mortality rate increased and in 1834, the British House of Commons recognised the impact of ethanol and reported that “children from alcoholic mothers have a starved, shrivelled and imperfect look”. In 1860, a French physician noted that children exposed to ethanol have nervousness, a small head, and strange facial features.(21)

Babies born to mothers who are suspected of misusing alcohol during pregnancy are at an elevated risk of developing foetal alcohol spectrum disorders (FASD) and Foetal Alcohol Syndrome (FAS).(23;24) Foetal exposure to alcohol can cause central nervous system dysfunction, pre- and post-natal growth problems, cardiac defects in neonates, attention deficit disorders and may also lead to mental retardation. Ethanol crosses the placenta and interferes with the brain and other organ development and can cause pre-term delivery, spontaneous abortion and foetal death. The effects associated with foetal alcohol exposure are permanent, irreversible and impact both the child and family for life. The greatest incidence of infant cognitive deficiency occurred in pregnant women who engaged in binge drinking.(23;25)

FASD is a non–diagnostic umbrella term identifying a range of disorders as a result of gestational alcohol exposure, including Foetal Alcohol Syndrome (FAS), Partial Foetal Alcohol Syndrome (PFAS), Alcohol-Related Birth Defects (ARBD) and Alcohol-Related Neurodevelopmental Disorders (ARND).(23;26) The severity associated with these disorders is thought to depend on the amount of alcohol consumed during pregnancy and at what time point during the pregnancy maternal consumption of ethanol occurred.

FAS is the most clinically recognized of the FASD conditions, exhibiting the full presentation associated with a pattern of anomalies including facial dysmorphism, permanent impairment of brain function due to damage to the central nervous system that may also lead to development disabilities, attention deficit disorder, hyperactivity, learning disabilities, receptive language deficits and pre and post growth deficiency.(23;26) The term partial FAS (PFAS) applies when there is a confirmed history of prenatal exposure and some characteristics

of the full syndrome but not enough to establish the diagnosis of FAS. The terms ARBD and ARND are two terms used when a complex pattern of behavioural and cognitive abnormalities are noted that could not be otherwise explained and when there is known significant maternal alcohol use.(26)

To facilitate early diagnosis and treatment and to prevent undesirable outcomes, clinical diagnosis and screening for alcohol biomarkers may confirm maternal alcohol consumption. Relying only on the mothers self-report is not an accurate means of confirming alcohol exposure, due to feelings of guilt, embarrassment, and fear of punitive action.(27;28)

Specific assessment techniques used to make definitive clinical diagnosis of FASD are still a matter of debate.(29) In order to prevent FASD it requires detection of in pregnancy maternal risk drinking. Surveys conducted in the UK have reported an increase in the percentage of women who gave up drinking during pregnancy, from 24% in 1995 to 34% in 2009.(16) However, in 2009, 61% continued to consume alcohol albeit to a lesser extent, while 4% admitted no change in their drinking pattern. In contrast, 11.6% of pregnant women aged 15-44 in the United States admitted to using alcohol in the month prior to the survey, while 16% of pregnant women aged 15-17 consumed approximately 24 drinks in the past month, with an average of 4 drinks on the day that they drank.(30;31)

The prevalence of FAS and FASD is underreported in the UK due to lack of reliable data collection methods and consistency. The likely reasons could be the complexity in diagnosing FASD disorders, the lack of available standardised methods to diagnose FASD and the associated costs. FAS is now widely known and accepted as a clearly diagnosed disorder, however, it is believed that the prevalence of FASD is three times greater than FAS.(31)

It has been reported that in Scotland there are as many as 300 babies a year who have been affected by maternal alcohol consumption during pregnancy, however the incidence of FAS in Scotland is based on estimates from other European countries and USA statistics.(25;32) Considering the difficulty of diagnosing FASD Than FAS, a global research suggests that the prevalence of FASD worldwide is 1 in 100 live births.(33) Using this to estimate FASD in Scotland would correspond

to a cumulative total of approximately 8000 children (infants to 16 years old). This figure could be generated if the total population of children (infants to 16 years old) in Scotland was around 800,000. Relying on the figures from the USA statistics, there are approximately 7 cases per 10,000 births according to the birth monitoring program in USA and when this is translated to Scotland, the cumulative figure is expected to be 560 children (infants to 16 years old) based on a population of 800,000.(32) Table 1-2 summarises the statistics on the prevalence of FAS and FASD by country.

Table 1-2: Summary of Statistics on Prevalence of Foetal Alcohol Exposure by Country

Country	Prevalence of FASD and FAS	References
USA	FAS - 0.3–5.6 per 1000 live births FAS - estimated 0.5-2.0 per 1000 live births FAS - 2-7 per 1000 live births	(25) (33) (34)
CANADA	FASD - 1 in every 100 live births	(35)
ITALY	FAS - 3.7-7.4 per 1000 children FASD - 20.3–40.5 per 1000 children	(36)
	FAS - 4.0-12.0 per 1000 children PFAS - 18.1-46.3 per 1000 children FASD - 2.3-6.3% of children	(37)
AUSTRALIA	FASD - 4.7 per 1000 live births	(23)
SOUTH AFRICA	FASD - 68.0–89.2 per 1000 children	(38)
ENGLAND	FAS - 95 in 2000–01, 90 in 2001–02 and 128 in 2002–03	(23)
SCOTLAND	FAS - 4 in 2000, 5 in 2001, 4 in 2002, 2 in 2003	(23)

In the USA, the recommendation from the Surgeon General is for pregnant women not to drink any alcohol during pregnancy to avoid the negative impacts on the health of both mother and foetus and to eliminate the chance of giving birth to a baby with FASD.(39) Fifteen states consider substance abuse during pregnancy to be child abuse under civil child-abuse.(40) Canada promotes alcohol-free pregnancy but has no law against consuming alcohol while pregnant.(41)

In the UK, there have been no universal strategies focusing specifically on preventing FASD.(23) The Department of Health and the National Institute for Health and Clinical Excellence (NICE) published guidance warning pregnant women or women trying to conceive to avoid drinking alcohol to protect the baby. If they choose to drink they should not drink more than 1-2 units of alcohol once or twice a week.(42);(43)

Several governments have introduced a label or logo as a health warning for pregnant women to be applied to all alcoholic drinks including different types of containers.(21) In the UK, voluntary labelling is preferred. For ethanol drink containers exported outside Britain labelling is sent out with necessary warnings as per the requirement of the importing country. However the same ethanol drink containers do not have the warning label when sold in Britain.(21)

1.3.5 Alcohol-Related Harm Reduction Plan in Scotland

In response to the growing scale of the problem associated with ethanol misuse, the Scottish Government published a new strategy for reducing alcohol related harm setting out proposed changes to legislation to build on those already in place, specifically the Licensing (Scotland) Act 2005 introduced in September 2009.(44;45) The Alcohol etc. (Scotland) Act 2010 was passed by the Scottish Parliament on the 10th November 2010 and bans quantity discounts on alcohol purchases, restricts alcohol-related promotions, introduces a Challenge 25 age verification scheme for licenced premises and aims to introduce social responsibility for those who profit from the sale of alcohol. The original Bill had also included minimum pricing for alcohol but this was not supported by all parties represented within the Scottish coalition government.(45)

1.4 Interpretative Challenges Associated with Medico-legal Investigations Involving Ethanol

Due to the prevalence of ethanol use and misuse across all areas of society, a wide range of case types and samples are submitted to forensic toxicology laboratories for ethanol analysis. Modern laboratories must develop methods which are suitably robust, sensitive and specific in accordance with national and international standards (e.g. ISO/IEC 17025) but must also consider additional factors for better interpretation of alcohol results in medico-legal cases. These include; the circumstances surrounding the death, whether there is a history of alcohol abuse, the type of sample and site of collection, whether the sample has been collected in an appropriate sample container with preservative and stored at the correct temperature. In addition, depending on the type of case and sample availability, the identification of specific ethanol biomarkers could provide significant insight into the role of ethanol.

It is therefore important for forensic toxicologists to understand in addition to alcohol distribution and elimination from the body, other factors affecting the stability of alcohol, alcohol biomarkers and related volatiles to aid interpretation of the measured concentration.

1.5 Pharmacokinetics of Ethanol

Ethanol can be absorbed into the body in different ways; through the skin, inhalation, intravenously or orally. The oral route is the most common with 80% of ethanol absorbed from the small intestine and 20% from the stomach into the blood by simple diffusion.(46) Ethanol enters the blood through the portal vein to the liver, and then travels to the right side of the heart and on to the lungs where gas exchange can occur. Ethanol dissolves in the blood and distributes into other tissues or fluids according to their water content, the greater the water content, the greater the ethanol concentration. Tissue with high water content will have more alcohol than tissues rich with fat.(46)

It is commonly assumed that women have less body water than men due to smaller body mass and a higher proportion of body fat. However, women achieve a higher blood alcohol concentration (BAC) than men after drinking the same

amount of alcohol. Equilibrium between the tissues and blood is achieved within 1-2 hours.(6) Absorption is most rapid when the stomach is empty. There is also considerable intra and inter individual variation in the absorption rate.(46-48) Figure 1-5 summarises the many factors affecting the BAC in the body after drinking.

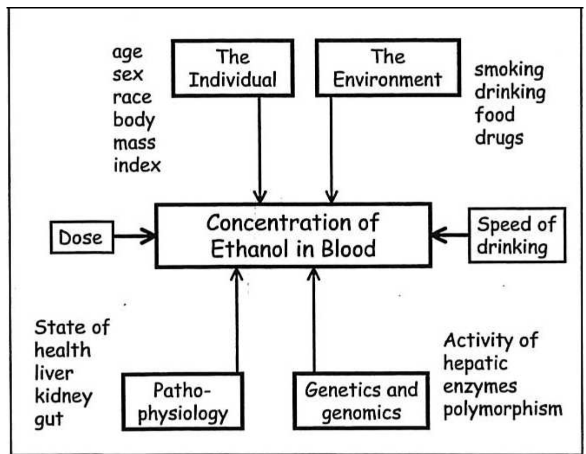


Figure 1-5: Factors Affecting Blood Alcohol Concentrations. "Reproduced with permission from Dr. Jones A. Wayne".(47)

The metabolism of ethanol as summarised in Figure 1-6, is predominantly via oxidation to acetaldehyde (CH_3CHO) by alcohol dehydrogenase (ADH) and then by aldehyde dehydrogenase (ALDH) to acetic acid (CH_3COOH). Approximately 5% is excreted unchanged in breath, urine, and sweat. Less than 0.1% undergoes non-oxidative metabolism in the liver in a phase II conjugation reaction to produce a water soluble metabolite ethyl-glucuronide, which is then excreted in urine.

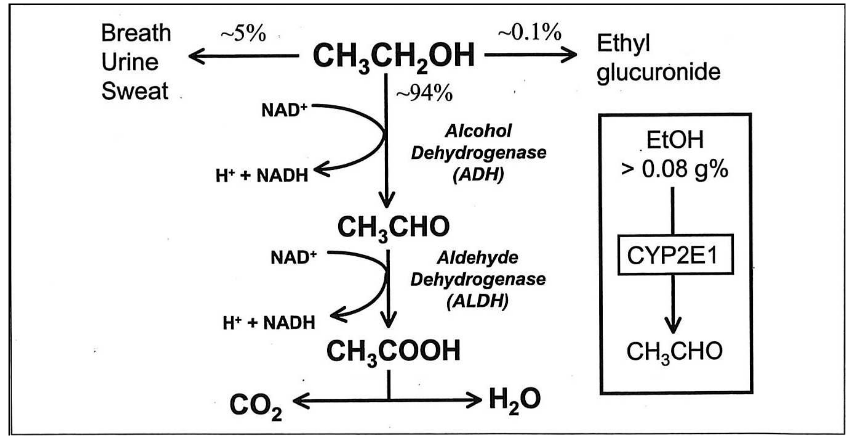


Figure 1-6: Ethanol Metabolism. "Reproduced with permission from Dr. Jones A. Wayne".(47)

The elimination of ethanol from the body occurs mainly by oxidative metabolism by a Class I isoenzyme of ADH in the liver. Ethanol is an example of a drug in which the Michaelis-Menten kinetic enzymatic model applies.(49)

ADH becomes saturated with substrate after the first few drinks and the rate of disappearance of ethanol therefore follows zero-order kinetics which is adequate to describe the decreasing phase of the BAC profile in most forensic situations (BAC >20 mg/dL).(49) When the BAC decreases below 10 mg/dL, the ADH is no longer saturated and the rate of disappearance changes to first–order kinetics in which only a fraction of ethanol is eliminated per unit time. Low BAC's are however not relevant in most forensic situations.(50)

The elimination rate on an empty stomach was reported to be lower than in non-fasting subjects. The presence of the food in the stomach boosts the rate of ethanol metabolism and the profile of BAC returns to zero earlier than with an empty stomach when the same dose of ethanol was consumed. It is spectaculted that ethanol binds with some type of amino acid such as glycine.(49)

Food in the stomach is an effective way not only to lower and delay the BAC peak but also to increase the rate of clearance of ethanol from the body. Food increases the blood flow in the liver, making the liver more efficient and facilitating exposure of ethanol to metabolizing enzymes in the liver.(50)

According to a survey on ethanol elimination rates carried out by Jones in 2010.(49) The survey was based on a large number of peer reviewed publications that met defined standards of experimental design, dose of alcohol given and blood sampling protocol. It was concluded that the physiological ethanol elimination rates range from 10-35 mg/100 mL/h. In moderate drinkers the rate is 15 mg/100 mL/h, while in arrested drivers 19 mg/100 mL/h, many of whom were binge drinkers or alcoholics. The results of this survey could be used as a guide when questions arise about elimination rates in alcohol-related crimes. Ultra-rapid elimination rates accounting for rapid metabolism could be related to the induction of the CYP2E1 enzyme pathway of ethanol oxidation as a consequence of heavy drinking, however, the dose and duration of drinking required to boost the CYP2E1 enzymes have not yet been established.(51)

Detoxification with high concentrations of ethanol and genetic predisposition of hypermetabolic rate could be another reasons for ultra-rapid ethanol elimination.(52)

1.6 Other Volatiles of Interest

Volatile compounds other than ethanol can be found in blood in both living subjects (acetone, isopropanol, n-propanol, isobutanol, butanone and ethyl esters (ethyl acetate), and at post-mortem (acetaldehyde, acetone, isopropanol, n-propanol, butanone, isobutanol, iso-amyl alcohol, d-amyl alcohol, acetate, propionate, butyrate, isobutyrate, and ethyl esters such as ethyl acetate and glycerol).(53)

The significance of the presence of these volatile compounds is particularly important as the presence of volatile compounds not found in living subjects could be good markers for post-mortem putrefaction or external contamination of the body.(53)

In many cases the presence of these volatiles is a result of microbial production postmortem but others are as a result of antemortem alcohol consumption, while the presence of acetone may be endogenous in individuals who are diabetic or fasting.(53) In addition, acetone concentrations can increase due to intoxication from barbiturates or alcohol, hypothermia, metabolic ketoacidosis and isopropanol intoxication. Isopropanol is rapidly oxidized to acetone, which can be measured for several days afterward.(54) Acetone is one of the ketone bodies that can be considered as a the potential markers of alcoholic ketoacidosis (AKA) in addition to β-hydroxybutyrate (BHB) and acetoacetate.(55)

Isopropanol or rubbing alcohol is commonly used as an antiseptic and disinfectant. Although it is less toxic than methanol and ethylene glycol, it produces greater central nervous system depression than ethanol.(54)

n-Propanol is produced in large quantities post mortem and may continue increasing in concentration even when ethanol has reached its peak. It has been reported that an n-propanol concentration greater than 10% of ethanol in muscle

and greater than 5% in blood confirms putrefaction.[53] n-propanol is considered a marker for ethanol formation as well as an indicator of putrefaction.(54)

Methanol or wood alcohol is commonly used as a solvent in the chemical industry and is highly toxic. It is first converted to formaldehyde and then to formic acid by reaction with ADH and ALDH, respectively, and causes metabolic acidosis that can be fatal.(54)

Ethylene glycol is the active constituent of antifreeze. It is not as volatile as methanol and isopropanol and is very toxic compound. The diol converts in the body to oxalic acid which then reacts with calcium in the body and produces insoluble calcium oxalate. Calcium oxalate precipitates in the kidney and the brain leading to death. Ethylene glycol intoxication is often identified without post-mortem toxicological analysis because the crystals of the calcium oxalate can be seen microscopically in the kidney or brain section.(46)

1.7 Sample Selection for the Analysis of Volatiles

The location from which a specimen is obtained from the body for analysis is important. During absorption, the alcohol concentration in different parts of the body may vary.[56] In living individuals, arterial blood is reported as an ideal blood sample for ethanol determination because the ethanol concentration correlates well with central nervous system (CNS) impairment.(57) Plasma, serum, urine, cerebrospinal fluid (CSF) and saliva contain more water than whole blood; therefore, they are expected to have a higher concentration of ethanol at equilibrium. Serum-blood ethanol concentration ratios vary from 1.12-1.17, whereas plasma-blood ratios range from 1.10-1.35, with an average of 1.18. There is no significant difference between the serum-blood and plasma-blood ratios.(58) The mean saliva-blood alcohol ratio is 1.08, and is independent of the sampling time.(59) The advantage of saliva is that it is easy to be collect by non-invasive methods and there is a high correlation between saliva alcohol concentration and venous blood, and breath-alcohol concentration.(60)

Many studies have evaluated the correlation between the urinary alcohol concentration (UAC) and the BAC. The majority of these studies suggest that the

ratio is 1.3:1, however, many factors may affect the concentrations in urine such as the time after drinking when the bladder is emptied and the remainder of any residue of alcohol-free urine present before the individual started to drink. This would tend to dilute the urine after ethanol consumption.(61) Collecting urine specimens for forensic purposes must be carefully supervised to avoid tampering with the specimen and must also be collected in a vial with an appropriate preservative.(62) In Road Traffic cases, two successive void samples are collected for urine, where the purpose of the first void is to empty the bladder from any old urine.(56)

In postmortem cases, interpretation of the significance of alcohol found is more difficult if there is insufficient information regarding the type of death, the stage of ethanol distribution in the body, storage conditions and temperature of the specimens available.

Whenever possible, femoral/peripheral blood should be collected at post mortem. Cardiac blood (aorta, vena cava) is commonly collected in the US forensic centres but interpreted with caution where contamination in a case is suspected (56;63), while in the UK and Ireland, collection of heart blood is only recommended when femoral blood is unavailable.(64) Blood collected from the chest or thoracic (pericardial blood, pleural cavity) cavity are not ideal as they may be susceptible to contamination from the stomach.(65) Cardiac blood and post-mortem femoral venous blood ethanol concentrations may reflect blood ethanol concentrations at the time of death, however, some peri-mortem physical factors associated with death, e.g. cardio pulmonary resuscitation (CPR) or suffering massive bleeding, may affect exogenous ethanol concentrations in cardiac blood.(66)

In many death situations including death from severe trauma, blood from central and peripheral sites is not available during autopsy, and other body fluids are used to investigate the ethanol concentration, such as urine, vitreous humour (VH) and bile.(46)

With urine, the presence of sugar and yeast can be converted to ethanol by fermentation.(61;67;68) There is a greater chance for bacterial contamination if

the specimen is not stored properly. Vitreous humour (VH) is the sample of choice in situations where the body has undergone extensive trauma.(69) Post-mortem formation of alcohol in vitreous humour is negligible and therefore any ethanol present in VH most likely reflects alcohol consumed and is not formed.(70;71) De Lima and Midio (1999)(72) reported that the vitreous alcohol concentration (VAC) is higher than the BAC during the post absorption phase due to the high water content in VH. According to study that was carried by Honey *et al.* 2005(73) the ratios varied between specimens ranging from 1.01-2.20. Possible reasons for the BAC ≥ VAC would be where death occurs before equilibrium is achieved, if endogenous ethanol forms in post-mortem, or if the blood sample is contaminated during sample collection.

It has been reported(56) that the bile-BAC is less variable and more reliable than other tissues to predict the blood alcohol concentration in both post-absorption and pre-absorption phases. A range of alternative biological fluids and tissues have been evaluated in comparison to blood for detecting ethanol in the living and after death and are summarised in Table 1-3.

Table 1-3: Ratio of Alcohol in Alternative Biological Specimens to BAC*.

Matrix	Ratio Mean (Range)		N	Ref.
Urine	1rst Void	Mean 1.345 (1.68-1.75)	450	(61)
	2nd Void	Mean 1.221 (1.43-1.47)		
	Acute Alcohol Poisoning	Mean 1.18 (0.87-1.53)	628	(67)
	Alcohol Death	Mean 1.30 (0.87-2.10)	647	
Serum/Plasma	Plasma	Mean 1.18 (1.10- 1.35)	20	(58)
	Serum	Mean 1.16 (1.12- 1.17)	-	(56)
Vitreous Humour		Mean 1.19 (0.63-1.75)	672	(71)
		Mean 1.24 (1.01-2.20)	322	(73)
Bile		Mean 1.01 (0.44-1.40)	33	(74)
		Mean 1.03 (0.32- 2.91)	-	(75)
Saliva		Mean 1.08 (1.065-1.088)	336	(75)
CSF		Mean 1.14 (0.79-1.64)	54	(70)
Brain		Mean 0.86 (0.64-1.2)	33	(70)
Liver	Violent Death	Mean 0.63 (0.51-0.83)	-	(76)
	Acute Alcohol Death	Mean 0.60 (0.54-0.72)		
Kidney		Mean 0.66 (0.57-0.76)	-	(76)
Bone Marrow	Actual	Mean 1.94 (1.34-3.22)	42	(74)
	Corrected	Mean 1.29 (0.94-1.64)	63	
Synovial fluids		Mean 1.01 (0.58-2.41)	12	(77)

***BAC- Blood Alcohol Concentration**

The following scenarios may help to differentiate whether the presence of ethanol is endogenous or exogenous;

1) the presence of ethanol in chest blood but not in vitreous humour or urine;

2) the presence of ethanol in body fluids, especially VH;

3) the presence of n-propanol and iso-propanol in the specimens;

4) the circumstances of the death.(72)

5) a history of alcohol abuse.

Finally, cases may be presented where blood transfusions have taken place resulting in antemortem dilution of the blood potentially leading to a low or false negative result for alcohol. Although rare in the UK, bodies that have been embalmed present a real challenge as embalming fluid replaces most of the blood and the embalming fluids contain both formaldehyde and methanol. Although ethanol may be present in some cavity fluid, vitreous humour would be the preferred matrix in these cases.(63)

1.8 Stability of Alcohol in Post-Mortem Blood

Understanding the source and stability of common volatiles in blood is important when interpreting alcohol results to help distinguish whether alcohol was endogenous or exogenous. The presence of alcohol (ethanol) in post-mortem samples can result from the ingestion of an alcoholic beverage or formation after death. The formation of ethanol may be due to physical contamination from embalming fluids or from microorganisms in the presence of an appropriate substrate and ideal temperature conditions.(78) Another source of contamination during the post-mortem examination could result from a blind external chest stick via cardiac fluid or from the stomach contents in cases where death occurred soon after alcohol consumption.(79) Ethanol formation in post-mortem blood has also been reported in the absence of a preservative in the collection vessel.(80)

There is also the potential for loss or gain of alcohol from the specimen analyzed depending upon the time delay from sample collection to analysis. The loss of ethanol is due mainly to evaporation, enzyme-mediated oxidation, or as a result of the action of micro-organisms.(81;82) Many different microbes utilize ethanol as a substrate for metabolism.(81-83) Loss of ethanol was reported from unpreserved specimen containers that contained a high number of microbes and a large headspace.(84) Oxidation of ethanol to acetaldehyde is another factor affecting ethanol loss, as reported by Brown *et al.* (1973).(81)

Dubowski *et al.* (1997) (85) found that alcohol loss was negligible in the presence of an azide, however, the azide-free specimen had losses of less than 5% over a one-year period and for that reason, researchers concluded that the use of azide

is unnecessary. In another study, ethanol production increased as glucose levels increased, indicating that *Candida albicans (C.albicans)* produced ethanol from glucose especially in blood diluted by ante-mortem intravenous infusions that included glucose.(86) Loss of ethanol from preserved blood samples stored in the refrigerator (ranging from 13 to 39 months) varied in 25 samples out of 32 samples, the most significant loss was 0.015 g/dL (15 mg/dL), which was observed in previously opened tubes compared to a mean loss of 0.010 g/dL in unopened tubes.(87)

Sodium fluoride (NaF) is commonly used as a preservative for biological samples. The fluoride ions prevent the formation of polysaccharides by the microbes which leads to the prevention of microbial growth.(88) Although NaF inhibits most organisms, ethanol production in the presence of *C.albicans* at room temperature has been reported.(89;90) Other reports indicate that 1% NaF is efficient at inhibiting *C.albicans* in urine specimen.(88;91;92) The concentration of NaF as a preservative in the ethanol specimens varied from 0.25 to 1%. Two percent NaF is reasonable in combination with a low temperature and might prevent alcohol degradation.(82) Sodium metabisulfite is used as a disinfectant, antioxidant, and preservative agent and has been used to prevent the oxidation of alcohol.(93)

1.9 Analysis of Ethanol and Related Volatiles

Ethanol is most commonly measured in breath by police officers at the road-side using a hand-held device and in blood or urine by scientists using large high-throughput immunoassay-based analysers or headspace gas chromatography coupled with a flame ionisation detector (HS-GC-FID).

Immunoassay-based analysers are used to screen large numbers of samples quickly to identify presumptive positive samples and eliminate negative samples. These screening assays are used by laboratories offering workplace drug testing where the majority of samples will be negative. Positive samples required to be confirmed by HS-GC-FID. Modern forensic toxicology laboratories offering analysis of medico-legal casework use HS-GC-FID systems to ensure accurate,

robust and legally-defensible identification and quantification of ethanol and related volatiles. (54;79;80;94;95)

1.9.1 Headspace Gas Chromatography-Flame Ionization Detection (HS-GC-FID)

Analysis of ethanol using HS-GC-FID (Figure 1-7) requires dilution of the sample with an aqueous solution of internal standard, mixed and allowed to equilibrate at approximately 50 to 60°C in an airtight glass vial with a rubber septa and crimp cap. The vapour of the diluted sample will be aspirated by the automated headspace device and then injected onto the chromatographic column. In forensic work, it is advisable to use dual columns made from two different stationary phases especially with putrefied blood or tissue samples, as this will help reduce the risk of obtaining coincident retention times.(50)

Figure 1-7: Gas Chromatogram with Headspace Autosampler (GC-HS)

Ionization of column eluent of the carrier gas and analyte molecules takes place where the hydrogen and air flame burns in the flame ionisation detectors (FID's). There is a constant electrical potential between the electrodes in the detector and the gap between them acts as a variable resistance. As the analyte molecules ionize, the resistance decreases and more current flows. This amplified current is the detector response. FID is known to be sensitive with broad applicability.(96)

1.9.2 Selection of a Suitable Internal Standard for Analysis of Volatiles

The use of n-propanol, isobutanone, butanone, and n-butanol as internal standards is not recommended, because these volatiles may be present in post-mortem blood. Instead, Acetonitile was recommended in literature as a promising compound to be used as an internal standard since it is not a product of microbial activity.(53)

n-propanol is a major putrefaction product that is not contained in alcoholic beverages or specimens from living subjects and could therefore be considered as a marker for post-mortem ethanol synthesis.(66) n-propanol is present in larger quantities because its production continues even when ethanol production has reached its maximum limit.(53) Ethanol that is formed postmortem is more likely to be lower than 0.7 g/L (equivalent to 70 mg/dL) and, more commonly, is less than 0.3 g/L (equivalent to 30 mg/dL). (83) The ratio of ethanol to n-propanol concentration has been used to confirm postmortem formation of ethanol.(97)

The presence of n-propanol in postmortem specimens renders it unsuitable as an internal standard when using HS-GC-FID for volatiles in post-mortem specimens but this does not apply to samples collected from the living.

Isobutanol is present in bodies recovered from water and has been also considered a good indicator of post-mortem ethanol production.(53)

It has been reported by Kuhlman, *et al.*(1991), (98) and Schuberth (1991),(99) that butanone was not produced in specimens as a result of microbial activity and that it, together with methanol, should be considered indicative of either ante-mortem exposure (e.g., consumption of alcoholic beverages) or contamination (e.g., methanol is found in embalming fluid).(100)

According to Canfield *et al.* (1998)(54) 2,800 blood samples from pilot fatalities were analysed over 8 years to assess levels of volatiles related to aviation accidents. An evaluation between three internal standards (propionaldehyde, propionic acid methyl ester, and t-butanol) was investigated and t-butanol met

the selection criteria, namely; the internal standard should not exist either in living or post-mortem samples; it should have similar properties to the analyte of interest; the retention time should be in the middle range of the retention time of the analytes being separated; and it should be separated from all components of the mixture.(54)

In addition, the standard deviation of replicate measurements of ethanol when using t-butanol as internal standard was better than n-propanol and the retention time for t-butanol was in the middle range of compounds being separated, while n-propanol was not. t-butanol was not found following examination of 2,880 post-mortem samples from pilot fatalities.(54)

Table 1-4: Published advantages and disadvantages of Different Internal Standards Used for the Analysis Volatiles by HC-GC-FID

Internal Standards	Advantages	Disadvantages	Ref
Methyl Ethyl Ketone	Resolved from ethanol, acetone, methanol & other putrefactive volatiles.	Used with Carbowax packed column, No information provided about using it with GC capillary column	(53;95)
Acetonitrile	Not a product of microbial activity- separated well using Rtx BAC1 or ALC1 capillary columns	Not commonly use. 100% overlapping with acetone using Rtx-BAC2 and ALC2 capillary columns	(53)
Propionaldehyde	Considering internal standard criteria, it was selcted as a potential replacement fo n-propanol Its retention time close to ethanol retention time	Does not have acceptable base line separation for compounds commonly found post mortem	(54)
Propionic acid methyl ester	Considering internal standard criteria , it was selcted as a potential replacement fo n-propanol Its retention time close to ethanol retention time	Not stable and decreases over time, affecting quantitation	(54)
t-butanol	Meets the criteria of selection for suitable internal standards	Concentration may decrease with increasing fat content.	(54;94)
n-propanol	Can be separated from acetone	Major putrefaction product and the retention time is not in the range of the analytes being separated.	(53;66;79)
Isobutanol	Well separated from other volatiles using using Rtx-BAC2 column	Could serve as a potential indicator of ethanol production (post mortem)	(53;80)
Methanol	Not produced in specimens as a result of microbial activity	Could be indicative of either ante-mortem exposure or due to contamination from embalming fluid	(98;99)

Although t-butanol could be a suitable internal standard for volatile analysis, it has been reported that the level of t-butanol as an internal standard might decrease when the fat content of the sample analysed is increased.(94) Table 1-4 summarises the advantages and disadvantages associated with using different internal standards for the analysis of volatiles.

1.9.3 Analytical Considerations for Method Development (Volatiles)

Before starting the process of developing a method for the analysis of volatiles in biological matrices, it is important to first establish the sensitivity and linear range required. Table 1-5 summarises the volatile concentrations reported in the literature from a range of cases where low levels of volatiles were measured through to high concentrations in volatile-related fatalities. These provide an indication of both the lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) for each volatile.

For legal purposes it is highly recommended that all determinations of blood-ethanol concentration are made in duplicate with a cut-off point of 10 mg/dL.

Table 1-5: Alcohol and Other Volatile Levels in Blood.

Alcohol Name	Endogenous blood level	Toxic (Fatal) blood level (mg/dL)	References
Ethanol	0.04-0.1 mg/dL	80-100 (>350)	(101;102)
Methanol	<10 mg/dL	20 (>89)	(101;102)
	(0.1-2.3) mg/dL	Two fatalities (219, 196)	(103)
Acetone	Serum 2.3-3.5 mg/L Non-diabetic (n=218) <10 mg/L	20-30 55	(55;102)
Isopropanol	0.1 - 0.2 mg/kg	>40 >150	(102;104)
n-Propanol	Post-mortem blood 3-7 mg/dL	UK workplace exposure limit 250ppm (625 mg/m^3)	(53;83;105)

1.10 Analytical Biomarkers of Alcohol Consumption

1.10.1 Introduction

Biological markers, or biomarkers, are parameters tested and measured in the blood, tissue or body fluids as indicators of biologic state and are commonly used for diagnostic testing to monitor the presence of disease or to predict the various stages of disease and mortality. Biomarkers have also been used to detect drug or alcohol consumption.(106) Biochemical biomarkers are usually evaluated in terms of specificity and sensitivity and the ideal marker should be 100% sensitive and specific but this has not as yet been achieved. (29)

Alcohol biomarkers are useful as indicators of chronic and acute alcohol consumption, due to the fact that the time frame for a positive identification of ethanol is limited with ethanol concentrations decreasing rapidly over time.(107) Also, ethanol can be produced naturally at very low concentration as a result of yeast proliferation after carbohydrate-rich meals, with formation also occurring after sampling due to microbial contamination and fermentation.(108)

The three major classes of alcohol biomedical markers include “relapse markers” which are used in tests sensitive enough to detect even a single intake of alcohol such as ethanol, methanol or 5- Hydroxytryptophol (5HTOL). The second class of maker are called “state markers” for hazardous alcohol consumption. This type of marker can indicate disturbed metabolic processes or malfunctioning body organs and tissue damage caused by long-term exposure to alcohol. Examples of these markers include, γ-Glutamyl transferase (GGT), aspartate (AST) and alanine aminotransferase (ALT), mean corpuscular volume (MCV) and carbohydrate-deficit transferring (CDT). “Trait markers” are used in tests that indicate whether or not a person has a genetic disposition for alcohol abuse, such as the monoamine oxidase (MAO) enzyme.(109) A lower activity of MAO in the blood platelets of alcoholics compared with those of control groups has been reported.[(110)] It is not only alcohol that may lower the activity of MAO as a separate study indicated that tobaco had the same effect on ethanol.(111)

1.10.2 Markers of Acute and Chronic Alcohol Consumption

In addition to ethanol, methanol and 5HTOL several biomarkers have been found in urine and blood that may indicate recent alcohol consumption. These include; acetaldehyde, acetate, the ratio of 5HTOL to 5-Hydroxyindole-3-acetic acid (5-HTOL/5-HIAA) and the direct ethanol metabolites ethyl glucuronide (EtG) and ethyl sulphate (ETS).

Acetaldehyde is present in low concentrations (<1% of ethanol concentration) and rapidly converts to acetate. Additionally, its binding to various endogenous molecules such as protein in the peripheral venous blood will further reduce the concentration of free acetaldehyde.(109) In the presence of ethanol, acetaldehyde may be formed artificially even after sampling. Acetate appears to be independent of blood ethanol concentration and remains elevated as long as ethanol is being metabolised.(109;112)

5-HTOL and 5-HIAA are the minor and major metabolites of serotonin respectively, 5-HTOL increases in urine after drinking alcohol and it is considered to be a marker for recent alcohol consumption. Results are expressed as the ratio of 5-HTOL/5-HIAA rather than the 5-HTOL/creatinine to compensate for variations in 5-HTOL concentrations caused by urine dilution and to eliminate a false positive of those two metabolites in urine due to dietary serotonin from food known to produce serrotinin such as banana, pineapple, kiwi fruit and walnuts. The advantage of 5-HTOL/5-HIAA ratio over ethanol and methanol to detect recent drinking is that the serotonin metabolites can persist for several hours longer. The disadvatages are that 5-HTOL/5-HIAA ratio can be also increased with treatment with aldehyde dehydrogenase (ALDH) inhibitors such as disulfiram and cyanamide.(109) Formation of ethanol in urine due to bacterial action may be a source of misinterpretation of the 5HTOH/5-HIAA ratio.(113) As much as 80 % of 5-HTOL is excreted as glucuronidated 5-hydroxytryptophol (GTOL) which offers promising results in the detection of previous alcohol consumption and can be determined by ELISA.(114)

Less than 0.1% of ingested ethanol undergoes non-oxidative metabolism in the liver in a phase II conjugation reaction to produce a water soluble metabolites

ethyl-glucuronide and ethyl sulfate which are then excreted in urine.(47) Both ETG and ETS are considered to be indicators for acute ethanol ingestion.(109)

The delayed elimination of EtG may lead to false positive results and in cases where the deceased died within a few hours of consuming alcohol, the positive detection of EtG may be due to a previous episode of drinking.(115) Secondly, the short time lag between the time ethanol ingestion took place and the possible detection of EtG in blood, may also lead to false negative results.(115)

In addition to the classic markers of chronic alcohol intake, namely; CDT, GGT and MCV(113), other markers of chronic alcohol consumption include, AST, ALT, phosphotidyl-ethanols (PEth), fatty acid ethyl esters (FAEEs) and β-hydroxybutyrate (BHB).

Carbohydrate–deficient transferring (CDT) is a reliable marker for the detection excessive alcohol consumption and refers to changes in the microheterogeneity of the iron transport glycoprotein transferring in serum after contenious excessive prolonged alcohol consumption. (116)

The main advantage of CDT, compared with other alcohol biomarkers, is its high specificity for alcohol and the associated lower risk of a false negative from heavy alcohol consumption.(109) Although CDT is able to identify heavy drinkers with a higher specificity than GGT, heavy drinkers can be detected only when daily alcohol consumption is greater than 60 g. (117) False positive results may be found for individuals with non-alcoholic related diseases, such as biliary cirrhosis, or chronic active hepatitis, and with rare defects such as glycoprotein defect.(106;109)

The advantage of GGT, AST and ALT markers is availability with low associated costs in most clinical laboratories, however GGT is non-specific for alcohol misuse and its level may increase due to other pathological conditions. MCV indicates the size of red blood cells and it is a part of routine blood count test. An elevated MCV is often observed in alcoholic patients and has a higher specificity than GGT and can be used in combination with other biochemical parameters. Cell size may take several months to return to normal.(109)

PEths are phosphatidylethanols and refer to a group of abnormal negatively charged diacyl phospholipids formed in the presence of ethanol from other naturally occuring phospholipids such as phosphatidylcholines. PEths are direct ethanol metabolites that may degrade depending on the storage of specimens. Blood samples that contain ethanol can be stored in the refrigerator for up to 72 hours or frozen in liquid nitrogen and stored at -80ºC without affecting PEth levels.(118);(119);(120) Disadvantages associated with PEths are that they are impossible to analyse in clotted post-mortem blood samples and can also be formed in the presence of ethanol.(121)

FAEEs have been analysed in meconium and hair as indicators of gestational alcohol exposure,(106) while both FAEEs and EtG have been measured in hair as markers for chronic alcohol consumption.(122) BHB is considered a potential marker of alcoholic ketoacidosis (AKA) and diabetic ketoacidosis (DKA). FAEE's and BHB will be discussed in more detail in the following sections.

Tables 1-6 summarises information relating to the many types of acute and chronic alcohol biomarkers, the matrices they are measured in and the detection window.

Table 1-6: Acute and Chronic Alcohol Biomarkers.

Alcohol Biomarkers	Matrix	Markers of Acute/Chronic	Detection time (hours)
Ethanol	Blood,serum, Plasma ,Urine, Saliva, sweat and Breath	Acute	1-2(Depends on dose)
Methanol	Blood,serum, Plasma ,Urine, Saliva, sweat and	Acute	2-3
Acetaldehyde	Blood	Acute	-*
Acetate	Blood	Acute	-*
EtG and EtS	Urine, serum, Hair, Vitreous Humor ,bone marrow,	Acute	EtG in urine was elevated 24-48 hours after ingestion.
5-HTOL/5-HIAA	Urine	Acute	Longer that ethanol and methanol.(113)
GTOL	Urine	Acute	-*
CDT	Serum	Chronic	Over 1.5-2 weeks(109)
GGT,AST and ALT	Serum, Blood	Chronic	-*
MCV	Blood	Chronic	-*
Phosphotidylethanols (PEth)	Blood	Chronic	14 days after sustained ethanol intake.(123)
Fatty Acid Ethyl Esters (FAEEs)	Serum, Hair and meconium	Chronic	Decrease within 29 hours, and can be detectable for long time after intake. (124)
ß-Hydroxybutyrate (BHB).	Serum,Postmortem Blood , Urine	Chronic	-*
Proteomics	Blood,serum, Plasma	Chronic	-*

***Detection time not documented**

1.11 β-Hydroxybutyrate (BHB)

BHB is considered a potential marker of alcoholic ketoacidosis (AKA), (125-128) a metabolic disturbance resulting from prolonged consumption of alcohol. AKA is attributed to the combined effects of alcohol and starvation in glucose metabolism. Alcohol blocks the hepatic gluconeogenesis which prevents glucose production, increases lipolysis, and fatty acid metabolism and results in the formation of ketone bodies as summarised in Figure 1-8.(129)

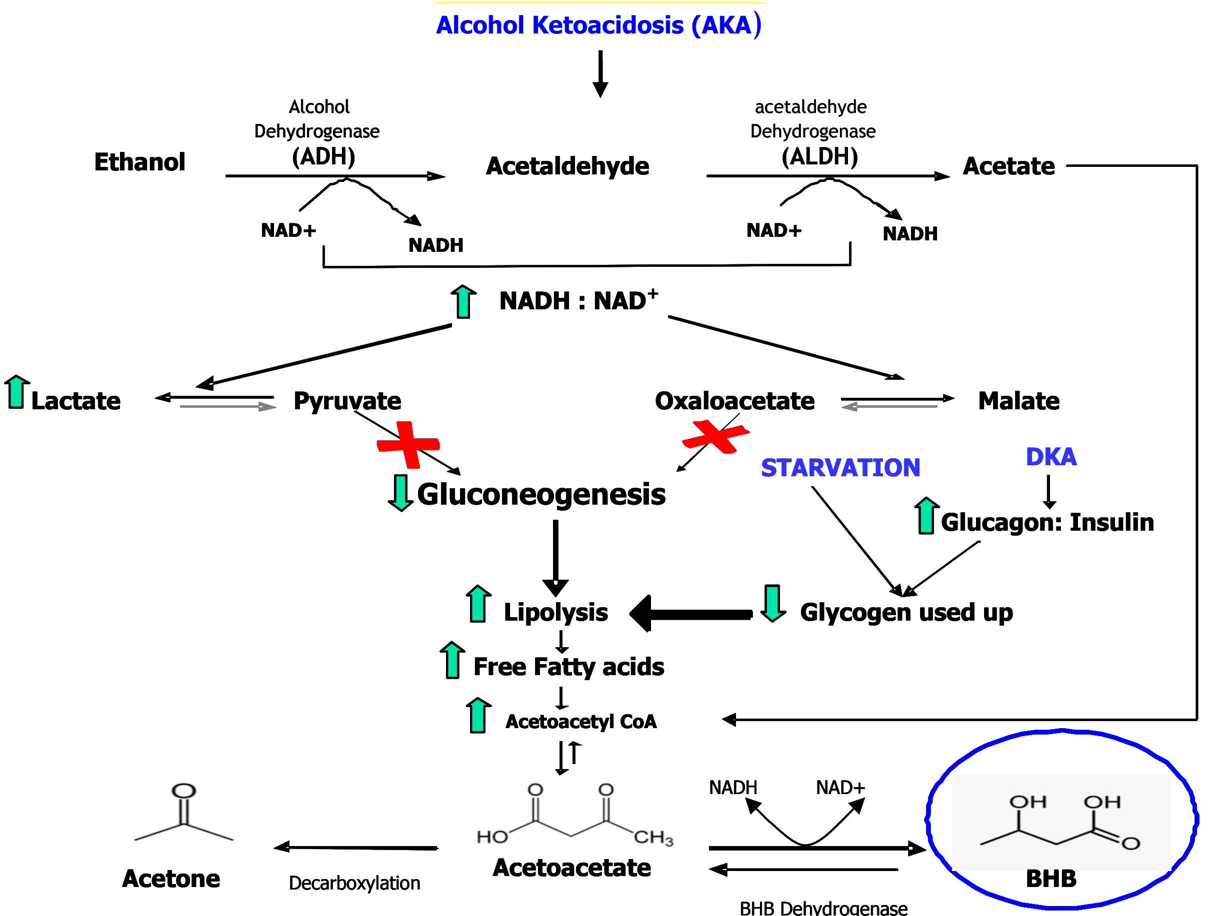


Figure 1-8: Formation of Ketone Bodies
[Reproduced from Iten&Meier (127) and Umpierrez, et al.(130)]

The accumulation of ketone acids reduces the pH of the body and results in ketoacidosis. The most common ketone bodies produced in the body are acetone, acetoacetate and β-hydroxybutyrate.

In many alcohol-related sudden death cases, the pathologist is unable to determine the cause of death with little findings of note at autopsy (other than a severely enlarged fatty liver) and no indications of the cause of death following routine histological, microbiological and toxicological investigations. In some cases the only positive findings, other than a fatty liver, are traces of ethanol in the blood and elevated acetone levels.(131) It is commonly believed that alcoholic cases of unexpected sudden death with no indication of the cause of death may be due to AKA.(108)

BHB is the analyte of choice among other ketone bodies when investigating AKA because BHB concentrations are higher (approximately two-thirds of all ketone bodies in healthy persons) compared with acetoacetate and acetone. There is a close association with the increase in $NADH:NAD^+$ and BHB. According to Iten *et al.*(127) there is no indication of BHB formation during storage or degradation in blood *in vitro* or in the corpse.(127) Acetone is neutral and does not contribute to an acidosis, and both acetoacetate and acetone are unstable in the blood. The production of volatile compounds due to putrefaction may interfere with acetone analysis. (55;127)

In contrast diabetic ketoacidosis (DKA) is a metabolic acidosis associated with type 1 diabetes and is related to insulin deficiency. Insulin controls the level of glucose in the body coupled with an increase in glucagon levels and produces maximum gluconeogenesis. This leads to severe hyperglycaemia and accumulation of ketone bodies due to the enhanced metabolism of free fatty acids that are librated from peripheral adipose tissue and may end with ketoacidosis.(129),(125) Both DKA and AKA are common medical emergencies and are life threatening if they are not treated promptly.(130)

Diabetic alcoholics may present with AKA; however, this distinction is difficult, particularly when the blood glucose level is moderately or mildly elevated. In

cases of starvation, ketosis alone usually causes a mild ketoacidosis, and the clinical presentation of AKA might distinguish it from starvation ketosis.(132)

Umpierrez *et al.*(130) concluded that differentiation between DKA and AKA should be based on the history of diabetes or alcoholism and on the blood glucose level at admission. The clinical diagnosis with AKA and DKA are similar; and they are both associated with vomiting, nausea, abdominal pain, dehydration, Kussmaul breathing (slow deep respiration associated with acidosis), and coma. A summary of the differences between AKA and DKA is shown in Table 1-7.

Table 1-7: Main Differences Between AKA and DKA.(55;130)

DKA	AKA
↑ Glucose level (Hyperglycaemia) 578± 67 mg/dL	↓ Glucose level (Hypoglycaemia) 118 ± 11 mg/dL
BHB/Acetoacetate Ratio 3:1	BHB/Acetoacetate Ratio 7:1
Lactate/Pyruvate Ratio 11:1	Lactate/Pyruvate Ratio 19:1
↑ Glycated Haemoglobin (HbA_1) 5-13%	Glycated Haemoglobin (HbA_1) <5.6%

Low or absent blood ethanol along with elevated levels of acetone do not in themselves determine the cause of death, but these findings, in combination with a history of chronic alcohol abuse, suggest alcoholic ketoacidosis.

The concentration of acetone in the blood could be considered as a indicator of the development or severity of AKA. Acetone blood levels can increase due to intoxication from barbiturates or alcohol, diabetes and hypothermia, metabolic ketoacidosis, and isopropanol intoxication. Isopropanol is rapidly oxidized to acetone, which can be measured for several days after isopropanol intoxication, while the isopropanol will disappear much earlier. Normal values in the serum of a living person range from 2.3-3.5 mg/L. In deaths due to diabetic coma the acetone level ranges from 16-864 mg/L with most values between 300-400 mg/L.(55) In non-diabetic controls acetone values are <10 mg/L. Out of 24 chronic

alcoholic cases, nine had acetone levels ranging from 74-400 mg/L with a mean value of 183 mg/L. (55)

It is generally believed that, in cases of alcoholic ketoacidosis, ethanol is low (<10 mg/dL) or absent, the absence of acetone does not preclude ketoacidosis; rather, it means that BHB still needs to be measured. Normal levels in post-mortem blood were reported as less than 52 mg/L, elevated to toxic levels ranged from 52 – 260 mg/L, while levels exceeding 260 mg/L are considered pathologically significant.(127) Other studies measured BHB in urine and vitreous humour from a range of cases including individuals who were known to be alcoholics or diabetics and are summarised in table 1-8.

Table 1-8: Levels of BHB Measured in post-mortem Blood, Urine and Vitreous Humour from a Range of Case Types.

Category	Blood (mg/L)	Urine (mg/L)	Vitreous Humour (mg/L)	N	Ref.
Elevated/Toxic	52-260	N/A	N/A	25	(127)
Fatal	>260	N/A	N/A	25	
Control Group	<52	N/A	N/A	69	
Alcoholics with unknown cause of death	N/A	267 - 850	106 - 850	6	(133)
Alcoholics with known cause of death	N/A	20 - 2000	0 - 850	6	
Control group	N/A	<100	<100	36	
Normal	N/A	8.5 - 34.7	N/A	30	(134)
Diabetic	N/A	4.9 - 4520	N/A	20	

Analysis of ketone bodies in both AKA and DKA may provide analytical evidence to support clinical diagnosis and may possibly provide information about the cause of death in the absence of other pathologically significant findings.(131;135)

1.11.1 Analysis of BHB in Biological Matrices

The small polar nature of BHB and compounds of similar structure, such as gamma-hydroxybutyrate (GHB), make the chromatographic and spectrophotometric analysis of this compound complicated. The presence of two hydroxyl groups in the structure of BHB makes it suitable for silyl derivatization as illustrated in Figure 1-9.

Figure 1-9: BHB and BHB-TMS-Derivative

Table 1-9 summarises published methods where BHB and related compounds were measured in biological matrices.

The majority of the methods utilised gas chromatography-mass spectrometry with electron ionization detection (GC/MS-EI) with full scan mode for ion identification. Separation was carried out for most of the GC/MS methods with similar types of capillary column and using deuterated internal standards for quantification. This combination provides greater specificity and accuracy unlike the methods utilising UV spectrometry. Head space-GC/MS has been also used for BHB determination however this involves many steps for sample preparation and involves conversion of BHB to acetone and a long incubation time. Ideally a wide linear range is preferable to eliminate additional work required to dilute and re-analyse the higher concentrations found in post mortem samples with confirmed AKA or DKA, however only a few methods have a linear range up to 500 mg/L.

1.11.2 Gas Chromatography/Mass Spectrometry (GC/MS)

Gas chromatography uses a gaseous mobile phase to transport the sample components through a packed column.

Before analysis of the analytes of interest by mass spectrometry, they must be converted into gas phase ions. Derivatization, which changes the chemical properties of the analytes of interest, is normally used to increase volatilization and ionization to promote chromatography, improve peak shape, and increase sensitivity and specificity. Analytes of interest are separated from the compound mixture by gas chromatography. The most common ionization techniques in GC/MS are electron impact (EI) (figure 1-10) and chemical ionization (CI).

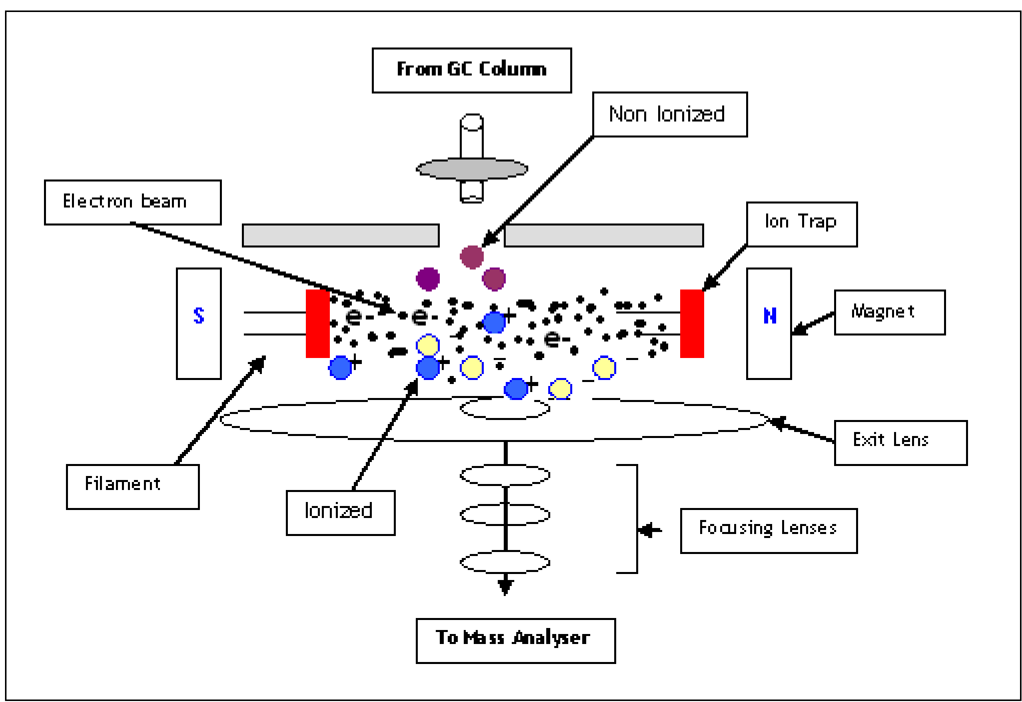


Figure 1-10: Electron impact ionization.

In electron impact (EI), the parent molecules usually enter the ionization chamber in the mass spectrometer ion source and are impacted by an electron beam of high energy around 70eV which is enough to ionize the molecules. This leads to the formation of positive, negative, and neutral molecular fragments.The fragments (usually positive) are introduced to the mass quadropole analyser to filter masses and to monitor ions by either scanning them in full scan, a selected m/z ratio range, a type of acquisition usually used for qualitative analysis or by selected ion(s) monitoring (SIM), a type of ionization known to be sensitive and specific. SIM is a scan mode used for quantification. (96;136)

Table 1-9: Summary of Published Methodologies for the Analysis of BHB in Biological Matrices

Analyte(s)	IS	Matrix	Instrument	Column	Identification	Linearity (BHB)	LOD/LLOQ	Ref.
BHB, acetone, acetoacetate	Acetone-$^{13}C_3$	B	HS-GCMS-EI	DB-6243 (30mx0.250mmi.d.X1 µm)	Full Scan	10-25000 µM	8 µM (LOD) 21 µM (LOQ)	(137)
BHB	D_6-GHB	U, B, VH, P	GC/MS-EI	DB-5 Zebron: ZB-5MS (30m x 0.25mm i.d. x 0.25 µm)	Full Scan 233 BHB and 239 D_6-GHB	31.25-500 mg/L	1 mg/L (LOD) 31.25 mg/L(LOQ)	(125)
BHB	D_6-GHB	U, B	GC/MS-EI	DB-5 (30m x 0.25mm i.d. x 0.25 µm)	Full Scan 233,191,117 BHB 239, 240, 241 D_6-GHB	50-500 mg/L	2 - 7 mg/L (Blood) 2 - 6 mg/L (Urine)	(138)
BHB	D_6-GHB	B	GC/MS-EI	HP-5MS (30m x 0.25mm i.d. x 0.25 µm	SIM 147, 117, 191, 91 BHB 239, 73, 240 D_6-GHB	50-500 mg/L	NIP	(139)
BHB, GHB, AHB and SA	HCA	U	GC/MS-EI	DB-17 (30m x 0.32mm i.d. x 0.25 µm).	Full scan 233, 191 and 147 BHB 147, 73 D_6-GHB.	1-300 µg/mL	0.03 ng/mL full scan 0.01 ng/mL SIM (LOD)	(134)
BHB	DNBP	P	HPLC-UV	55mm x 4.0mm i.d. x 3 µm	UV detection at 320nm	0.05-2.0 µmol/L	5 nmol/mL (LOQ) 50nmol/mL	(140)
BHB, acetone, acetoacetate	NIP	B	HS-GC-FID	DBWAX megabore (30m x 1 µm)	FID	NIP	NIP	(126)
BHB, acetoacetate	NIP	B	GC/MS-EI	Packed (4m x 6mm)	SIM 275 BHB 273 Acetoacetate	BHB 0-18 Molar ratio x100 Acetoacetate 0-24 Molar RatioX100	NIP	(141)

Key: AHB (Alpha-Hydroxybutyrate); B (blood); DNBP (2,2-dinitrobiphenyl); HCA (2-hydroxycaproic acid); LLOQ (lower limit of quantification); LOD (limit of detection); P (plasma); SA (Succinic Acid); SIM (selected ion monitoring);U (urine); VH (vitreous humour), NIP (No Information Provided)

1.12 Fatty Acid Ethyl Esters (FAEEs)

FAEEs are non-oxidative products of ethanol metabolism and are formed by the esterification of ethanol with free endogenous fatty acids and acyl-CoA/fatty acids. These reactions are catalyzed by the two enzymes FAEEs synthase and acyl/CoA/ethanol O-acyl-transferase.(142-145) After being synthesized in the liver and the pancreas, FAEEs are transported through the blood, while bound to lipoprotein and albumin and to the adipose tissue.(146) FAEEs persist in the blood for approximately 24 hours after ethanol intake has ceased.(147) Figure 1-11 illustrates the structure of common FAEEs and deuterated analogues commercially available to purchase as analytical standards. A shorter alternative nomenclature is commonly used for FAEEs, including one letter and two numbers separated by a colon. This corresponds tothe chain length and number of double bonds: for example; ethyl oleate is (E18:1), with 18 being the number of carbons and the number 1 being the number of double bonds.(148)

Ethyl Laurate (E12:0)

Ethyl Linoleate (E18:2)

Ethyl Myristate (E14:0)

Ethyl Linolenate (E18:3)

Ethyl Palmitate (E16:0)

Ethyl Arachidonate (E20:4)

Ethyl Palmitoleate (E16:1)

Deuterated Ethyl Palmitate

Ethyl Stearate (E18:0)

Deuterated Ethyl Stearate

Ethyl Oleate (E18:1)

Deuterated Ethyl Oleate

Figure 1-11: FAEES and D5-FAEES Structures

1.12.1 Sample Selection for the Analysis of FAEEs

FAEEs can be analysed in variety of biological samples such as blood, plasma, hair and meconium. FAEEs in serum correlate well with blood alcohol concentrations (BAC) and start to decrease within 2 hours after ethanol administration has stopped.(146) FAEEs persist in the blood for approximately 24 hours after ethanol intake.(147)

In patients with a positive BAC, ethyl palmitate (E16:0) was detected in all plasma samples. Ethyl stearate (E18:0) was detected in 49% of the patients, ethyl oleate (E18:1) in 64%, ethyl linoleate (E18:2) in 13% and ethyl arachidonate (E20:4) was found in only 5% of patients. FAEEs were not detected in the control group who screened positive for ethanol.(149)

According to Borucki et al.(124), FAEEs in serum decrease rapidly within 29 hours following ethanol intake; then they stabilize at values ranging from 0.007–0.025 mg/L with no further decrease and remain detectable for long periods of time after intake. FAEEs take time to be released from the storage in adipose tissues, thus, FAEE determination in serum is limited to detecting recent ethanol consumption.

It has been observed that the total FAEE concentration of ethyl palmitate (E16:0), ethyl stearate (E18:0), ethyl oleate (E18:1), and ethyl linoleate (E18:2) in the plasma of patients with a history of chronic ethanol abuse was significantly higher than in patients with a history of acute ethanol abuse.(149)

Refaai et al.(150) found good correlation between BAC and FAEEs in the liver and between BAC and fat. Ethyl arachidonate (E20:4) is thought to be a highly specific marker of premortem alcohol ingestion, and has been detected at concentrations greater than 0.066mg/kg in individuals with a positive BAC.

When ingested, FAEEs are rapidly hydrolysed in the duodenum but remain intact in the stomach; therefore, FAEEs may reach the bloodstream after absorption from the stomach.(151) It was concluded that kinetics of FAEEs are different in heavy drinkers compared with healthy subjects and that FAEEs are of limited value for the detection of a prior single ethanol intake.(124)

Meconium is a complex composition of greenish mucus material excreted by newborns approximately 2–3 three days after birth (may be delayed in premature infants). The advantage of meconium is that the collection is easy and non-invasive and it is a cumulative matrix in which xenobiotics and endogenous substances accumulate from the 13th week of gestation till birth, providing a wider window for detection of long-term exposure.(28) All samples must be stored immediately after collection in the freezer.

Although it has been shown that FAEEs do not cross the human placenta unlike ethanol, FAEEs can be produced in vivo in the human placenta.(28)

In a study by Chan et al. 2004, approximately 14% of meconium samples (N=142) tested positive for prenatal alcohol exposure with a level greater than 2.0 nmol/g, equivalent to between 458-666 ng/g of corresponding FAEEs. Ethyl oleate, linoleate, palmitate, and arachidonate were detected most often and at the highest levels. In 95% of positive samples, a minimum of 3 FAEEs were detected.(27) A high correlation was found between FAEEs in meconium collected from 124 infants and maternal ethanol. It has also been reported that ethyl linoleate (E18:2) and ethyl arachidonate (E20:4) are biomarkers with high specificity for detecting prenatal alcohol exposure. Ethyl arachidonate levels were significantly higher in the alcohol-exposed groups than in the control groups.(152)

In a separate study, 17 of 682 meconium samples tested positive for significant prenatal ethanol exposure (>2.0 nmol/g). The 95% confidence interval of FAEEs results that were less than the cut-off was 0.38–0.49 nmol/g [87-163 ng/g] versus those that were positive and greater than the cut-off which ranging between 7.74–151.28 nmol/g [1772-50376 ng/g].

Meconium FAEEs analysis demonstrates a fivefold increase in sensitivity over self-report–based screening methods for the detection of ethanol-exposed pregnancies in a clinical setting.(152)

The total FAEEs in the study carried out by Moore et al. 2003 (153) was an accumulation of 7 FAEE concentrations not including ethyl laurate and ethyl

myristate. Cumulative FAEE acid concentration greater than 10,000ng/g may indicate that the newborn has been exposed to significant amounts of ethanol during the pregnancy. The presence of laurate and myristate ethyl esters in meconium is considered non-specific.(153)

Ethyl linoleate (E18:2) was positive in a meconium specimen of a neonate whose mother admitted to an intake of at least 10 drinks per week during the last month of gestation. It was also reported in negative cases where the mother had less than 3 drinks per week. Ethyl oleate (E18:1) is the most prevalent individual ester in meconium, followed by ethyl palmitate (E16:0) and ethyl palmitoleate (E16:1); whereas ethyl laurate (E12:0) and ethyl myristate (E14:0) were also detected in alcohol-free mothers.(154)

In addition It has been reported that ethyl linoleate (E18:2) is the best marker of foetal alcohol spectrum disorder FASD.(155)

Maternal FAEEs in hair has also been recommended as an alternative to meconium. The reason behind this is due to the additional cost of developing an effective meconium assay with the limitation of providing information on maternal drinking only after childbirth, and even then only for the latter two trimesters. Also, other research is on-going on a range of other biomarkers, such as potential proteomic and metabolic marker.(26)

1.12.2 Analysis of FAEEs in Biological Matrices

Kulig *et al.*(156) utilised GC-MS with a nonpolar dimethylpolysiloxane column to analyse FAEEs in plasma. FAEEs analysis methodology includes acetone precipitation and extraction with hexane, using amino propyl silica solid phase extraction. These methods have been shown to have high sensitivity, specificity and accuracy (156)

The internal standard used was either ethyl heptadecanoate (for several studies carried out using LC/MS/MS), or in-house prepared deuterated FAEE standards with other analytical techniques such as headspace-gas chromatography mass spectrometry (HS-GC/MS) coupled with or without solid-phase microextraction. The methods showed high sensitivity, specificity, and accuracy.(157-159)

The recommended method to be used for FAEEs determination by the Society of Hair Testing (SOHT) is head space (HS)-solid-phase microextraction (SPME) in combination with GC/MS with a deuterated internal standard.(160)

Table 1-10 is a summary of the advantages and disadvantages of using different techniques for determining the FAEEs in different matrices.

Table 1-10: The Difference of the Techniques Used for the Determination of FAEEs in Biological Samples

Techniques	Advantage	Disadvantage
LC/MS/MS	High specificity	Many sample preparation steps
	Sensitive	High solvent volume used
	Same chromatographic method column can be used to detect opiates, cocaine, and FAEEs in meconium	Large sample size
HS-SPME-GC-MS	Eliminating the need for the organic solvent	Needs optimization of the HS-SPE
	Minimal sample preparation	Equal or less specificity than LC/MS/MS
	Obtaining clean extract less matrix effect	
	Require small amount of sample	
	Using deuterated internal standard improve the accuracy, sensitivity Ideal for routine analysis.	
GC/MS	Alternative method when no LC/MS/MS or HS-SPME available in the laboratory Using CI is better than EI mode because EI yields identical fragments for various FAEEs	Less specific than LC/MS/MS and less sensitive than LC/MS/MS and HS-SPME method
GC-FID	Can be used as screening method	Not specific

Table 1-11: Summary of Techniques for the Analysis of FAEEs in meconium.

Ref.	FAEES	Internal Standard	Matrix	Instrument	Extraction/Column	Identification	Linearity LOD/LLOQ
(Kwak *et al.* 2010) (158)	ethyl laurate ethyl myristate ethyl palmitate, ethyl palmitoleate ethyl linoleate ethyl linolenate ethyl oleate ethyl stearate ethyl arachidonate	Ethyl Heptadecanoate	Meconium	LC/MS/MS-MRM-ESI-Positive Mode	Liquid liquid extraction Solid Phase extraction X Bridge C8 column (150mm, 1.0mm, 3.5μm)	Laurate 229.4→201.4 Myrisate 257.4→229.4 Palmitate 285.4→257.4 Palmitoleate 283.5→219.4 Linoleate 309.5→263.4 Linolenate 307.4→261.5 Oleate 311.4→265.4 Stearate 313.5→285.4 Arachidonate 333.5-281.5 Heptadecanoate 299.4→271.5	0.01-0.08 nmol/g 0.02-0.27 nmol/g
(Pichini *et al.* 2008) (159)	ethyl laurate ethyl myristate ethyl palmitate, ethyl palmitoleate ethyl linoleate ethyl linolenate ethyl oleate ethyl stearate ethyl arachidonate	Ethyl Heptadecanoate	Meconium	LC/MS/MS-MRM ESI-Positive Mode	Liquid liquid extraction Solid Phase Extraction Eclipse XDB-C8 column (150x4.6mm,5 μm)	Laurate 229→201 Myristate 257→229 Linoleate 309→245 Linolenate 307→261 Palmitate 285→257 Palmitoleate 283→237 Oleate 311→265 Stearate 313→285 Arachidonate 333→287 Heptadecanoate 299→271	LOQ-50.0 nmol/g (LOQ- to 16,625 ng/g) 0.04-0.07 nmol/g (12.4-15.9 ng/g) 0.12-0.20 nmol/g (40.2-45.6 ng/g)

Most techniques used deuterated FAEEs-D_5 internal standards prepared in-house with HS-SPME-GCMS instruments for analysis of FAEEs in hair and meconium. Only recently did four deuterated internal standards become commercially available to purchase, namely; ethyl myristate-D_5, ethyl palmitate-D_5, ethyl oleate-D_5 and ethyl stearate-D_5. Although HS-SPME-GCMS has more advantages as previously indicated in Table 1-10, and is recommended by Society of Hair Testing for use when analysing FAEEs in hair, it has also been used for the analysis of FAEEs in meconium. Only two LC-MS/MS methods have been published for the analysis of FAEEs in meconium and both used a non-deuterated internal standard (ethyl heptanoate) as listed in Table 1-11. A summary table of all of the different techniques used to analyse FAEEs in different matrices can be found in Appendix 1-1.

1.12.3 Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS)

A triple quadropole LC/MS/MS consists of an HPLC coupled with a triple quadropole mass spectrometry. HPLC uses a suitable liquid mobile phase to transport the sample through a column packed with stationary phase material.

A triple quadrupole mass spectrometer (QQQ) consists of two mass analysers (MS1 and MS2) separated by a collision cell arranged in series. The first mass analyser allows only the ions of interest (precursor or parent ions) to pass through, the ions enter the collision cell where fragmentation takes place and produces product or daughter ions which are analyzed in the second mass analyser (third quadrupole) and detected by the mass detector using specialist software for analysing and processing the data for quantification and identification Figure 1-12.(161;162)

Five scan modes can be used in the triple quadrupole mass analyser. The product ion scan to detect all fragments produced by a single precursor, the precursor ion scan to detect all precursors producing a common fragment ion, neutral loss scan to detect all precursors undergoing a loss of a common neutral moiety, the single reaction monitoring (SRM) to detect a specific fragment ion produced from

a single precursor, and the multiple reaction monitoring (MRM) to detect multiple specific fragment ions produced from a single precursor. Both SRM and MRM are the most scan mode used in LC/MS/MS for identification and quantification. (161;162)

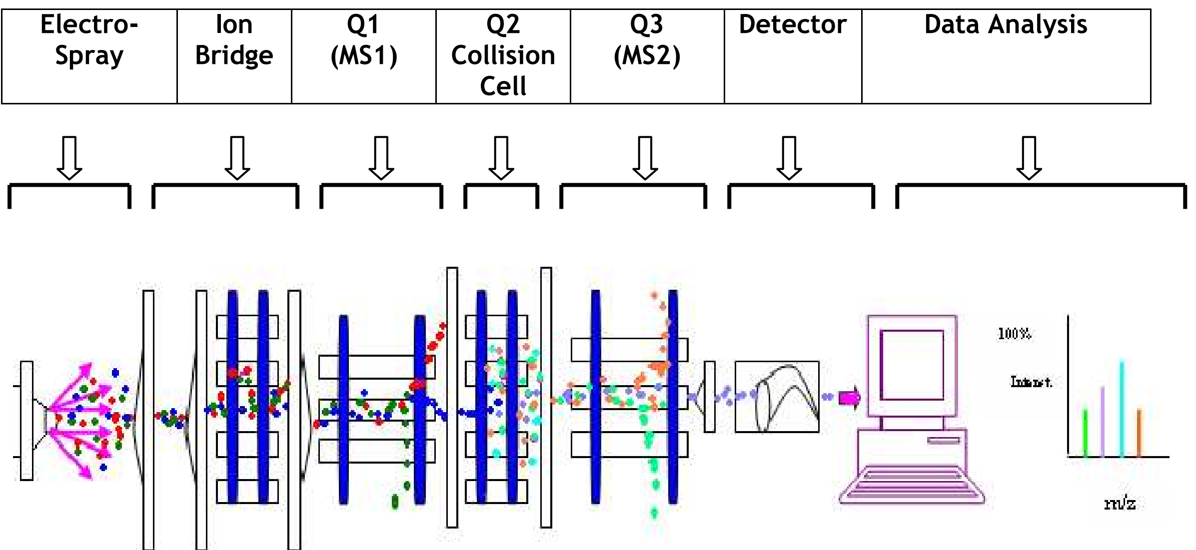


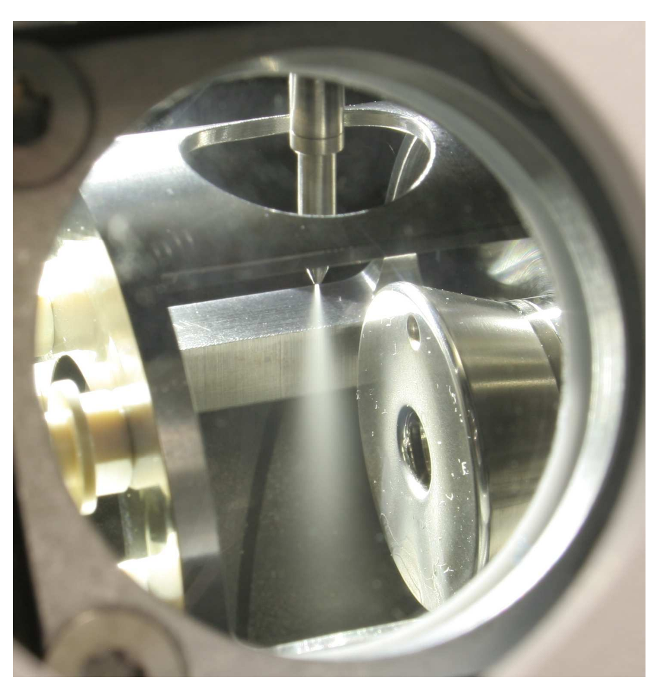
Figure 1-12: Illustration of Triple Quadrupole Mass Spectrometry.
This figure has been modified and redrawn(161)

The most common atmospheric pressure ionization techniques (API) in LC/MS/MS are electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo ionization (APPI). Both ESI and APCI provide high sensitivity and specificity for a wide range of analytes(163)

Electrospray ionization (ESI) is an ionization process whereby ions are formed from liquid phase droplets when sprayed through a small tube into a strong electrical field in the presence of a flow of warm gas (nitrogen) to assist in formation of ions Figure 1-13. As the solvent vaporizes, the droplets disappear, leaving highly charged analyte molecules. Nebulisation, desolvation and further evaporation are three important steps for transferring sample molecules from a liquid to gas phase in the presence of a flow of warm gas (nitrogen) to assist in the formation of ions (162;163).

The first step is nebulisation and charging the sample nebulises through a needle at ground potential surrounded with electrodes with a high voltage. The potential difference between the nebuliser and the electrode produces a strong

electric field that charges the surface of the sample droplets. In the second step, desolvation occurs by shrinking the charged droplets with a flow of heated drying nitrogen. The uncharged material will be carried out of the system. Ion evaporation further shrinks the droplets until the analyte ions are desorbed into a gas phase and then passed through the sample capillary into a low pressure region of the ion source on the mass analysed. (162;163)



(A)

Curtain Gas
Vacuum
1.Formation of Charged Droplets
3.Coulomb-explosion
High voltage
Mass Analyser
LC
Nebulizer Gas
2.Evaporation
Orifice
Curtain Gas

(B)

Figure 1-13: Electrospray Ionization (ESI) (A) and (B).
"(A) This media is in public domain Permission is granted (164)"

1.13 Aims and Objectives:

From the literature it is clear that there are many variables that must be considered when interpreting the presence of volatiles and alcohol biomarkers in medico-legal investigations. A better understanding of the stability of these analytes in samples collected from the living and at post-mortem, and an evaluation of their prevalence in different matrices will greatly aid interpretation. The aims of this study and how they will be achieved are listed below:

1. To investigate the prevalence of ethanol and other volatiles (methanol, acetone, isopropanol and n-propanol) in different matrices by:

    - Developing a dual-column HS-GC-FID method for the determination of ethanol and other volatiles in different biological matrices, involving the optimisation of GC conditions and the selection of a suitable internal standard;

    - Validating the developed method in accordance with ISO/IEC 17025;

    - Selecting and analysing post-mortem cases where femoral blood, urine, vitreous humour and bile samples are available to investigate the presence of volatiles in these matrices.

2. To investigation the stability of volatiles in blood under different conditions (temperature and preservative) by:

    - Analysing blank (volatile-free) blood spiked at different volatile concentrations, with or without preservative following storage at room temperature, in a refrigerator and a freezer;

    - Analysing preserved blood samples collected from individuals arrested under section 5 of the Road Traffic Act (1988) after storage at room temperature for a time span ranging from 1-5 years;

- Analysing paired preserved and unpreserved postmortem blood samples following storage in the refrigerator and freezer.

3. To investigate the role of beta-hydroxybutyrate (BHB) in diagnosing alcoholic ketoacidosis by:

    - Developing and validating a GC-MS method for the determination of BHB in blood in accordance with ISO/IEC 17025;

    - Quantifying BHB concentrations in postmortem blood samples and comparing these findings with case histories and incidence of alcoholic ketoacidosis (AKA);

    - Collating all cases on the Forensic Medicine and Science in-house database (GUFM) that have been analysed for BHB, ethanol and acetone and evaluate the relationship between these analytes, the case history, the cause of death and the potential role of AKA.

4. To investigate the role of fatty acid ethyl esters (FAEEs) as an indicator of foetal alcohol exposure by:

    - Developing and validating an LC-MS-MS method for the determination of FAEEs in accordance with ISO/IEC 17025;

    - Analysing meconium samples collected from babies born to mothers (some of whom were on a methadone maintenance programme) at the Princess Royal Maternity Hospital, Glasgow.

Chapter 2 - Evaluation of Common Volatiles in Post-Mortem Blood by HS-GC-FID

2.1 Introduction

Use of a dual-column system is advisable in forensic casework especially when analysing highly putrefied samples. This involves using two columns with different stationary phases, providing two sets of data and reducing the number of false positive results due to other post-mortem volatiles that may be eluting at the same retention time as the target analytes.(50) In addition, many forensic toxicology laboratories also offer blood alcohol analysis for road traffic cases where accuracy and precision are of higher importance than issues relating to co-elution of volatiles. A summary of published methods for the analysis of volatiles in biological matrices is summarised in Appendix 2-1.

The main aim of this study was to optimise the current FMS in-house method for the determination of ethanol, acetone, isopropanol, methanol and n-propanol in blood and other body fluids. Method development was achieved by improving separation of the volatiles through use of different temperature programmes; to evaluate use of two different internal standards (n-propanol and t-butanol) and different capillary column stationary phases. The developed method was validated and applied to the analysis of real case samples.

2.2 Materials and Methods

2.2.1 Statistical methods

Excel Windows 2003 data analysis was used to calculate average, median, standard deviations and regression. The chart wizard was also used for drawing histograms, pie charts and bar charts. Minitab software manufactured by Minitab, Inc.USA, version 15.1.2 and 15.1.3 and Minitab16 were obtained from the University of Glasgow Computing Services. Various statistical tests were used in this thesis including paired–t test, regression and correlation calculations, scatter plot and bestfit line plots.

The P value was also calculated to decide whether there is a significance difference between measured and standard amounts. A statistical test known as the significance test can be employed by testing the truth of the hypothesis that is known as null hypohesis (H_0). The term null is used to imply that there is no difference between two sets of data under test. If null hypothesis is rejected the difference between the two sets of data is real. Usually the null hypothesis is rejected when the probability of difference is less that 1 in 20 (i.e. 0.05 or 5%) in such cases the difference is said to be significant at 0.05 (or 5%) level. (165) The criteria used to assess the null hypothesis in normally distributed data:

Calculated value<Tabulated value	Accept null hypotheis
Calculated value $\geq$ Tabulated value	Reject null hypothesis

The tabulated t value can be found in t distribution tables in statistics books. Calculated value can be calculated manually as following:

In order to decide whether the difference between $\bar{x}$ (sample mean) and μ (true value or population mean) is significant to test the H_0. The statistic t is calculated, where s= sample standard deviation and n=sample size

$$t = \frac{\overline{x} - \mu_0}{s/\sqrt{n}},$$

In order to decide whether the difference between two sample means $\bar{x}_1$ and $\bar{x}_2$ is significant to test H_0: $\mu_1 = \mu_2$. The statistic t is calculated

$$t = \frac{\bar{X}_1 - \bar{X}_2}{S_{X_1X_2} \cdot \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

Paired t tests are used to test whether n paired results are drawn from the same population, that is H_0: $\mu_d = 0$, The statistic t is calculated. Paired t-test compare if there is a significant difference between the results obtained by two methods. The $\bar{x}_D$ and S_D indicate the mean and standard deviation respectively of D, the difference between paired values.

$$t = \frac{\overline{X}_D - \mu_0}{s_D/\sqrt{n}}.$$

2.2.2 Chemicals and Reagents

Ethanol 99.9% was purchased from Joseph Mills (Denaturants) Limited, Liverpool, methanol 99.8% from Sigma Aldrich, acetone 99.9% and n-propanol from BDH-VWR International, isopropanol and acetonitrile (HPLC grade) from VWR-PROLAB-BDH and t-butanol 99.8% (GC) from Fluka. Sodium chloride (NaCl) was purchased from BDH Chemical Ltd (Poole, England). Double distilled water was obtained from the in-house Millipore system.

2.2.3 Preparation of Blank (Volatile-Free) Blood

Expired packed red blood cell pouches were obtained from the Blood Bank at the Western Infirmary Hospital, Glasgow to be used for preparing quality control samples. The red blood cells were collected according to the United Kingdom Blood Services guidelines,(166;167) and were frozen on receipt within Forensic Medicine and Science (FMS). The volume of packed red blood cells was measured once fully defrosted and diluted 1:1 with 1% saline solution, which was prepared by adding 9.5g sodium chloride into a volumetric flask and dissolving in 1 litre of deionized water.

2.2.4 Preparation of Working Standards and Quality Controls

Internal standard working solutions were prepared for n-propanol and t-butanol at concentrations of 33 and 40 mg/dL respectively by preparing a 10 g/dL w/v standard of each and then by adding 3.3 and 4 mL to 1 litre of deionised water, respectively. Separate mixed volatile working standard solutions were prepared for use with each internal standard, both internal standards stored at room temperature for 1 month.

Table 2-1: Preparation of Mixed Working Standard Solutions

		Method 1	Method 2
Analyte	Final Concentration	Volume from Stock (10g/dL) in 100 mL water	
Ethanol	500 mg/dL	5	5
Methanol	500 mg/dL	5	5
Isopropanol	500 mg/dL	5	5
Acetone	500 mg/L	0.5	5
n-Propanol	500 mg/dL	N/A	5

Preparation of mixed volatile working standard for use with n-propanol: These solutions were prepared from stock standards (each at a concentration of 10 g/dL w/v) to contain 500 mg/dL of ethanol, isopropanol and methanol and 500 mg/L of acetone as summarised in Table 2-1 below. The calibration solutions were diluted from the freshly prepared working standard with deionized water. The final concentration of the calibration solutions were 10, 25, 50, 80, 100, 200, 300, 400, and 500 mg/dL for ethanol, isopropanol and methanol and the equivalent mg/L for acetone. The preparation of these calibration solutions is summarised in Table 2-2.

Table 2-2: Preparation of Calibration Standards

Calibrator Levels	Volume of working standards (mL)	Volume of Water (mL)	[Analytes] (mg/dL)	[Acetone] (mg/L)
BLANK	0	2	0	0
LEVEL 1	0.02	1.98	5	5
LEVEL 2	0.04	1.96	10	10
LEVEL 3	0.1	1.9	25	25
LEVEL 4	0.2	1.8	50	50
LEVEL 5	0.32	1.7	80	80
LEVEL 6	0.4	1.6	100	100
LEVEL 7	0.8	1.2	200	200
LEVEL 8	1.2	0.8	300	300
LEVEL 9	1.6	0.4	400	400
LEVEL 10	2	0	500	500

Preparation of mixed volatile working standard for use with t-butanol: prepared as described above but also including n-propanol at a concentration of 500 mg/dL as summarised in Table 2-1. The calibration solutions were also diluted as described above and their preparation summarised in Table 2-2

A second set of mixed volatile working standard solutions were prepared as described above to make the quality control (QC) samples. QC material was prepared in expired (volatile-free) blank blood screened negative for volatiles at concentrations of 30, 80, and 300 mg/dL for all volatiles except for acetone, which was spiked at 30, 80, and 300 mg/L. The quality control materials were aliquoted and transferred to 1.5 mL polypropylene tubes and stored at -22°C. Table 2-3 summarises the preparation of the control material.

Table 2-3: Preparation of QC Material

QC Points	Volume of working standards (mL)	Volume of blood (mL)	[Analyte] (mg/dL)	[Acetone] (mg/L)
QC 1	1.5	23.5	30	30
QC 2	4	21	80	80
QC 3	15	10	300	300

2.2.5 Post-Mortem Case Samples

One hundred and twenty one preserved post-mortem blood samples were selected where there was a history of decomposition as indicated in the post-mortem report or specimens stored within the laboratory were noted as putrefied. All preserved post-mortem blood samples were stored within recommended screw-capped vials containing 2 mg/mL of sodium fluoride and 3 mg/mL of potassium oxalate.

2.2.6 Instrumentation

HS-GC-FID analysis was carried out using a Thermo Trace GC 2000 series instrument supported with Thermo Co. Chromoquest software version 2.41. The instrument has dual column capability connected with a y-splitter and was fitted with DB-ALC1 (30 m x 0.53 mm id x 3 µm) and DB-ALC 2 (30 m x 0.53 mm id x 2

μm) supplied by J&W Scientific, Agilent technologies, USA. A Compudil 300 automatic diluter was used for sample preparation.

2.2.7 Optimisation of GC Conditions

The FMS in-house method for the analysis of ethanol and acetone using the Thermo Trace GC 2000 series achieved separation under isothermal conditions, with an oven temperature set at 60°C. In order to achieve optimum separation of all six volatiles, several GC program were investigated in combination with changes to the flow rate.

2.2.8 Sample Preparation

200 μL of blank blood, calibration standard, quality control (QC) or case sample was aspirated with 500 μL of internal standard solution using an automatic diluter. The mixture was dispensed into a 20 mL head-space vial, then capped and loaded onto the HS-GC-FID auto-sampler.

2.2.9 Method Validation

Two methods were validated; method 1 involved the use of n-propanol as an internal standard, while method 2 involved the use of t-butanol as an internal standard. As n-propanol was being used as the internal standard in method 1, it was only validated as a volatile of interest using t-butanol as an internal standard in method 2.

Linearity was assessed over a concentration range of 5-500 mg/dL for ethanol, methanol, n-propanol and isopropanol and from 5-500 mg/L for acetone. The limit of detection (LOD) and limit of quantification (LOQ) were evaluated by preparing samples and carrying out a serial dilution of the volatiles in duplicate Regression analysis was undertaken, and the LOD and LOQ were calculated in accordance with Miller and Miller.(168)

LOD was calculated by using Equations 1 and 2:

$$Y_{LOD}=Y_B+3SB \quad \text{Equation 1}$$

$$LOD = (Y_{LOD}-Y_B)/m \quad \text{Equation 2}$$

where YLOD is the standard error, YB is the intercept, and m is the gradient.

LLOQ values are calculated using the same method of LOD but using 10 times the standard error of the regression line. LOQ was calculated using Equations 3 and 4:

$$Y_{LOQ}=Y_B+10SB \quad \text{Equation 3}$$

$$LOQ= (Y_{LOG}-Y_B)/m \quad \text{Equation 4}$$

where YLOQ is the standard error, YB is the intercept, and m is the gradient .

Intra-day precision (within run precision) was determined at five independent preparations of spiked blood control samples at three different concentration levels (30, 80, and 300 mg/dL) for ethanol, isopropanol, methanol and n-propanol, and equivalent in mg/L for acetone. Controls were prepared in duplicate and analysed on the same day in the same analyser and same run. The percentage coefficient of variance was calculated (%CV), and acceptance criteria was set at <15%.(169)

Inter-day precision (between day precision) was determined at eight independent preparations of spiked blood and urine control samples at three different levels (30, 80, and 300 mg/dL) for ethanol, isopropanol, methanol and n-propanol, and equivalent mg/L for acetone. Controls were prepared in duplicate and analysed over eight days. The percentage coefficient of variance was calculated (%CV), and acceptance criteria was set at < 15%.(169)

The recovery and accuracy of the methods were assessed at concentrations of 30, 80, and 300 mg/dL (n=5) for ethanol, isopropanol, methanol, and n-propanol equivalent in mg/L for acetone. Recovery was assessed by calculating the percentage of the ratio of the measured value of spiked blood to the measured value in water. The accuracy was assessed by calculating the percentage of the ratio of measured value of the spiked blood to the expected theoretical value.

2.3 Results and Discussion

2.3.1 Optimisation of GC Conditions

Table 2-4 summarises the different GC conditions used to optimise the separation of the volatiles in both capillary columns.

Table 2-4: Summary of GC Conditions Used to Optimise Separation of Volatiles

Conditions	(1)		(2)		(3)		(4)		(5)		Final	
	RD	LD	RD	LD	RD	LD	RD	LD	RD	LD	RD	LD
Oven Temperature (˚C)	60	60	40	40	40	40	40	40	40	40	40	40
Right inlet Base Temperature (˚C)	200	200	200	200	200	200	200	200	200	200	200	200
Hydrogen Flow (mL/min)	35	35	35	35	35	30	30	35	30	35	30	35
Air Flow (mL/min)	350	350	350	350	350	300	300	350	300	350	300	350
Right and Left FID Base temperature (˚C)	250	250	250	200	250	250	250	250	150	150	250	250
Split injection mode flow rate (mL/min)	48	48	24	24	20	20	20	20	24	24	24	24
Carrier gas flow rate(mL/min)	16	16	12	12	10	10	10	10	12	12	8	8
Run Time (min)	6	6	8	8	9	9	10	10	9	9	9	9

***RD-Right Detector and LD-Left Detector**

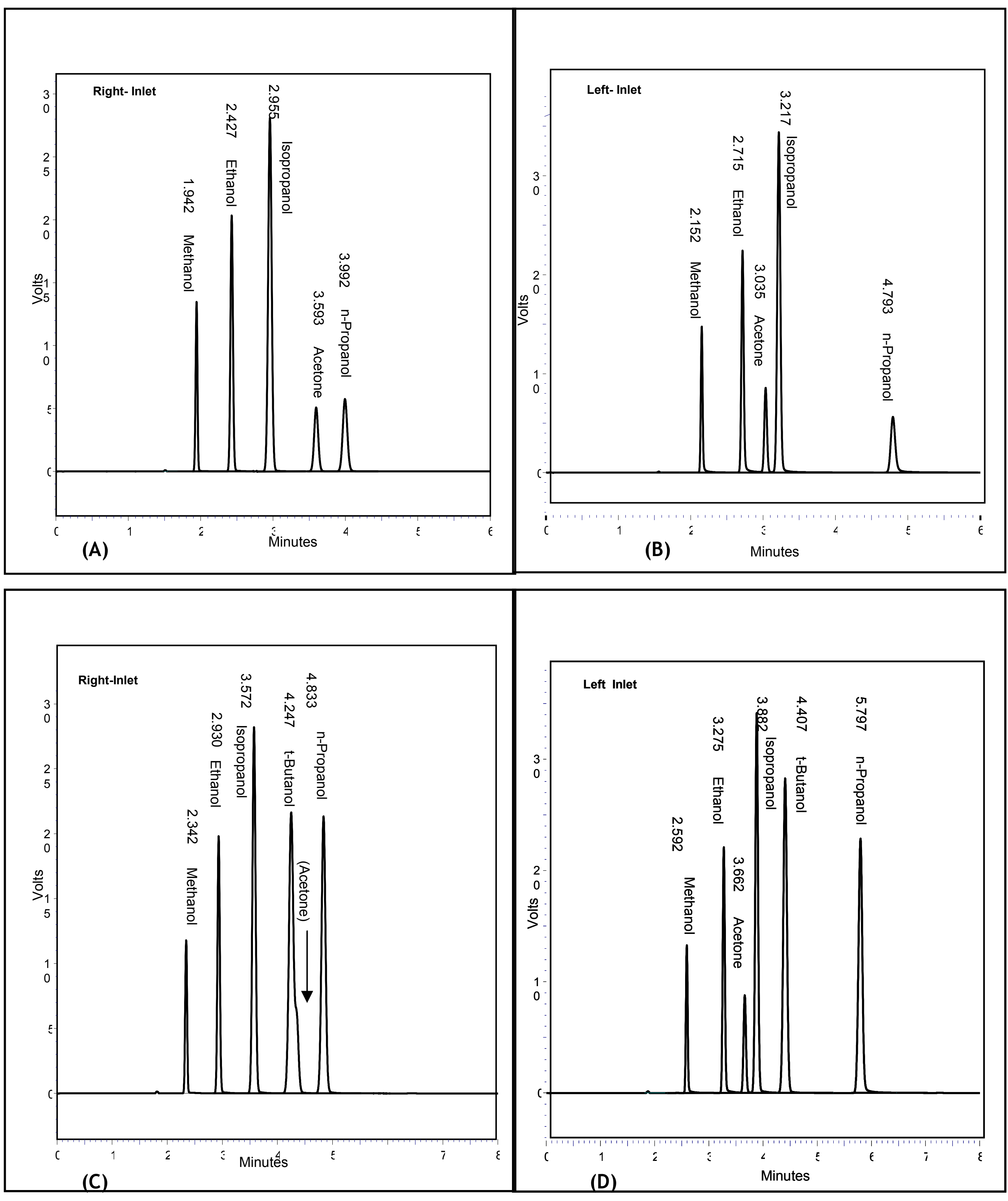


Figure 2-1: Separation of Volatiles by HS-GC-FID

Figure 2-1 (A-D) illustrates the separation achieved for each of the volatiles of interest in a standard alcohol mixture (water spiked at 300 mg/dL or mg/L for acetone) using HS-GC-FID and the final conditions listed in Table 2-4. (A) and (B) are chromatograms of the right and the left inlet respectively using n-propanol as

an internal standard (method 1). (C) and (D) are chromatograms of the right and left inlet respectively using t-butanol as an internal standard (method 2). (A) and (C) were connected to the right inlet with DB-ALC1 while (B) and (D) were connected to the left inlet with DB-ALC2.

Good separation was achieved when using n-propanol as an internal standard using DB-ALC1 (Figure 2-1A) and DB-ALC2 (Figure 2-1B). Co-elution of t-butanol and acetone could not be resolved with DB-ALC-1 (as illustrated in Figure 2-1C) even after adapting the oven and base temperatures, gas and split flow rates and trying numerous combinations, as summarised in Table 2-4.

From the literature, it has been reported that separation of the volatile mixtures including t-butanol and acetone can be achieved using RTX-BAC2 (80;95) (30m x 0.53mm x 2um) and DB-ALC2(170)(see Figure 2-1D) as they are similar stationary phases. Separation on RTX-BAC1 (30m x 0.53mm x 3um) was not indicated. Although a similar stationary phase to DB-ALC1, the RTX-BAC1 was investigated to see if separation of t-butanol and acetone could be achieved, as expected, resolution of t-butanol and acetone was not achieved with RTX-BAC1. Separation of t-butanol and acetone has been reported using carbowax,(54) in a 6-foot x 1/4 inch OD glass-packed column.(54)

Acetonitrile was also included at the start of the project to assess its potential as an alternative internal standard but as it also co-eluted with acetone and so no further investigation was undertaken.

The optimised GC parameters are summarised in Table 2-5. The only difference (indicated * in Table 2-5) was in relation to the carrier gas flow which was set at 10 mL/min for method 1 (n-propanol) and 8 mL/min for method 2 (t-butanol). This resulted in a slightly different run time of 6 and 9 minutes respectively.

Table 2-5: Optimised GC Parameters used to Evaluate Methods 1 and 2

Conditions	Column 1	Column 2
GC Parameters	DB-ALC1 (30m x 0.53 mm id x 3µm)	DB-ALC 2 (30m x 0.53 mm id x 2µm)
	Right Detector	Left Detector
Oven Temperature(˚C)	40ºC	40ºC
Right Inlet Base Temperature(˚C)	200	200
Hydrogen Flow (mL/min)	30	35
Air Flow (mL/min)	300	350
Right and Left FID Base temperature(˚C)	250	250
Post temperature(˚C)/time(minute)	120 for 1 min.	120 for 1 min
Split/Splitless mode	Split	Split
Split injection mode flow rate (mL/min)	24	24
Incubation temperature(˚C) and time(minute)	60 for 4 min.	
Carrier gas flow rate(mL/min)	10* 8	10* 8
Injection Volume (mL)	2	2
Run Time (min)	6* 9	6* 9

2.3.2 Method Validation

The correlation coefficients (R^2) were >0.999 for ethanol, isopropanol, methanol, acetone, and n-propanol over the linear range of 10–500 mg/dL (mg/L for acetone) using either internal standard, as demonstrated in (Figure 2-2).

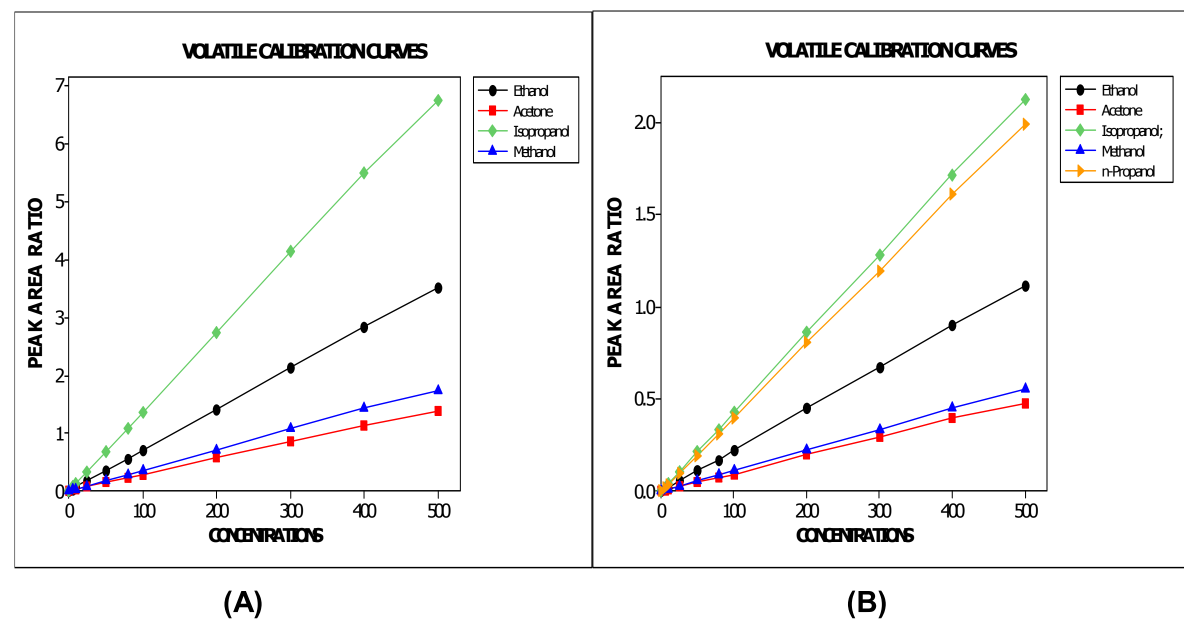


Figure 2-2: Volatile Calibration Curves
(A) Ethanol, acetone, isopropanol, and methanol using n-propanol as an internal standard (Method 1). (B) Ethanol, acetone, isopropanol, methanol and n-propanol using t-butanol as an internal standard (method 2).

The LOD for both methods 1 and 2 for ethanol, isopropanol and methanol was 1 mg/dL and 1 mg/L for acetone for both methods. The LOQ for all volatiles was slightly lower when using t-butanol as an internal standard as summarised in Table 2-6.

Table 2-6: LOD and LOQ for Validated Methods

Method	Ethanol mg/dL		Acetone mg/L		Isopropanol mg/dL		Methanol mg/dL		n-propanol mg/dL	
	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ
Method 1 (n-propanol)	1	3	1	4	1	3	1	4	N/A	
Method 2 (t-butanol)	1	2	1	3	1	2	1	2	1	2

When analysing case samples, results less than 10 mg/dL for ethanol and less than 10 mg/L for acetone were reported as negative, in accordance with FMS reporting criteria. Results of isopropanol, methanol, and n-propanol that were less than the LOQ were reported as negative. Determinations greater than the highest calibration point were reported as present >500 for all analytes. A cut-off of 10 mg/dL was not applied to isopropanol, methanol, and n-propanol to allow a

thorough evaluation of the expected concentration range of these analytes in the post-mortem blood.

The intra- and inter-day precision ranged from 1-5% in blood for both methods as summarised in Table 2-7. In general, the %CV when using n-propanol as an internal standard, was higher compared with using t-butanol as an internal standard.

Table 2-7: Intra-day and Inter-day Precision for Volatiles in Blood

Analyte	Internal Standard	30		80		300	
		Intraday %CV (n=5)	Interday %CV (n=8)	Intraday %CV (n=5)	Interday %CV (n=8)	Intraday % CV (n=5)	Interday %CV (n=8)
Ethanol	n-propanol	3.17	2.51	3.23	3.34	2.08	3.81
	t-butanol	2.32	1.56	0.77	3.10	1.25	1.15
Acetone	n-propanol	3.92	3.22	3.91	3.98	1.93	3.86
	t-butanol	3.41	4.49	1.96	2.56	2.81	1.04
Isopropanol	n-propanol	3.28	2.64	3.39	3.46	1.97	2.87
	t-butanol	2.63	2.07	1.04	2.48	1.95	1.04
Methanol	n-propanol	3.48	2.66	3.32	3.41	2.28	4.81
	t-butanol	2.46	2.53	0.80	3.65	1.25	1.23
n-propanol	t-butanol	2.50	1.22	0.83	2.68	1.64	1.07

*** The unit is mg/dL for all except acetone in mg/L**

The average recovery for spiked blood controls (30, 80, and 300 mg/dL and equivalent mg/L in acetone) was 99-107% for method 1 (n-propanol) and 99-109% for method 2 (t-butanol). Corresponding accuracy ranged from 95-98% and 95-101%, for method 1 and 2 respectively. Table 2-8 contains a full list of the recovery and accuracy data for each volatile by method.

Table 2-8: Recovery and Accuracy for Methods 1 and 2 in Blood

	Method (2) t-butanol (%)						Method (1) n-propanol (%)			
BLOOD (N=5)	**Control mg/dL or mg/L**	**EtOH**	**ACET**	**IPOL**	**MeOH**	**N-PROP**	**EtOH**	**ACET**	**IPOL**	**MeOH**
Recovery	30	99	104	99	99	99	99	104	99	99
Accuracy	30	98	96	97	98	98	98	96	97	98
Recovery	80	102	107	102	101	101	102	107	102	101
Accuracy	80	95	95	95	96	96	95	95	95	96
Recovery	300	105	109	103	106	94	105	109	103	106
Accuracy	300	95	97	97	95	101	95	97	97	95

Although the validation data indicated that t-butanol was marginally better with respect to accuracy and precision compared with n-propanol as an internal standard, both methods were acceptable for the analysis of volatiles in blood.

2.3.3 Prevalence of Volatiles in Post-Mortem Blood

Post-mortem blood samples (n=121) were tested using both methods to evaluate the prevalence of each volatile and to compare the results to assess the effect of n-propanol present due to post-mortem putrefaction.

Ethanol was present in 63 samples with concentrations ranging from 10 – 397 mg/dL (see Table 2-9) and acetone in 46 samples with concentrations ranging from 10 – 443 mg/L (see Table 2-10). Methanol and isopropanol were not identified in any of the samples tested and n-propanol was present in all samples at concentrations ranging from 2 – 14 mg/dL.

A total of 7 samples were mismatched for ethanol when tested by both methods and are highlighted in Table 2-9 and they are in the range from 10-20 mg/dL, while ten samples were mismatched for acetone and are highlighted in Table 2-8 and they are with in the range of 10-12 mg/L. All mismatched results for ethanol and acetone are close to the cut-offs established provide likely explanation for the contradictory result.

Table 2-9: Ethanol Concentrations Measured in Post-Mortem Blood by two Methods

(Methods1=n-propanol; Method2=t-butanol)

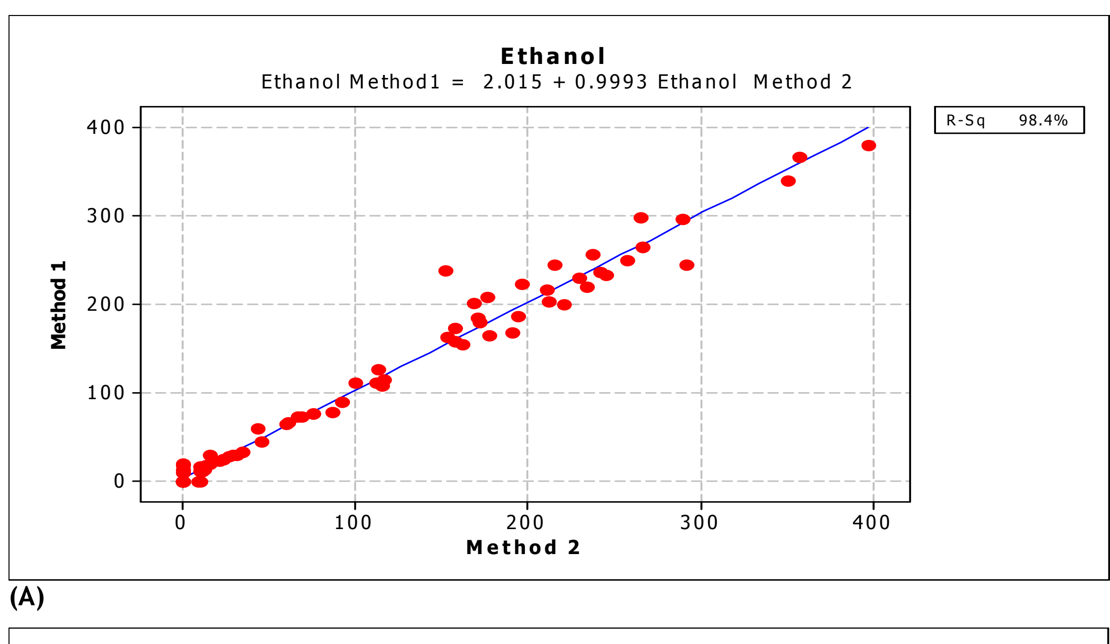
Ethanol (mg/dL)								
Case Number	Method (1)	Method (2)	Case Number	Method (1)	Method (2)	Case Number	Method (1)	Method (2)
1	110	100	38	76	75	78	155	162
2	12	10	39	65	60	82	202	212
3	10	NEG	40	10	NEG	83	250	257
5	208	177	43	16	11	86	11	11
7	13	NEG	45	29	17	87	340	349
8	297	264	46	20	NEG	93	245	291
9	NEG	10	47	17	NEG	95	236	241
10	73	67	50	200	169	96	230	229
13	60	44	51	73	70	97	265	266
14	65	61	52	89	92	99	220	234
15	365	357	54	157	157	101	45	46
17	173	158	55	162	153	102	28	28
18	24	23	56	216	210	103	114	117
20	125	113	58	12	11	109	111	112
21	30	29	59	233	245	110	78	87
26	179	172	61	167	191	113	237	152
27	223	196	62	30	32	115	NEG	11
28	18	13	64	186	194	116	165	178
29	19	16	67	296	289	117	380	397
31	244	216	68	22	22	118	107	115
33	257	237	72	199	221	121	33	35
37	12	13	74	185	171			

Table 2-10: Acetone Concentrations Measured in Post-Mortem Blood two Methods

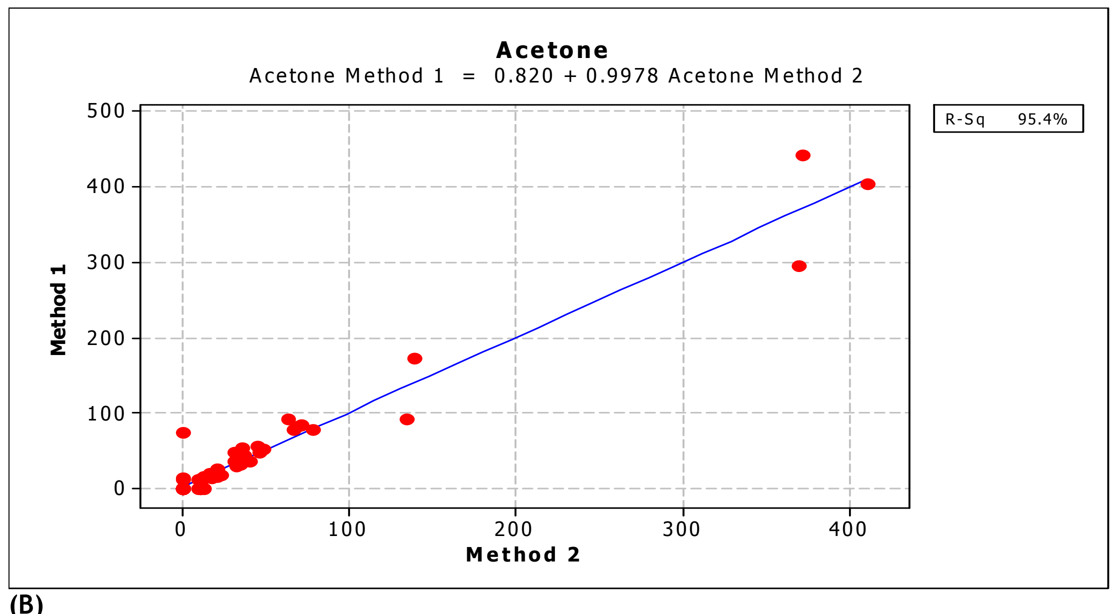
Acetone (mg/L)								
Case Number	Method (1)	Method (2)	Case Number	Method (1)	Method (2)	Case Number	Method (1)	Method (2)
1	NEG	13	41	NEG	11	83	16	13
7	14	11	42	NEG	10	86	25	21
9	405	411	45	296	369	88	21	21
13	74	59	46	92	134	91	53	36
14	NEG	11	58	443	372	97	35	40
21	17	24	60	12	NEG	100	16	13
22	48	31	62	29	33	102	12	10
25	NEG	11	64	NEG	10	103	20	17
26	13	17	65	NEG	11	107	13	12
31	NEG	11	69	20	21	109	32	34
32	83	72	70	11	12	110	173	139
33	NEG	11	71	51	48	111	79	67
34	36	31	74	11	12	115	48	46
36	17	18	75	93	64	118	44	38
37	17	21	76	55	45	121	82	70
			81	78	79			

The correlation coefficient (R^2) for ethanol was 0.984 (P = 0.084) and 0.954 (P = 0.536) for acetone. As the P values are greater than 0.05 for both ethanol and acetone this indicates that there is no significant difference between the two sets of results.

Figure 2-3 illustrates the correlation between post-mortem blood volatile concentrations for ethanol (A) and acetone (B) when measured using n-propanol as an internal standard (Result 1) compared with t-butanol (Result 2).



(A)



(B)

Figure 2-3: Correlation of Ethanol (A) and Acetone (B) concentrations in post-mortem blood using n-propanol (Method 1) versus t-butanol (Method 2) as an Internal Standard.

Looking more closely at the levels of n-propanol found in post-mortem blood, the majority of samples were positive at concentrations close to the LOQ of 2 mg/dL. Six cases had elevated n-propanol concentrations ranging from 5 – 14 mg/dL as summarised in Table 2-7.

Table 2-11: Elevated Post-mortem Blood n-propanol Concentrations.

Case Number	Ethanol (n-propanol)	Ethanol (t-butanol)	n-propanol (t-butanol)	Ethanol: n-propanol Ratio
18	24	23	**5**	5
21	30	29	**5**	6
26	179	172	**5**	34
72	199	221	**9**	25
93	245	291	**14**	21
110	78	87	**11**	8

It has been reported that n-propanol is produced in larger quantities than other volatiles and can be detected in postmortem cases when ethanol production is suspected. n-propanol concentrations continue to increase even when ethanol concentrations have peaked and start to decline. The reason being that n-propanol can be produced postmortem from fermentation of amino acids, glycerol, fatty acids and could last until the advanced stage of putrefaction.(53)

It is expected that the presence of n-propanol in putrefied post-mortem blood samples will increase the peak area of n-propanol when used as an internal standard and this may lead to an underestimate in the calculation of ethanol and other volatile concentrations. This is certainly the case with the 3 samples (# 72, #93 and #110) in Table 2-7 with higher concentrations of n-propanol. The ethanol concentrations are higher by between 11 and 19% when using t-butanol compared with n-propanol as an internal standard. However, interpretation of the ethanol concentrations in all six cases would not be affected by the presence of n-propanol due to the putrefaction process.

The concentrations in this study are much lower than those reported in previous studies, where n-propanol was present in post-mortem blood at concentrations ranging from 30 - 70 mg/L. (60;83) However, it should also be noted that there is no mention in these publications regarding validation of the methods used to quantify n-propanol.

Nanikawa and Kotoku(171), Nanikawa *et al.*(172) and Moriya and Hashimoto(97) have considered n-propanol as an indicator of post-mortem ethanol production and have evaluated the ratio of n-propanol to ethanol in post-mortem blood to identify ethanol formation. In these studies, n-propanol was detected in all cases of suspected post-mortem ethanol production. Nanikawa and Kotoku(171) also suggested that the antemortem ethanol concentration could be estimated from the postmortem n-propanol concentration. The authors state that in previous studies they found that the potential range of ethanol produced postmortem was 20 times the amount of n-propanol produced.

Moriya and Hashimoto(97) concluded that n-propanol cannot be used as an internal standard for post-mortem blood samples unless a screen has first been conducted to confirm its absence from the specimen.

Ziavrou *et al.*(173) suggested that due to significant variability, no valid estimation of antemortem ethanol production can be made from the postmortem n-propanol and ethanol concentrations. In another study of the relationship between ethanol and n-propanol in 62 post-mortem cases, only 17 of the cases samples had a detectable level of n-propanol. This finding is in variance with Nanikawa *et al.*(172) whose study indicated that n-propanol could be used as an indicator for ethanol formation and further showed that all positive results for ethanol were also positive for n-propanol. (83)

The ratios were calculated for the cases with elevated n-propanol concentrations in this study and are summarised in Table 2-7. The ratios range from 5 to 34 but with conflicting evidence on the suitability of this ratio for estimating post-mortem production of ethanol, no further conclusions can be made.

Although studies have concluded that n-propanol is produced in all specimens along with ethanol(171;172;174), it may in fact be detected in only a small percentage of postmortem cases. Further, Schuberth(99) and Kronert *et al.*(175) identified n-propanol in living subjects along with other common volatiles at concentrations below the limit of quantitation (10 mg/dL) utilised by many forensic toxicology laboratories today.

The majority of samples in this study had n-propanol concentrations below 5 mg/dL and must be interpreted with caution as the presence of n-propanol in these cases may or may not be as a result of post-mortem production.

2.4 Conclusion

Two methods have been developed and validated for the quantitative analysis of common volatiles (methanol, ethanol, isopropanol, n-propanol and acetone) utilising dual-column HS-GC-FID and two internal standards, namely n-propanol and t-butanol. Both methods demonstrated excellent linearity, precision and accuracy. The analysis of real case samples to evaluate the meaning of the presence of n-propanol in putrefied blood samples found no significant effect with levels in this study much lower than previously reported in the literature. The correlation between both methods was excellent and although the presence of elevated levels of n-propanol resulted in lower ethanol concentrations, the decreases had no impact on the interpretation of ethanol in the cases.

It is however recognised that this study looked at a relatively small data set (N=121) and that a much larger study is required to investigate the prevalence of n-propanol in putrefied post-mortem blood samples. The findings in this study do not support the findings reported by other authors that n-propanol should not be used as an internal standard. n-propanol is an ideal internal standard for use with the dual-column system used in this study and when applied to the analysis of ethanol in blood from living subjects, e.g. road traffic arrests. Although using n-propanol as an internal standard in post-mortem cases may result in an underestimate of the concentration of other volatiles, the risk associated with this appears to be minimal with the majority of samples in this study having n-propanol concentrations close to the LOQ and therefore negligible. It is

impractical to consider screening all post-mortem blood samples for the presence of n-propanol prior to its use as an internal standard, as has been suggested by other authors, but further investigations are required into the prevalence and range of concentrations of n-propanol to fully assess the risks associated with the use of this volatile as an internal standard to quantify volatiles in post-mortem blood.

The use of ethanol to n-propanol ratio to determine whether ethanol formation has occurred is unlikely to be an effective indicator and other criteria must be considered, including the case history, condition of the specimens, distribution of ethanol in different matrices, presence or absence of ethanol in different specimens of the case sample, presence of volatile compounds and the concentration of ethanol. (83)

The internal standard t-butanol offers an effective alternative internal standard for the quantification of volatiles in post-mortem blood, including the ability to measure n-propanol. However, co-elution with acetone when using DB-ALC1 or RTX-ALC1 rules out the use of this internal standard with these types of columns. It has also been reported that the level of t-butanol as an internal standard may decrease when the fat content of the sample analysed increases. The fat content, as well as protein and salt, may affect the accuracy of the head-space method. Increased concentrations of ethanol may be a consequence of the stronger affinity of isobutyl alcohol when used as an internal standard for fatty aqueous slurry. The peak height of t-butanol decreases as the amount of fat in the sample analysed is increased.(94)

Chapter 3 - Application of a Validated Method for the Analysis of Volatiles in Biological Matrices

3.1 Introduction

Following validation of a method for the analysis of common volatiles (ethanol, methanol, isopropanol, n-propanol and acetone) with t-butanol as an internal standard (detailed in Chapter 2), the method was utilized to investigate:

- The stability of volatiles in blood;
- The prevalence and correlation of volatiles measured in different biological matrices;
- The evaluation of a commercially available enzymatic assay for screening ethanol in blood.

The aims of the stability study were three-fold; to evaluate the effect of preservative and antioxidant on the stability of volatiles at different concentrations during short-term storage at different temperature conditions; to evaluate the stability of ethanol and acetone in paired preserved and unpreserved post-mortem blood samples, and to evaluate the stability of ethanol in preserved blood after long-term storage at room temperature.

The aims of the study involving the analysis of volatiles in different biological matrices were; to assess the prevalence of volatiles in femoral blood, vitreous humour, urine and bile and to evaluate the correlation between the different matrices for ethanol and acetone.

The final study aim was to evaluate the performance of the enzymatic assay manufactured by Immunalysis Corporation as a semi-quantitative screening method for ethanol in blood, in comparison to the validated method utilising HS-GC-FID.

3.2 The Effect of Preservative and Antioxidant Reagents on the Stability of Volatiles in Blood

3.2.1 Introduction

An evaluation of the stability of common volatiles in blood was carried out by spiking blood at two concentrations with ethanol, isopropanol, acetone, methanol and n-propanol and adding different amounts of preservative and antioxidant. Spiked samples were stored at room temperature (25±2°C), refrigerated (4±2°C), and frozen (-22±2°C). Tests were carried out at regular time intervals over a period of 50 days.

3.2.2 Sample Preparation

Two 250 mL class A volumetric flasks were labelled (A) and (B). To flask (A), 0.5 mL of each volatile stock standard (10% w/v) and 0.05 mL of acetone stock standard (10% w/v) were added for a final concentration of 20 mg/dL for ethanol, isopropanol, n-propanol and methanol, and 20 mg/L for acetone in blood.

To Flask (B), 2 mL of each each volatile stock standard (10% w/v) and 0.2 mL of acetone stock standard (10% w/v) were added for a final concentration of 80 mg/dL for ethanol, isopropanol, n-propanol and methanol, and 80 mg/L for acetone. Each flask was further sub-divided into five 50 mL class A volumetric flasks. The flasks were labeled as groups 1, 2, 3, 4, and 5, containing varying amounts of preservative (sodium fluoride) and antioxidant (sodium metabisulfite) as summarised in Table 3-1.

Table 3-1: Preservative and Antioxidant Concentrations by Group

Groups	PRESERVATIVE (Sodium Fluoride)	ANTIOXIDANT (Sodium Metabisulfite)
GROUP 1	NO	NO
GROUP 2	0.2%	NO
GROUP 3	0.2%	0.2%
GROUP 4	1%	NO
GROUP 5	1%	0.2%

The concentration of sodium fluoride (NaF) at 0.2% was selected to be representative of the commercially available specimen containers submitted to Forensic Medicine and Science (FMS) for blood alcohol analysis. From each group, 1 mL aliquots were placed into labeled 1.5 mL polystyrene screw cap tubes. The aliquots for each group were stored under three different conditions; at room temperature (RT) at 25±2 °C, in the refrigerator at 4±2°C, and in the freezer at -22 ±2°C as illustrated in Figure 3-1. Temperatures were monitored by using a certified calibrated thermometer.

Stability Study Design

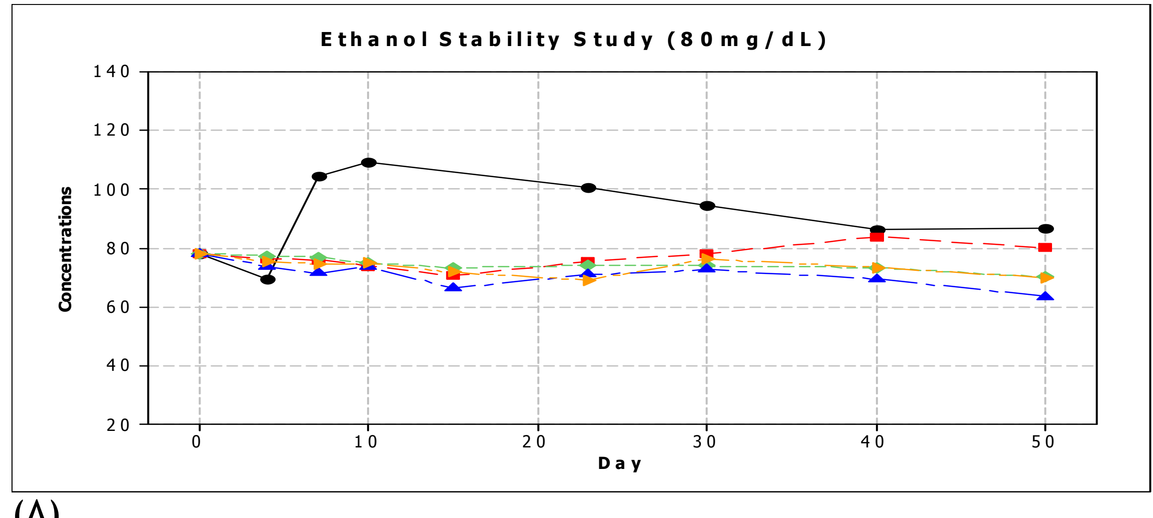
20 and 80 mg/dL for all in mg/L for acetone		
Groups	NaF	ANTIOXIDANT
G 1	NO	NO
G 2	0.2%	NO
G 3	0.2%	0.2%
G 4	1%	NO
G 5	1%	0.2%

20 mg/dL
80 mg/dL
250
250
DAY
[1] [4] [7] [10]
[15] [23] [30]
[40] [50]
50 mL
50 mL
G1 G2 G3 G4 G5
G1 G2 G3 G4 G5
-22±2°C
RT@25±2°C
4±2°C

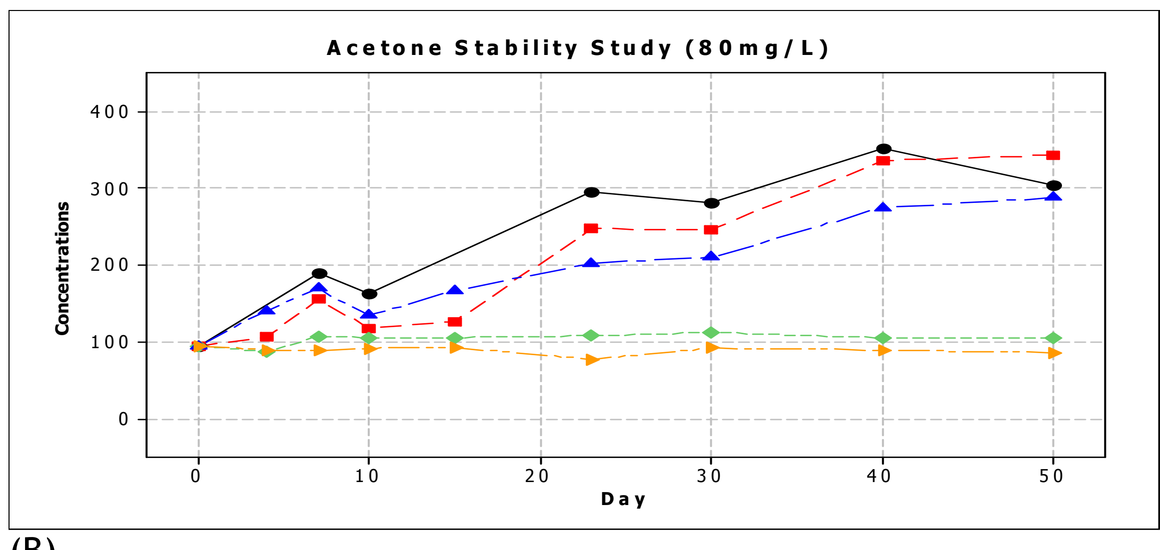
Figure 3-1: Stability Study Design and Spiked Sample Preparation Guide

3.2.3 Results and Discussion

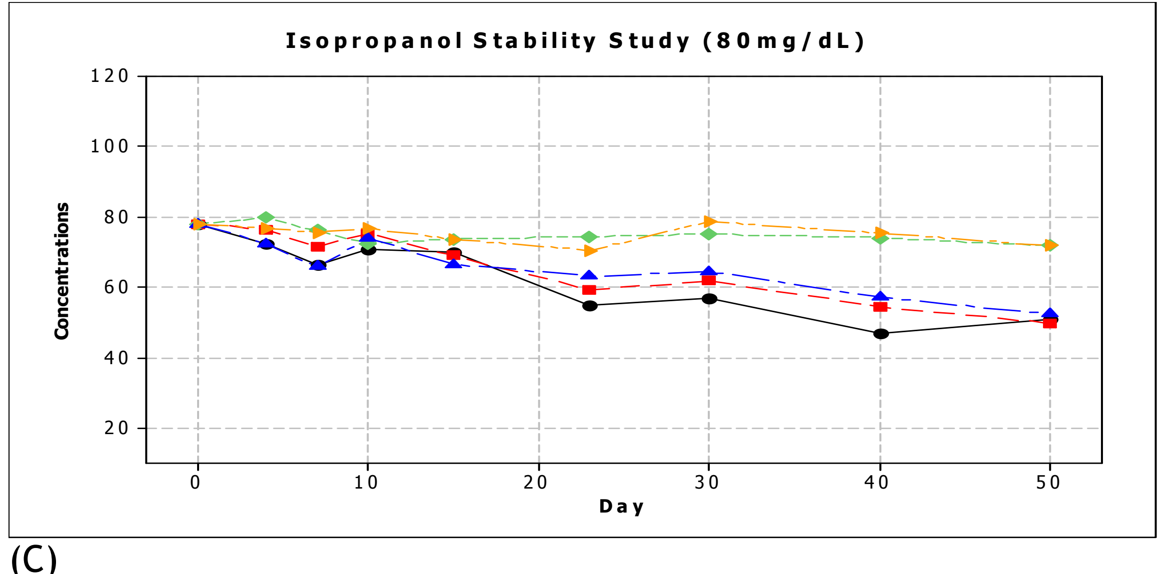
Figure 3-2 illustrates the changes observed in volatile concentrations for blood spiked at 80 mg/dL (mg/L for acetone) and stored at room temperature (25±2˚C).



(A)

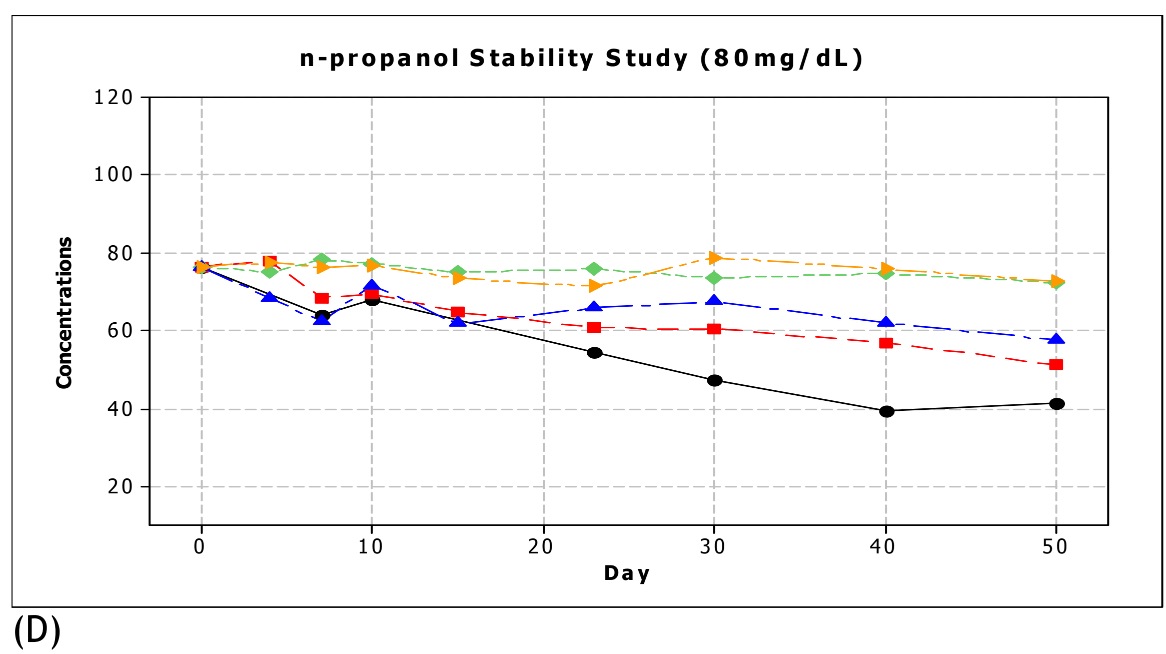


(B)

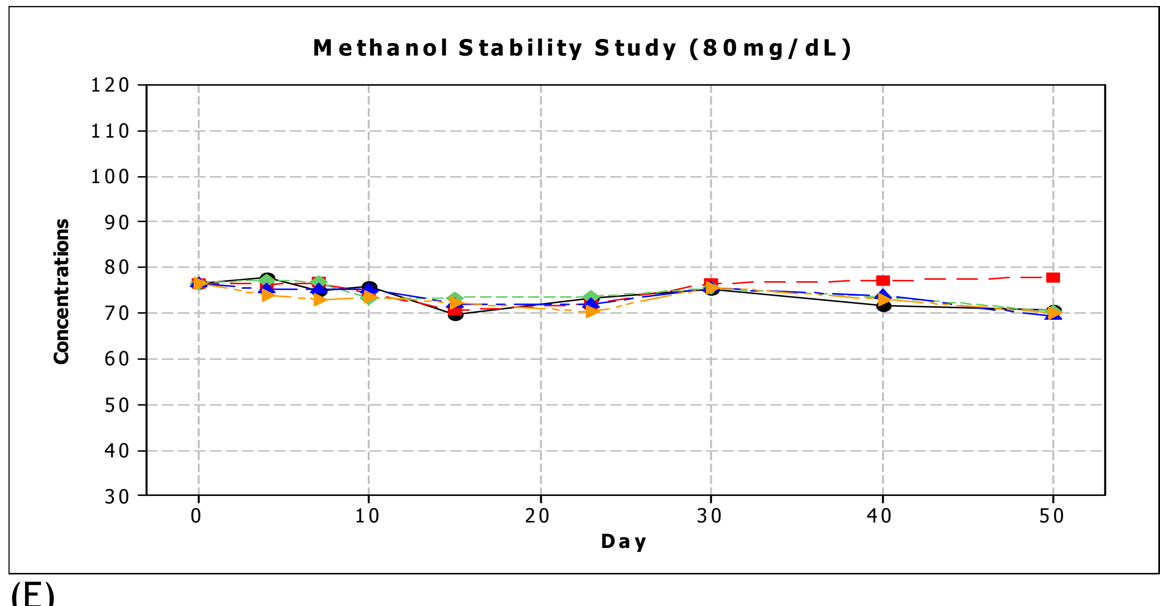


(C)

(A) Ethanol, (B) Acetone, (C) Isopropanol related to figure 3-2 continued next page
Stability of Volatiles Spiked in Blood at Room Temperature (25 ±2°C)



(D)



(E)

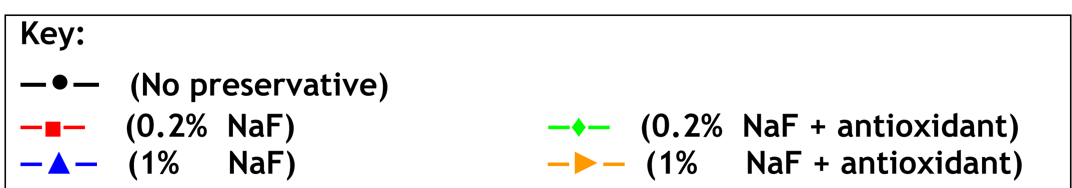


Figure 3-2: Stability of Volatiles Spiked in Blood at Room Temperature (25 ±2°C) (Spiked at 80 mg/dL and equivalent mg/L for acetone for (A) Ethanol, (B) Acetone, (C) Isopropanol, (D) n-propanol and (E) Methanol).

The corresponding figures and tables summarizing the changes observed in volatile concentrations for blood spiked at 20 and 80 mg/dL (mg/L for acetone) following storage at different temperatures are summarized in Appendix 3-1.

Ethanol levels initially increased and then decreased when stored in the refrigerator or at room temperature with or without preservative. The observed increase was greater at room temperature when no preservative or antioxidant was present as illustrated in Figure 3-2 A. The subsequent ethanol decrease is most likely due to oxidation of ethanol to acetaldehyde. However, the improved stability of ethanol in the presence of the sodium fluoride preservative was unaffected by the presence or absence of the antioxidant sodium metabisulphite.

At room temperature, acetone concentrations increased and isopropanol levels decreased from the start of the stability study for all groups. This inversely proportional relationship between acetone and isopropanol is most likely explained by the oxidation of isopropanol to acetone. This is further supported by the less-pronounced increase in acetone concentrations for the samples containing the antioxidant, sodium metabisulphite as illustrated in Figures 3-2 B and 3-2 C.

n-Propanol mirrored the same profile as that observed with isopropanol, namely a decreasing concentration as seen in Figure 3-2D. The decrease was less pronounced when the samples were stored in the refrigerator or freezer and when both sodium fluoride and sodium metabisulphite were present.

Methanol was stable under all conditions and was the only volatile of those studied whose stability was unaffected by temperature, preservative or antioxidant as illustrated in Figure 3-2 E.

All volatiles were stable in blood over the study period of 50 days when stored in the freezer (-22±2°C) whether preservative or antioxidant was present or not. Samples spiked at 20 mg/dL (mg/L for acetone) had a similar pattern to samples spiked at 80 mg/dL (mg/L for acetone).

The stability study was also carried out using method 1 (n-propanol as an internal standard and not an analyte of interest). Similar results were also observed and are summarized in Appendix 3-2.

3.3 Stability of Ethanol and Acetone in Paired Preserved and Unpreserved Post-Mortem Blood

3.3.1 Introduction

Post-mortem examinations carried out in the West of Scotland requiring toxicological investigation are routinely tested for alcohol and drugs. Femoral blood samples are collected and submitted as both preserved and unpreserved samples. The preserved samples are stored within screw cap vials containing 2 mg/mL of sodium fluoride and 3 mg/mL of potassium oxalate. Following submission to the toxicology laboratory within FMS, the preserved sample is tested for alcohol and may also be tested for known labile drugs, e.g. cocaine, while the unpreserved blood sample is tested for a wide range of prescription and illicit drugs. Samples are stored in the refrigerator for approximately 1 to 2 months while investigations are on-going before being moved to a freezer for long-term storage.

The analysis of paired preserved and unpreserved post-mortem samples from a range of cases will provide an insight into the role of the preservative on the stability of ethanol and acetone in real cases.

3.3.2 Methodology

Seventy-one paired preserved and unpreserved post-mortem blood samples were re-analyzed after storage in the refrigerator for six months and a further 10 months following storage at -22 ±1°C. The selection criteria required paired preserved and unpreserved post-mortem blood, stored in the refrigerator for 6 months with a selection of cases that were positive and negative for ethanol in post-mortem blood.

The initial analysis was carried out by FMS Technical Staff in accordance with the ISO/IEC 17025 accredited method for the analysis of ethanol in post-mortem blood and urine. Acetone was quantified in these cases using a positive control and the calibration curve for ethanol which was linear over the range of 0-400 mg/dL.

The first re-test of the preserved blood and paired unpreserved blood six months later was carried out using the validated method described in Chapter 2 utilising n-propanol as internal standard. The second re-test after a further 10 months was carried out using the validated method described in Chapter 2 utilising t-butanol as internal standard including n-propanol as an analyte.

3.3.3 Results and Discussion

Table 3-2 and 3-3 summarizes the results of the paired samples positive for ethanol and/or acetone. All samples with volatile concentrations lower than 10 mg/dL (or 10mg/L for acetone) were classified as negative. A total of ten cases were negative for all volatiles. The stability of methanol, isopropanol and n-propanol could not be assessed as too few samples were positive for these volatiles. Four cases were positive for methanol, two for isopropanol and two for n-propanol at low concentrations ranging from 11 to 29 mg/dL.

The results for the original test of the preserved blood sample (OP) are included for ethanol and acetone along with subsequent analysis (after 6 months refrigeration) of both preserved and unpreserved blood (P1, UP1) and re-analysis (after a further 10 months in the freezer) of both the preserved and unpreserved blood (P2, UP2).

Table 3-2: Ethanol Concentrations in Paired Post-Mortem Blood Samples.

Ethanol (mg/dL)											
Case Number	OP	P1	P2	UP1	UP2	Case Number	OP	P1	P2	UP1	UP2
1	158	73	70	141	120	**37**	374	340	349	412	355
2	136	89	92	118	131	**38**	N	N	N	N	N
3	N	N	N	11	10	**39**	N	N	N	11	11
4	186	157	157	128	142	**41**	N	N	N	N	N
5	223	162	153	222	228	**42**	16	N	N	N	10
6	220	216	210	196	209	**43**	260	245	291	212	272
7	N	N	N	N	N	**44**	26	N	N	N	N
8	23	12	11	24	23	**45**	325	236	241	N	N
9	280	233	245	286	340	**46**	226	230	229	222	237
10	N	N	N	22	15	**47**	308	265	266	149	153
11	323	167	191	256	270	**48**	40	N	N	N	N
12	71	30	32	45	54	**49**	191	220	234	49	52
14	246	186	194	223	211	**50**	17	N	N	N	N
15	N	N	N	N	N	**51**	20	45	46	80	94
17	362	296	289	365	377	**52**	70	28	28	73	81
18	44	22	22	43	46	**53**	128	114	117	126	138
19	N	N	N	N	N	**54**	N	N	N	21	24
20	N	N	N	N	N	**57**	N	N	N	N	N
21	N	N	N	N	N	**58**	N	N	N	21	30
22	231	199	221	172	189	**59**	134	111	112	138	145
24	278	185	171	256	264	**60**	131	78	87	126	157
25	17	N	N	24	21	**61**	N	N	N	N	N
26	N	N	N	N	N	**62**	N	N	N	N	N
27	N	N	N	17	17	**63**	174	237	152	169	173
28	187	155	162	141	146	**64**	N	N	N	N	10
29	N	N	N	N	N	**65**	23	N	11	53	56
31	N	N	N	N	N	**66**	212	165	178	185	185
32	236	202	212	14	46	**67**	391	380	397	406	436
33	297	250	257	268	277	**68**	N	107	115	33	36
34	10	N	N	21	24	**71**	54	33	35	30	27
36	19	11	11	N	N						

(OP - Original results for the preserved sample; (P1 & P2) preserved blood retested after 6 months and a further 10 months; (UP1 & UP2) corresponding unpreserved blood samples).

Table 3-3: Acetone Concentrations in Paired Post-Mortem Blood Samples

Acetone (mg/L)											
Case Number	OP	P1	P2	UP1	UP2	Case Number	OP	P1	P2	UP1	UP2
1	N	N	N	15	15	37	N	N	N	N	N
2	N	N	N	N	N	38	N	21	21	10	11
3	N	N	N	N	N	39	N	N	N	N	N
4	N	N	N	10	12	41	43	53	36	70	55
5	N	N	N	23	N	42	N	N	N	N	N
6	N	N	N	N	N	43	N	N	N	N	N
7	N	N	N	16	11	44	N	N	N	N	N
8	260	443	372	310	258	45	N	N	N	10	12
9	N	N	N	N	N	46	N	N	N	N	N
10	N	12	N	10	11	47	N	35	40	41	42
11	N	N	N	N	N	48	N	N	N	N	N
12	93	29	33	N	N	49	N	N	N	N	N
14	N	N	10	10	11	50	N	16	13	162	101
15	N	N	11	14	18	51	N	N	N	N	N
17	N	N	N	N	N	52	N	12	10	N	N
18	N	N	N	N	N	53	N	20	17	17	19
19	N	20	21	11	12	54	N	N	N	N	N
20	N	11	12	22	18	57	N	13	12	10	11
21	N	51	48	54	50	58	N	N	N	N	N
22	N	N	N	N	N	59	N	32	34	25	29
24	N	11	12	N	N	60	N	173	139	24	32
25	30	93	64	40	31	61	94	79	67	70	73
26	N	55	45	40	43	62	N	N	N	19	10
27	N	N	N	N	N	63	N	N	N	N	N
28	N	N	N	N	N	64	N	N	N	N	N
29	N	N	N	21	18	65	38	48	46	46	49
31	63	78	79	107	75	66	N	N	N	N	10
32	N	N	N	N	N	67	N	N	N	N	N
33	N	16	13	13	15	68	N	44	38	N	N
34	N	N	N	N	N	71	N	82	70	13	15
36	28	25	21	32	27						

(OP - Original results for the preserved sample; (P1 & P2) preserved blood retested after 6 months and a further 10 months; (UP1 & UP2) corresponding unpreserved blood samples).

3.3.3.1 Ethanol Stability in Preserved Blood After 6 Months Refrigeration and 10 Months in the Freezer

The mean ethanol concentration of the original results (OP) was 109 mg/dL. The mean ethanol results for preserved and unpreserved samples stored for 6 months refrigeration and 10 months in the freezer were 90, 92, 90 and 96 for P1, P2, UP1 and UP2 respectively. The percentage of mean loss ranged between 22-27% of preserved and unpreserved samples.

A total of 40 preserved blood samples originally tested (OP) positive for ethanol. Following re-analysis after a period of 6 months in the refrigerator, 90% (N=36)

had lower levels of ethanol (P1), indicating loss of ethanol during the six-month storage period. A loss of greater than 20% was evident in 58% (N=21) of these samples. Ethanol production was also evident in five cases: four samples had higher levels of ethanol (#46, #49, #51 and #63), while case #68, originally negative had an ethanol concentration of 107 mg/dL when retested. This concentration increased further to 115 mg/dL after an additional 10 months storage in the freezer (P2). Thirty cases were negative for all tests, indicating no ethanol formation.

The correlation coefficient (R^2) was calculated when comparing the different data sets and fitted line plots were plotted as illustrated in Figure 3-3 (A-D) for ethanol and Figure 3-4 (A-D) for acetone. A summary of the values are listed in Table 3-3 along with the P values.

An assessment of the correlation between the results generated for the original preserved blood ethanol concentrations (OP) compared with those measured in the same preserved blood sample after 6 months (P1) and a further 10 months (P2) storage are summarized in Table 3-3. Although the values for the original test (OP) versus P1 and P2 were 92.6% and 92.9%, respectively, the P values were less than 0.05 at the 95% confidence interval, indicating significant differences between the initial analysis and the two subsequent analyses.

Figure 3-3A illustrates the correlation between the ethanol concentrations in the original preserved blood (OP) and the reanalysis after 6 months in the refrigerator. Although ethanol loss was observed in most of the cases over the initial 6 months refrigeration, no significant differences in ethanol concentration were observed following a further 10 months storage in the freezer (P1 versus P2, = 98.5%, p = 0.993).

Table 3-4: Summary of Correlation Data for Ethanol in Blood.

	Ethanol			Acetone		
		Regression Equations	P value		Regression Equations	P value
OP vs. P1	0.926	OP=8.353+1.110P1	0.00	0.724	OP=-1.528+0.5136P1	0.006
OP vs. P2	0.929	OP=8.398+1.088P2	0.00	0.738	OP = - 2.081 + 0.6196P2	0.005
P1 vs. P2	0.985	P1=0.754+0.9743P2	0.334	0.993	P1=-0.8029+1.192P2	0.037
P1 vs. UP1	0.766	P1=11.38+0.8500UP1	0.947	0.696	P1 = 1.256 + 1.108 UP1	0.422
P2 vs. UP2	0.775	P2=9.366+0.8436UP2	0.570	0.787	P2=-0.577+1.220UP2	0.330
UP1 vs. UP2	0.984	UP1=-1.291+0.9580UP2	0.334	0.973	UP1=-1.053+1.220UP2	0.075

(P value of paired preserved (P) post-mortem blood and unpreserved(UP). OP: Original preserved blood. P1: Preserved blood retested after 6 months in fridge. P2: Preserved blood retested after additional 10 months in freezer.

3.3.3.2 Ethanol Stability in Unpreserved Blood after 10 Months in the Freezer

An excellent correlation (R^2= 98.4%) was observed (see Figure 3-3D) for the unpreserved blood ethanol concentrations measured following 10 months of storage in the freezer (UP1 versus UP2) with a P value of 0.334 indicating no significant differences. The unpreserved blood samples were not analysed at the same time as the original preserved blood so no assessment of the stability of ethanol in unpreserved blood following 6 months refrigeration can be undertaken.

3.3.3.3 Ethanol Stability in Preserved Blood versus Unpreserved Blood

When comparing the unpreserved blood (UP) ethanol concentrations with those of the original preserved samples (OP), 70% (N=28) of preserved samples and 60% (N=24) of unpreserved samples had lower levels of ethanol, with about 54% (N=15) of these samples recording a loss of greater than 20% over the six-month period. Ethanol production was also evident in 18 and 23 cases respectively for both UP1 and UP2 with 7 of these cases originally negative having ethanol concentrations ranging from 10 to 36 mg/dL when retested.

As would be expected, the preserved samples demonstrated less variability than the unpreserved samples regarding formation of ethanol; however, both preserved and unpreserved samples had significant loss of ethanol over the period of six months when stored in a refrigerator. There are a number of factors that must also be considered when interpreting the data. The unpreserved samples are commonly retained within Forensic Medicine and Science for analysis of a range of analytes and as such the vials are opened and closed on numerous occasions depending on the number of tests carried out, increasing the risk of ethanol loss due to evaporation. In addition, the circumstances surrounding the cases were not considered (i.e., body badly decomposed).

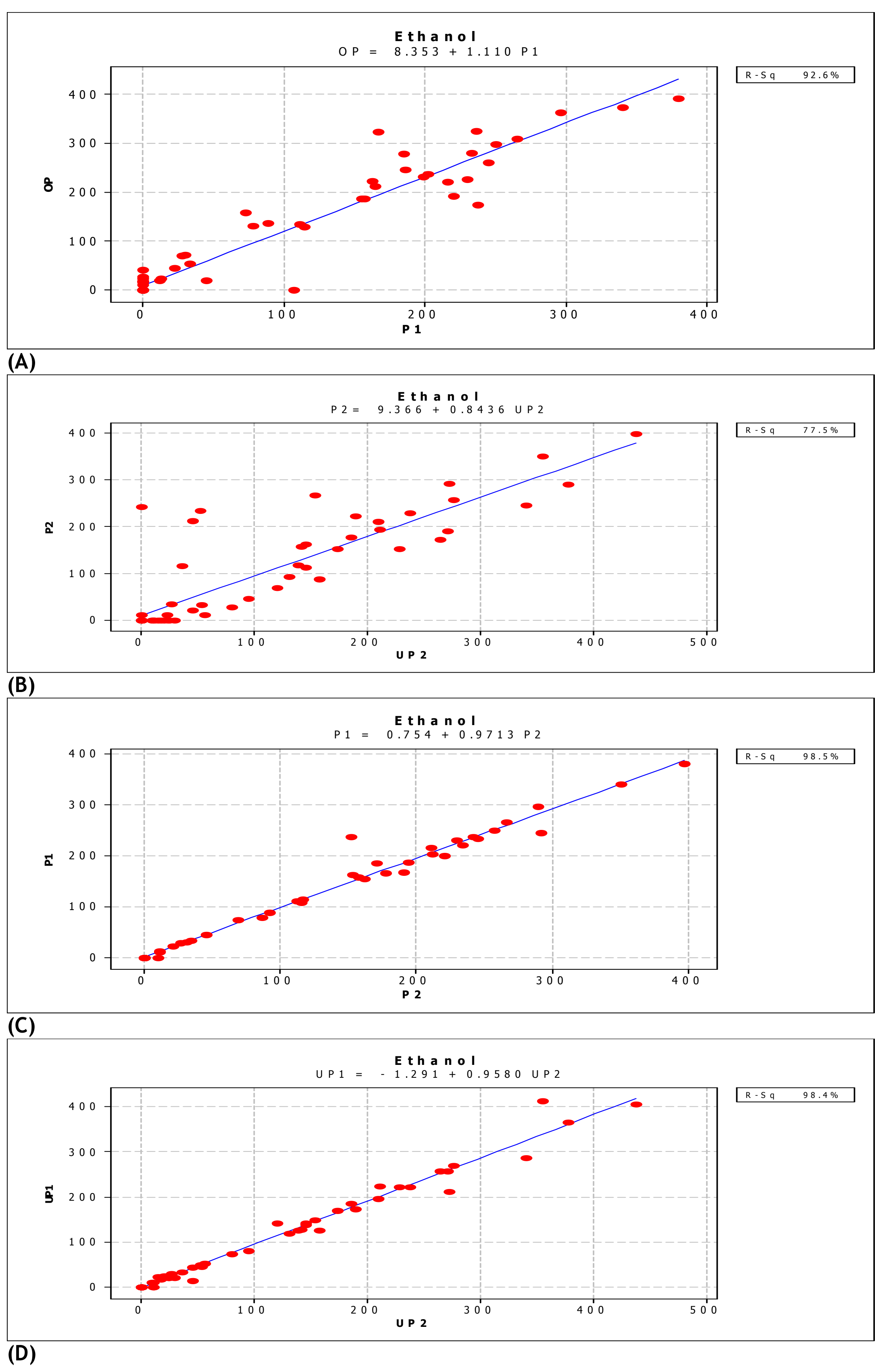


Figure 3-3: Fitted Line Plots of Ethanol concentrations in Paired Blood Samples. (A) Original [EtOH] in preserved blood(OP) versus preserved blood [EtOH](P1) after 6 months in the refrigerator (B) Preserved blood [EtOH] versus unpreserved [EtOH] following 16 months storage(6 months fridge+ 10 months freezer). (C) preserved blood [EtOH](P1) versus preserved blood [EtOH](P2) after 10 months storage in freezer. (D) Unpreserved blood[EtOH] (UP1) versus Unpreserved blood [EtOH](UP2) after 10 months storage in freezer.

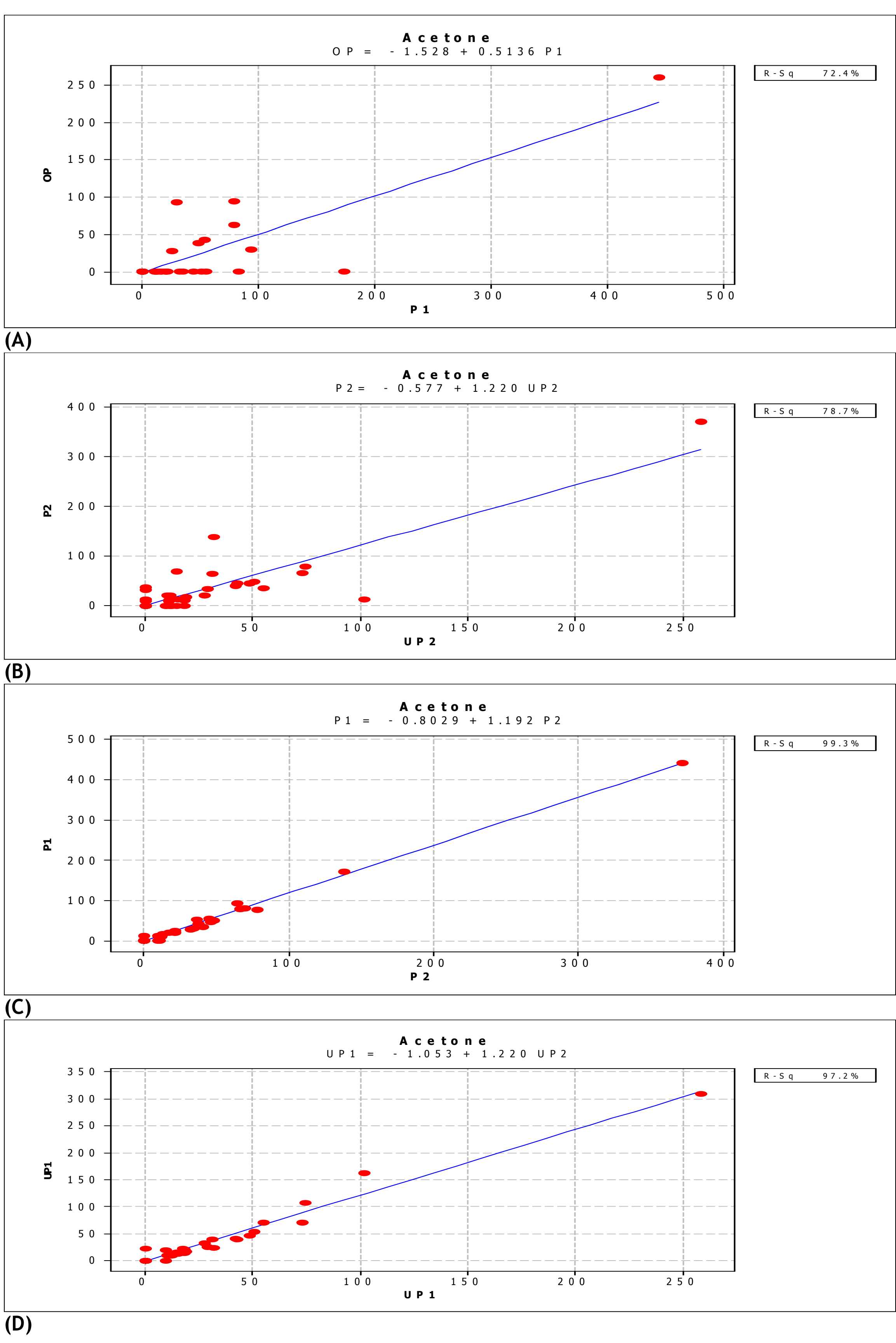


Figure 3-4: Fitted Line Plots of Acetone concentrations in Paired Blood Samples. (A) Original [Acetone] in preserved blood(OP) versus preserved blood [Acetone](P1) after 6 months in the refrigerator (B) Preserved blood [Acetone] versus unpreserved [Acetone] following 16 months storage(6 months fridge+ 10 months freezer). (C) preserved blood[Acetone](P1) versus preserved blood [Acetone](P2) after 10 months storage in freezer. (D) Unpreserved blood[Acetone EtOH] (UP1) versus Unpreserved blood [Acetone EtOH](UP2) after 10 months storage in freezer.

3.3.3.4 Acetone Stability in Preserved Blood versus Unpreserved Blood

The mean acetone concentration of the original results (OP) was 11 mg/L. The mean acetone results for preserved and unpreserved samples stored for 6 months refrigeration and 10 months in the freezer were 24, 21, 21 and 18 for P1, P2, UP1 and UP2 respectively.

Acetone was detected in 35 cases at concentrations ranging from the LOQ to 443 mg/L, however acetone was not present in all of the paired samples. The correlation between the original acetone concentration (OP) and the re-analysis after 6 months (P1) and 10 months (P2), as illustrated in Figures 3-4 A and C respectively, was greater than 70% but with significant differences ($p<0.05$) in the measured concentrations. However, the linear range and LOQ of the original analysis were not comparable with the current method developed and reported here. As a consequence, further comparison of the significance of the results in relation to stability of acetone is not possible.

When comparing acetone concentrations in preserved blood (P1 versus P2) and unpreserved blood (UP1 versus UP2) after a period of 10 months storage in the freezer, 17 preserved and 13 unpreserved samples showed acetone loss of on average 20% and 26%, respectively. Acetone production was evident in 7 preserved samples and 17 unpreserved samples with gains in the range of 9-14%. The values for P1 versus P2 and UP1 versus UP2 were 99.3% and 97.3% respectively with corresponding P values >0.05 indicating that there is a good correlation between the two results. This in turn means that acetone concentrations may not increase or decrease significantly when samples are stored for over 10 months in the freezer.

3.4 Stability of Ethanol in Preserved Blood Following Long-Term Storage at Room Temperature

3.4.1 Introduction

Blood samples collected from individuals arrested under section 5 of the Road Traffic Act 1988 (United Kingdom) are regularly submitted to Forensic Medicine and Science for quantitative analysis of ethanol. Prior to 2007, on completion of the case, the samples were removed from cold storage and kept at room temperature as no disposal process was in place. This provided an ideal study to evaluate the stability of ethanol in preserved blood samples that were stored in crimped cap vials containing sodium fluoride and potassium oxalate at concentrations ranging from 1 to 2%.

3.4.2 Methodology

Two hundred and nineteen preserved blood samples collected from individuals arrested under section 5 of the Road Traffic Act 1988 (United Kingdom) were analysed for the presence of alcohol on receipt within the laboratory of Forensic Medicine and Science (Result 1). The blood samples were refrigerated until the case was heard in court (approximately 1 month after analysis). Following completion of the court case the samples were removed from the refrigerator and stored at room temperature for varying periods of time spanning 5 years. The blood samples were removed from long-term storage at room temperature and re-analysed (Result 2).

The initial analysis (Result 1) was carried out by FMS Technical Staff prior to attaining ISO/IEC 17025 accreditation for the analysis of ethanol in blood. Acetone was quantified in these cases using a positive control and the calibration curve for ethanol (linear over the range of 0-400 mg/dL).

The re-test (Result 2) of the blood was carried out using the validated method described in Chapter 2 utilising n-propanol as internal standard and HS-GC-FID.

3.4.3 Results and Discussion

Preserved blood samples (N=219) collected from individuals arrested under section 5 of the Road Traffic Act were initially tested following short-term storage (Result 1) in a refrigerator and then re-tested after long-term storage of between 1 and 5 years (Result 2) at room temperature. The correlation coefficient (R^2) between the ethanol concentrations measured was 0.85 as illustrated in Figure 3-5 with a P value of <0.05. Out of the 219 samples tested, 208 when retested were positive at concentrations >10 mg/dL. The mean and median ethanol concentration of the original results were 102 and 88 respectively and for retested results the mean and median were 71 and 60 respectively. The vast majority (85%) had an ethanol recovery greater than 50% as summarized in Figure 3-6.

Five samples were negative for ethanol at both the initial and second analysis times. Only 1% (N=3) of samples demonstrated ethanol formation Figure 3-7. One sample was originally negative for ethanol but when retested had an ethanol concentration of 76 mg/dL and two samples positive for ethanol at concentrations of 19 and 65 mg/dL after the initial analysis had significant gains when retested with ethanol concentrations of 46 (247%) and 138mg/dL (212%) respectively. This is most likely as a result of a lack of sufficient preservative in the collection vial or the blood in the collection vial was not mixed appropriately with preservative and anticoagulant powder.

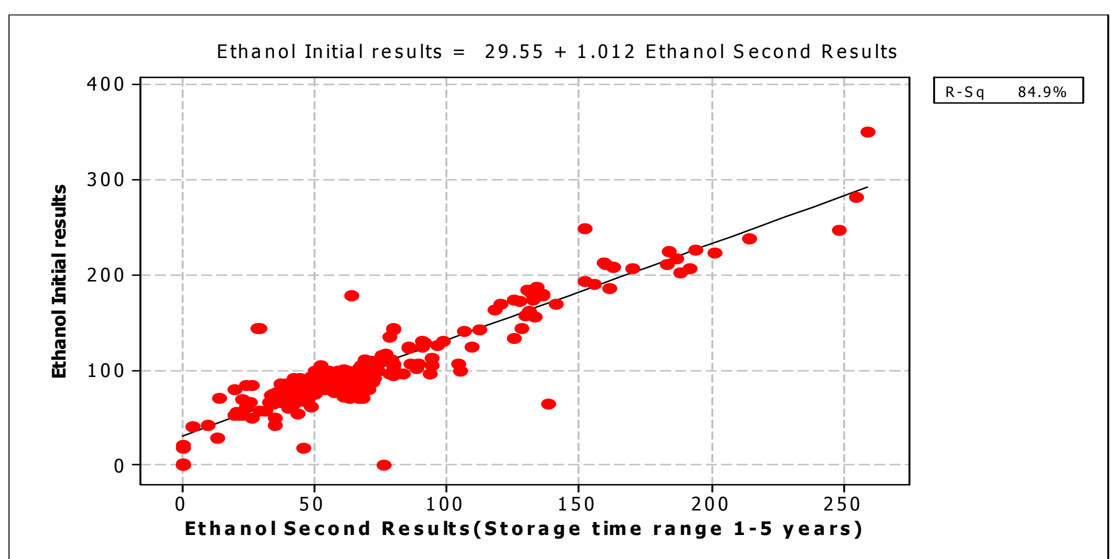


Figure 3-5: Correlation Between Road Traffic [Ethanol] following Long-Term Storage (Result 1 versus Result 2)

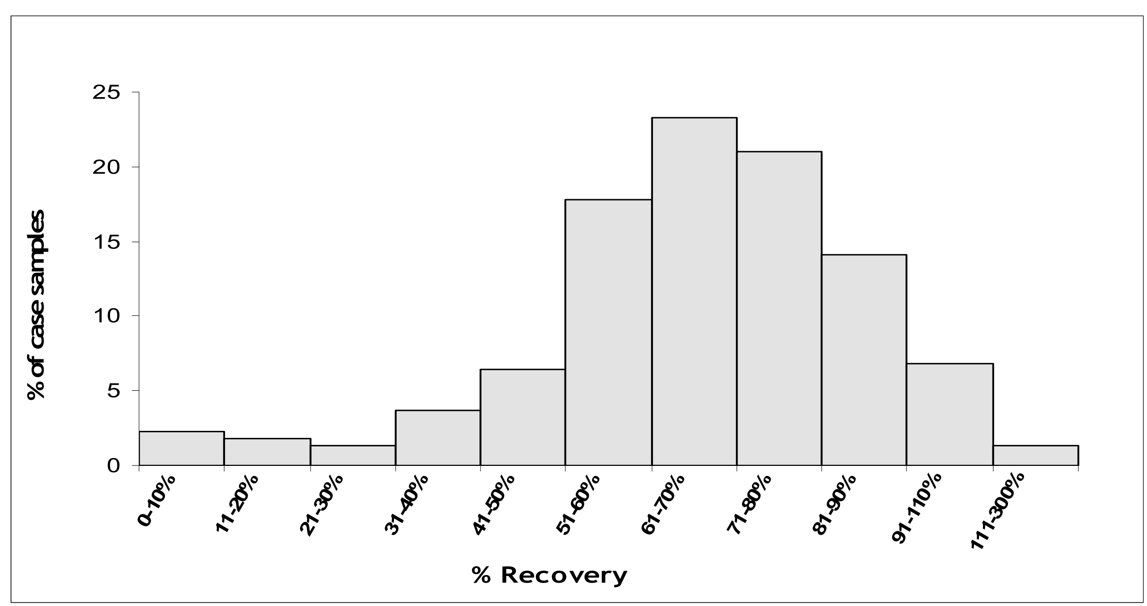


Figure 3-6: Road Traffic Preserved Blood Percentage Recovery of Ethanol (Result 1 versus Result 2).

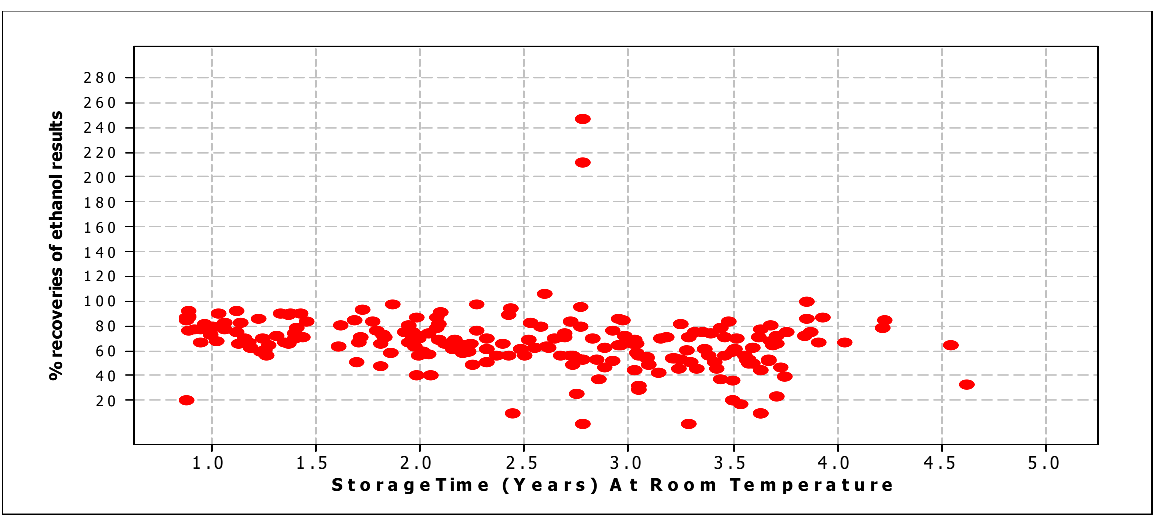


Figure 3-7: Road Traffic Preserved Blood - Percentage Recovery of Ethanol following Long-Term Storage at Room Temperature.

With the exception of the two samples with excessively high gains in ethanol concentration, the average recovery of ethanol was 71% with a standard deviation of 48.3, following long-term storage from between 1 and 5 years. As can be seen in Figure 3-7 the variation in recovery increases in proportion with the increasing number of years in storage. Only 23% of the samples retested after more than 3 years in storage at room temperature had recoveries of greater than 50% of the initial ethanol concentration.

3.5 Evaluation of the Prevalence of Volatiles in Urine, Vitreous Humour and Bile and their Correlation with Femoral Blood.

3.5.1 Introduction

Interpretation of alcohol concentrations in post-mortem cases is more challenging if there is insufficient information about the type of death, the stage of the ethanol distribution in the body, incubation period, storage conditions and temperature of the specimens. Analysis of other body fluids, mainly vitreous humour, bile and urine, in addition to blood samples, may help with interpretation. Ethanol distributes throughout other tissue or fluids according to their water content. The body fluids and tissues containing more water will contain more alcohol. Even within the blood, there may be differences in ethanol concentrations depending on the stage of absorption.(46)

Samples of blood and urine are regularly submitted to FMS for the determination of volatiles, with ethanol and acetone most commonly identified but vitreous humour and bile are only submitted in a small proportion of cases. The sample database within FMS was used to extract all cases where alternative matrices (vitreous humour and bile) were analysed for volatiles in addition to femoral blood. The prevalence of volatiles in these cases was evaluated and any correlations between urine, vitreous humour, bile and femoral blood investigated.

3.5.2 Methodology

The method used for the analysis of ethanol and acetone was fully validated, robust, and sensitive and was carried out by FMS Technical Staff. Acetone was quantified in these cases using a positive control and the calibration curve for ethanol (linear over the range of 0-400mg/dL).(132)

Paired vitreous humour and blood (N=109), bile and blood (N= 20) and urine and blood (N=942) post-mortem case samples were analysed using HS-GC-FID.

Comparisons between the paired matrices were carried out by calculating the correlation coefficient (R^2) and P values to evaluate if there were significant differences between the volatile concentrations in both matrices.

The ratio of the ethanol and acetone concentrations in vitreous humour, bile and urine versus those in femoral blood were calculated. The ratio range, at a 95% 95% confidence interval (95% CI), were also calculated for both ethanol and acetone. The calculated ratios were compared with those published in the literature.

3.5.3 Volatile Standards

n-Propanol was used as the internal standard as described in section 2.2.3. Certified solutions of ethanol were purchased from LGC Standards (Teddington, UK) at concentrations of 10, 25, 50, 80, 100, 200, 300 and 400 mg/dL. Quality control (QC) samples were purchased from Medichem (Germany) at concentrations of 30, 80 and 300 mg/dL. Acetone was quantified in these cases using a positive control (water spiked at 250 mg/L) and extrapolated using the ethanol calibration curve.

3.5.4 Results

3.5.4.1 Comparison of Ethanol Concentrations in Paired Blood and Vitreous Humour Samples

Of the 109 paired blood and vitreous humour (VH) samples, 89% of the pairs were positive (N=43) or negative (N=54) for ethanol in both matrices as summarised in Table 3-5. The remaining 12 paired case samples were negative in vitreous humour and positive in blood with ethanol concentrations ranging from 12-70 mg/dL. The P value for VH versus blood was >0.05 with a value of 94.4% as summarised in Table 3-6 and illustrated in Figure 3-8. The mean ratio of VH:BAC in this study was 1.13 with a range of 1.00-1.26 in comparison with published ratios ranging from 0.63 – 1.75 (n=672) with mean value 1.19, median 1.18.(71) in another study the ratio ranged from 1.0 to 2.20(n=209) mean value 1.24, median 1.19.(73).

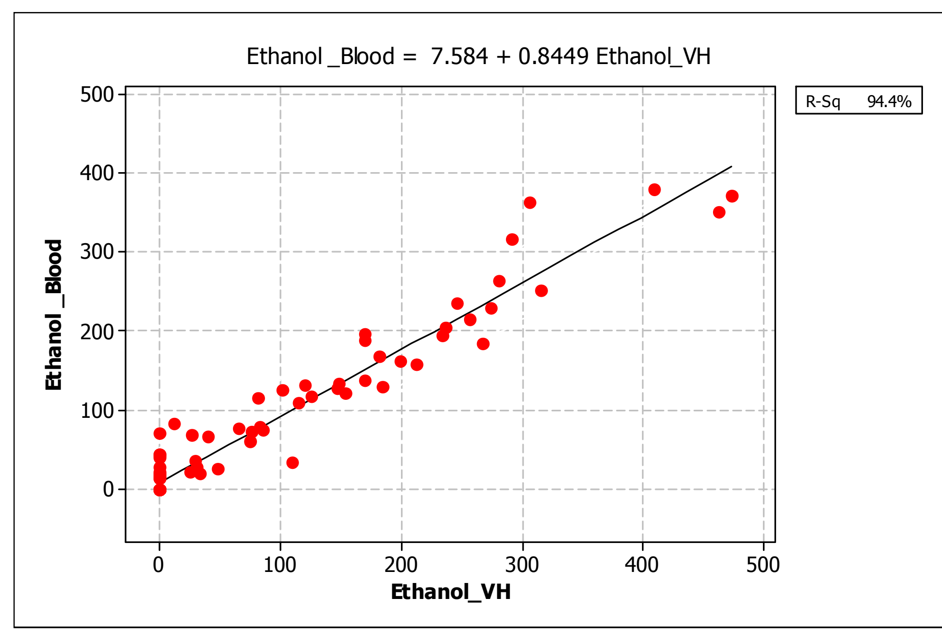


Figure 3-8: Comparison of Ethanol Concentrations in Paired Blood and Vitreous Humour Samples

Table 3-5: Comparison of Positive and Negative Results for Ethanol in BloodVersus Vitreous Humour

ETHANOL		
N= 109	**Positive Vitreous Humour**	**Negative Vitreous Humour**
Positive Blood	**43**	**12**
Negative Blood	**0**	**54**

Table 3-6: Summary of Mean Ethanol Ratio and Correlation for Blood in Comparison to Vitreous Humour (VH), Bile and Urine.

Ethanol Ratio	Mean Ratio/(n)	Median	St Dev.	SE Mean	95% CI	Regression Equations		P value
VH/Blood	1.13 (n=43)	1.11	0.4319	0.0659	1.00-1.26	EtOH _Blood = 7.584 + 0.8449 EtOH_VH	94.4	0.356
Bile/Blood	1.04 (n=13)	1.07	0.469	0.135	0.74-1.33	EtOH _Blood = 7.30 + 0.7739 EtOH_Bile	92.0	0.122
Urine/Blood	1.35 (n=461)	1.31	1.0917	0.0491	1.25-1.44	EtOH _Blood = 0.559 + 0.7607 EtOH_Urine	90.0	0.0001

3.5.4.2 Comparison of Ethanol Concentrations in Paired Blood and Bile Samples

Of the 20 paired blood and bile samples, 80% of the pairs were positive (N=13) or negative (N=3) for ethanol in both matrices as summarised in Table 3-7. The remaining 4 paired case samples were negative in blood and positive in bile with ethanol concentrations ranging from 10-39 mg/dL.

The P value for bile versus blood was >0.05, this indicates no significant difference between the ethanol concentrations in blood and bile. The value of 92% as summarised in Table 3-6 and illustrated in Figure 3-9. The mean ratio of bile to blood in this study was 1.04 with a range of 0.74-1.33 in comparison with published ratios ranging from 0.32 -2.91.(75)

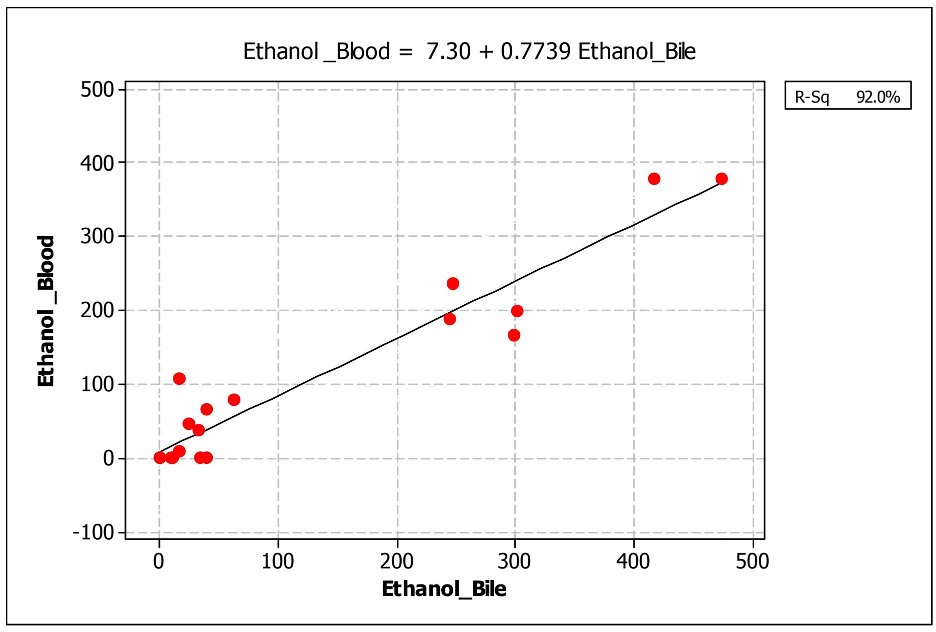


Figure 3-9: Comparison of Ethanol Concentrations in Paired Blood and Bile Samples

Table 3-7: Comparison of Positive and Negative Results for Ethanol in Blood Versus Bile

ETHANOL		
N = 20	Positive Bile	Negative Bile
Positive Blood	13	0
Negative Blood	4	3

3.5.4.3 Comparison of Ethanol Concentrations in Paired Blood and Urine Samples

Of the 942 paired blood and urine samples, 85% of the pairs were positive (N=461) or negative (N=338) for ethanol in both matrices as summarised in Table 3-8. Of the remaining paired case samples, 34 were negative in urine and positive in blood with ethanol concentrations ranging from 10-303 mg/dL, and 109 were negative in blood and positive in urine with ethanol concentrations ranging from 10-174 mg/dL. The P value for urine versus blood was <0.05 with a value of 90% as summarised in Table 3-6 and illustrated in Figure 3-10. The mean ratio of urine to blood in this study was 1.35 with a range of 1.25-1.44 in comparison with published ratios ranging from 0.53 -2.17.(61;67;68). In another study the first void ethanol mean ratio was 1.34 with the range of 1.68-1.75 and the second void ethanol urine to blood ratio was 1.22 with a range of 1.43-1.47. (61)

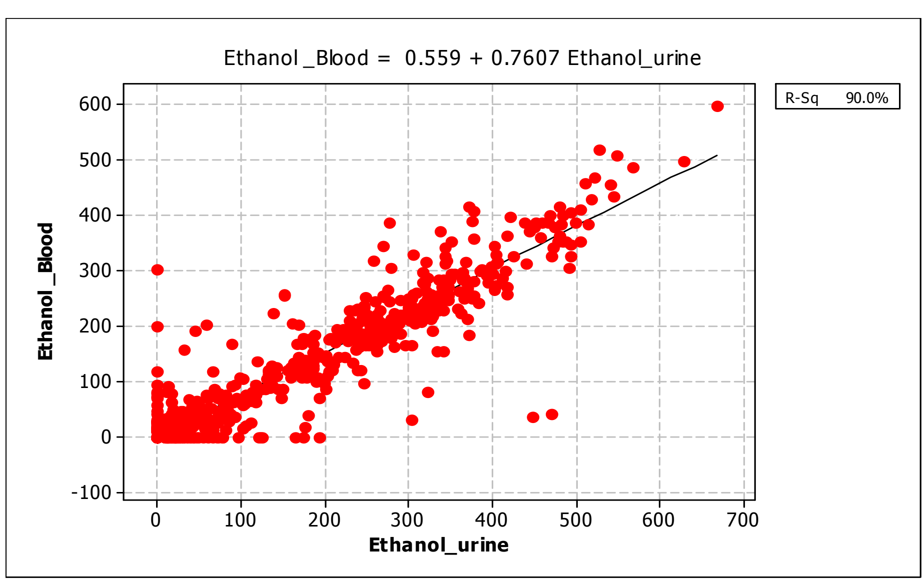


Figure 3-10: Comparison of Ethanol Concentrations in Paired Blood and Urine Samples

Table 3-8: Comparison of Positive and Negative Results for Ethanol in Blood Versus Urine

ETHANOL		
N = 942	Positive Urine	Negative Urine
Positive Blood	461	34
Negative Blood	109	338

3.5.4.4 Comparison of Acetone Concentrations in Paired Blood and Vitreous Humour and Paired Blood and Bile Samples

Tables 3-9 and 3-10 summarise the results for acetone in paired blood and vitreous humour samples and paired blood and bile samples respectively. Of the 104 paired blood and vitreous humour samples, 100% of the pairs were positive (N=2) or negative (N=102) for acetone in both matrices, while 84% of the paired blood and bile samples were positive (N=3) or negative (N=13) for acetone in both matrices. Of the remaining paired blood and bile samples, 2 were negative in bile but positive in blood with acetone concentrations of 44 and 62 mg/L. One case was negative in blood <10 mg/L but positive in bile with an acetone concentration of 15 mg/L.

Table 3-11 summarises the ratios and correlations values for both vitreous humour and bile acetone concentrations versus blood acetone concentrations. The P values for both were >0.05 for both which indicates that there is no significant difference between two sets of results (VH-Blood and Bile-blood). The mean ratio values which was 2.61 for VH: blood acetone concentrations and 0.54 for bile: blood acetone concentrations.

Table 3-9: Comparison of Positive and Negative Results of Acetone in Blood versus Vitreous Humour

ACETONE		
N = 104	Positive Vitreous Humour	Negative Vitreous Humour
Positive Blood	2	0
Negative Blood	0	102

Table 3-10: Comparison of Positive and Negative Results of Acetone in Blood Versus Bile

ACETONE		
N = 19	Positive Bile	Negative Bile
Positive Blood	3	2
Negative Blood	1	13

Table 3-11: Summary of Mean Acetone Ratio and correlation for Blood in Comparison to Vitreous Humour (VH), Bile and Urine.

Acetone Ratio	Mean Ratio/(n)	Median	St Dev.	SE Mean	95% CI	Regression Equations		P value
VH/Blood	2.61 (n=2)	2.61	1.85	1.31	0-19.27	Acetone _Blood=0.2351+0.3820 Acetone_VH	74.4	0.248
Bile/Blood	0.54 (n=3)	0.64	0.281	0.126	0-1.08	Acetone _Blood =6.083+1.749 Acetone_Bile	0.41	0.064
Urine/Blood	2.11 (n=70)	1.56	2.262	0.270	1.57-2.65	Acetone _Blood =1.101+0.5021 Acetone_Urine	0.69	0.0001

3.5.4.5 Comparison of Acetone Concentrations in Paired Blood and Urine Samples

Of the 934 paired blood and urine samples, 96% of the pairs were positive (N=70) or negative (N=828) for acetone in both matrices as summarised in Table 3-12. Of the remaining paired case samples, 7 were negative in urine and positive in blood with acetone concentrations ranging from 56-339 mg/L, and 29 were negative in blood and positive in urine with acetone concentrations ranging from 25-861 mg/L. There were three samples not included in the calculation and were considered as outliers and they were at concentrations of 1482 mg/L in urine with a corresponding negative blood), 2879 mg/L in urine and corresponding negative blood) and 1581 mg/L in urine and 117 mg/L corresponding in blood). (The P value for urine versus blood was <0.05 with a value of 68.6% as summarised in Table 3-11 and illustrated in Figure 3-11. The mean ratio of urine to blood in this study was 2.11 with a range of 1.57-2.65.

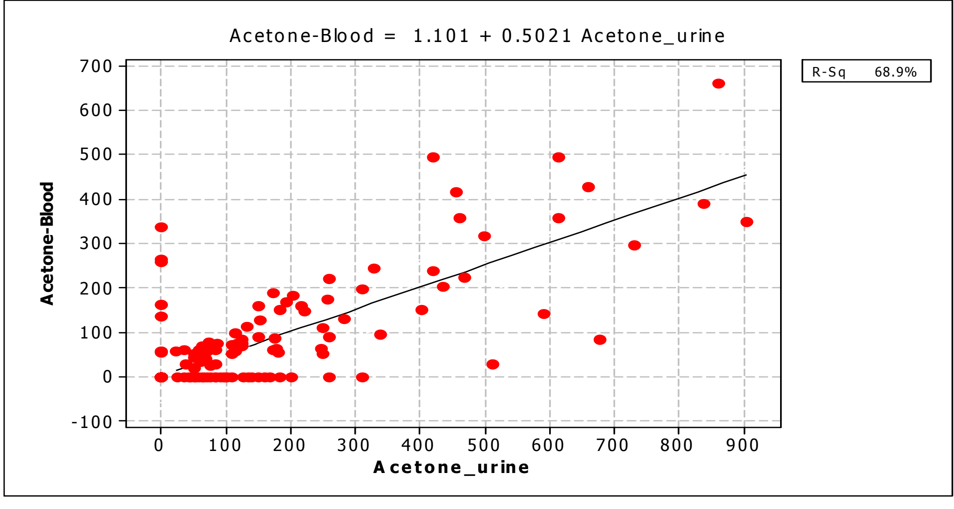


Figure 3-11: Comparison of Acetone Concentrations in Paired Blood and Urine

Table 3-12: Comparison of Positive and Negative Results for Acetone in Blood Versus Urine

ACETONE		
N = 934	Positive Urine	Negative Urine
Positive Blood	70	7
Negative Blood	29	828

3.5.5 Discussion

Ethanol concentrations in vitreous humour and bile samples were not significantly different from the blood ethanol concentrations. The ratio of VH ethanol concentration to blood ethanol concentration was reported to be higher during the post absorption phase as a result of the higher percentage of water in VH than in blood.(73) The mean ratio of VH/Blood in this study was 1.13 which is in agreement with published ratios as previously indicated. It was also observed that 12 of the 109 case samples were negative for ethanol in VH but positive in the paired blood sample. The most likely explanation for this is neo formation of ethanol in blood by microorganisms or that the death occurred before equilibrium was achieved.

The bile to blood ratio range of ethanol concentration obtained from this study was within the published range. Four bile samples were positive for ethanol but negative in paired blood samples. A possible reason behind this could be that the sample was collected during the post-absorption phase after ethanol was eliminated from the blood. There was a significant difference between urine and blood ethanol concentration, although the R^2 was approximately 90% and the ratio of urine to blood was in agreement with published ratios. 34 out of 942 case samples were negative for ethanol in urine but positive in blood. This may be due to several factors, such as formation of ethanol in blood due to bacterial action; or death occurred in the pre-absorption phase; or the urine may be diluted or contaminated. It could also be due to loss of ethanol from the urine sample due to microorganisms especially in non-preserved samples or the container may not have been sealed properly or was an unsuitable size. Another

reason might be that there is still a residue of negative urine in the bladder that diluted the urine after alcohol consumption.

A total of 109 urine samples were positive for ethanol in urine but negative in blood. This could be due to the sample being collected in the post-absorption phase, formation of ethanol due to microorganism action in the urine sample or if the sample was collected and stored in unpreserved samples.

The P values were > 0.05 for acetone concentrations in vitreous humour and bile paired with blood, indicating that there is no significant difference in these matrices. However, a large proportion of the samples tested were negative and as such any conclusions from this study must be made with caution and it is recommended that a much larger study be carried out with more positive paired samples.

The two samples that were negative for acetone in bile but positive in blood could be because the sample was collected in the pre-absorption phase, due to acetone formation or as a result of oxidation of isopropanol to acetone.

A significant difference was indicated between acetone concentrations in blood and urine. Seven cases were negative for acetone in urine but positive in blood and this may be due to formation of acetone in blood due to microorganisms, or due to oxidization of isopropanol to acetone. Twenty nine cases were positive for acetone in urine but negative in blood. This may be due to the samples being collected after elimination of acetone from blood or due to loss of acetone due to evaporation. There are no published ratios for acetone in body fluids versus blood to make any further comparison with those calculated in this study.

3.6 Evaluation of the Immunalysis Ethyl Alcohol Enzymatic Assay as a Semi-Quantitative Test in Comparison to HS-GC-FID

3.6.1 Introduction

Testing each sample received in the forensic laboratory by complicated methods and instrumentations will need more manpower, time, and high cost especially when the workload is high. The purpose of the immunoassay screening tests is to eliminate the negative samples from the more lengthy confirmation testing process. There are different types of immunoassays including radioimmunoassay (RIA), fluorescence polarization immunoassay (FPIA), enzyme multiplied immunoassay technique (EMIT), and enzyme linked immunosorbant assay (ELISA).

Several biological matrices can be tested with immunoassays, including blood, hair, oral fluid, plasma, post-mortem blood, serum, urine, and sweat. Sample pre-treatment is required before applying the samples to the microplate.

The basic principle is that immunoassays can be used to screen biological samples for an antigen, such as a drug of interest. Establishing the cut-off point in an enzymatic method is important in order to distinguish between positive and negative results and provide a balance between false positive and false niegative results.

Cross-reactivity may occur in tested specimens with structurally similar compounds or due to interference from the matrix. In terms of result interpretation, a false negative result means that it was negative by immunoassay and positive with a confirmatory test. A false positive result means that the screening was positive and the confirmatory test was negative.

The signal changes associated with binding to form a labelled "antibody and drug–derivative immune complex" can be measured either qualitatively or semi-quantitatively. The qualitative mode is used as a screening test to identify the presence or absence of the analyte of interest relative to the cut-off value. The semi-quantitative mode is conducted by comparing the signal value of a known sample to a calibration curve to estimate the concentration of in the sample.

The Immunalysis ethyl alcohol assay is an enzymatic method used for the semi-quantitative analysis of ethyl alcohol in human blood. Positive alcohol results generated from using the enzymatic method should be confirmed with an alternative non-enzymatic method such as head space gas chromatography flame ionization detection (HS-GC-FID).(176)

Enzymatic assays used for the detection of ethanol in humans are based on the same reaction that occurs *in-vivo* (see Figure 3-12). Ethanol is converted to acetaldehyde in the presence of alcohol dehydrogenase (ADH) with the accompanying formation of the nicotinamide adenine dinucleotide reduced form (NADH). NADH is then measured spectrophotometrically at a wavelength of 340 nm.(176;177)

Figure 3-12: Enzymatic Alcohol Reaction

3.6.2 Chemicals and Reagents

Certified ethanol standard solutions were purchased from LGC Standards (Teddington, UK). The concentrations were: 25 mg/dL 50 mg/dL, 80 mg/dL, 100 mg/dL, 200 mg/dL, 300 mg/dL, and 400 mg/dL. The Enzymatic Kit for alcohol determination in blood was manufactured by the immunalysis Corporation (Pomona, CA, USA) and contained one bottle of 0.5 N perchloric acid, Reagent A (RA) containing 0.6 M tris buffer, and Reagent E (RE) containing ADH and NAD in tris buffer as a stabilizer. Both RA and RE contain 0.1% sodium azide preservative. Other materials included 96-well flat-bottom polysterene microtiter plates; pipettes with volumes of 10, 20, and 100 µL with disposable tips, disposable 1.5 polypropylene conical bottom centrifugal tubes with caps, and a timer.

3.6.3 Methodology

A total of 60 post-mortem blood samples, 50 preserved in 0.2% sodium fluoride (NaF) and 10 unpreserved were analysed for ethanol, acetone, isopropanol, and methanol utilizing a validated HS-GC-FID as described in Chapter 2. The same samples were screened for ethanol using the enzymatic assay.(176)

The cut-off point used to distinguish positive from negative utilizing HS-GC-FID was 10 mg/dL for ethanol and 10 mg/L for acetone. Although the LOD was established by the immunalysis manufacturer at 10 mg/dL, the cut-off point used for the ethanol results generated from the enzymatic method was 25 mg/dL, equivalent to the lowest point of the calibration curve.

A tabulated cross-match between the positive and negative results was carried out in addition to evaluating any correlation between the two tests.

3.6.3.1 Enzymatic Ethyl Alcohol Assay

An aliquot of 100 µL of the standard, sample or quality control material was added with 400 µL 0.5N perchloric acid to each centrifuge tube. The tubes were vortexed and left at room temperature for 5 minutes. All tubes were centrifuged at 4000 RPM for 5 minutes. A 10 µL aliquot of the clear solution was transferred to a microplate well in duplicate and 100 µL each of RA and RE added to each well. The plate was covered and placed in the dark for 30 minutes, then the plate was read at 340nm using microplate RXM reader.

3.6.3.2 HS-GC-FID

The validated method utilizing HS-GC-FID and n-propanol as internal standard was carried out as described in chapter 2.

3.6.4 Method Validation

For the enzymatic assay, validation was carried out by the Immunalysis manufacturer as per the manufacturers insert package instructions.(176) The linearity was assessed by preparing the calibrators at 0, 20, 100, 200, and 400 mg/dL. Intra-assay (within-run) and inter-assay (between-run) precision was

obtained by preparing 8 replicates of treated samples at concentrations of 0, 20, 100, 200, and 400 mg/dL. In terms of accuracy, two sets of data (n=50) generated from the HS-GC-FID and enzymatic method were compared and the R^2 was calculated. Specificity was assessed with various structurally similar organic compounds tested at different levels, such as acetaldehyde, acetone, n-butane, ethylene glycol, isopropanol, methanol, n-propanol, and urea. The linearity was assessed by preparing a concentration range of 25-400 mg/dL for ethanol. The cut-off point used in this method was at the concentration of the first calibrator (25 mg/dL).

3.6.5 Results and Discussion

The cross-reactivity studies were carried out by the manufacturer and were reported as low for all volatiles assessed: n-butanol (9%), ethylene glycol (2.5%), isopropanol (6.5%) and n-propanol (11.5%).

Linearity was assessed using certified standards and ranged from 25-400 mg/dL with an >0.997 (Figure 3-13).

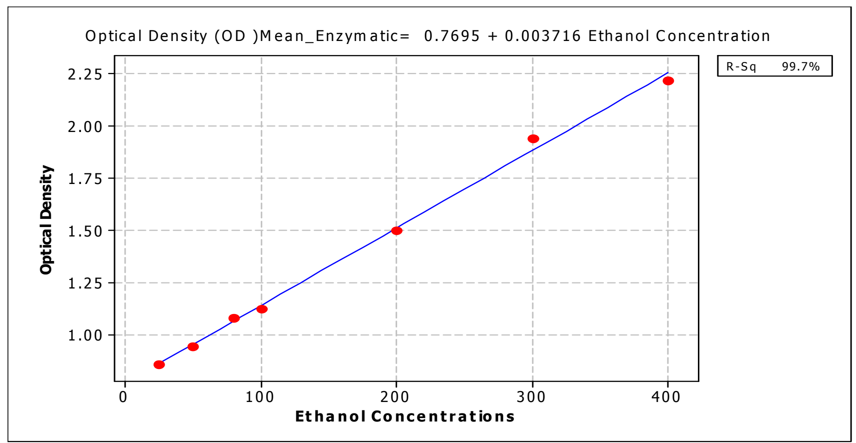


Figure 3-13: Calibration Curve for Ethanol using the Enzymatic Assay

Table 3-13 summarises the semi-quantitative results for the samples tested using the enzymatic assay and the quantitative results following analysis by HS-GC-FID. Of the 60 samples, 24 (21 preserved and 3 unpreserved) samples were positive in both methods, while 31 (26 preserved and 5 unpreserved) were negative in both. These results are summarised in Table 3-14 and highlight four

samples (3 preserved and 1 unpreserved) that were positive by HS-GC-FID but negative by the enzymatic assay, and 1 unpreserved blood sample that was negative by HS-GC-FID but positive by the enzymatic assay.

Table 3-13: Ethanol Results for HS-GC-FID versus Enzymatic Assay highlighted mismatched cases in yellow

Case No.	Enz (mg/dL)	GC (mg/dL)	Case No.	Enz (mg/dL)	GC (mg/dL)	Case No.	Enz (mg/dL)	GC (mg/dL)
1	33	73	**16**	<25	<10	**34**	<25	<10
1UP	57	141	**16UP**	<25	<10	**35**	<25	<10
2	48	89	**17**	355	296	**36**	<25	11
3	<25	<10	**18**	25	22	**36UP**	<25	<10
4	118	157	**19**	<25	<10	**37**	366	340
5	109	162	**20**	<25	<10	**38**	<25	<10
5UP	161	222	**21**	<25	<10	**39**	<25	<10
6	187	216	**22**	231	155	**40**	<25	<10
7	<25	<10	**23**	<25	<10	**41**	<25	<10
8	<25	12	**24**	<25	<10	**42**	<25	<10
8UP	<25	24	**25**	<25	<10	**43**	351	245
9	226	233	**26**	370	199	**44**	<25	<10
10	<25	<10	**27**	<25	<10	**45**	319	236
11	166	167	**28**	329	185	**45UP**	<25	<10
12	<25	30	**29**	<25	<10	**46**	259	230
13	<25	<10	**30**	<25	<10	**47**	342	265
13UP	<25	<10	**31**	<25	<10	**48**	<25	<10
14	213	186	**32**	362	202	**49**	321	220
15	<25	<10	**32UP**	134	14	**50**	<25	<10
15UP	<25	<10	**33**	247	250	**50UP**	29	<10

UP-Unpreserved

Table 3-14: Ethanol in Blood (Enzymatic-v-HS-GC-FID)

Ethanol in Blood GC vs. Immunoassay Method		
N = 60	Positive (Enzymatic)	Negative (Enzymatic)
Positive (GC result)	24	4
Negative (GC result)	1	31

The correlation coefficient (R^2) between the two sets of the results was 87.6% as illustrated in Figure 3-14). The P value was 0.056, indicating that there is no significant difference between the results of the two methods.

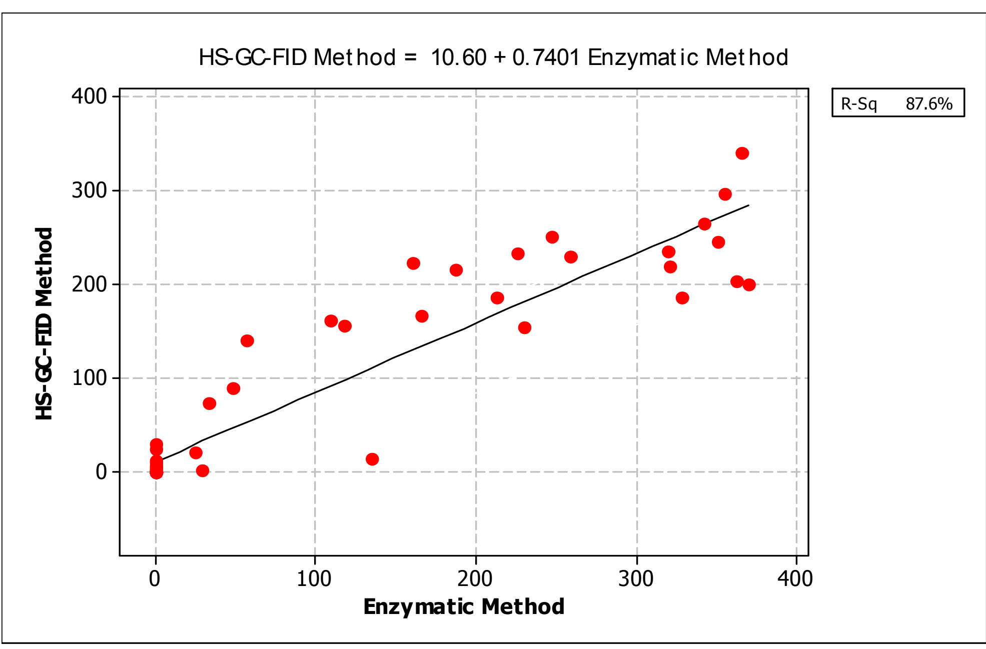


Figure 3-14: Blood Ethanol Correlation between HS-GC-FID and the enzymatic method

Table 3-15 highlights the five unmatched ethanol results. The first four samples were all positive by HS-GC-FID and negative by the enzymatic assay, however the different cut-off points used for each method provide an explanation for the difference in these results. Although the manufacturer's cut-off was 10 mg/dL for ethanol and 10 mg/L for acetone and because the immunoassay method used for the purpose of comparison with HS-GC-FID, validation of this method has not been carried out. Instead the lower point used for the immunoassay calibration curve which are 25 mg/dL for ethanol and 25 mg/L for acetone was used as a cut-off for the immunoassay method.

Table 3-15: Mismatched Ethanol Results (Enzymatic versus HS-GC-FID)

Case No.	Sample Condition	Acetone HS-GC-FID (mg/L)	Ethanol HS-GC-FID (mg/dL)	Ethanol Enzymatic Method (mg/dL)
8	Preserved	443 (POS)	12 (POS)	(2) <25 (NEG)
8	Unpreserved	310 (POS)	24 (POS)	(9) <25 (NEG)
12	Preserved	29 (POS)	30 (POS)	(22) <25 (NEG)
36	Preserved	25 (POS)	11 (POS)	(11) <25 (NEG)
50	Unpreserved	162 (POS)	<10 (NEG)	(29) (POS)

One sample, number 50, was negative using HS-GC-FID and positive using the enzymatic method. Two possible explanations are that as the sample contains no preservative and there was a delay between the initial test by HS-GC-FID and the enzymatic assay, the formation of ethanol may have taken place, or it may be due to interference from other volatile compounds that were present in the sample. The cross-reactivity of acetone was not reported by the manufacturer so this may provide an explanation. However, acetone was also present in the other samples and is a particularly high concentration in both preserved and unpreserved samples of case 8 with corresponding negative enzymatic assay response.

3.7 Conclusion

All volatiles investigated in this study, with the exception of methanol, had improved stability when stored in containers with preservative (0.2 or 1% sodium fluoride) and antioxidant (0.2% sodium metabisulphite). Storage at -22°C is preferred for optimal stability rather than at room temperature (25°C) or in the refrigerator (4°C). Isopropanol concentrations decreased and acetone concentrations increased over the 50-day testing period, confirming oxidation of isopropanol to acetone. The oxidation process for both isopropanol and n-propanol was significantly reduced with the addition of the antioxidant, sodium metabisulphite.

Blood samples collected following suspected drink-driving offences which contain both preservative and antioxidant were stored at room temperature for a period of between 1 to 5 years following initial tests for ethanol. Recoveries of

50% or more of the original ethanol concentration were achieved in 85% of the samples tested. However, recoveries decreased with increasing number of years of storage with only 23% of the samples retested after more than 3 years with recoveries achieving 50% or more.

Over a period of six months refrigerated storage, significant loss and formation of ethanol occurred in both preserved and unpreserved paired post-mortem blood samples from cases submitted to the toxicology laboratories of Forensic Medicine and Science. However, further losses were not significant following an additional storage period of 10 months in the freezer at -22°C.

Further investigations are needed to evaluate the effect of anti-coagulant in terms of anti-oxidation and to investigate the optimum antioxidant concentration with the additional evaluation of a longer period of storage to optimize the conditions required to prevent loss or formation of volatiles.

These findings support the conclusions of the author of a 2007 report who highlighted the importance of understanding the stability of analytes of interest and ultimately interpretation for medico-legal casework.(178)

Several different specimens should be collected from different sites of the body, mainly vitreous humour, or bile and urine if they are available for better interpretation of alcohol results. Many other factors might be useful for better interpretation of alcohol results (e.g. the circumstances of the death and the peri-mortem status, medical history of the deceased if any ante-mortem dilution occured due to transfusions of blood or fluids at the hospital, contamination of specimen by diffusion from the stomach to surrounding organs in case of trauma, or stage of absorption in the body, post- mortem decomposition and embalming effect). Collection of post-mortem samples for alcohol analysis should be under hygienic procedures, with proper storage in a container that includes a suitable preservative and is stored at a low temperature. These measures may help to eliminate the alteration of the alcohol level from the time of collection until analysis, however this will not prevent the action of microorganisms between death and time of sample collection, especially in cases of advanced decomposition.

Although femoral blood is recommended for the analysis of alcohol, this sample is not always available. In this study, the comparison showed a correlation between bile, vitreous humour and blood, which may indicate that the distribution in the majority of the case samples was in post-absorption. Vitre Humor, urine and bile/blood ratios were within the published range with the urine alcohol results significantly different with blood for both ethanol and acetone. Vitreous humour and bile seem to be more reliable than urine as alternative samples in a post-mortem casewoek for alcohol and acetone analysis. A wide range of data needs to be analysed for alcohol and acetone with consistent sample conditions in order to confirm this conclusion; also many factors (as mentioned previously) that may affect the alcohol level should be considered when using urine as an alternative sample. More investigation needs to be carried out in relation to acetone such as: cut of points in the post-mortem formation, stability and the ratio between blood and other body fluids. Analysis of ethanol biomarkers may help with interpretation.

The enzymatic assay demonstrated good correlation with HS-GC-FID. Further evaluation and optimization of the LOD and LLOQ is required. However, its application to post-mortem or criminal toxicology is of limited value.

Chapter 4 - Determination of β-Hydroxybutyrate (BHB) in Post-mortem Blood and Urine Using Gas Chromatography -Mass Spectrometry (GC-MS)

4.1 Introduction

β-Hydroxybutyrate (BHB) has the potential as a biomarker for sudden deaths in chronic alcoholics caused by alcoholic ketoacidosis (AKA). The aim of this study was to develop and validate a method for the determination of BHB in postmortem blood and urine using GC-MS following silyl derivatisation. The validated method was then applied to the analysis of real case samples to investigate the prevalence of BHB in medico-legal cases.

A further database study of all samples tested for BHB, ethanol and acetone within Forensic Medicine and Science (FMS) was carried out to evaluate the relationship between BHB, ethanol, and acetone levels in post-mortem blood and urine.

4.2 Method Development

4.2.1 Materials

4.2.1.1 Chemicals and Reagents

The internal standard used was deuterated γ-hydroxybutyrate (GHB-d_6, 1 mg/mL) purchased from LGC Standards (Teddington, UK). β-Hydroxybutyrate (BHB) and o-bis (trimethylsilyl) trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) were sourced from Sigma-Aldrich. (Basingstoke, England). Acetonitrile (ACN), methanol (MeOH) and ethyl acetate (EtOAc) were all HPLC grade, sulphuric acid was 98% and were all purchased from VWR International Ltd. Disodium hydrogen orthophosphate anhydrous (Na_2HPO_4; MW 141.96) and sodium dihydrogen orthophosphate monohydrate (NaH_2PO4H_2O; MW 137.99) were sourced from BDH. Sodium chloride 99+% was sourced from Aldrich. Ammonia solution 35% was sourced from Fisher Scientific. ZSGHB20 Clean Screen SPE column size 200 mg/10 mL were purchased from United Chemical Company

UCT, USA. Distilled water came from the Millipore purification system within FMS.

4.2.1.2 Preparation of Blank (Drug and Volatile-Free) Blood and Urine

Prepared using expired packed red blood cell pouches and 1% saline solution as described in section 2.2.2. Blank urine was obtained from a healthy adult volunteer with no history of alcohol consumption. Triplicate samples of each blood and urine were screened for BHB and the level was less than the LOD limits. Preparation of Standards and Quality Controls

Two separate stock standard solutions (A) and (B) were prepared at concentrations of 2 g/L. Stock standard solution (A) was used to prepare the standards for the calibration curve. The calibrator points were prepared in blood and urine as summarised in Table 4-1 from the stock standard to give final BHB concentrations of 0, 5, 10, 25, 50, 100, 200, 300, 400, 500 mg/L.

Table 4-1: Preparation of BHB Calibration Standards

Calibrator Concentrations (mg/L)	**Calibration Level**	**Volume of Stock A Added** (µL)	**Final Volume Blood/Urine** (mL)
0	Level 1	0	5
5	Level 2	12.5	5
10	Level 3	25	5
25	Level 4	62.5	5
50	Level 5	125	5
100	Level 6	250	5
200	Level 7	500	5
300	Level 8	750	5
400	Level 9	1000	5
500	Level 10	1250	5

Stock solution (B) was used for preparing the quality control samples as summarised in Table 4-2. Quality control material was prepared in blood and urine at concentrations of 50 and 300 mg/L and then aliquoted and transferred to 1.5mL polypropylene tubes and stored at -22±2°C.

Table 4-2: Preparation of BHB Quality Control Samples

Concentrations (mg/L)	QC's level	Volume of Stock B Added (μL)	Final Volume Blood/Urine (mL)
0	Level 1	0	5
50	Level 5	125	5
300	Level 8	750	5

The working internal standard (GHB-D_6) was prepared at 10 mg/L in methanol from a stock solution of (1 mg/mL). This was achieved by adding 1 mL of the stock internal standard solution (1 mg/mL) to a 100 mL volumetric flask and diluting with methanol.

4.2.1.3 Preparation of Solutions

0.1M monobasic sodium phosphate was prepared by weighing and transfering 2.76g of sodium dihydrogen orthophosphate monohydrate ($NaH_2PO_4H_2O$; MW 137.99) to a 200 mL volumetric flask, dissolving in 100 mL deionized water and then making up to the mark with water. The solution was mixed and then transferred to a labelled clean glass bottle to be stored for 1 month at 5±1°C.

0.1M dibasic sodium phosphate was prepared by weighing and transferring 2.84g of disodium hydrogen orthophosphate anhydrous (Na_2HPO_4; MW 141.96) to a 200 mL volumetric flask, dissolving in 100 mL deionized water and then making up to the mark with water. The solution was mixed and then transferred to a labelled clean glass bottle to be stored for 1 month at 5±1°C.

0.1 M phosphate buffer (pH 6) was prepared by dissolving 1.7g disodium hydrogen orthophosphate anhydrous (Na_2HPO_4) and 12.14g sodium dihydrogen orthophosphate monohydrate ($NaH_2PO_4H_2O$) in 800 mL of water. The pH was adjusted to pH 6±0.1 with 0.1M monobasis sodium phosphate (lowers pH) or 0.1M dibasic sodium phosphate (raises pH) and made up to 1 litre with water. The buffer was mixed and then transferred to a labelled clean glass bottle to be stored for 1 month at 5±1°C.

Methanol (CH_3OH)/Ammonia NH_4OH (99:1) was prepared by measuring 99 mL of methanol using a measuring cylinder and was then transferred to a clean labelled reagent bottle and 1 mL of ammonia added and mixed. This solution was prepared fresh daily

A solution of 0.025M H_2SO_4 was prepared by dissolving 0.695 mL of concentrated (18 M) sulphuric acid in approximately 200 mL of deionized water and then made up to the mark (500 mL volumetric flask) with water. The solution was stored at room temperature after being transferred to a clean labelled bottle.

4.2.2 Instrumentation

Gas chromatography-mass spectrometry (GC-MS) was carried out using a Thermoquest, CE Instrument Trace GC with X-calibur software coupelled with Finnigan Trace MS and AS 800 autosampler purchased from (Thermo Fisher Scientific, UK). The column used was a DB-5 capillary column (5% phenyl/95% methylsiloxane, 30m x 0.25 mm I.D. 0.25 mm film thickness) purchased from Agilent Technologies Ltd, UK.

4.2.2.1 GC/MS Conditions

Optimization of the GC conditions was carried out by comparing two programs:

Initial GC program - Initial oven temperature of 60ºC was held for two minutes and then ramped to 180ºC at 15ºC/min, followed by a further ramp to 300ºC at 50ºC/min and held for 5 minutes. The total run time was 10 minutes. The source temperature was 200ºC; the transfer line temperature was 250ºC; the injector base temperature was 250ºC using splitless mode and the carrier gas flow rate was 1.2 mL/min. The mass detector multiplier voltage was 500 volts and the scan range was 40-300 amu.

Final GC program - Initial oven temperature of 60ºC was held for two minutes and then ramped to 180ºC at 20ºC/min, followed by a further ramp to 250ºC at 50ºC/min and held for 1 minute. The total run time was 10 minutes. The source temperature was 200ºC; the transfer line temperature was 250ºC; the injector base temperature was 250ºC using splitless mode and the carrier gas flow rate

was 1.2 mL/min. The mass detector multiplier voltage was 500 volts and the scan range was 40-300.

Data was collected in full scan mode and the ions monitored were: m/z **233**, 191, and 117 for BHB and m/z **239** and 240 for the internal standard GHB-d_6. The bolded underlined ions were used for quantification.

4.2.3 Method Evaluation

Methods previously developed by other authors(179-181) were modified slightly, including optimisation of the extraction, derivatization and GC-MS conditions and were then applied to blood and urine samples. The three methods involved: (A) clean screen solid-phase extraction (SPE); (B) liquid-liquid extraction (LLE) and (C) protein precipitation. A summary of the methods are listed in Table 4-3.

Table 4-3: Methods Evaluated for the Determination of BHB in Blood and Urine.

Method	Conditions	Extraction Solvent	Derivatization Conditions
A (SPE)	Original(181)	Acetone, pH 6 Phosphate buffer MeOH: Ammonia 99:1	100 µL EtOAc:100 µL of BSTFA+1%TMCS at 70ºC for 30 minutes
B (LLE)	Original(179)	EtOAc	75 µL ACN: 25 µL BSTFA+1% TMCS at 60ºC for 60 minutes
	Condition 1	EtOAc	75 µL ACN: 25 µL BSTFA+1% TMCS at 60ºC for 60 minutes
	Condition 2	EtOAc	50 µL EtOAc: 50 µL BSTFA+1% TMCS at 70ºC for 30 minutes
	Condition 3	EtOAc	25 µL EtOAc: 75 µL BSTFA+1% TMCS at 70ºC for 30 minutes
C (PP)	Original(180)	ACN	75 µL BSTFA+1% TMCS, at 90ºC for 10

SPE - Solid Phase Extraction, LLE - Liquid-Liquid Extraction, PP - Protein Precipitation, EtOAc - Ethyl Acetate and ACN - Acetonitrile

Two of the methods (A and C) were originally used for analysing GHB in different post-mortem matrices and were applied to the detection of BHB in blood and urine due to the similarity of their chemical structure.

Peak shape, reproducibility, time of extraction, linearity range and the cleanliness of the derivative were evaluated when comparing the above methods. The LLE extraction method (B) was also used to evaluate and optimize different derivatization conditions (incubation temperature and time and volume of derivatising agent) as summarised in Table 4-3.

4.2.3.1 Method A – CLEAN SCREEN SPE

The CLEAN SCREEN SPE is a mixed-mode cartridge packed with hydrophobic and phenyl/ propylsulfonic acid, which is a strong cationic exchange sorbent.(182) It was designed to be used for filtration and extraction of GHB. Isolation of BHB from blood and urine was achieved using acetone, after adding 100 µL of internal standard (GHB-D_6) as follows; after vortexing for 15 seconds and centrifugation at 1500 rpm for 5 minutes, the upper layer was collected and transferred to a clean vial and evaporated at 70ºC to dryness with nitrogen. The dried extract was dissolved in 200 µL of 0.1M phosphate buffer (pH 6.0) and was vortex mixed for 10 seconds. The reconstituted extract of the samples was applied to the conditioned SPE columns. Clean test tubes were added to the rack and then 1 mL of methanol/ammonia (99:1) was added to the original sample test tube, vortex mixed and then decanted onto the SPE column. The extract was collected, removed from the vacuum manifold and evaporated at 70ºC to dryness with nitrogen. For the derivatization step, 100 µL of ethyl acetate and 100 µL of BSFTA +1% TCMS were added to all samples, mixed, and heated at 70ºC for 30 minutes. Samples were transferred to GC vials and 2 µL injected onto the GC-MS.

4.2.3.2 Method B – Liquid-Liquid Extraction (LLE)

A 100 µL aliquot of blood or urine was extracted by adding 100 µL of internal standard (GHB-d_6), 1 mL ethyl acetate and 100 µL 0.025 M sulphuric acid. After mixing in the sample shaker for 10 minutes, the samples were centrifuged at 1500 rpm for 5 minutes. The upper layer was collected and transferred to a clean vial and evaporated to dryness with nitrogen then derivatized with BSFTA +1% TCMS. Evaporation and derivatization was carried out at different temperatures and using different ratio mixtures of ethyl acetate and BSFTA +1%

TCMS as per Table 4-3. Samples were transferred to GC vials and 2 µL injected onto the GC-MS.

4.2.4 Results and Discussion

Good separation was achieved between BHB and the internal standard (GHB-D_6) in blood and urine using the "Final GC Program" conditions as illustrated in Figures 4-1 and 4-2 respectively.

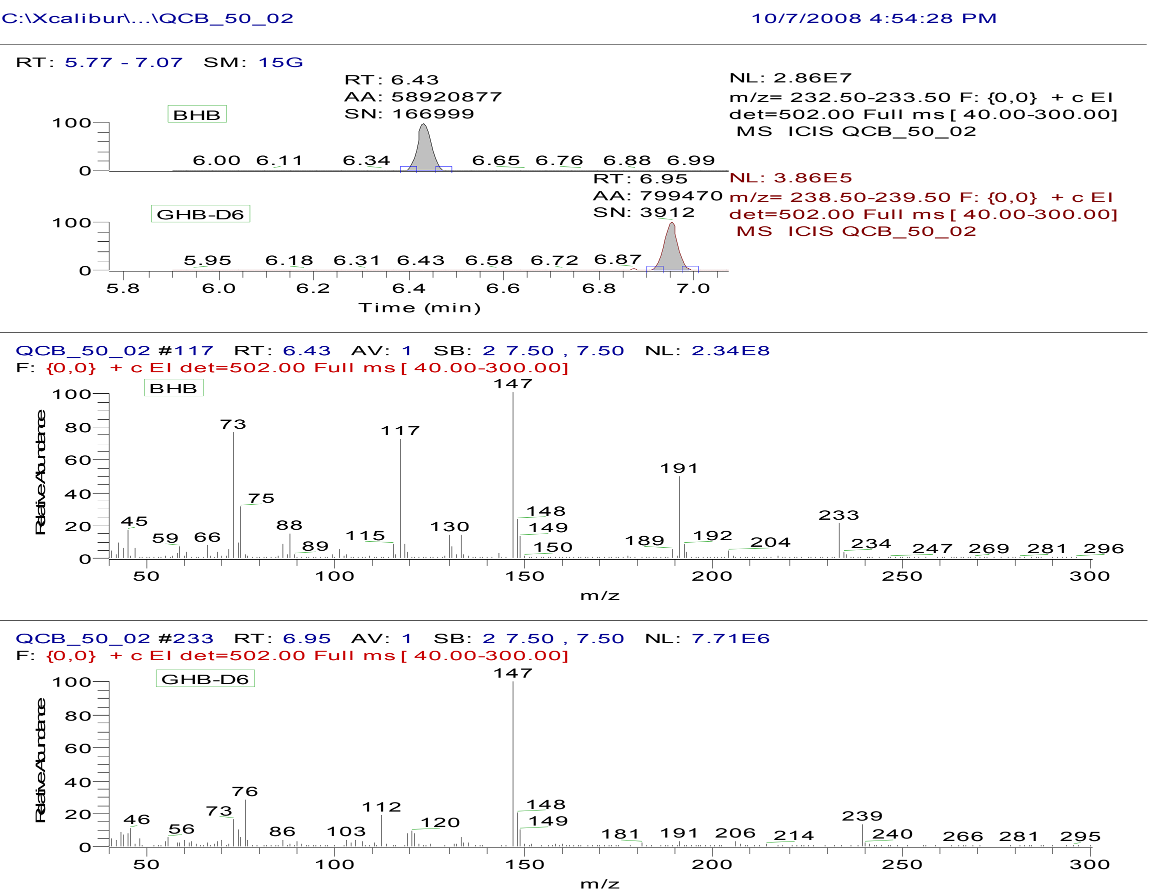


Figure 4-1: Chromatogram and Spectra for BHB and GHB-D6 from a Blood Control (50 mg/L)

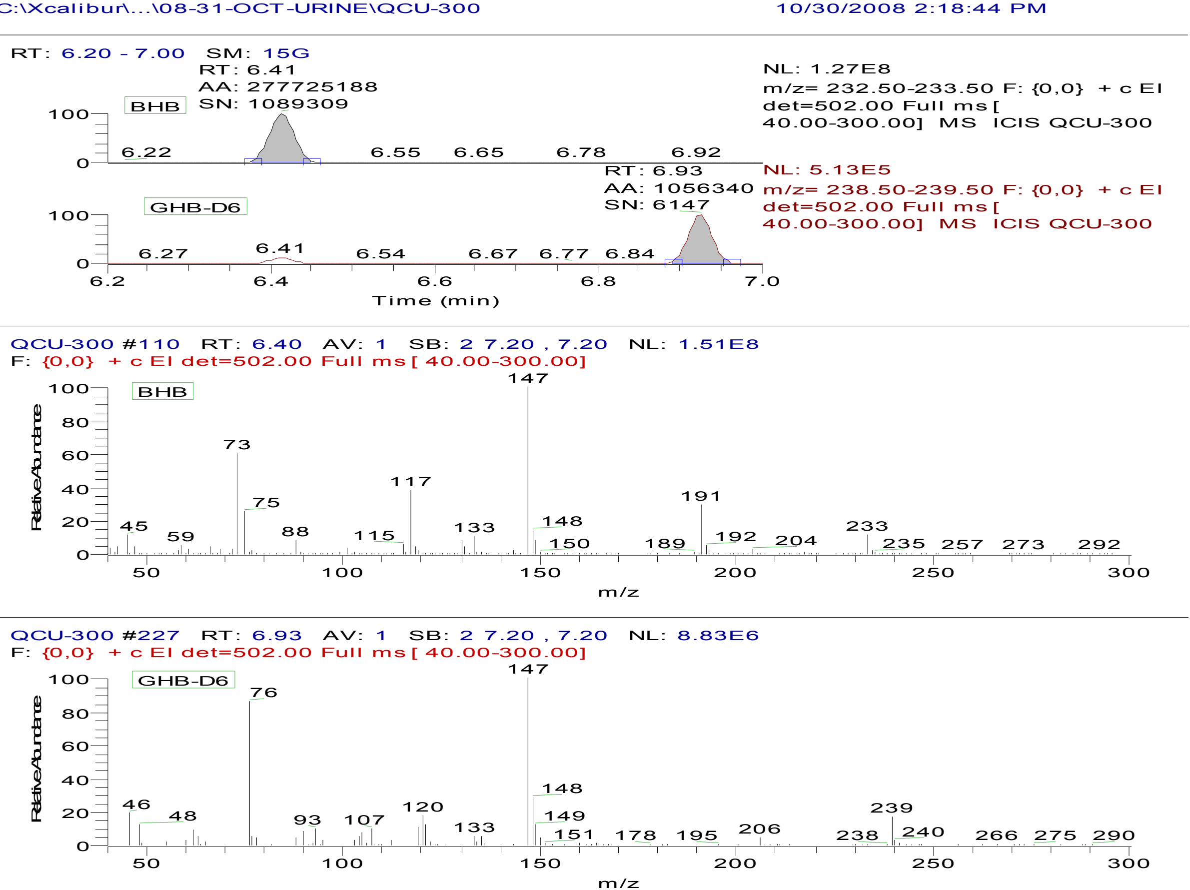


Figure 4-2: Chromatogram and Spectra for BHB and GHB-D6 in Post-Mortem Urine (300 mg/L)

Poor peak shape was observed with Method B (LLE) using derivatisation conditions (1): 75 µL ACN and 25 µL BSTFA+1% TMCS at 60°C for 60 minutes as illustrated in Figure 4-3.

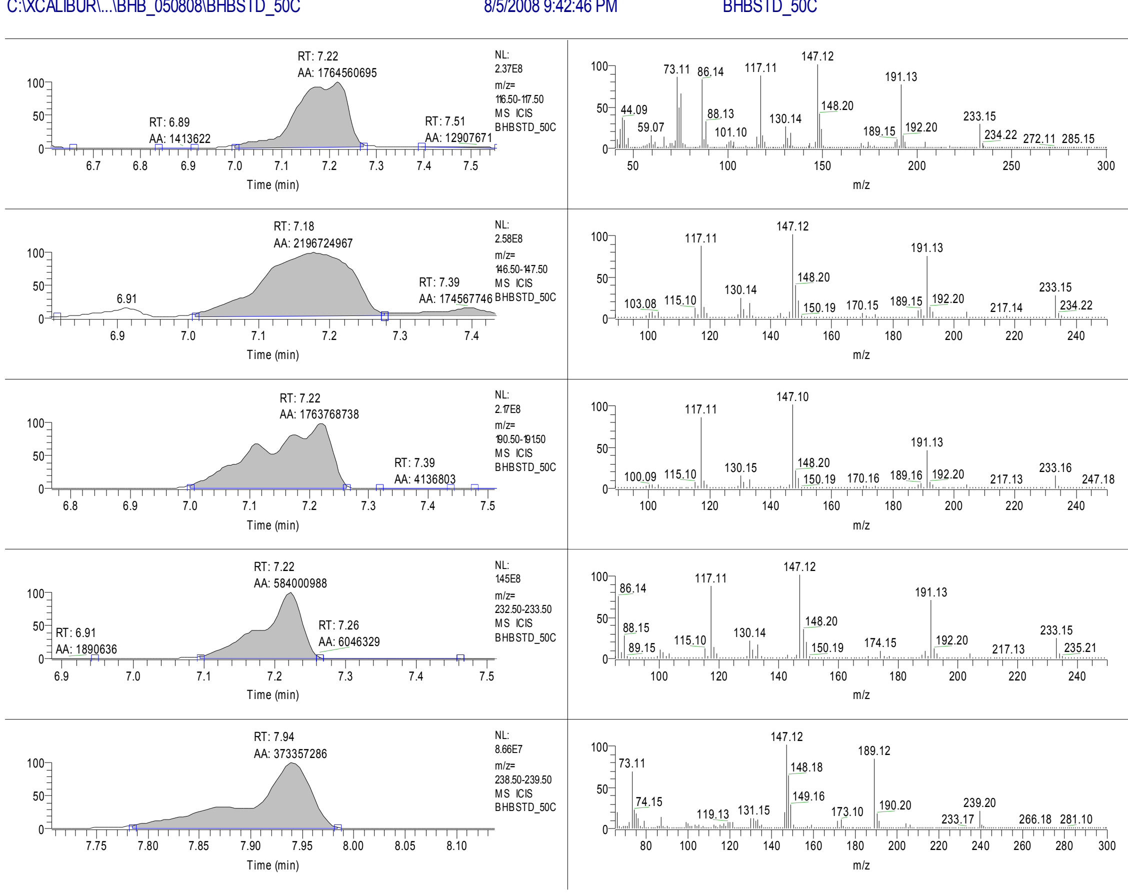


Figure 4-3: BHB and GHB-D6 Extracted Ions for Spiked Blood using LLE and derivatization Conditions (1)

This may be due to the long derivatization time or as a consequence of incomplete derivatisation due to the low volume of the derivatizing reagent. Symmetric peak shapes were observed when using derivatisation conditions (2) and (3). Figure 4-4 illustrates the excellent peak shapes achieved with the LLE method and derivatisation conditions (3). The derivatisation time was shorter and the volume of BSTFA+1%(TMCS) greater.

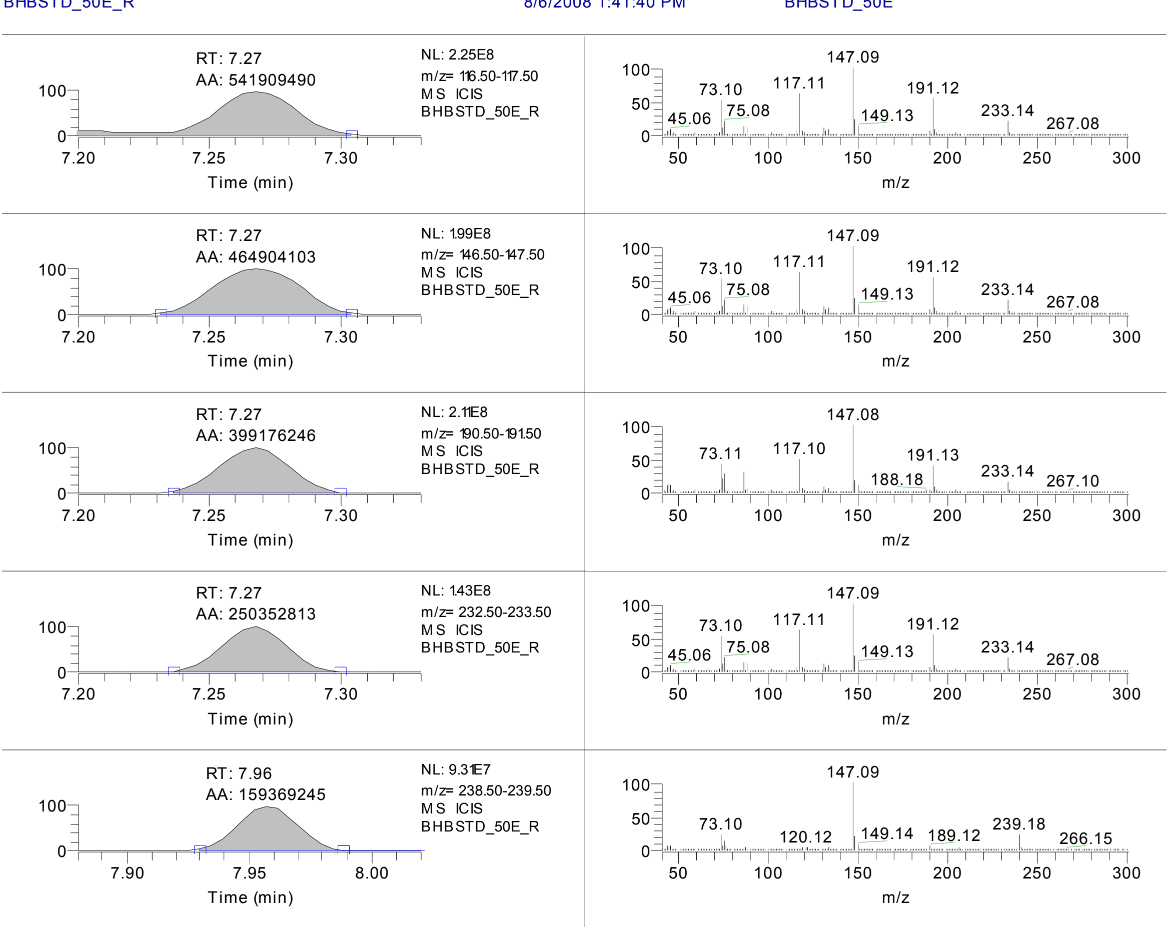


Figure 4-4: BHB and GHB-D6 Extracted Ions for Spiked Blood using LLE Method and Derivatisation Conditions (3).

The correlation coefficient (R^2) and linearity ranges are summarized in Table 4-4. The linearity of BHB using the SPE method (A) was up to 200 mg/L and 100 mg/L in blood and urine, respectively. The values were not always > 0.99 when the experiment was repeated and the sample preparation was found to be time-consuming. The main limitations of the SPE method were: the number of steps involved with sample preparation and the linear range only extended to 200 and 100 mg/L in blood and urine, respectively. The upper limit of quantification (ULOQ) was not high enough and would therefore require reanalysis and dilution of real case samples in order to achieve accurate quantitative results within the linear range. Also interference from uric acid is another problem and this is also observed by telepchak, *et al*. 2004.(183)

Table 4-4: R^2 and Linear Range of BHB for Different Methods

Method	Conditions	Matrix	BHB (R^2)	Linear Range (mg/L)
A (SPE)	Original	Blood	0.9878	LOQ-200
		Urine	0.9335	LOQ-100
B (LLE)	Original	N/A	Poor peak shape	
	Condition 1	N/A	Poor peak shape	
	Condition 2	Blood	0.9877	LOQ-500
		Urine	0.9877	LOQ-500
	Condition 3	Blood	0.9991	LOQ-500
		Urine	0.9987	LOQ-500
C (PP)	Original	Blood	0.9994	LOQ-500
		Urine	0.9983	LOQ-400

Although the linearity using the LLE method (B) was up to 500 mg/L using both derivatizing conditions 2 and 3, the value was better using conditions (3). The limitation of derivatisation conditions (2) was that the derivative, following extraction of the blood sample, was brownish in colour and the presence of uric acid interfered with the urine extraction.

Using the protein precipitation method (C) the linear ranges in blood and urine for BHB were LOQ-500 mg/L and LOQ-400 mg/L, respectively. The ULOQ in the published method was 300 mg/L for GHB. Interference with uric acid in urine samples was a problem with this method. Comparing the protein precipitation methods with LLE, the protein precipitation method in blood was more robust and used less solvent. The final derivatised blood extract was cleaner, the sample preparation was faster due to fewer and shorter steps. However, using this method to extract urine was problematic, due to high interference with uric acid crystals in the final derivative.

The SPE method (A) using clean screen SPE column was not selected due to the narrow linear range and the interference with uric acid which was also reported by Telepchak.(183) The LLE method (B) with condition 3 was selected for the

extraction of BHB from urine as the final extract was cleaner than the protein precipitation method with a linear range up to 500 mg/L. The protein precipitation method (C) was used for the extraction of BHB from post-mortem blood because it was more robust, the final derivatised extract was cleaner compared with the LLE method and the linear range was up to 500 mg/L while using less solvent. The sample preparation was fast due to the fact that it contained fewer and shorter steps. Method validation was carried out for both selected methods as summarised in Table 4-5.

Table 4-5: Summary of Blood and Urine Extraction Methods for the Analysis of BHB

Matrix	Step 1	Step 2	Step 3	Step 4
	Sample Volume	**$GHB-d_6$** (10 mg/L)	**Extraction**	**Derivatization Conditions**
Blood	100 µL	100 µL	0.5 mL ACN Vortex mix 30s Centrifuge @ 1500 rpm Evaporate @ 45ºC±1 to dryness	75 µL BSTFA+1% TMCS, at 90ºC for 10 minutes
Urine	100 µL	100 µL	100 µL 0.025 M H_2SO_4 + 1 mL EtOAc Vortex 30s Centrifuge @ 1500 rpm Evaporate @ RTºC±1 to dryness	25 µL EtOAc: 75 µL BSTFA+1% TMCS at 70ºC for 30 minutes

4.3 Method Validation

4.3.1 Linearity

Calibration standards were prepared by spiking blank blood and urine with BHB at concentrations ranging from 50-500 mg/L as detailed in section 4.2.1.3. The peak area ratio of the BHB to internal standard ($GHB-D_6$) was calculated, and the calibration curve was generated by plotting the area ratio against the concentration. The equation of line and correlation of coefficient (R^2) was measured and should be greater than 0.99.

4.3.2 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ were determined by diluting the spiked samples with decreasing concentrations of BHB within the expected range of the LODs (1.25–25 mg/L). Regression analysis was undertaken and the LOD and LOQ were calculated was explained in chapter 2 section 2.2.9 in accordance with Miller and Miller.(168)

Case samples for BHB at concentrations that are above or below the linear range (50-500 mg/L) were reported as less than the LOQ (<50 mg/L) or greater than the highest calibrator (>500 mg/L).

4.3.3 Precision and Accuracy

Intra-day precision (within-day precision) was evaluated with urine (N=6) and blood (N=8) spiked controls, prepared at two different concentrations (50 and 300 mg/L) and extracted in a batch with case samples. Inter-day precision (between-day precision) was evaluated with urine and blood spiked controls, prepared at two different concentrations (50 and 300 mg/L) and extracted in batches with case samples on different days (N=8). The percentage of coefficient of variance (%CV) acceptance criteria was <20%. Accuracy was assessed by preparing and extracting triplicate spiked blood and urine control samples at concentrations of 50 and 300 mg/L. The accuracy was determined by comparing the calculated amount extracted to that of the theoretical expected concentration of both control levels.

4.3.4 Recovery and Matrix Effect

The recovery of BHB from blood and urine was assessed by preparing spiked blood and urine controls at concentrations of 50 and 300 mg/L. The controls were extracted as described above however, 100 µL of the internal standard (GHB-D_6) at a concentration of 10 mg/L was added prior to evaporation after extraction. The recovery was determined by comparing the extracted BHB:GHB-D_6 ratio to that of the unextracted standards at the same concentration. All analyses were carried out in triplicate and the average calculated. The absolute recovery was calculated by comparing the peak area ratio of extracted sample

when the internal standard was added at the beginning of the extraction versus the unextracted standards at the same concentration.

The relative recovery was obtained by extracting water spiked at concentrations of 50 and 300 mg/L, calculating the BHB: GHB- D_6 ratio and comparing that to the ratio calculated for blood and urine, expressed as a percentage. All analyses were carried out in triplicate and the average calculated. The relative recovery was carried out to check the matrix effect and the absolute recovery to check the efficiency of the method.

4.3.5 Stability

Two small studies were carried out to assess the stability of the derivatized extracts and to assess the stability of BHB in blood. The stability of the derivatized extract was evaluated by repeatedly injecting the same two levels (50 and 300 mg/L) of processed samples for 4 days following derivatization and storage at room temperature (22±2°C).

The stability of BHB in blood was assessed by extracting replicate spiked samples (50 and 300 mg/L) from the same quality control lot stored at 2-5ºC and analysed at time intervals of days 1, 3, 11, 13 and 15. The percentage recovery was calculated from the mean concentration of QC samples injected in days 3, 11, 13 and 15 and compared as a percentage to day one (normalised as 100%). The 95% confidence interval (CI) from the mean concentration (n=6) for day one was calculated and stability was assumed when the percentage of the mean of the QC samples was within 80-120%.(169)

4.3.6 Case samples

Post-mortem blood (N=13) and urine (N=12) were selected from fourteen cases where the cause of death was not known and there was a history of alcohol abuse. Preserved samples (0.2% sodium fluoride) were analysed in preference to unpreserved samples but these were not available for all cases. All unpreserved and preserved samples were stored at 4±1°C until analysis.

4.3.7 Results and Discussion

Both methods for blood and urine were linear over the range of 50-500 mg/L and resulted in average coefficients of correlation (R^2) > 0.99 as illustrated in Figure 4-5). The LOD was 2 mg/L in blood and urine, while the LOQ was 6 and 7 mg/L respectively for urine and blood. Although the LOQ is lower than the first calibrator point (50 mg/L), this point was used as a cut-off point to distinguish between positive and negative results. The endogenous level in serum has been reported in healthy adults as being 30-60 mg/L.(133) Urinary endogenous levels of BHB in healthy humans ranged 8.51-34.7 mg/L (mean is 18.4 mg/L), it has been also observed that the urinary endogenous levels of BHB is approximately 20 times higher than its related compounds such as GHB.(134) Triplicate samples of blood and urine were screened for BHB to be used to spike quality conrol samples and the results were less than LOD of the method also they very low compared to the cut-off points (50 mg/L). Urine and blood blanks were included in each calibration curve to take into account any endogenous BHB.

(A) **(B)**

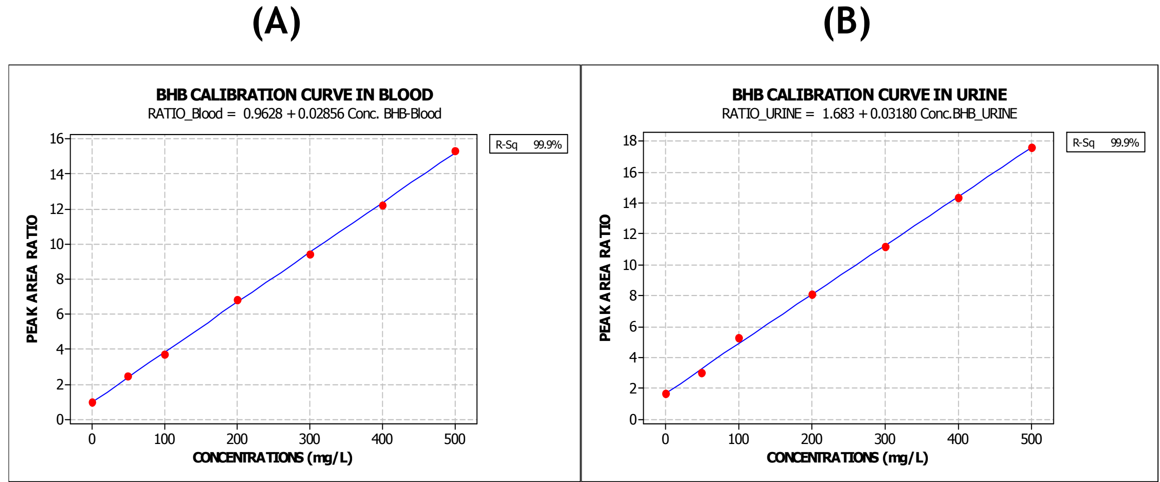


Figure 4-5: Calibration Curves for BHB in Blood (A) and Urine (B)

The inter-day and intra-day precision was measured by coefficients of variation (%CV) for blood and urine and ranged from 1.40 to 12.41% for quality control samples spiked at level of 50 and 300 mg/L as summarised in Table 4-6.

Table 4-6: BHB Intra day and Interday Precision in Blood and Urine

Matrix	Intraday Precision (%CV)		Interday Precision (%CV)		Accuracy (%)	
	50 mg/L	300 mg/L	50 mg/L	300 mg/L	50 mg/L	300 mg/L
Urine	6.75 (N=6)	5.23 (N=6)	12.16 (N=8)	6.03 (N=8)	111 (N=3)	100 (N=3)
Blood	4.94 (N=8)	1.40 (N=8)	12.41 (N=8)	6.79 (N=8)	97 (N=3)	99 (N=3)

Table 4-7 summarises the recovery of BHB from blood and urine. The recovery of BHB from blood was greater than or equal to 82% while the relative recovery (matrix effect) was 129% and 106% at concentrations of 50 and 300 mg/L, respectively. The recovery of BHB from urine was 134 and 105% at concentrations of 50 and 300 mg/L, respectively. The efficiency (Absolute recovery) for both urine and blood were all within the range of 88-106% at both concentrations except in urine at 50 mg/L (135%). The high % recoveries may be explained by matrix effects, e.g. interference from uric acid in urine, but can also be affected by poor extraction or the fact that the internal standard, (GHB-D_6) although structurally related to BHB is not the same.

Table 4-7: BHB Recovery and Matrix Effect Data

Method	Matrix effect (Relative Recovery) %		Efficiency (Absolute Recovery) %		Recovery %	
	50 mg/L	300 mg/L	50 mg/L	300 mg/L	50 mg/L	300 mg/L
Urine	101	131	135	89	134	105
Blood	129	106	106	88	82	83

Table 4-8 summaries the stability data for BHB in blood over a period of 15 days and a separate study following derivatization and storage for 4 days.

Table 4-8: BHB Stability Data for Spiked Blood Stored at 2-5ºC for 15 Days

(A) BHB stability in spiked blood stored at 2-5º for 15 days		
n=3	Recovery %[Measured value mg/L]	
Day	**50 mg/L**	**300 mg/L**
1	100 [55]	100 [297]
3	103 [56]	117 [348]
11	106 [58]	105 [313]
13	98 [53]	106 [315]
15	77 [42]	100 [298]
Range [Mean of day 1±SD	52-57	262-332

(B) BHB stability in processed samples stored at room temperature for 4 days		
n=3	Recovery % Measured value mg/L]	
Day	**50 mg/L**	**300 mg/L**
1	100 [61]	100 [330]
2	109 [66]	112 [369]
3	102 [62]	99 [327]
4	113 [69]	99 [326]
Range [Mean of day 1±SD	56-66	283-378

(A) and (B) for Derivatised Extracts Stored at Room temperature for 4 Days

Although there was some variation in the measured concentrations, BHB remains stable in blood spiked at a concentration of 300 mg/L for 15 days when stored within a refrigerator (2-5°C). There was some loss of BHB in the control samples spiked at 50 mg/L on day 15 and a longer stability study is required to investigate this further. BHB remains stable for approximately four days at room temperature after derivatization.

Fourteen case samples(details summarised in Table 4-9) consisting of blood (N=13), urine (N=12) and vitreous humour (N=1) samples were analysed for BHB. The cause of death as reported by the pathologist is also included.

Table 4-9: BHB Summary of Case Results

No.	BHB mg/L			Acetone mg/L			Ethanol mg/dL			History /Cause of Death
	Blood	Urine	VH	Blood	Urine	VH	Blood	Urine	VH	
1	205	>500		145*	431	152	19*	81	30	Alcoholic/fatty liver, cause of death undetermined
2	343	>500		35*	452	41	NEG*	NEG	NEG	Fatty liver
3	239	429		694*	780		30*	NEG		Diabetic/possible ketoacidosis or drug/alcohol related
4		>500			201	325		12	25	Known alcohol abuse
5	<50	>500		20*	164		12*	58		Chronic alcohol abuse
6	203	>500		40*	54		NEG*	NEG		Fatty liver
7	328			387*			NEG*			Diabetic/possible ketoacidosis or drug/alcohol related
8	214*	>500		19*	89		NEG*	NEG		Alcoholic/Fatty liver, cause of death undetermined
9	198*	432		194*	342		12*	NEG		Alcohol abuse
10	138*	323	341	76*	103	71	67*	88	72	Undetermined cause of death, fibrosis
11	233	307		157*	225		340*	538		Liver failure, known alcoholic, smell of alcohol, micronodular cirrhosis, heart disease
12	211			311*			NEG*			Suspected drug related death/alcohol abuse but no smell of alcohol
13	246	252		50*	76		297*	347		Found dead in his room
14	267*	>500		250*	314		NEG*	NEG		Fatty degeneration of the liver, chronic alcohol abuse, fatty liver

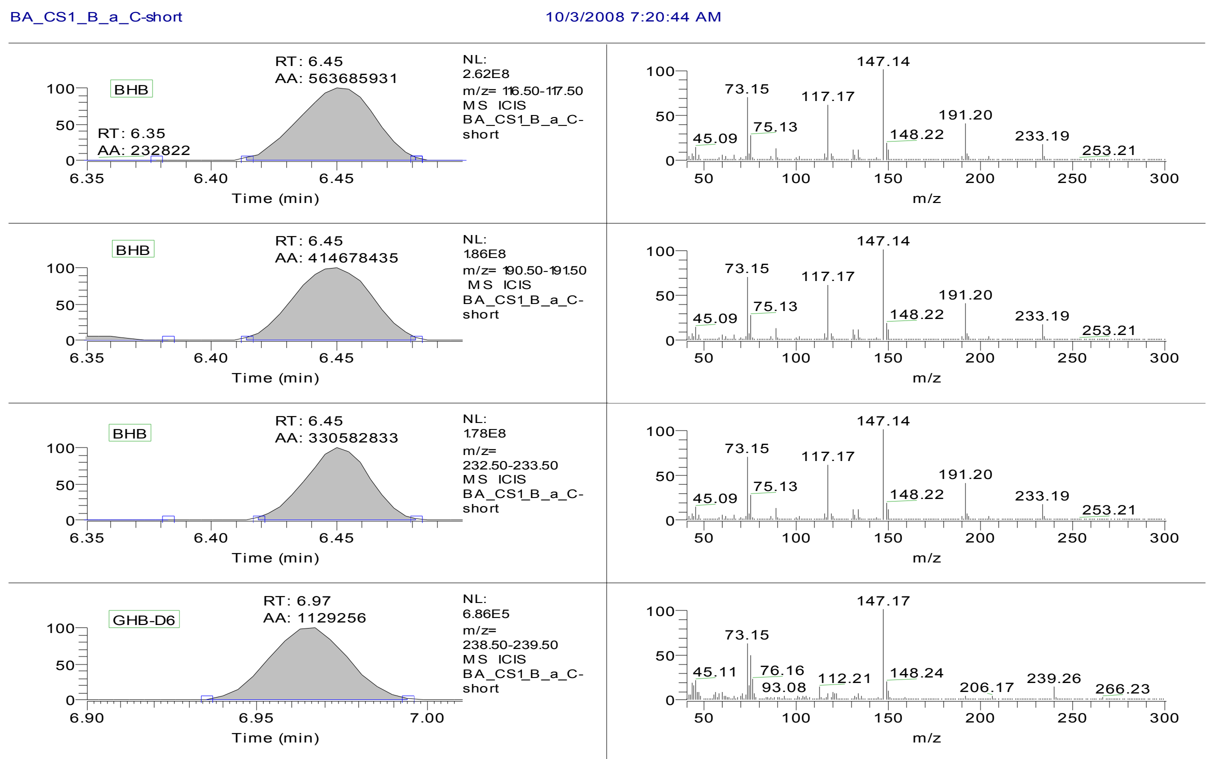
***Indicates preserved samples.**

BHB and acetone are reported in mg/L while ethanol is reported in mg/dL. Results are reported with no decimal places. Blood BHB results for alcoholics above 260 mg/L or 2500 μmol/L are considered to be consistent with alcoholic ketoacidosis (AKA).

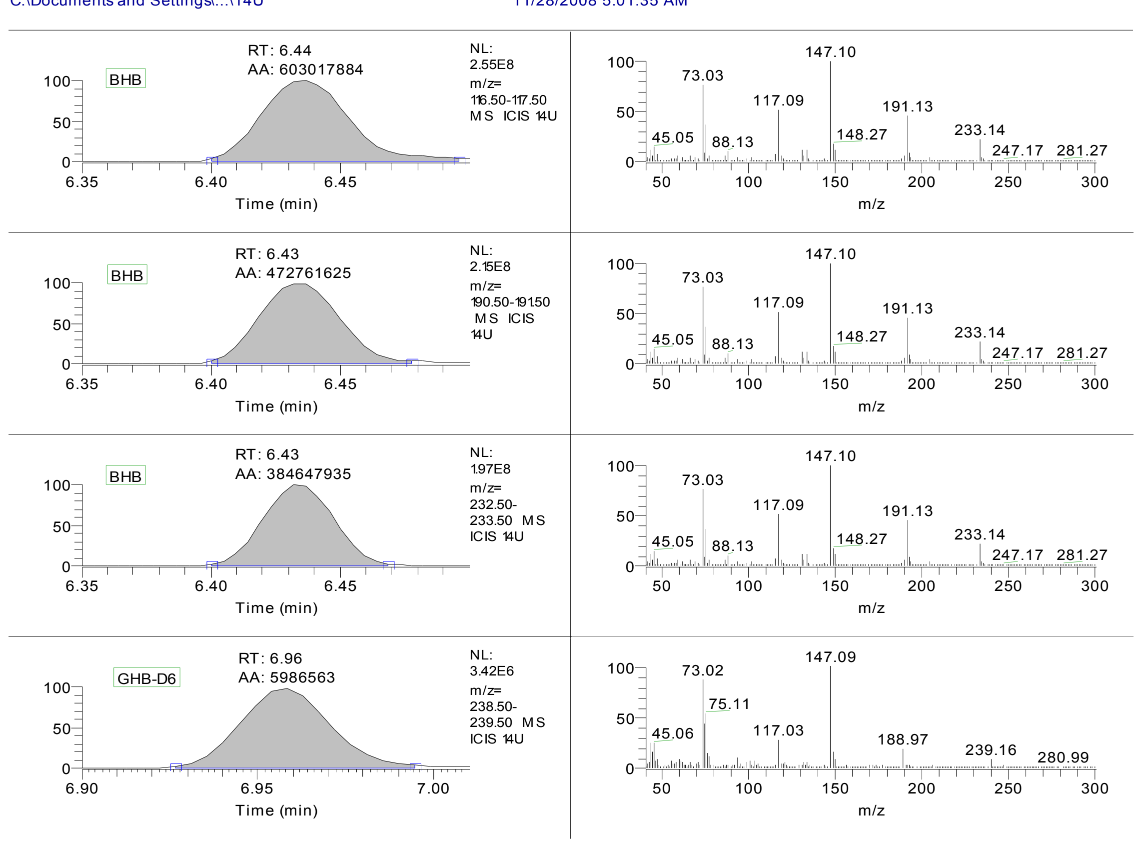
The cases were selected on the basis of high acetone levels and the cause of death was either undetermined or the deceased had a history of chronic alcohol abuse. Two of the fourteen cases were known diabetics (Cases #3 and #7), but in all cases the deceased had a history of alcohol abuse and post-mortem findings included fatty degeneration or fibrosis of the liver.

BHB levels in post-mortem blood ≥260 mg/L have been associated with fatalities attributed to alcoholic ketoacidosis.(127) BHB was elevated within the toxic or fatal ranges in blood for all the cases investigated except one (case sample #5) where the BHB level was normal <50 mg/L. The range of BHB in urine in all 12 cases was between 252 to >500 mg/L.

Figures 4-6 illustrates a chromatogram and spectra for BHB and the internal standard GHB- D_6 extracted from a case with both blood and urine samples.



(A)



(B)

Figure 4-6: Post-mortem Case Samples with BHB in (A) Blood (343 mg/L) and (B) Urine (>500 mg/L)

Blood acetone levels were elevated in all cases although, in five cases, acetone levels were ≤ 50 mg/L. Ethanol levels in all case samples were low or less than the LOD, except for case samples # 11 and 13, where the ethanol level for both urine and blood samples was high.

The relationship between BHB, acetone and ethanol levels requires further investigation with a larger data set.

4.4 Study Investigating the Relationship between β-hydroxybutyrate (BHB), Acetone and Ethanol in Post-Mortem Cases

4.4.1 Introduction

A reduced blood pH (ketoacidosis) from the production of ketone bodies as a result of alcoholic ketoacidosis (AKA) or diabetic ketoacidosis (DKA) can feature in many fatalities and analytical evidence can be used to support a pathological diagnosis, or provide a possible cause of death in the absence of other pathologically significant findings. Existing beliefs concerning the relationship of BHB concentrations, acetone and ethanol needs to be re-examined by the analysis of these analytes in a range of different post-mortem cases. BHB, acetone and ethanol concentrations measured in post-mortem blood will be grouped according to whether or not the deceased had a history of alcohol abuse or diabetes.

The method developed and described earlier in the chapter for the determination of BHB in blood was adapted and revalidated using a newer instrument from a different manufacturer. Acetone and ethanol were analysed with a dual–column head space gas chromatography flame ionisation detector (HS-GC-FID) in accordance with the FMS ISO/IEC 17025 accredited method.(132)

The existing belief that in cases of AKA, ethanol is always low (<10 mg/dL) or absent will be evaluated in addition to whether the absence of acetone will preclude a high BHB (>250 mg/L). The levels of BHB measured in this study will be categorised according to the published data indicating endogenous levels are <50 mg/L, toxic levels are within the range of 51–249 mg/L and pathologically significant levels are >250 mg/L.

4.4.2 Methodology

4.4.2.1 Instrumentation

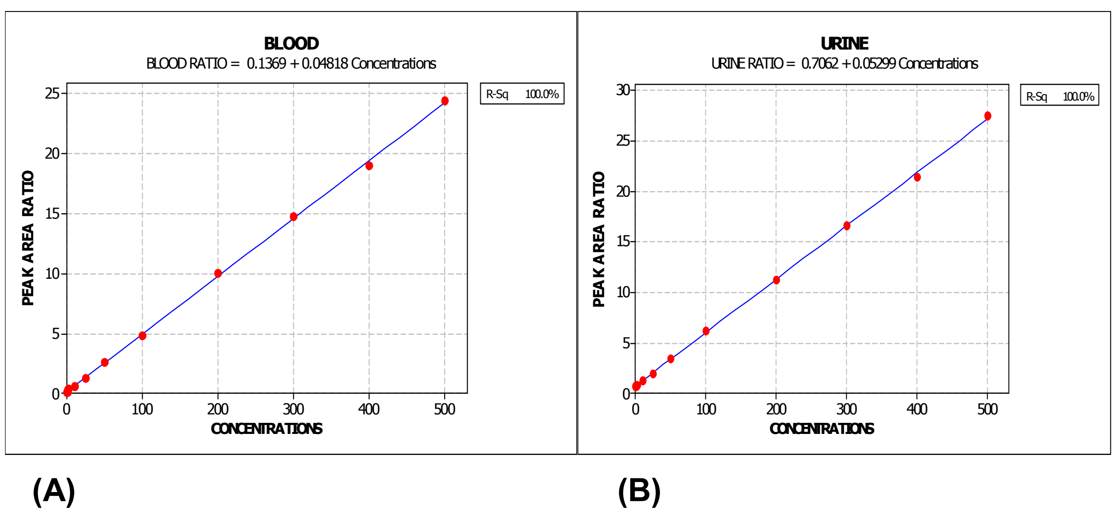
GC-MS analysis was carried out using an Agilent 7890A GC system coupled with a 5975 mass spectrometry detector (MSD) and ChemStation software (Agilent, UK).

A DB-5+ DG capillary column (30m x 0.25 mm I.D., 0.25 µm film thickness) was purchased from Agilent Technologies Ltd., UK.

The conditions optimised using the Thermo instrument and described in 4.2.2 were used on the Agilent instrument with only one alteration. Due to the sensitivity of the newer Agilent instrument the samples were injected in split mode as opposed to splitless mode.

4.4.2.2 Summary of Validation

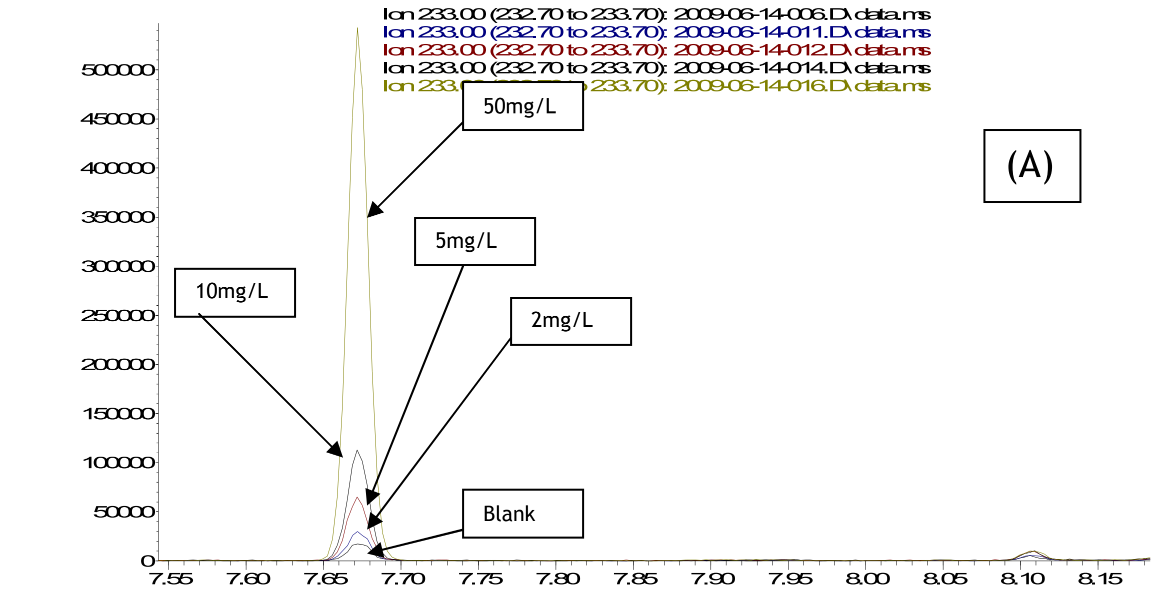
The validation was carried out as described in sections 4.3.1 to 4.3.4 above. In summary, BHB in both blood and urine was found to have a linear response over the concentration range of 10-500 mg/L as illustrated in Figure 4-7.



(A) (B)

Figure 4-7: BHB Calibration Curves for Blood (A) and Urine (B) using the Agilent GC-MS.

The LOD and LOQ was 2 and 6 mg/L respectively for blood and 1 and 2 respectively for urine. An overlay of chromatograms for BHB in samples of spiked blood and urine at concentrations of 2, 5, 10, and 50 mg/L in comparison to a blank sample is illustrated in Figure 4-8.



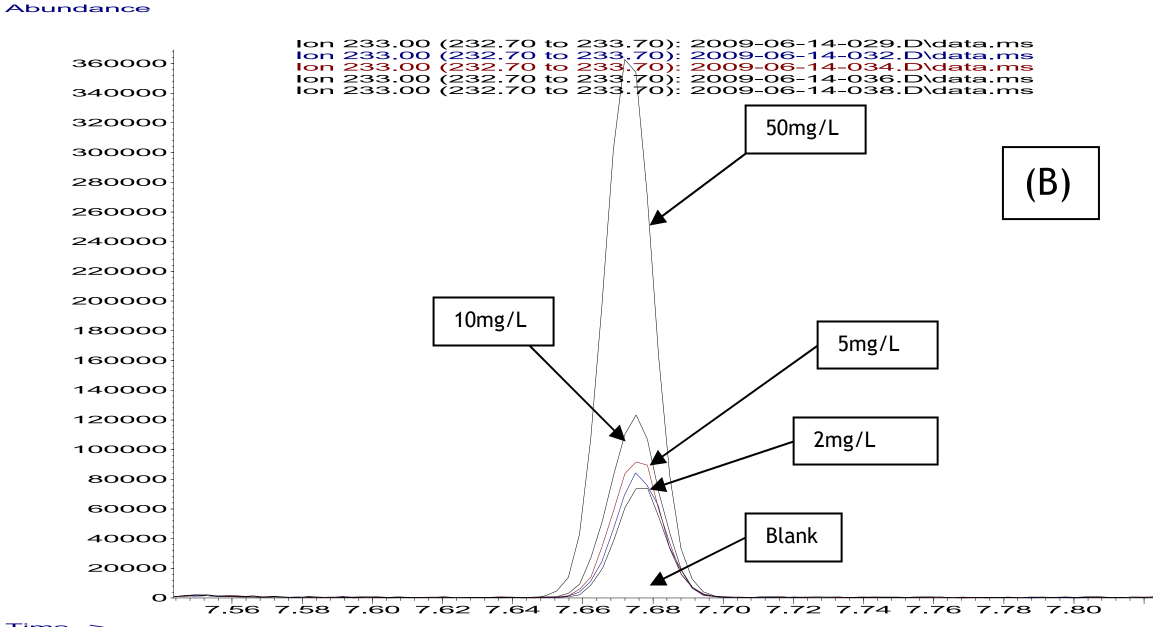


Figure 4-8: BHB Overlaid Chromatograms for Spiked Blood (A) and Spiked Urine (B) at Different Concentrations.

The intra and inter-day precision was less than 20%. Although the recovery was between 50-61% for BHB in blood and urine, the efficiency of the method was between 93-137% and its accuracy between 84-98%. Table 4-10 summarises the validation data for BHB in blood and urine.

Table 4-10: BHB Intraday, Interday Precision, Accuracy and Recovery Data

	Intraday precision CV% (n=6)		Interday precision CV% (n=10)		Accuracy %		Recovery %		Efficiency %	
Matrix	50 mg/L	300 mg/L	50 mg/L	300 mg/L	50 mg/L	300 mg/L	50 mg/L	300 mg/L	50 mg/L	300 mg/L
Urine	7.5	4. 6	13.2	5.8	84	98	61	50	137	105
Blood	1. 3	1. 4	2.8	5.3	95	97	56	60	100	93

Quality control samples (QC's) were prepared at two different concentrations (50 and 300 mg/L) and were then extracted with each batch and injected on different days. QC's were evaluated according to the acceptable range which is the mean ± 2 standard deviations. Quality control charts were used to record and monitor the acceptability of each batch and are included in Appendix 4-1. QC results were within the acceptable range for all batches.

4.4.2.3 Case Samples

Post-mortem blood (N=120) and urine (N=82) from 122 cases were identified within the FMS in-house database to be tested for BHB. The cases had already been analysed for volatiles and had varying causes of death recorded. BHB had not been tested routinely within FMS prior to this study and as such the cause of death was determined by the pathologist without knowledge of the BHB concentrations. Preserved case samples were analysed in preference to unpreserved samples but were not available for all cases; namely case numbers 99, 101, 102, 104, 105, 108 and 109. Full details of each case are listed in Appendix 4-2. "Preserved" blood samples were blood samples collected and stored in commercially available vials containing a preservative (0.2% sodium fluoride). All unpreserved and preserved samples were stored at 4ºC ±1 until analysis.

4.4.3 Results and Discussion

The majority of the cases (72%, N=79) have blood BHB levels between 0 and 50 mg/L with 10% of cases having blood BHB levels >250 mg/L as illustrated in Figure 4-9.

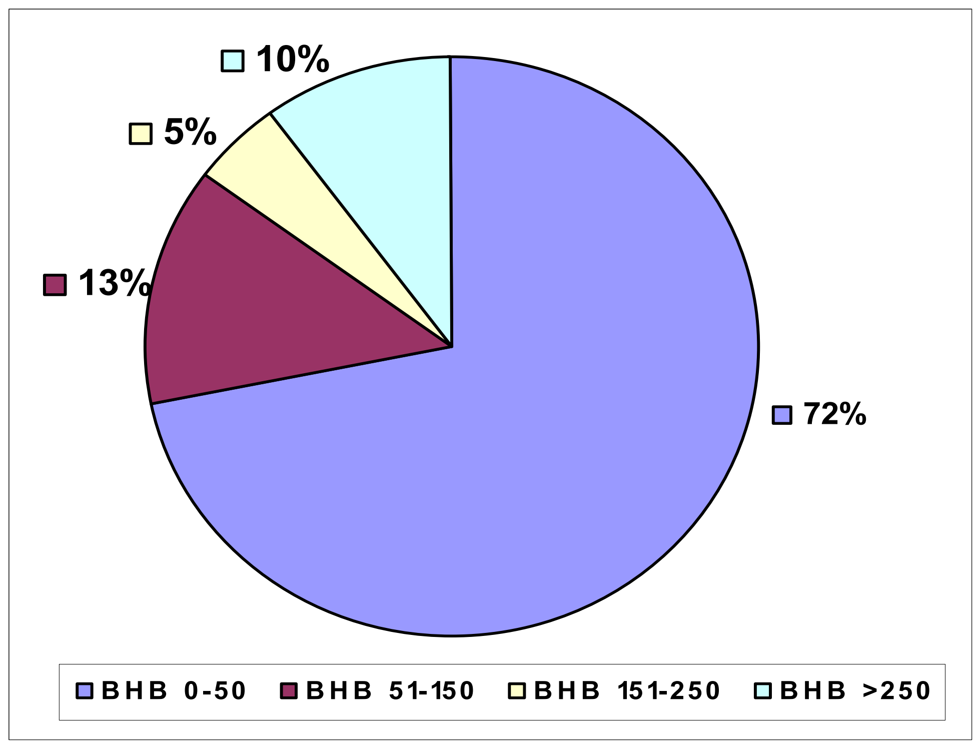


Figure 4-9: Prevalence of BHB at Different Concentrations in Post-Mortem Blood

The cases were grouped according to the cause of death and the level of BHB present in blood. Twelve cases were excluded on the basis that the cause of death was not known (N=11), as these were carried out by external pathologists or only urine was available (N=1). Table 4-11 summarises the number cases categorised by cause of death and blood BHB concentration, while Figure 4-10 illustrates the percentage of cases at each blood BHB concentration by cause of death.

Table 4-11: Post-Mortem Cases Classified by Cause of Death and Blood BHB Level

		Cause of Death						
BHB	N	Natural	Alc	Drug	Accident	Suicide	Homicide	Unasc.
0-50	79	18	6	23	9	16	3	4
51-150	16	6	5	1	3	0	0	1
151-250	6	3	2	1	0	0	0	0
>250	9	3	6	0	0	0	0	0

Alc: alcohol-related; Drug: drug-related; Unasc.: unascertained/undetermined

Of the 79 cases with blood BHB levels at 50 mg/L or below, 27 cases (34%) were negative (<10 mg/L) for BHB in blood. The positive blood samples ranged from 10 to 48 mg/L with a mean and median of 22 and 19 mg/L respectively. The cause of death in these cases was predominantly un-related to alcohol (92%) but it was not possible to determine from the case files whether there was a history of alcohol misuse or diabetes in these cases.

Although the number of cases with blood BHB levels ranging between 51-150 mg/L, was considerable smaller, there was observed, an increase in the percentage of alcohol-related deaths in this group.

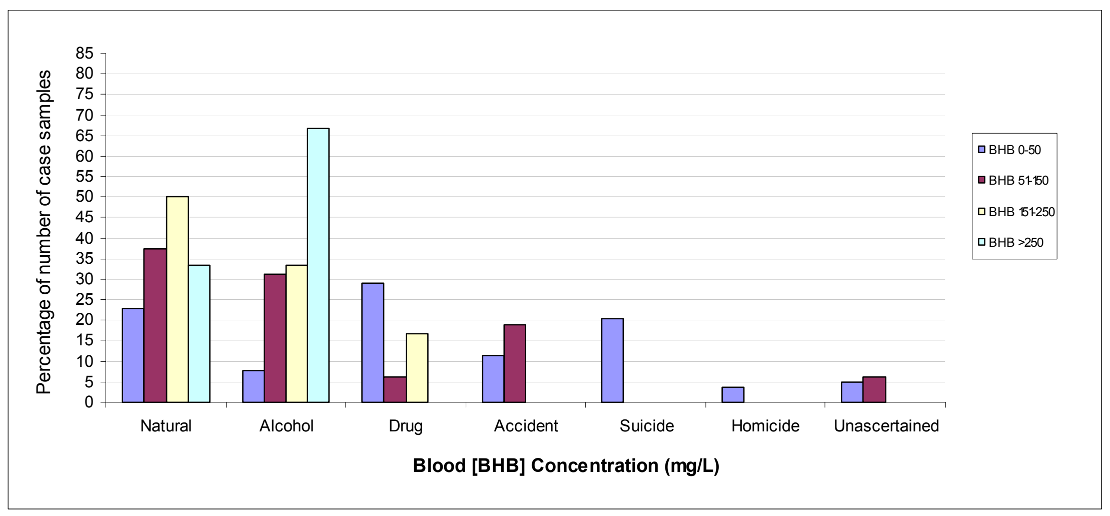


Figure 4-10: Percentage of Cases with Varying Blood BHB Concentrations by Cause Death

Table 4-12 summarises details of the six cases with elevated levels of BHB ranging from 165 - 249 mg/L. The cause of death in two cases was alcohol-related. A history of alcohol misuse was recorded for one of the four cases where the cause of death was not related to alcohol.

Table 4-12: Post-Mortem Cases with Blood BHB Concentrations Between 151-250 mg/L

Case #	Gender	Age	BHB (mg/L)		Acetone (mg/L)		Ethanol (mg/dL)		Cause of Death
			Blood	Urine	Blood	Urine	Blood	Urine	
16	M	73	195		50	56	NEG	NEG	1a- Ischaemic heart disease 1b- Coronary artery atheroma
30	F	66	237	452	15	26	NEG	NEG	1a- Acute hepatic necrosis; 1b- paracetamol Toxicity
73	M	71	208	308	67	84	NEG	NEG	1a- Acute pyelonephritis; 1b- Possible hypothermia
81	F	40	249	498	121	145	NEG	66	1a- Fatty degeneration of the liver 1b- Chronic alcoholic abuse
100	M	80	215	497	82	111	23	14	1a-Hypothermia
113	F	36	165		40		NEG		1a. Fatty degeneration of the Liver; 1b. Chronic Alcohol Abuse

F: female, M: male, NEG: negative

Table 4-13 summarises the cases where blood BHB concentrations are >250 mg/L which is associated with pathological findings and considered a marker for ketoacidosis. Three deaths (#17, 89, 112) were categorised as natural and the cause of death in each of these was related to insulin dependent diabetes mellitus (IDDM) and was specifically recorded as diabetic ketoacidosis in two cases (#17. 89). In both cases, the pathologist had requested BHB analysis, while in the third case (#112) BHB analysis was not requested even though the individual had a history of diabetes and alcohol misuse.

The remaining six cases were all alcohol-related and in all cases the individual had a history of chronic alcohol misuse. However, alcoholic ketoacidosis was not recorded as a contributing factor to the cause of death. The role of alcoholic ketoacidosis should be considered in these cases and those listed in Table 4-12 in light of the elevated blood BHB concentrations.

Table 4-13: Post-Mortem Cases with Blood BHB Concentrations >250 mg/L

Case #	Gender	Age	BHB (mg/L)		Acetone (mg/L)		Ethanol (mg/dL)		Cause of Death
			Blood	Urine	Blood	Urine	Blood	Urine	
5	F	44	>500	>500	167	224	NEG	16	1a- Fatty degeneration of the liver 1b- Chronic alcoholic
17	F	18	>500	>500	>400	>400	NEG	NEG	1a- Diabetic ketoacidosis; 1b. Insulin Dependent Diabetic Mellitus
89	M	34	>500	>500	297	>400	NEG	NEG	1a- Drowning ; 1b- Diabetic ketoacidosis; 1c. Insulin Dependent Diabetic Mellitus
98	M	67	383	358	72	85	338	392	1a. Alcohol related death
104	M	61	263	>500	42	147	193	286	1a. Ischaemic heart disease and gastro intestinal haemorrhage from erosive oesophagitis. 2a. Fatty degeneration of the liver
105	F	56	396		177		34		1a. Fatty degeneration of the liver due to; 1b. chronic alcohol abuse.
110	F	57	>500	>500	399	620	NEG	NEG	1a-Alcoholic Stetosis and Ischaemic heart disease; 1b Fatty degeneration of the liver; 1c- Chronic Alcoholic
112	M	42	>500		469		30	NEG	1a-Ischaemic heart disease 1b- Coronary artery atheroma 2a- Insulin dependent diabetes mellitus
122			>500		169	247	NEG	NEG	1a. Fatty degeneration of the Liver; 1b. Chronic Alcohol Abuse

F: female, M: male, NEG: negative

Alcoholic ketoacidosis is commonly associated with high blood BHB concentrations, elevated blood acetone concentrations and low alcohol concentrations.

Figure 4-11 illustrates the concentrations of acetone found relative to the blood BHB concentration. In all cases with blood BHB >250 mg/L, acetone was >10 mg/L. In two cases (# 98, 104) the acetone concentrations were below 100 mg/L (42 and 72 mg/L respectively) with corresponding BHB >250mg/L.

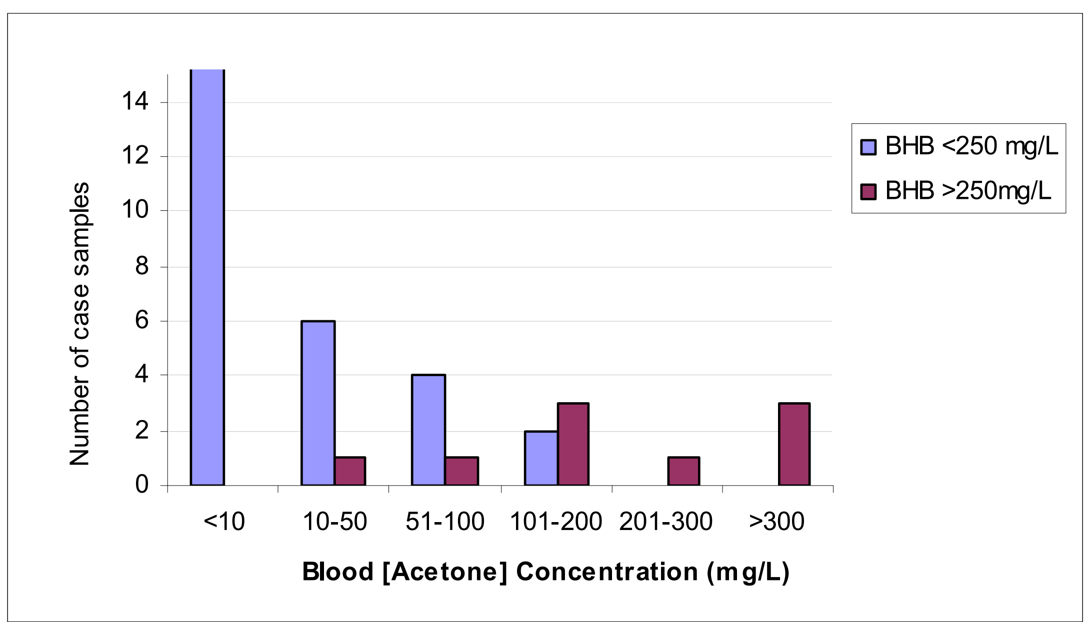


Figure 4-11: Acetone Concentrations in Post-Mortem Blood Relative to BHB Levels
Note: The scale of y axis has been reduced to 15 instead 100 to show the case number in lower scale

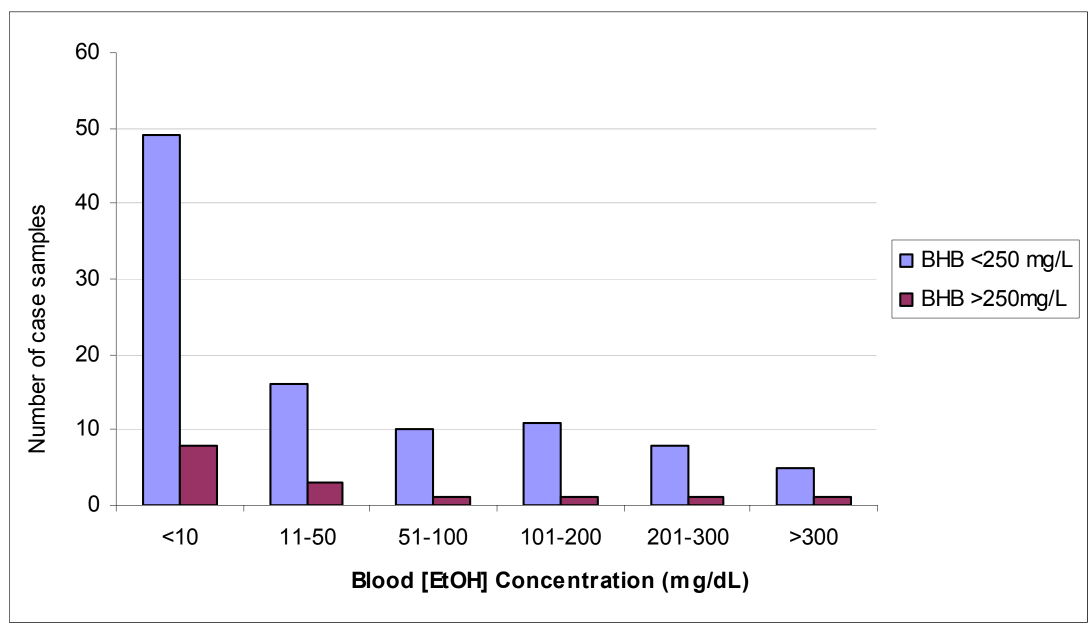


Figure 4-12: Ethanol Concentrations in Post-Mortem Blood Relative to BHB Levels

In five of the nine cases with blood BHB >250 mg/L, no ethanol was detected. Two cases had low ethanol concentrations of 30 and 34 mg/dL, however the remaining two cases had high ethanol concentrations of 193 and 338 mg/dL, as illustrated in Figure 4-12.

4.5 Conclusion

A robust and sensitive method for the analysis of BHB in postmortem blood and urine was validated. The method was utilised to investigate the stability of BHB in blood and to investigate the potential of BHB as a biomarker for AKA. BHB was found to be stable in blood for 15 days when stored in the refrigerator and initial findings support the use of BHB as a biomarker for AKA. The majority of samples analysed were preserved, there were not enough unpreserved results to evaluate the affect of preservative on BHB. AKA does not always equate to low or negative ethanol levels. High levels of ethanol were reported in case samples with blood BHB levels >250 mg/L, which is in agreement to other studies.(55;184) In all case samples with high blood BHB level (>250 mg/L), the acetone concentration was high. Acetone can be used as an initial biomarker for AKA but the concentrations measured are not well correlated to BHB. Acetone is volatile and may be unstable in the blood and the production of other volatiles may interfere with it due to putrefaction, (55;127) therfore, its absence does not preclude a pathologically significant ketoacidosis (BHB >250 mg/L). Therefore, BHB should still be analysed in case samples associated with a history of chronic alcoholism, even if acetone is low or absent. From the literature, vitreous humour could be an alternative substitute for blood.(125)

Chapter 5 - Application of the Method Validated for the Analysis of β-Hydroxybutyrate (BHB) to the Analysis of γ-Hydroxy-butyrate (GHB) and β-Hydroxy-β-Methylbutyrate (HMB)

5.1 Introduction

Due to the similarity of the structures of β-hydroxy-β-methylbutyrate (HMB) and γ-hydroxybutyrate (GHB) to β-hydroxybutyrate (BHB), as illustrated in Figure 5-1, the method validated for the analysis of BHB (as described in Chapter 4) was applied to the analysis of GHB in blood and urine, and the analysis of HMB in plasma and urine.

β-hydroxybutyrate **(BHB)**   β-hydroxy-β-methylbutyrate **(HMB)**   γ-hydroxybutyrate **(GHB)**

$HO-CD_2-CD_2-CD_2-C(=O)-O^-Na^+$

Deuterated γ-hydroxybutyrate sodium salt

(GHB-D_6)

Figure 5-1: Chemical Structures of BHB, HMB, GHB and GHB-D_6

This chapter has two parts, the first part will summarise the validation of the BHB method for the analysis of GHB in postmortem blood and urine. The aims of this study are to develop a robust method for the determination of GHB in post-mortem blood and urine, to identify GHB-related fatalities, and to evaluate the level of GHB in post-mortem bodies that are not associated with a history of GHB intoxication. These results will then be compared with the published data.

The second part of the chapter will summarise the validation for the analysis of all three analytes (BHB, GHB & HMB) in plasma and urine. The aims of this study

were to develop and validate a method for the determination of HMB in plasma and urine in normal individuals at baseline and after oral administration of a dose of 3 grams of HMB.

5.2 Determination of GHB in Post-Mortem Blood and Urine using GC-MS

5.2.1 Introduction

GHB is a minor metabolite of γ-aminobutyric acid (GABA) which is an inhibitory neurotransmitter, and is a central nervous system depressant. The two main metabolic precursors of GHB are γ-butyrolactone (GBL) and 1,4-butanediol (1,4B) Figure 5-2.

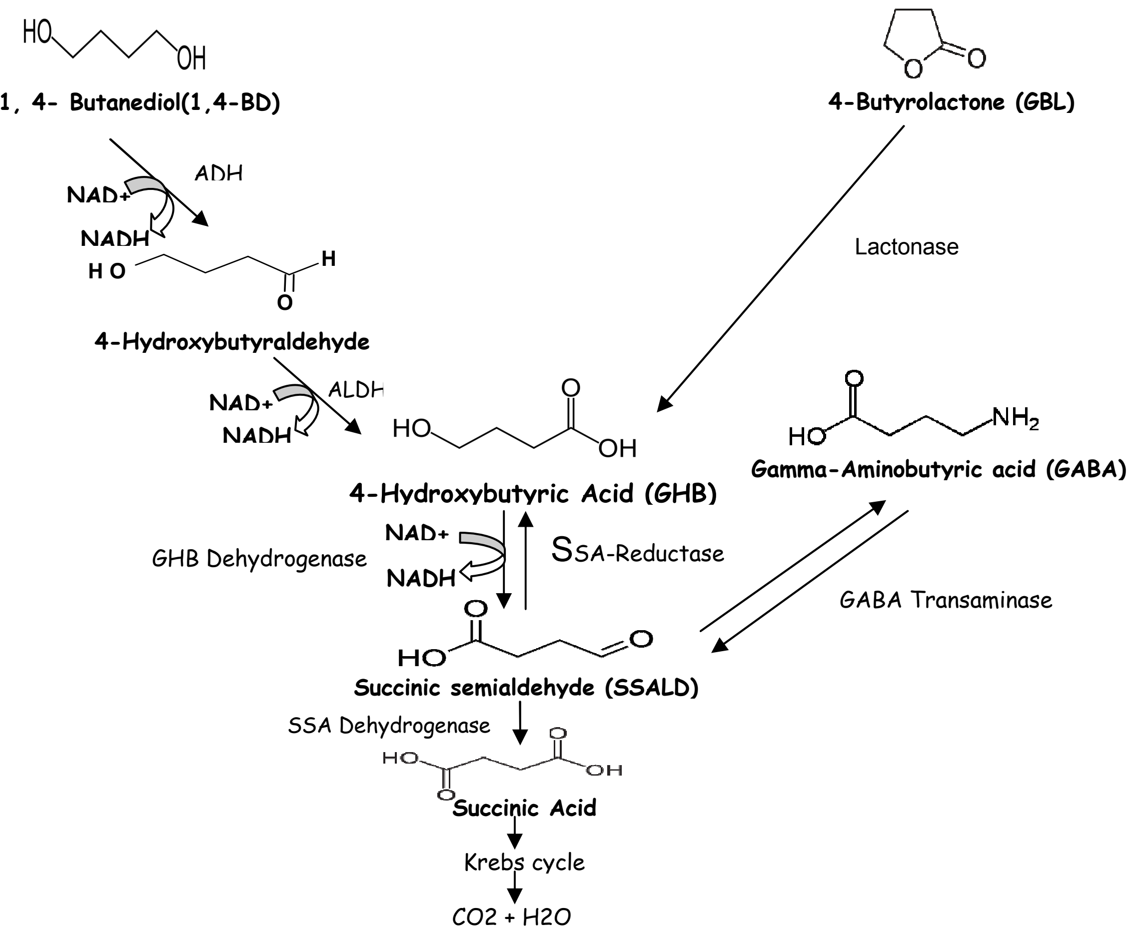


Figure 5-2: In vivo metabolism of GHB, GABA, GBL and 1,4BD [Redraw from Marinetti(185)]

GHB was therapeutically used as an anaesthetic and antidepressant agent. It causes relaxation and has the capacity to induce euphoria, short-term amnesia, and sedation at high concentrations.(185) In the USA, has been used in the form of a sodium oxybate under the name of Xyrem to treat narcolepsy. It is also used in the treatment of alcohol withdrawal syndrome. (185) Misuse of GHB is common among body builders, as it is believed that GHB stimulates the release of the growth hormone; it is also used by dance club attendees, for its sedative and muscle relaxant effects and has been detected in drivers and victims of drug-facilitated sexual assault.(186) The use of GHB has been associated with fatalities when administered alone or in combination with other drugs. GHB is commonly referred to as a "date-rape drug", with street/slang names for GHB including "scoops," "liquid ecstasy," "easy lay," "salt water," "vita G," "Georgia home boy," "grievous bodily harm" and "great hormones at bedtime." (185)

The slang name "liquid ecstasy" used for GHB is often confused with MDMA. "Ecstacy," or “XTC” is the slang name commonly associated with methylene-dioxymethamphetamine (MDMA), which is not in the same classification as GHB. (185) MDMA scheduled as class A and GHB as Class C.(187)

5.2.2 Prevalence of GHB in Biological Matrices

GHB is a small molecule. It behaves similar to ethanol due to being highly solubility in water and lacking the ability to bind to plasma protein. As expected GHB concentrations are higher in plasma than blood due to a higher percentage of water in plasma than blood. The distribution ratio between blood and serum is probably similar to ethanol which is 1.6:1. The fatal concentration of GHB in plasma ranged between 250-280 mg/L and therefore it is expected that whole blood GHB concentration will be around 16% lower in plasma. [Cited by Kugelberg, *et al. 2008* (188) from Iffland *et al 1999]*. Due to the endogenous nature of GHB, the interpretation of GHB levels in biological matrices is a challenge in forensic toxicology.(188)

In living individuals, toxicological analysis of exogenous GHB in body fluids is time critical, since GHB disappears very quickly from the body after ingestion.

The cut-off time for detection in blood is approximately 8 hours post-ingestion and 12 hours and not more than 48 hours after offence in the urine.(189)

An elevation of the urinary levels of GHB has been reported in the case of a genetic disorder called GHB aciduria, due to a deficiency of succinic semialdehyde dehydrogenase. This leads to an accumulation of GHB in the urine. The reported level of GHB in this type of disorder was around 200 mg/L in urine.(184;190-192)

Characteristics of GHB toxicity include euphoria, dizziness, visual disturbances, vomiting, nausea, bradycardia, hypotension, confusion, agitation, hypothermia, coma, respiratory depression, and death.(185)

In postmortem blood samples, GHB is frequently detected at levels that range from physiological to pharmacological concentrations, even in cases when GHB use is not suspected. Stephens *et al.* (1999) (193) observed a relatively minimal formation of GHB in blood samples stored in a refrigerator without sodium fluoride. It has been reported GHB detected in the blood of corpses could be due to production during the interval between death and autopsy, rather than during sample storage at 4°C.(194)

GHB concentrations may increase after death in the case of advanced decomposition or when the sample is heart blood. The common cut-off level of urinary GHB used in several studies to differentiate endogenous from exogenous is 10 mg/L. This can be applied to the samples that were properly stored but excludes cases of GHB aciduria.(134;192;195) Some laboratories use a cut-off concentration of 50 mg/L in the case of post-mortem cardiac blood.(186) In another study, the cut-off concentration of GHB for reporting positive results in post-mortem femoral blood and urine was 30 mg/L.(188)

It is expected that the concentrations of GHB produced *in vitro* during storage will be more prevalent in post-mortem than in antemortem material. No levels of GHB above 3 mg/L were found in antemortem serum specimens, whereas in post-mortem blood, GHB levels up to 100 mg/L were reported, followed by a subsequent drop to unmeasurable values. (196) GHB production is less likely in

post-mortem urine samples and therefore collection and analysis of urine could provide an ideal control for the analysis of GHB in suspected drug related death. Freezing or preservation of specimens with sodium fluoride may improve stability of GHB from the time of the collection until the time of analysis.(196)

It has been suggested that enzymatic conversion of succinic acid, GABA and putrescine are probably responsible for endogenous GHB production. In addition, glycolysis by bacteria may enhance endogenous GHB production.(185) Bacteria can metabolize glucose to succinic acid via phosphoenolpyruvate and oxaloacetate. Succinic acid is converted to succinic semialdehyde that can be reduced to GHB by succinic semialdehyde reductase.(185) Table 5-1 summarises published GHB levels in both living and post-mortem samples.

Table 5-1: GHB Published Levels in the Living and Post-Mortem

Reference	Matrix	Population/Status	GHB (mg/L)	N
Endogenous GHB				
Elian, 2002.(197)	Urine	Ante mortem urine	0.34-5.75	670
Fieler, Coleman and Baselt, 1998.(198)	Blood	Postmortem	3.2-168	20
Rahbeeni *et al*. 1994; Divry *et al*. 1983; Elliott, 2003. (190-192)	Urine	Patients with GHB aciduria	200	6
Exogenous GHB				
Kavanagh, Kenny and Feely, 2001(199)	Urine	Volunteers after 10 hours after administration of 1 gram GHB	4	1
Jones, Holmgren and Kugelberg, 2008. (200)	Blood	People arrested for driving under the influence of GHB	Mean=89, highest 340	548
Fatal GHB level				
Kugelberg *et al*. 2010. (188)	Blood	Postmortem	In excess of 300	49

5.2.3 Analysis of GHB

Several techniques have been used to analyse GHB in biological samples, such as GC-MS, high performance liquid chromatography with UV detection (HPLC-UV) and liquid chromatography tandem mass spectrometry (LC/MS/MS). Appendix 5-1

lists a summary of the published methods for the analysis of GHB and related compounds in different biological matrices.

The method originally developed and validated for the analysis of BHB in post-mortem blood and urine(138) as described in Chapter 4, was adapted and revalidated for the determination of GHB in post-mortem blood and urine and this validation is described below.

5.2.4 Materials

The internal standard used was deuterated gamma hydroxybutyrate (GHB-D_6 1 mg/mL), purchased from Cerilliant Corporation (Round Rock, TX) and gamma-hydroxybutyrate (GHB) was obtained from Sigma-Aldrich Co. Ltd. (Basingstoke, England). All other materials used are as described in sections 4.2.1.1 – 4.2.1.4. Drug and volatile-free blood and urine blanks used for preparing quality controls and calibrators were tested for endogenous interference.

Post-mortem blood (N=120) and urine (N =64) samples selected from the same cases analysed for BHB (as described in section 4.5.2.3) were also analysed for GHB. Basic demographic information is also summarised in Appendix 5-2 for these cases, including gender, age, other drugs detected at post-mortem and the cause of death. This information was not always available for every case as the post-mortem was carried out for some cases by a forensic pathologist who was not based within Forensic Medicine and Science. These cases are indicated as “External Case”.

5.2.5 Methodology

The instrumentation, GC conditions utilised and extraction methods developed and validated in Chapter 4 for the analysis of BHB were also used for the analysis of GHB in blood and urine. Data was collected in full scan mode and the ions monitored were m/z **233**, 117, and 204 for GHB and m/z **239** and 240 for the internal standard GHB-D_6. The underlined ions were used for quantification.

5.2.6 Method Validation

Method validation for GHB in post-mortem blood and urine was carried out as previously described in Section 4.3 for the validation of BHB in blood and urine.

5.2.7 Results and Discussion

Figures 5-3 and 5-4 illustrate typical chromatograms and spectra for GHB and the internal standard GHB- D_6 extracted from blood and urine, respectively.

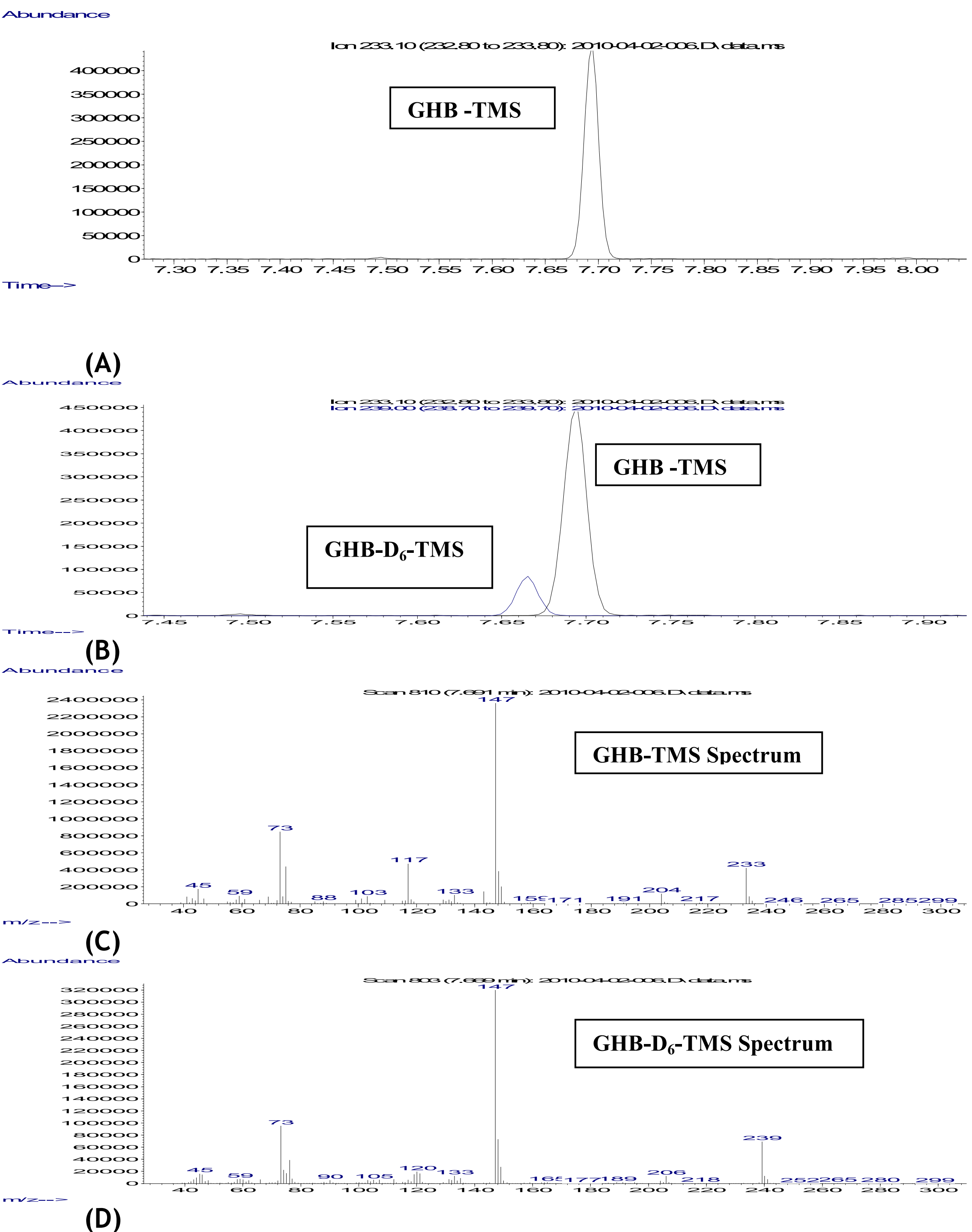


Figure 5-3: Chromatogram and Full-Scan Spectrum of GHB extracted from spiked blood (50 mg/L).
[(A) GHB-TMS extracted ion chromatogram, (B) GHB-TMS and GHB-d$_6$-TMS overlaid extracted ions chromatogram. (C) and (D) Full-scan spectra of GHB-TMS and GHB-D$_6$ TMS, respectively]

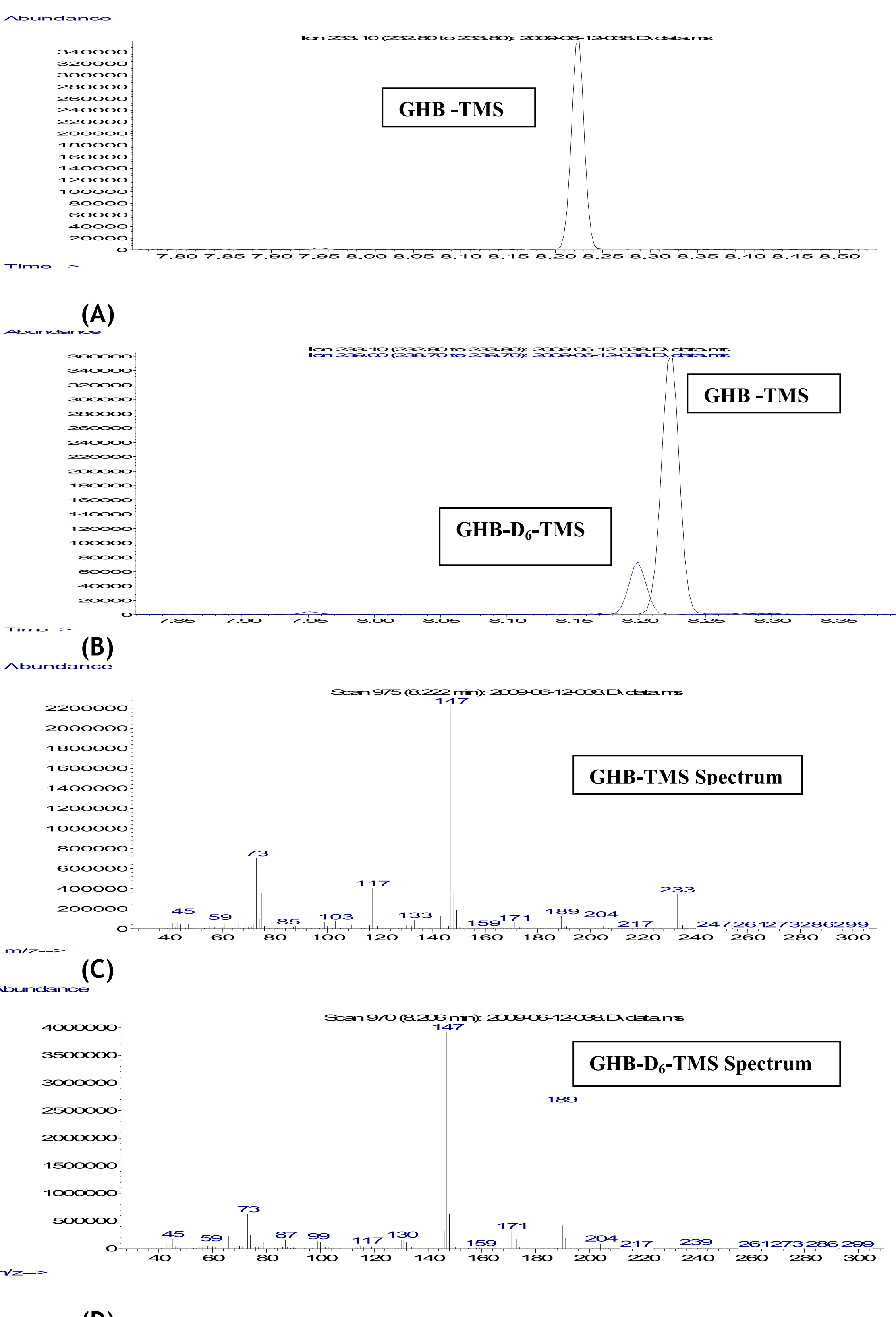


Figure 5-4: Chromatogram and Full-Scan Spectrum of GHB extracted from spiked urine (50 mg/L).
[(A) GHB-TMS extracted ion chromatogram, (B) GHB-TMS and GHB-d$_6$-TMS overlaid extracted ions chromatogram. (C) and (D) Full-scan spectra of GHB-TMS and GHB-D$_6$ TMS, respectively]

GHB was linear over the concentration range of 10-500 mg/L in both blood and urine as illustrated in Figure 5-5.

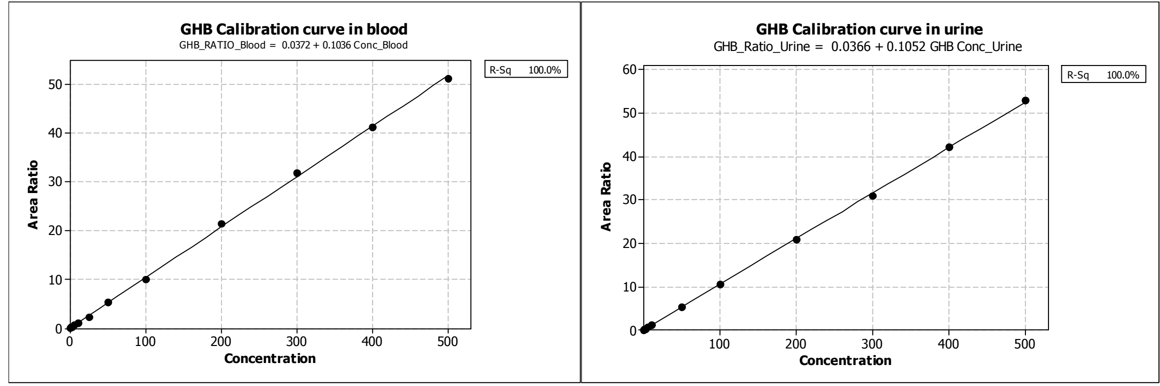


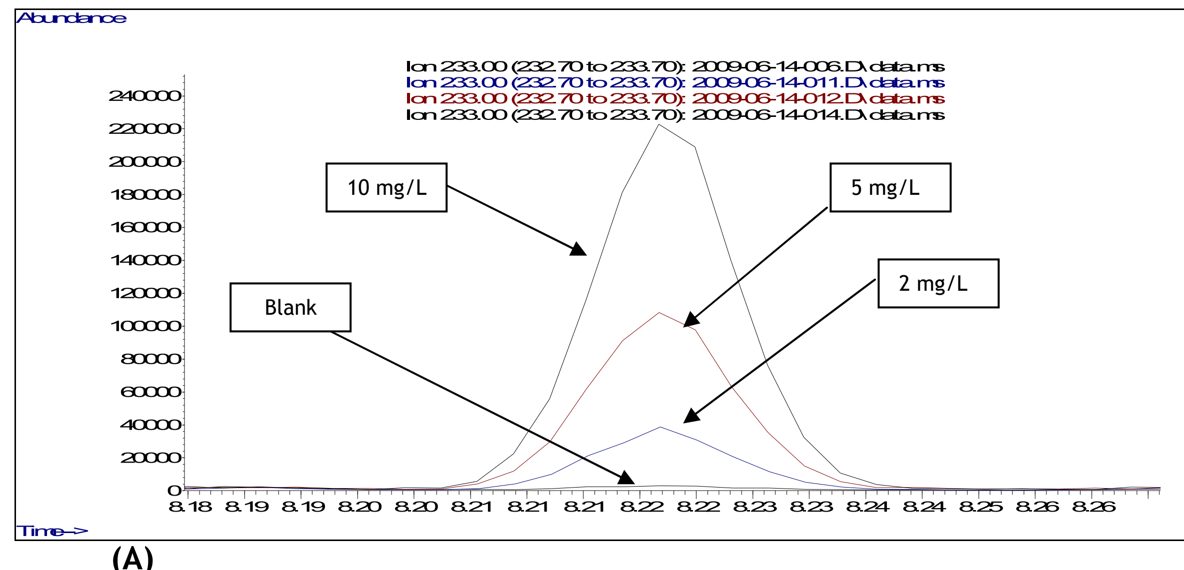
Figure 5-5: GHB Calibration Curves for Blood and Urine

The LOD of GHB in urine and blood was 1 mg/L and the LOQ was 2 and 4 mg/L respectively. Figure 5-6 involves a series of overlaid chromatograms for GHB spiked in blood and urine at low concentrations (blank, 2, 5 and 10 mg/L) to demonstrate that there was no interference from the matrix due to endogenous GHB. GHB levels in the blank samples were less than the LOD in both matrices.

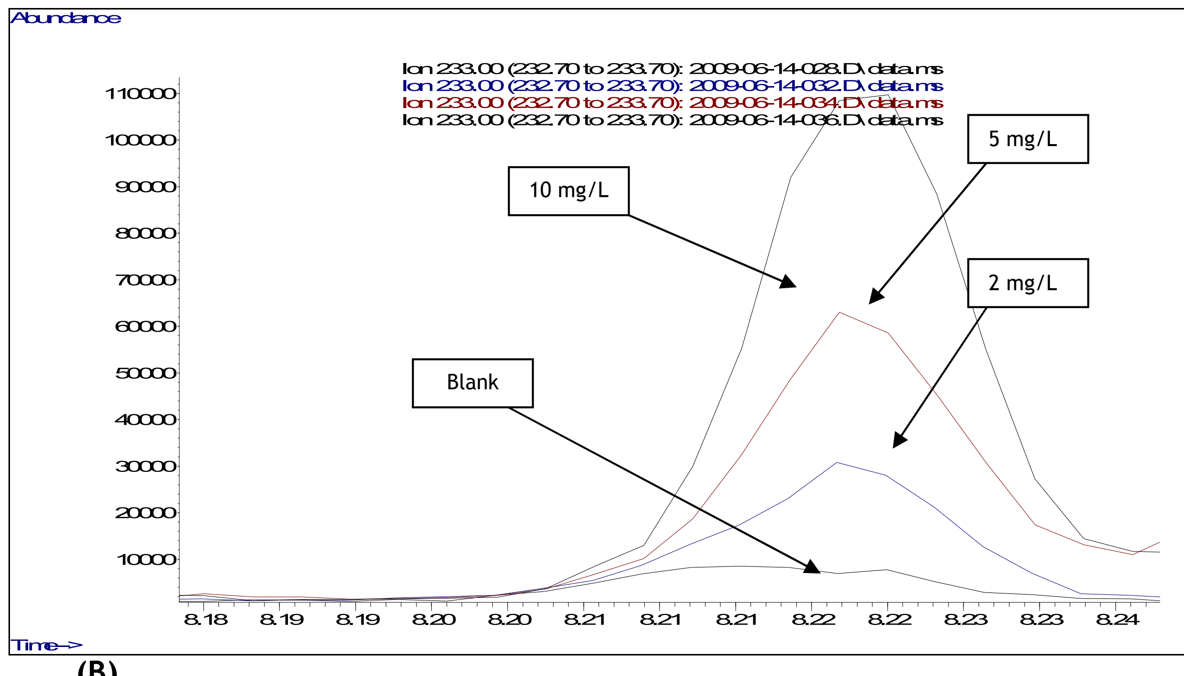
The intra-day and inter-day precision were less than 10% as summarised in Table 5-2. QC results were within the acceptable ranges as illustrated in Appendix 5-3.

Table 5-2: GHB Intra-day and Inter-day Precision for Blood and Urine

	Intra-day precision %CV (n=6)		**Inter-day precision %CV** (n=10)	
Matrix	**50** (mg/L)	**300** (mg/L)	**50** (mg/L)	**300** (mg/L)
Urine	1.7	1.5	2.9	2.7
Blood	2.3	3.1	3.3	5.6



(A)



(B)

Figure 5-6: GHB Extracted Ion Chromatograms for Blank Blood (A) and Urine (B) [Spiked at Concentrations of 2, 5 and 10 mg/L]

Recovery of GHB ranged from 40-50% in blood and urine. The efficiency (absolute recovery) of the method was between 83 and 99% and the accuracy was between 89 and 104%, as summarised in Table 5-3.

Table 5-3: Recovery, Efficiency and Accuracy of GHB

Matrix	Recovery (%)		Efficiency (%)		Accuracy (%)	
	50 (mg/L)	**300** (mg/L)	**50** (mg/L)	**300** (mg/L)	**50** (mg/L)	**300** (mg/L)
Urine	40	41	99	96	95	89
Blood	47	50	86	83	95	104

Although the recovery of GHB was low (40 and 50%) in blood and urine, the efficiency (absolute recovery) of the method was between 83 and 99%, and the accuracy was between 89 and 104%.

Appendix 5-2 summarises the 120 cases tested for GHB and included five cases (case # 36, 103, 106, 108 and 118) with high levels of GHB in post-mortem blood and urine (where available). These five specific cases are summarised in Table 5-4 and Figure 5-7 illustrates the chromatograms and spectra for the high levels GHB detected in blood and urine samples from Case number 36.

Table 5-4: Case Samples with High/Toxic Levels of GHB in Post-Mortem Blood and Urine

Case No.	Gender	Age	GHB (mg/L)		Background/ Cause of Death
			Blood	Urine	
36	Male	34	>500	>500	1a- GHB intoxication
103	Male		369		Suspected GHB/GBL intoxication (External Case)
106	Male		300		Suspected GHB/GBL intoxication (External Case)
108	Female		>500	>500	Suspected GHB/GBL intoxication (External Case)
118	Female	37	264		Suspected GHB/GBL intoxication (External Case)

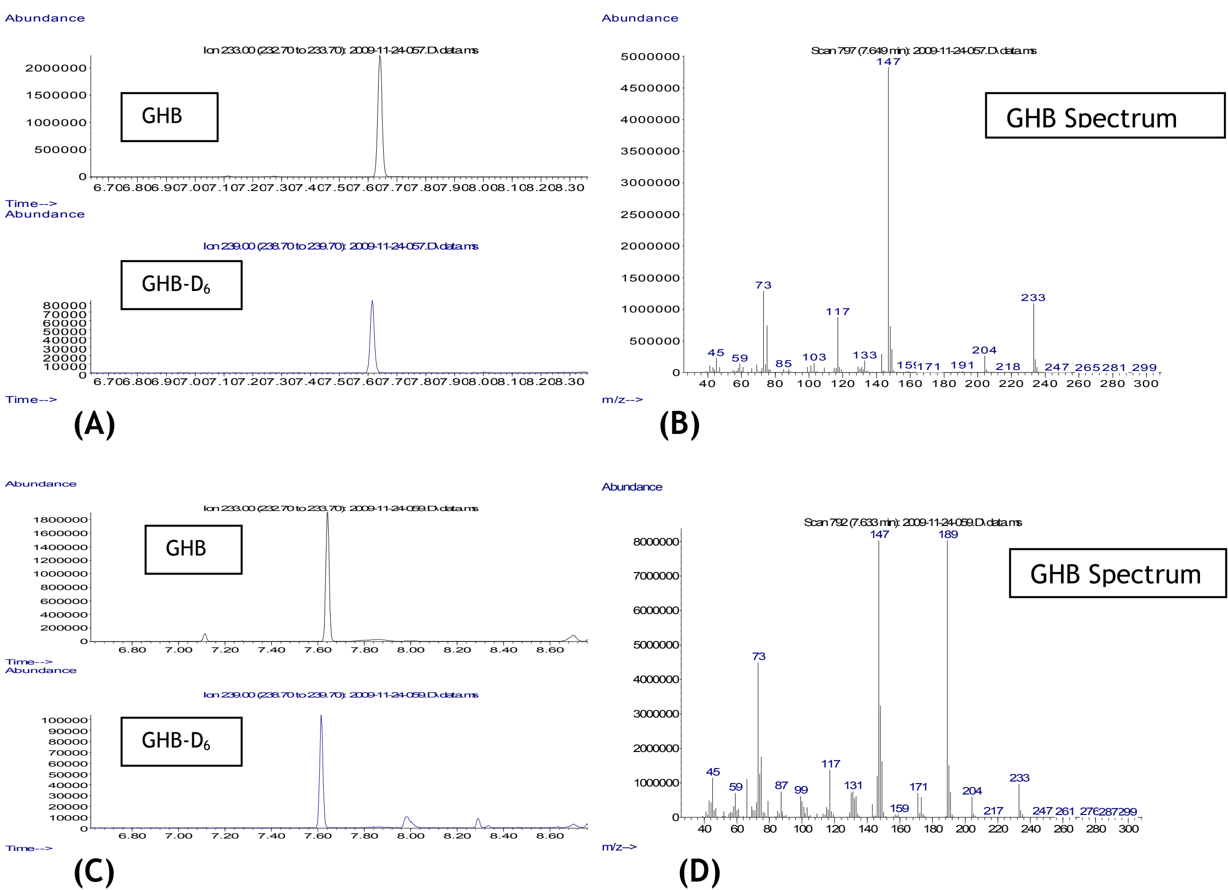


Figure 5-7: Chromatograms (A) and (C) and Full-Scan Spectra (B) and (D) for GHB Extracted from Blood and Urine, respectively, from Case # 36 at Levels >500 mg/L.

The mean, median and 95% confidence interval (95% CI) of GHB in post-mortem blood and urine were calculated from table of results for GHB in Appendix 5-2, with no history of misuse of GHB or its precursor and excluding the five cases listed in Table 5-4. The mean GHB concentration in blood case samples where there was no history of GHB intoxication, was 20 mg/L, the median was 19 and the 95% CI range 17-23 mg/L. In urine the mean was 4 mg/L, the median was 0 and the 95% CI range 2-8 mg/L in urine. The majority of the urine samples were negative (<10 mg/L), while the majority of blood samples (83%) were positive for GHB at concentrations ranging from 10-50 mg/L as illustrated in Figure 5-8.

Significant variations in the GHB levels in postmortem blood have been noted, even in cases when GHB use is not suspected. Table 5-1 summarises published studies reporting endogenous GHB levels in living individuals and in different postmortem biological matrices. The broad range of GHB levels in postmortem blood could be due to formation of GHB between death and sample collection, from enzymatic or bacterial actions as reported earlier. The mean concentration

of GHB in urine was 4 mg/L, the majority of the results were negative. Concentrations of 50 mg/L in postmortem blood and less than 10 mg/L in urine may be due to endogenous levels of GHB, but this is not sufficient by itself to distinguish between natural endogenous levels and exogenous use.

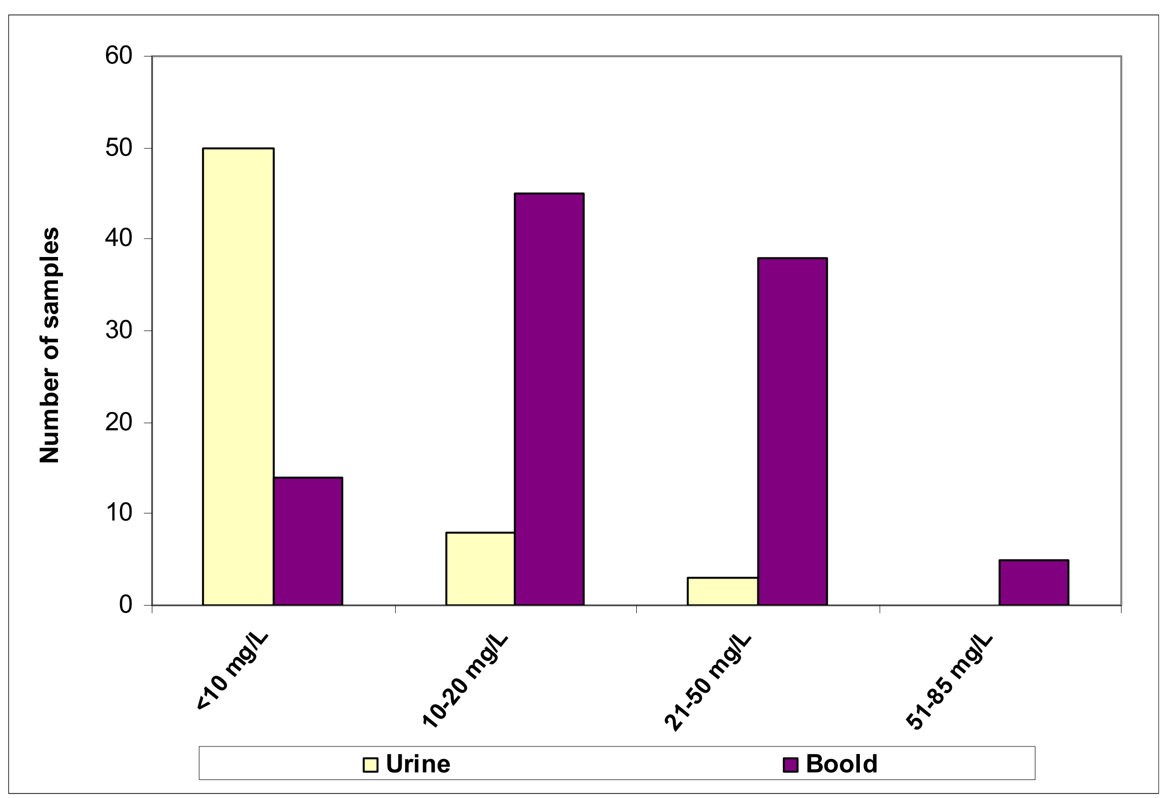


Figure 5-8: GHB Levels in Post-Mortem Blood and Urine in Cases with no History of GHB and/or GBL Misuse.

Earlier observations suggested that GHB was present in postmortem blood of non-GHB related fatalities, it was therefore suggested that the presence of GHB in urine could indicate the exogenous ingestion of GHB before death.(198) A study detected GHB in postmortem blood and urine in non GHB related fatalities with higher levels in blood than urine.(193) The exact source of the GHB in such instances has not as yet been identified.

Although some studies indicated that the GHB concentration due to formation postmortem may reach 409 mg/L(186) and 168 mg/L,(198) Berankova *et al.*(196) found levels up to 100 mg/L of GHB concentration in postmortem blood. The exogenous increase of GHB levels in post-mortem blood was only temporary, reaching the maximum with a subsequent drop to immeasurable values. Another

study reported that 43 mg/L was the highest GHB level in postmortem blood.(194)

The levels of GHB in postmortem blood and urine that were associated with GHB/GBL intoxication in this study had high or fatal levels of GHB ranging from 264 to >500 mg/L. A high GHB concentration ranging from(was also reported in case samples where there was no evidence of GHB and or GBL exposure.Fieler *et al*.1998 reported that GHB post-mortem blood can be detected at concentration ranges between (3.2-168 mg/L)in post-mortem femoral blood. According to a study carried out by Kintz *et al*. 2004 (186) seventy one autopsy cases were reported where cardiac blood GHB concentrations were >50 mg/L with range of (0.4-409 mg/L).This makes the interpretation more difficult to distinguish between endogenous and exogenous levels of GHB. Analysis of other biological fluids postmortem, such as vitreous humour or femoral blood, in addition to the knowledge of the circumstances surrounding the death may help in interpreting the results. Additionaly information relating to the post-mortem interval, the time of sample collection and the condition of storage of the corpse may also provide supportive information for result interpretation. Elliott, 2001(184;201) reported that GHB in urine was elevated in GHB unrelated fatalities and suggested that the presence of GHB in urine does not necessarily indicate GHB ingestion. Furthermore in living subjects although urine was an accepted specimen in most of drug-facilated sexual abuse (DFSA) at a cut off 10 mg/L, it has been reported by Le Beau *et al*. 2006.(202) that there is potential for an artificial increase in GHB concentrations in urine and reported results from 31 volunteers who never used GHB thought to occur during storage in refrigerator at 5ºC without preservative for a period over 6 months.

5.3 Simultaneous Determination of HMB, BHB and GHB in Plasma and Urine using GC/MS

5.3.1 Introduction

β-Hydroxy-β-methylbutyrate (HMB), or β-hydroxyisovaleric acid (β-HIV), has recently been considered a promising dietary supplement; the claim is that it increases strength and muscle mass and that it is the sixth most commonly used supplement. In 1999, the International Olympic Committee's medical advisory board categorized HMB as a legal substance.(203) It was patented by Iowa State University Research Foundation in 2000. HMB has been found useful for reducing cholesterol levels and improving low lipoprotein density (LDL), enhancing the immune response of mammals (204), and it may lead to increase muscle mass and decrease muscle break down and proteolysis. It is considered an anticatabolic agent(204). It has been proposed that the HMB nutritional supplement may increase the strength and lean mass in humans involved in resistance training programs.(205) HMB can be synthesised through oxidation of diacetone alcohol in a free acid form which is then converted to the salt.(204) Other studies indicate that HMB can be produced through the conversion of β–methylbutyric acid by the action of bacteria (Galactomyces reesii). (205) HMB doses used in research was between 1.5 to 3.0 grams per day. (206) It has been concluded from review studies carried out by Slater and Jenkins, 2000 (203) that supplementation of HMB at doses of 1.5 to 3 g/day may enhance gains in strenghth and lean body associated with resistance training and there may be a reduction in skeletal muscle damage. The mechanism by which this may occur is unknown. It has been suggested that the further controlled studies to confirm this finding.

HMB is a metabolite of the essential amino acid (leucine). Transamination of leucine is through branched chain acid transaminase to produce alpha-ketoisocaptoate (KIC). KIC is involved in the formation of β-hydroxyisovalorate (β-HIV or HMB) in 3 different ways. The first way is through the action of cytosolic α-ketoisocaproate oxygenase. The second way is through decarboxylation of the branched chain ketoacid dehydrogenase to isovaleryl-CoA, which is partially hydrolyzed to isovalerate and then oxidized to HMB. The

third way is through further oxidization of isovaleryl-CoA to β-methyl crotonyl-CoA by isovaleryl-CoA-dehydrogenase and by hydration, followed by enoyl-CoA hydratase, followed by hydrolysis of β-hydroxyisovaleryl-CoA. β-methyl crotonyl-CoA is metabolized to acetyl CoA and ketone bodies via β-methylglutaconyl-CoA and β-hydroxy-β-methylglutaryl-CoA(206).as illustrated in Figure 5-9. Approximately 2–10% of leucine oxidation is converted to HMB.(207)

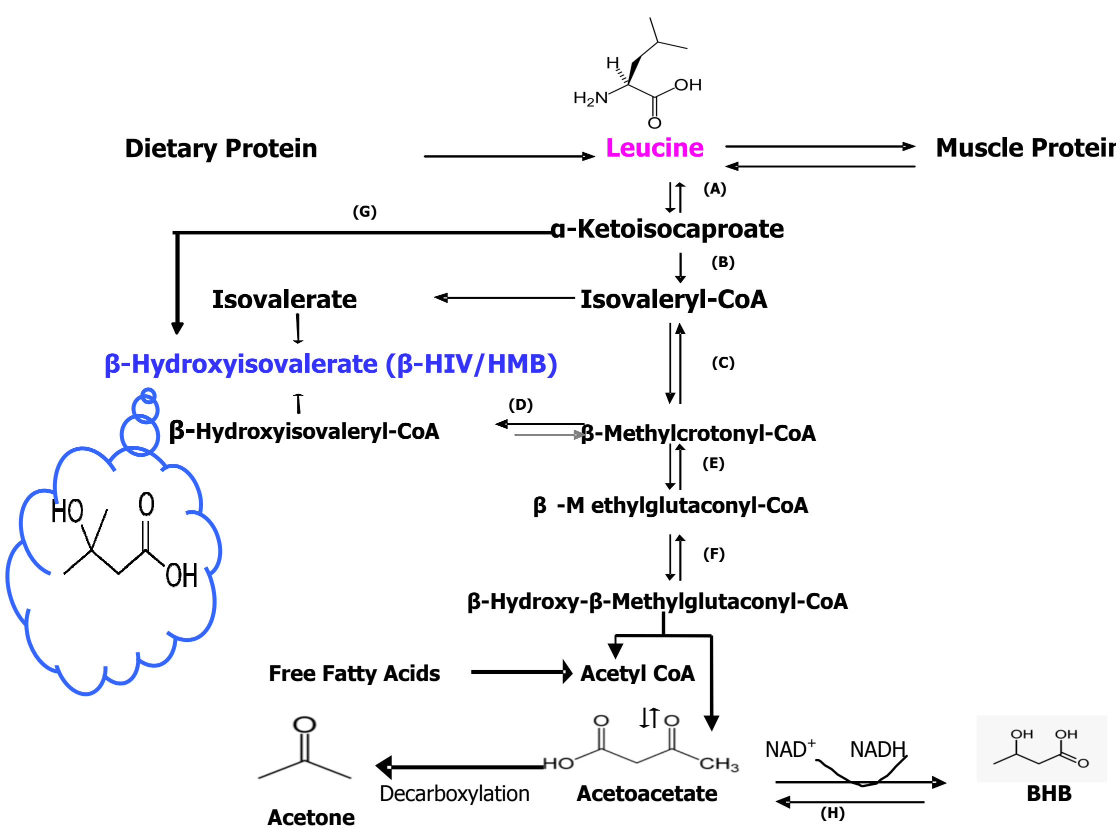


Figure 5-9: Leucine Metabolism and Formation of β-hydroxy-β-methylbutyrate (HMB) [(A) Branched chain amino acid transaminase, (B) branched chain ketoacid dehydrogenase, (C) isovaleryl-CoA, (D) enoyl-CoA hydratase, (E) β-methylcrotonyl-CoA carboxylase,(F) β-methylglutaconyl-CoA hydratase, (G) cytolic α-ketoisocaporate oxygenate, (H) β–hydroxy butyric acid dehydrogenase. [Redraw from Yu et al (206)and Landaas(208)]

Leucine hypoglycaemia and maple-syrup urine disease (MSUD) are known to be the only inborn error disease related to leucine metabolism. Several studies have indicated that a new genetic disorder related to leucine metabolism has been discovered in which the defect of leucine metabolism results in the accumulation of isovaleric acid, HMB and β-methylcrotonyl aciduria. (206;209-212) It was reported that the urinary increase of HMB in the inborn error disease

could be due to the defect of enzymes involve in the degradation of leucine.(209)

In ketoacidosis, usually characterized by the accumulation of ketone bodies (acetone, acetoacetate and BHB), this may occur with several clinical disorders in which fatty acids replace glucose for energy. Production of acetyl CoA is as a result of fatty acid oxidation. Acetyl CoA has an important role in ketogeneis.(208) Landaas reported the urinary increase of HMB in all ketotic patients and a positive correlation between HMB and BHB. High levels of BHB and acetoacetate and increased oxidation of fatty acids may influence the degradation of leucine, which may lead to increased HMB levels.(208)

In 1975, Landaas(213) reported that in ketoacidosis, a close relationship was demonstrated between the excretion of HMB in urine and the degree of ketoacidosis. He also indicated that another two hydroxy acids (β-hydroxyisobutyric acid and 2-methyl-3-hydroxybutyric acid) increased, in addition to HMB. Two possibilities have been considered in relation to the formation of HMB during ketoacidosis. The first one indicates that either the leucine might be the precursor or the increased pool of 3-hydroxy-3-methylglutaryl-CoA(3-HMG-CoA) might give rise to metabolites.(213)

According to the study of Weimin, *et al.*(206), it is also known that type 1 diabetes mellitus (insulin deficiency) may accelerate the breakdown of proteins, leading to enhanced leucine metabolism, and the same effect might occur in cases of diabetes melitus type 2 (insulin resistance). It was also reported that urinary levels of HMB and serum leucine were higher in ketotic patients than in nonketotic patients. The amount that HMB increases in nonketotic patients depends on the degree of hyperglycemia. It has also been observed that the leucine levels in nonketotic patients was almost the same as in the control group. This study leads to the conclusion that urinary HMB levels in diabetes mellitus might be useful in the evaluation of protein metabolism even in the absence of ketosis and marked hyperglycemia.

Weimin, *et al.*(206) calculated the HMB and BHB urinary concentrations listed in Table 5-5 were from the ratio of the HMB peak area to that of the internal

standard and expressed as μg per mg creatinine as a correction for urinary dilution or concentration by kidneys which may affect endogenous HMB concentrations.

Table 5-5: Urinary Levels of HMB, BHB and Serum Leucine in Diabetic and Non-Diabetic Patients(206)

Group	Urinary HMB	Urinary BHB	Serum leucine μmol/L	N
Diabetic with ketosis	27.1-87.5 ug/mg creatinine	0-1274 ug/mg creatinine	212-260	9
Diabetic without ketosis	21.7-51.3 ug/mg creatinine	undetectable	141-187	12
Control Group	4.1-16.3 ug/mg creatinine	undetectable	135-168	21

5.3.2 Analysis of HMB

Analysis of HMB has been conducted using a variety of different analytical techniques such as GC/MS and LC/MS/MS as summarised in Table 5-6.

Table 5-6: Summary of the Published Methods for the Analysis of HMB.

Ref.	Analyte	Matrix	Extraction and Internal Standard	Technique	Ions/identification
(206)	HMB, BHB, MMA	Urine serum	PP for serum (n-heptadecanoic acid). LLE for urine then SPE (MMA D_3)	GC/MS-SIM	HMB (247) MMAD_3 (250)
(208)	HMB, BHB and Lactate	Urine serum	PP for serum LLE for urine (2-HIV).	GLC/MS	Retention Time/Mass spectra
(214)	HMB	Plasma	LLE (MMA D_3) 98% pure synthesized from acetone D_6 and acetic acid	GC/MS	HMB (117,175, 217) HMB-D_6 (181)
(162)	Amino acid, Fatty acids Organic acids	Blood	Sample dissolve in water and organic solvent then derivatized.	LC/MS/MS	Different scan mode depends on analytes

PP (Protein precipitation), LLE (Liquid Liquid Extraction), MMA D_3 (Deuterated methyl malonic acid), 2-HIV (2- Hydroxy isovaleric acid), HMB-D_6 (Deuterated HMB)

The method originally developed and validated for the analysis of BHB and GHB in post-mortem blood and urine,(212) as described in Chapter 5, was further adapted and revalidated for the determination of HMB in plasma and urine as described below.

5.3.3 Materials

β-hydroxyisovaleric acid (HMB), liquid form, purity, ≥97% was purchased from Sigma Aldrich Co. (St Louis, USA). Blank plasma was obtained from the blood bank at the Western Infirmary Hospital, Glasgow. Blank urine was obtained from a healthy adult volunteer. Both blank plasma and urine were tested in triplicate for the presence of HMB, BHB and GHB. The internal standard used was deuterated gamma hydroxybutyrate (GHB-d_6, 1 mg/mL), purchased from Cerilliant Corporation (Round Rock, TX). All other materials used are as described in Sections 4.2.1.1 – 4.2.1.4. Drug and volatile-free plasma and urine blanks used for preparing quality controls and calibrators were tested for endogenous interference.

One hundred and forty five plasma and 201 urine samples were received from the Center for Human Toxicology, University of Utah, USA as part of collaboration. The samples were collected from subjects (N=8) as part of a clinical study, at time zero (baseline), and after 2 hours for plasma and between 0–4 hours for urine after oral administration of 3 grams of HMB. All samples were tested for HMB, BHB and GHB. Results less than the LOQ were reported as <LOQ. Samples greater than the ULOQ were diluted in water and reanalysed, then multiplied by the dilution factor after analysis. All samples were stored at -20±2°C during the time of analysis.

A statistical calculation of mean, standard deviation and 95% confidence intervals (CI%) was calculated at baseline and after 2 hours for plasma and at 0–4 hours for urine for all analytes (HMB, BHB and GHB).

The method was applied to two post-mortem cases in which the results of initial analysis using the previous method indicated a high BHB level in one case and

high GHB level in the other case. Whole blood and urine for both cases were analysed using this method.

5.3.4 Methodology

The instrumentation, GC conditions utilised and extraction methods developed and validated in Chapter 4 and section 5.2 for the analysis of BHB and GHB were also used for the analysis of HMB in plasma and urine. Data was collected in full-scan mode and the ions monitored were: for HMB m/z **247**, 131 and 205; BHB m/z **233**, 117 and 191; GHB m/z **233**,117 and 204 and m/z **239** and 241 for the internal standard GHB-d_6. The bold underlined ions were used for quantification.

A 50µL aliquot of plasma or urine calibrators, QCs and samples were transferred to 2 mL snap top polypropylene microcentrifuge tubes and extracted using the same blood method described in section 4.2.4 and Table 4-5.

5.3.5 Method Validation

Method validation for HMB, BHB and GHB in plasma and urine was carried out as previously described in Section 4.3 for the validation of BHB in blood and urine, with the exception of the assessment of stability which is summarised below.

The assessment of the long-term stability of HMB, BHB and GHB in plasma and urine at two concentrations (50 and 300 mg/L) was carried out by re-analysing three sets of QC samples stored for 12 months at -20±2°C. The percentage recovery was calculated by dividing the mean peak area ratio of the extracted QC samples versus the mean peak area ratio of the same spiked QC samples extracted and analysed 12 months previously. Analytes were considered as stable when the percentage recovery was between 90-110% of control samples and the 95% confidence interval (CI) was within 80-120% of the mean of the control samples.(169)

5.3.6 Results and Discussion

Figure 5-10 illustrates a typical chromatogram and spectra of an unextracted derivatized standard for HMB, BHB, GHB and internal standard GHB-D_6.

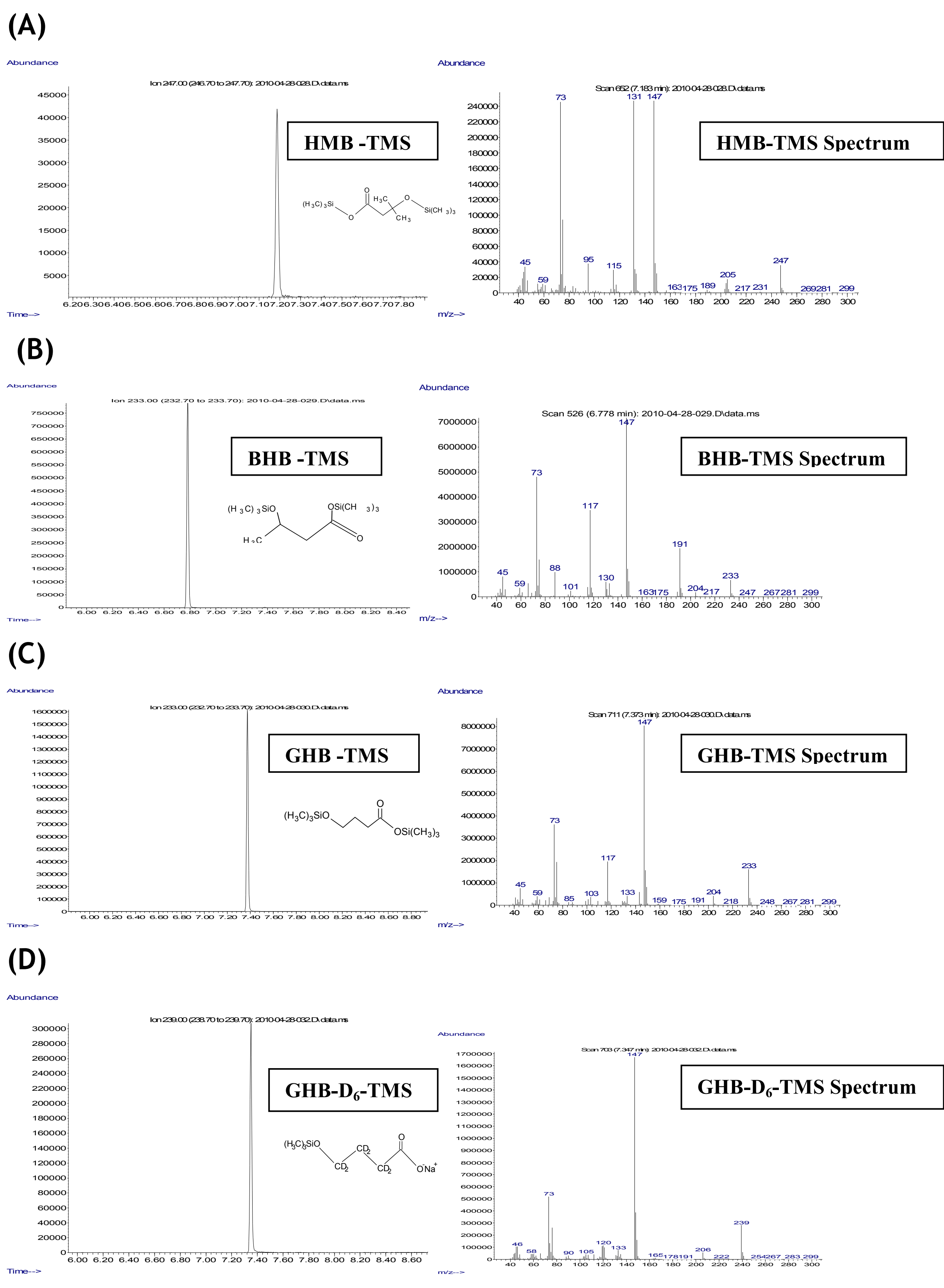


Figure 5-10: (A) HMB, (B) BHB, (C) GHB and (D) GHB-D6Chromatograms and Full-Scan Spectra of Unextracted Derivatized Standards (100 mg/L).

Excellent separation and peak shape was achieved as illustrated in figures 5-11 and 5-12 which includes both chromatograms and full scan spectra for the three

analytes in plasma and urine respectively. The retention time of all analytes, including the internal standards was between 6-8 minutes.

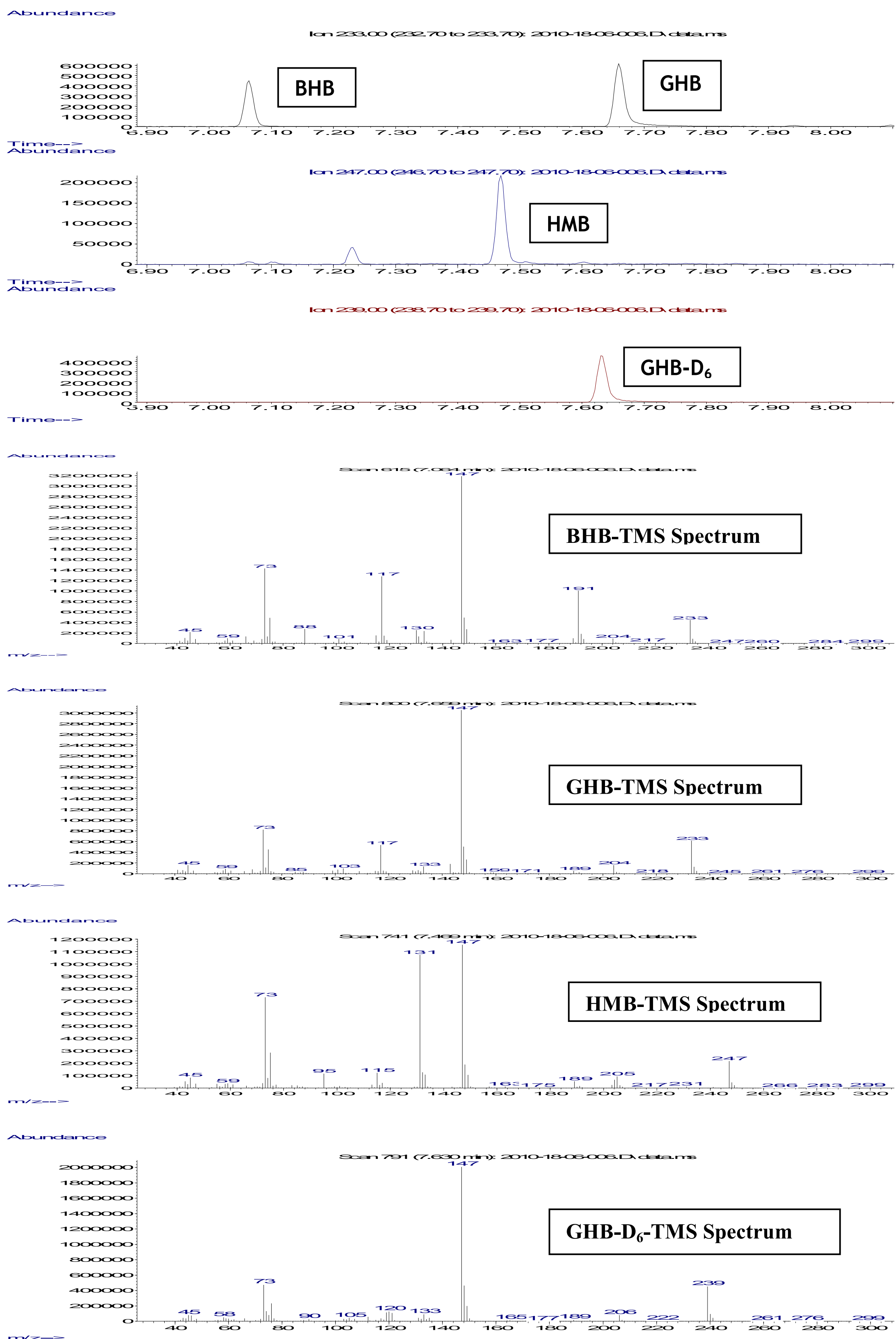


Figure 5-11: Chromatogram and Full-Scan Spectrum for HMB, BHB and GHB Extracted from Spiked Plasma (50 mg/L).

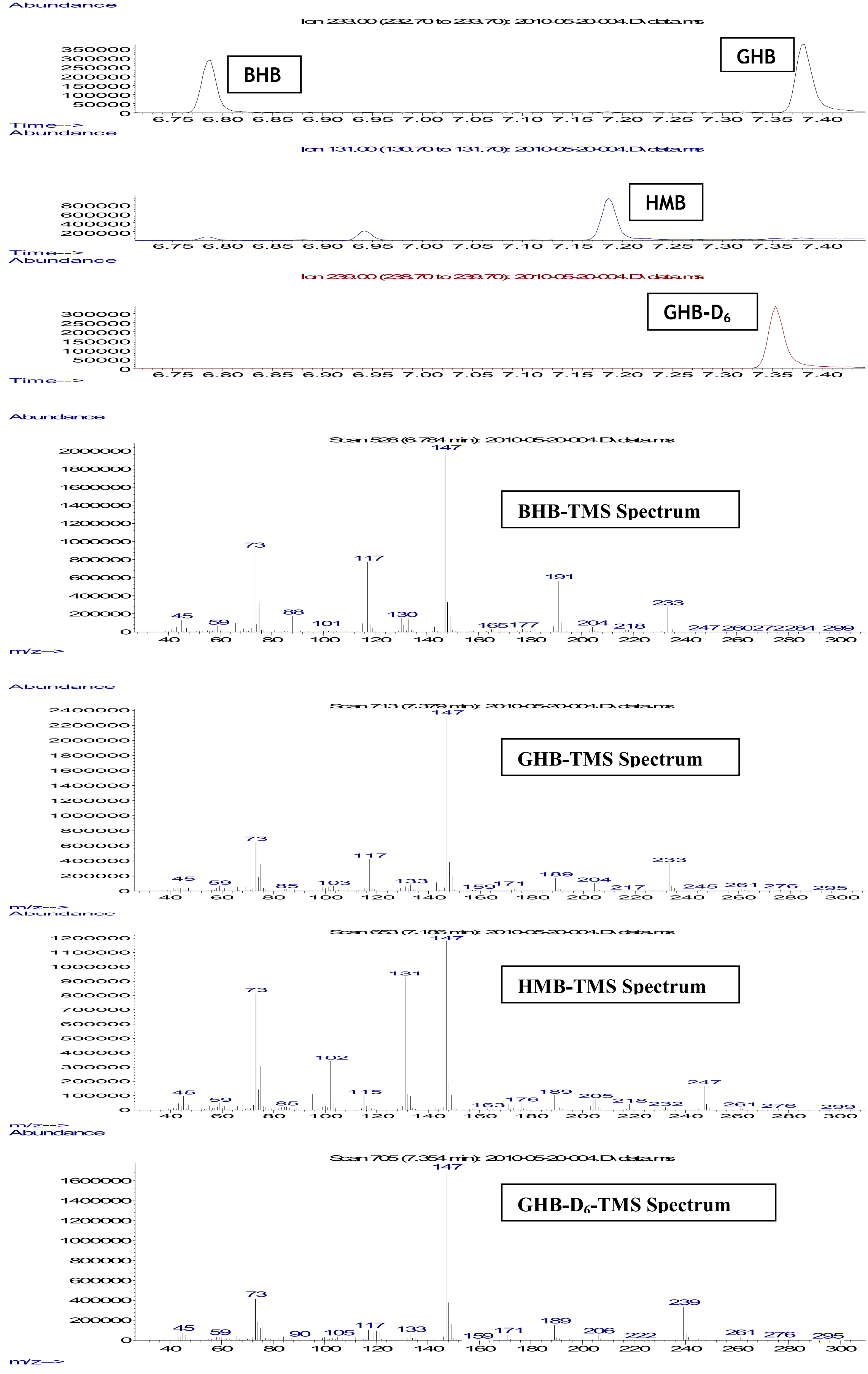
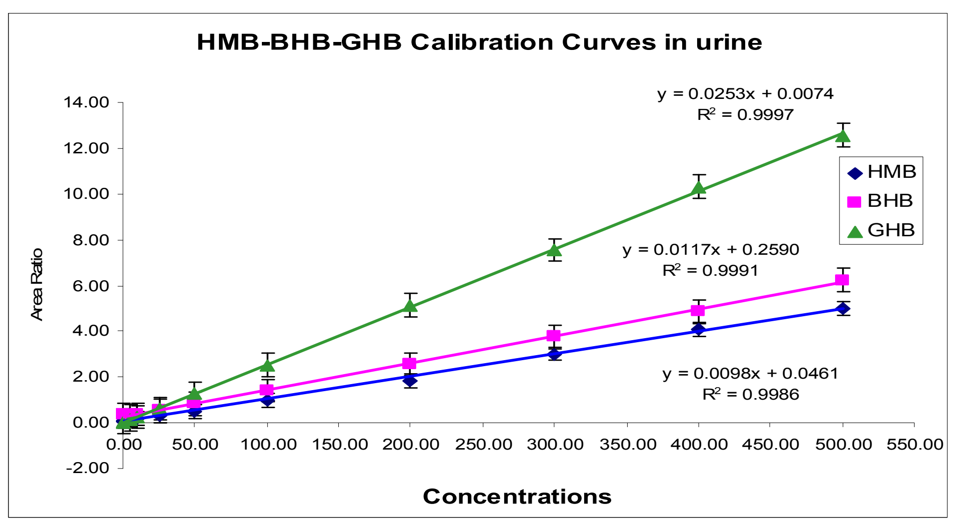
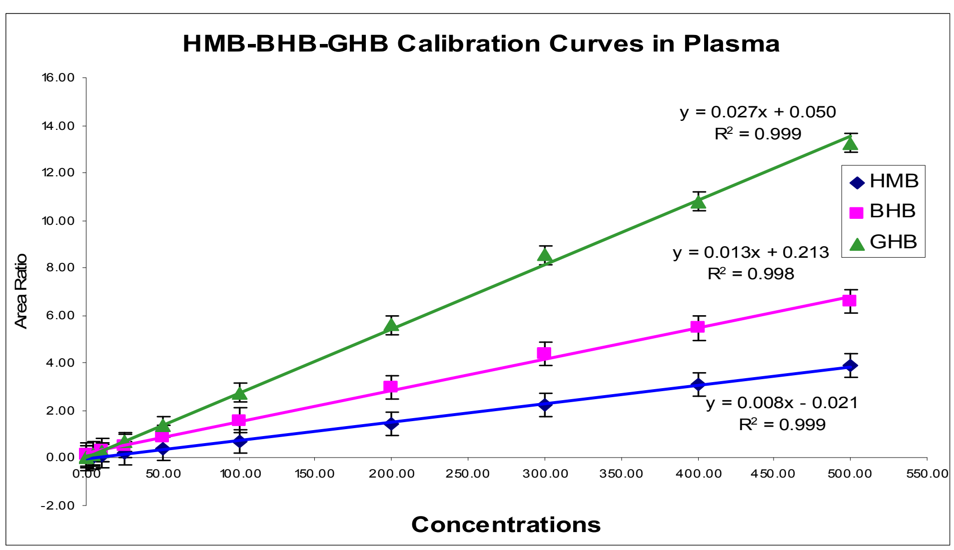


Figure 5-12: Chromatogram and Full-Scan Spectrum for HMB, BHB and GHB Extracted from Spiked Urine (50 mg/L).

The correlation coefficients (R^2) were >0.99 for HMB, BHB and GHB over the linear range of 10–500 mg/L as illustrated in figure 5-13. Table 5-7 summarises the linearity data for each analyte in both matrices and also includes the LOD and LOQ. Due to the endogenous nature of HMB (212), BHB and GHB, the first point of the calibration curve was used as the lower limit of quantitation (LLOQ). Results less than 10 mg/L were reported as negative and results above 500 mg/L were reported as >500 mg/L.



(A)



(B)

Figure 5-13: HMB, GHB and BHB Calibration Curves for Urine (A) and Plasma (B)

Table 5-7: LOD, LOQ and Linearity for HMB, BHB and GHB

Matrix	Analyte	LOD (mg/L)	LOQ (mg/L)	Linearity (mg/L)	R^2
Urine	HMB	2.3	7.7	10-500	0.994
	GHB	1.0	3.3	10-500	0.999
	BHB	2.2	7.3	10-500	0.999
Plasma	HMB	2.3	7.8	10-500	0.990
	GHB	1.5	5.1	10-500	0.999
	BHB	0.9	2.9	10-500	0.998

Inter-day and intra-day precision was less than 10% for all three analytes in both matrices. The average accuracy ranged from 98-102% and recovery from 42-77% in spiked controls as summarized in Table 5-8.

Table 5-8: HMB, BHB and GHB, Intraday and Interday Precision, Recovery and Accuracy

Matrix	Analyte	QC conc. (mg/L)	Intraday %CV (n=5)	Interday %CV (n=12)	Accuracy (%)	Recovery (%)
Urine	HMB	50 (300)	3.7 (1.7)	12.4 (8.5)	120 (102)	75 (77)
	GHB	50 (300)	3.2 (1.8)	5.5 (5.2)	98 (100)	67 (74)
	BHB	50 (300)	1.8 (1.5)	11.1 (5.3)	100 (99)	64 (68)
Plasma	HMB	50 (300)	6.2 (9.2)	9.5 (7.9)	100 (101)	42 (53)
	GHB	50 (300)	0.9 (3.6)	2.9 (4.0)	100 (102)	73 (74)
	BHB	50 (300)	8.9 (2.8)	3.4 (4.4)	98 (101)	57 (66)

The quality control samples were analysed on several occasions as illustrated in Appendix 5-4 and although some variation was noted, specifically in the lowest QC level of 10 mg/L, the majority of the QC results were within the acceptable limit of the mean ± 2 standard deviations (±2SD).

HMB, GHB and BHB were all stable when stored for over 12 months at -20±2°C with recoveries of greater than 91% achieved for the 300 mg/L QC as summarised in Table 5-9.

Table 5-9: HMB, GHB and BHB Concentrations Measured in Spiked Plasma and Urine [following (A) Initial tests and (B) after 12 months storage at -20°C].

			PLASMA (mg/L)			URINE (mg/L)		
Sample Conditions	**Conc. (mg/L)**		**HMB**	**GHB**	**BHB**	**HMB**	**GHB**	**BHB**
A	**50** (n=12)	Mean	50	50	49	60	49	50
		SD	4.26	1.78	2.54	8.14	2.96	5.03
		%CV	**8.51**	**3.54**	**5.17**	**13.59**	**6.05**	**10.02**
	300 (n=12)	Mean	304	305	304	307	299	296
		SD	26.96	13.71	14.26	44.53	10.34	10.97
		%CV	**8.87**	**4.49**	**4.68**	**14.51**	**3.46**	**3.71**
B	**50** (n=6)	Mean	47	56	43	33	54	43
		SD	6.53	1.62	6.78	4.90	1.70	5.77
		%CV	**13.77**	**2.90**	**15.61**	**14.73**	**3.16**	**13.54**
	300 (n=6)	Mean	282	334	296	279	358	316
		SD	21.68	14.83	18.35	14.09	9.91	7.40
		%CV	**7.68**	**4.44**	**6.20**	**5.06**	**2.77**	**2.34**
% Recovery	**50**		**95**	**111**	**88**	**56**	**110**	**85**
	300		**93**	**109**	**97**	**91**	**120**	**107**

GHB concentrations increased across both matrices and at the two concentrations investigated. The recoveries for BHB were lower for the 50 mg/L QC in both plasma and urine although still greater than 85% after 12 months. The %CV was much higher for the 50 mg/L QC when retested for both plasma and urine and may account for this variability. However, this does not explain the considerable decrease in HMB concentration for the 50 mg/L spiked urine QC sample (56%) however this is not reflected in the recoveries at 300 mg/L urine QC sample or either of the plasma samples which are all greater than 91%.

Figures 5-14 and 5-15 provide examples of HMB identified in plasma and urine respectively in both spiked and real case scenarios when compared with blank matrices. Full scan spectra are also provided.

The method was applied to two post-mortem cases where both whole blood and urine were analysed. The first case (Figure 5-16) had high concentrations of BHB in blood and urine and elevated levels of GHB, while the second case (Figure 5-

17) had high concentrations of GHB in blood and urine and low levels of BHB. In both cases HMB was less than the LOQ.

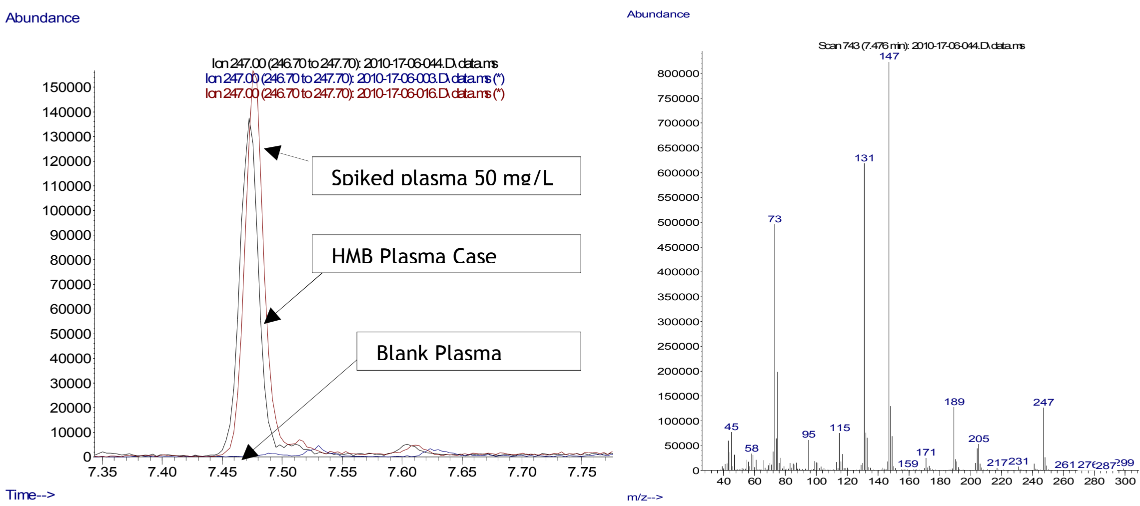


Figure 5-14: Chromatogram and Full-Scan Spectrum for HMB Extracted from a Plasma Case Sample (37 mg/L) Versus the Spiked Plasma Control at 50 mg/L and Blank Plasma.

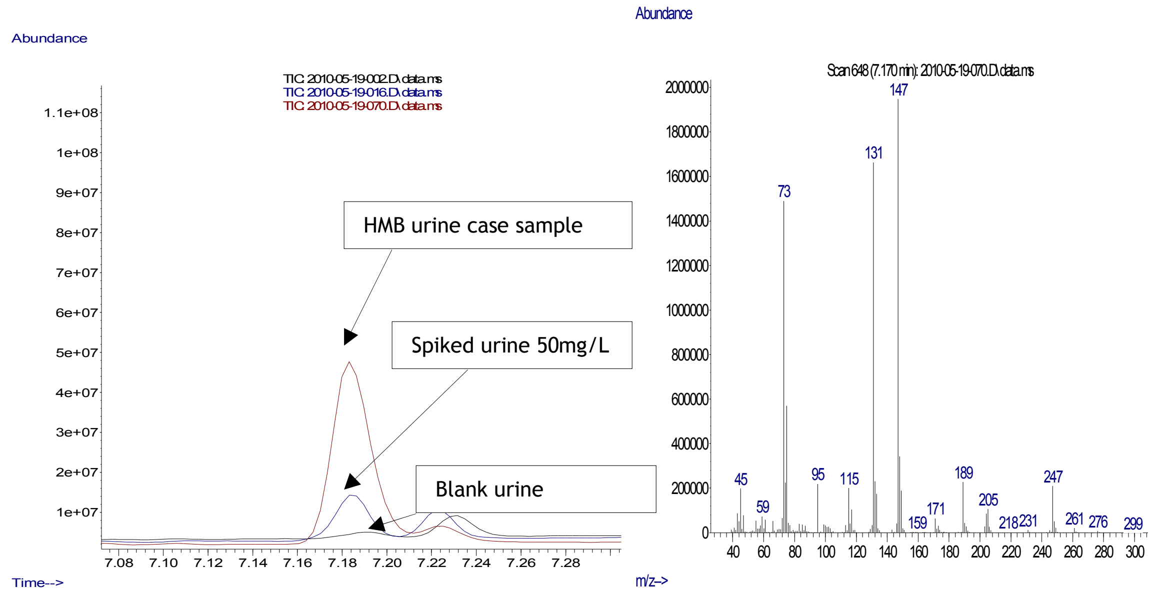
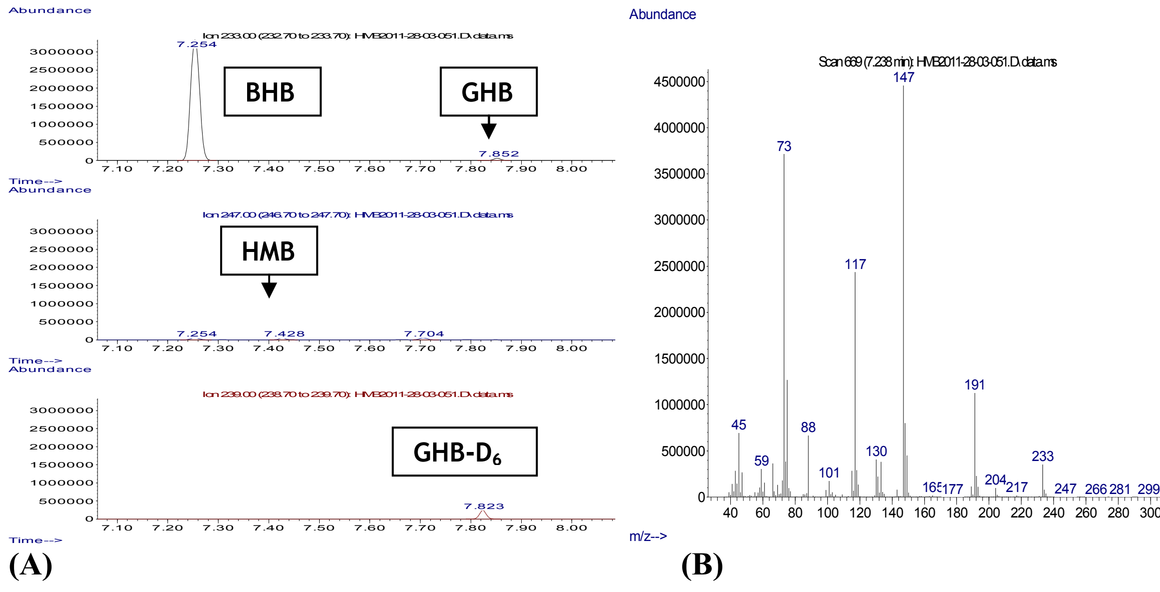


Figure 5-15: Chromatogram and Full-Scan Spectrum for HMB extracted from a Urine Case Sample (500 mg/L) versus the Spiked Urine Control at 50 mg/L and Blank Urine.



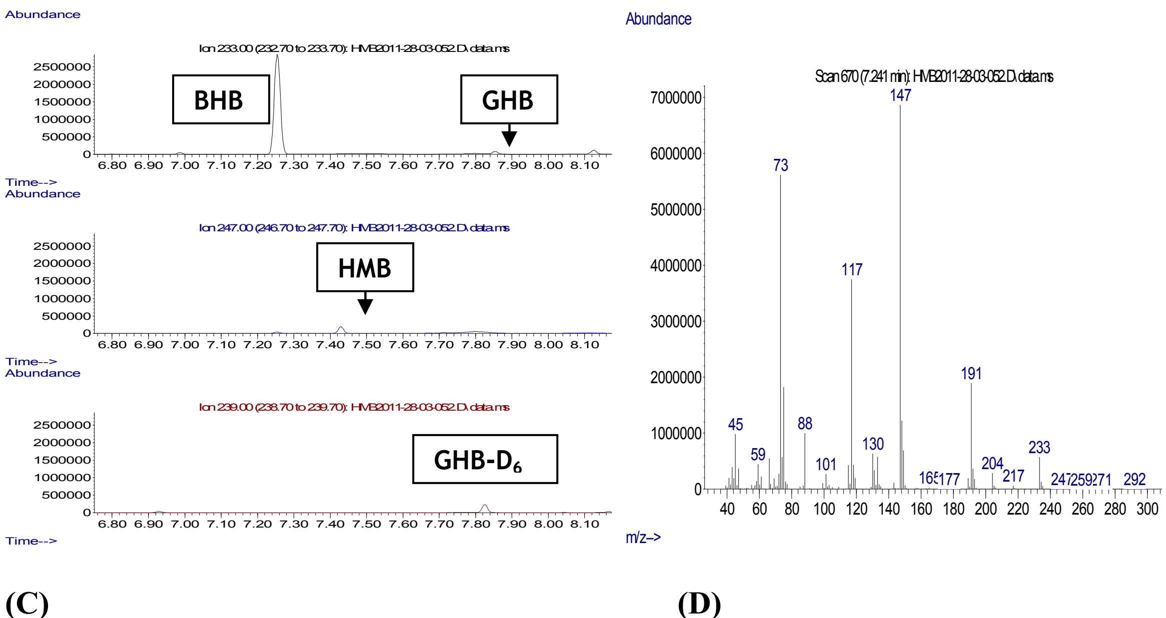
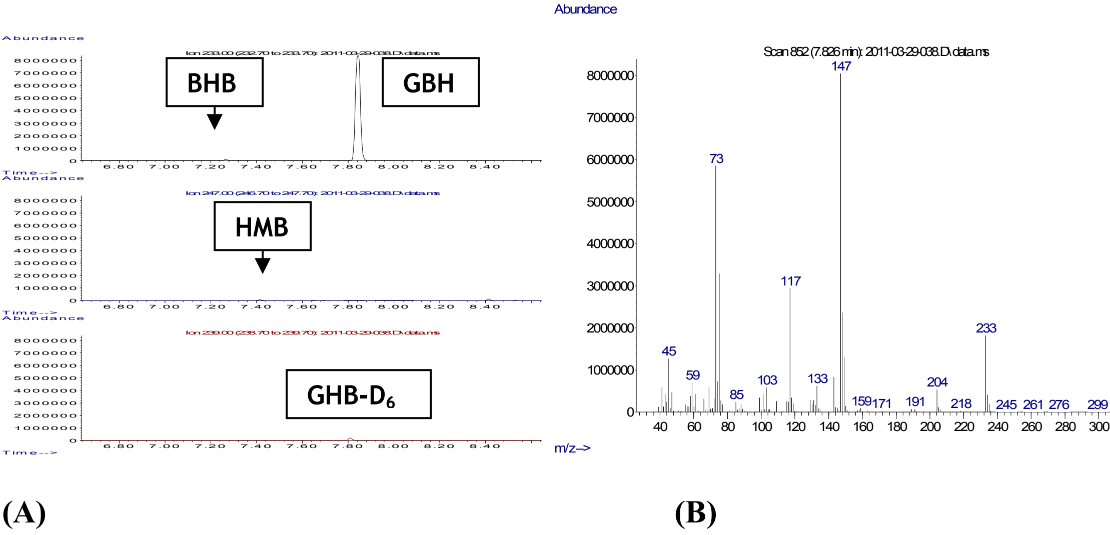
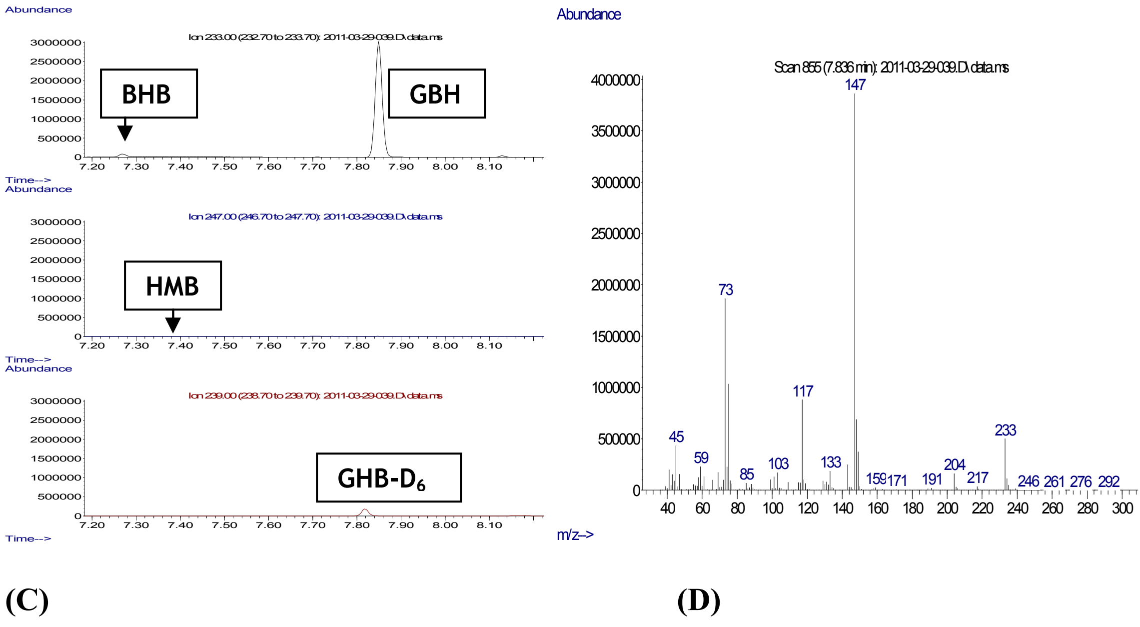


Figure 5-16: (A) Chromatogram and (B) Full-Scan Spectrum for BHB [Extracted from Urine Case Sample (>500 mg/L), HMB (< LOQ) and GHB (13 mg/L). (C) Chromatogram and (D) Full-Scan Spectrum for BHB extracted from Blood Base Sample (>500 mg/L), HMB (< LOQ) and GHB (18 mg/L)].



(A) (B)



(C) (D)

Figure 5-17: (A) Chromatogram and (B) Full-Fcan Spectrum of GHB [Extracted from Urine Case Sample (>500 mg/L), HMB (< LOQ) and BHB (12 mg/L). (C) Chromatogram and (D)Full-Scan Spectrum for GHB Extracted from Blood Case Sample (>500 mg/L), HMB (14 mg/L) and BHB (12 mg/L)].

The use of GC-MS provides a specific and selective technique for determining HMB and structurally related compounds such as BHB and GHB in biological samples. Modification of the method used for the determination of BHB and GHB in postmortem samples did not present any challenges. The reason for using blank plasma to dilute the urine and plasma samples is to obtain a cleaner extraction as interference with urea is a problem in this method. The recovery

of HMB at a concentration of 50 mg/L was less than 50%, but the accuracy was between 98% and 102%.

Table 5-10 summarises the mean and range of HMB, GHB and BHB at baseline and after administration of 3 g of HMB. Baseline concentrations for HMB in plasma and urine were less than the LOQ (<10 mg/L) before drug administration. Following administration of the 3g dose of HMB, plasma and urine were positive respectively.

Table 5-10: HMB, GHB and BHB level in urine and plasma at baseline and after 0-4 hours(urine) and 2hours(plasma) following 3g dose of HMB

Matrix	Analyte	Baseline (mg/L)			0-4 hours (urine) or 2 hours (plasma) after 3g oral HMB (mg/L)		
		Mean	Median	95% CI	Mean	Median	95% CI
Urine (n=8)	HMB	4	4	1-7	123	111	52-194
	GHB	0	0	0-1	0	0	0-1
	BHB	5	4	1-9	1	0	0-3
Plasma (n=7)	HMB	0	0	0	20	23	17-24
	GHB	0	0	0	0	0	0
	BHB	6	0	0-15	0	0	0-1

5.4 Conclusion

A robust and sensitive method for the analysis of gamma-hydroxybutyrate (GHB) in postmortem blood and urine was validated. The method was utilised to investigate GHB intoxication in postmortem case samples. In all case samples, there are no data to support GHB exposure with the exception of five cases. Concentrations of GHB less than the proposed published cut-off point of 50 mg/L in postmortem blood may be due to endogenous levels of GHB, but this is not sufficient by itself to distinguish between natural endogenous levels and exogenous use. Other matrices, such as vitreous humour, femoral blood, urine and hair, must also be considered when interpreting postmortem GHB levels, as recommended by Kintz *et al*. (186)Knowledge of the time intervals from the

time of death until the time of sample collection and the condition of the storage of the corpse before collection of the samples might also provide some information for better interpretation of the results.

It is important to evaluate the effect of HMB on the body because it is the sixth most commonly used dietary supplement and because it is legal.(203) There was a significant increase of HMB levels in urine and plasma 20–30 times more than the baseline level after consumption of 3 grams of HMB. No increase of BHB and GHB was observed. There was no correlation between the HMB levels and BHB or GHB in plasma and urine after administration of the HMB drug.

The use of GC-MS provides a specific and selective technique for determining HMB and structurally related compounds such as BHB and GHB in biological samples. Dilution of the urine and plasma samples with blank plasma provided a cleaner extract minimising the interference from urea in urine samples. A rapid, specific and robust method was developed for the simultaneous determination of HMB, BHB and GHB in plasma and urine. This method was successfully applied to post-mortem blood and urine, and plasma and urine from healthy subjects. For long-term storage, samples should be stored at -20±2ºC to minimise degradation of HMB, BHB or GHB.

Chapter 6 - Fatty Acid Ethyl Esters

6.1 Introduction

Fatty acid ethyl esters (FAEEs) are non-oxidative metabolites of ethanol which are currently used as biomarkers for direct ethanol consumption in different biological matrices including, hair, blood and skin surface lipids. Elevated FAEEs in meconium, the infant's first bowel movement, have also been reported as a biomarker for the detection of heavy prenatal ethanol exposure. The aim of this project was to develop and validate a method for the determination of FAEEs in meconium using liquid chromatography tandem mass spectrometry (LC-MS/MS). Commercially available deuterated internal standards were used for the first time for the detection of FAEEs using LC/MS/MS. The validated method was then applied to the analysis of eighty- four meconium case samples collected from babies born at the Princess Royal Maternity Hospital, Glasgow to investigate neonatal exposure to alcohol in the absence of maternal drinking history.

6.2 Materials and Methods

6.2.1 Chemicals and Reagents

Ethyl laurate 99.5% GC grade (E12:0), ethyl myristate (E14:0) 99% GC grade, ethyl palmitate 99% GC grade (E16:0), ethyl stearate (E18:0) 99% GC grade, ethyl oleate 98% GC grade (E18:1), ethyl linoleate 99% GC grade (E18:2), ethyl linolenate (E18:3) minimum 98%, and ethyl arachidonate 99% (E20:4) esters were obtained from Sigma Aldrich Company Ltd., Dorset, UK. The deuterated internal standards (IS) ethyl myristate-D_5 99%, ethyl palmitate– D_5 99%, ethyl oleate– D_5, and ethyl stearate-D_5 were obtained from LGC Standards, Teddington,UK. Ethyl heptadecanoate (E:17) >97 % GC, ethyl palmitoleate (16:1) >99%, and the methyl esters standards were all obtained from Sigma Aldrich Company Ltd., Dorset, UK. The methyl esters included, methyl arachidonate (99% GC grade), methyl heptadecanoate (99% GC grade), methyl linoleate (minimum 99%), methyl lenolenate, methyl myristate (99% GC grade), methyl oleate (≥ 99% GC grade), methyl palmitate (≥ 99% GC grade), methyl palmitoleate (99% GC grade), methyl stearate (99%) and methyl laurate (99.5% GC grade).

The deuterated internal standards purchased from LGC Standards were not available at the start of this project and so deuterated internal standards were initially prepared in-house, the details of which are summarised in Appendix 6-1.

Cyclohexane, acetone, acetonitrile and 2-propanol were all purchased from VWR International Ltd; ultrapure water was obtained from the in-house Millipore purification system. Amino propyl-silica solid phase extraction (SPE) columns (100 mg sorbent amount, 1 mL volume, 40µm particle size) were obtained from Sigma Aldrich Company Ltd., Dorset,UK.

6.2.2 Instrumentation

Analysis was carried out using an Agilent 6410 LC/MS/MS triple quadropole (Agilent, USA) coupled with an Agilent 1200-series binary pump, high performance autosampler, and thermostated column compartment. An electrospray ionization (ESI) source was used in positive ionization mode. The column used was Eclipse XDB-C8 column (150 x 4.6mm, 5µm) with a guard column of the same packing material (4.6 x 12.5) from Agilent Technologies, USA. An LC/MS/MS LCQ DECA XP Plus ion trap instrument from Thermo Finnigan (San Jose, CA) was used to tune the analytes of interest for collision energy optimization and product ion optimization to compare with the triple quadropole LC/MS/MS instrument.

6.2.3 LC-MS-MS Operating Conditions

Isocratic elution was chosen using a mobile phase with the ratio of water: isopropanol: acetonitrile of 20:40:40, v/v/v, at a flow rate of 0.7 mL/min. The total run time was 25 minutes with a column temperature maintained at 30ºC. The source parameters to obtain optimum sensitivity and specificity were the as follows capillary voltage, 4500v, source temperature 120ºC, gas temperature 300ºC, gas flow 8 L/min, and nebulizer pressure 15 psi. LC and source parameters are summarized in Table 6-1.

Table 6-1: Summary of LC and Ion Source Parameters

LC Parameters	
Column	XDB-C8 RP column (150x4.6 mm,5 µm) with guard column of the same packing material
Mobile phase	ACN:IPA:H_2O-40:40:20 (V:V:V)
Flow rate	0.7 mL/min isocratic
Injection volume	50 µL
Column Temperature	30ºC
Run Time	25 minutes
Mass Spectrometry Parameters	
Operating mode	ESI-positive mode
Gas temperature	300ºC
Gas flow	8 L/min
Nebulizer Pressure	15 PSI
Capillary Voltage	4500 V
Scan mode	MRM

6.2.4 Meconium Samples

Drug and alcohol free blank meconium samples used for development and validation of the method were obtained from a previous project from babies of drug and alcohol-free mothers and were stored at -20±2°C within FMS. Three pretested pooled meconium blank samples were prepared by pooling 6 to 10 blank meconium specimens and mixing them thoroughly; the approximate total weight for each pooled blank sample was between 40–50 g. The pooled meconium blanks were stored at -20±2°C to be used for preparation of calibratior and quality control specimens.

Eighty-four meconium case samples were collected from diapers of newborn infants delivered at the Royal Maternity Hospital in Glasgow. The meconium was

recovered using wooden spatulas and stored in plastic tubes at -4°C until transported to the laboratory. All samples were shipped in ice packs in closed containers, and they were stored immediately in the freezer at -20±2°C.

The stability of FAEEs in meconium as previously reported by Moore *et al.* (2003)(153) and Pichini *et al.* (2008)(159) is summarised in Table 6-2. FAEEs degrade if not stored properly and therefore specimens should be immediately shipped packed in ice and stored in the freezer to eliminate the risk of degradation of FAEEs. The longer-chain FAEEs appear to be more susceptible to heat and light; this was hypothesised as no ethyl arachidonate was detected in a study of 436 specimens analysed. This loss could be from exposure to light and heat, because the specimens were not shipped in ice and stored at a proper temperature.(153)

Table 6-2: Summary of FAEEs Stability Study by Moore et al.(153)

% loss of FAEEs	Temperature/Conditions	Hours/Days
86%	Room temperature in light	24 hours
60%	Room temperature in dark	24 hours
40%	Room temperature in dark under argon	24 hours
10%	Refrigerator	24 hours
18%	Refrigerator	24 hours
11%	Freezer	6 days
≤ 50%	Freezer	43 days

It has been reported that around 10% degradation of FAEEs from the initial concentration occurs after 3 freeze/thaw cycles.(159)

6.2.5 Preparation of Stock Standard Solutions

Individual stock standards were prepared at a concentration of 10 mg/mL for each analyte in cyclohexane. The intermediate standard was prepared from the individual stock standards at a concentration of 1 mg/mL. The working standard mixture solution was prepared at concentrations of 1, 10, and 100 mg/L from

the intermediate standard solution to be used for spiking the calibrators. The working internal standard mixture (including ethyl-myristate-D_5, ethyl palmitate-D_5, ethyl oleate– D_5, ethyl stearate-D_5, and ethyl heptadecanoate) was prepared at a concentration of 100 mg/L in cyclohexane from a stock standard of 10 mg/mL. Internal standards used for the quantification of ethyl palmitate was ethyl palmitate-D_5, for ethyl oleate was ethyl oleate–D_5, for ethyl stearate was ethylstearate-D_5. Deuterated ethyl oleate-D_5 was used for the remaining analytes namely; ethyl arachidonate, ethyl palmitoleate, ethyl laurate, ethyl myristate, ethyl linolenate and ethyl linoleate as deuterated internal standards of these FAEEs were not commercially available for purchase.

Tuning solutions were prepared at 10 mg/L in mobile phase from the mixed stock standard solution (1 mg/mL for all analytes). Cyclohexane was evaporated and the mobile phase solutions reconstituted with mobile phase. All standards and tuning solutions were stored at -20± 2ºC.

The international unit used for the concentration of FAEEs in meconium is nmol/g which has been converted to ng/g for this project and the conversion is summarised in Table 6-3.

6.2.6 Preparation of Quality Control (QC) Material

Two QC's samples were prepared at two concentrations (400 and 8000 ng/g). An aliquot of 0.5 g from pooled meconium blank which was screened to be used for spiking QC were weighed into 15 mL screw cap round-bottom glass tubes and centrifuged at 2500rpm for 5 minutes. After centrifugation 250 µL of deionised water was added. QC samples were vortexed for 0.5–1 minute until a homogeneous suspension was formed after spiking with a specified amount of the FAEEs standard mixture QC samples were vortexed for 0.5–1 minute and then internal standard will be added and follow the same steps of sample extraction procedure. The QC's were prepared and extracted fresh with each batch and the mean and standard deviation calculated to evaluate the acceptability of each batch.

6.2.7 Sample Extraction Procedure

Samples of 0.5 g of meconium were weighed into 15 mL screw cap round-bottom glass tubes, and centrifuged at 2500rpm for 5 minutes. After centrifugation 50 µL of internal standards mixture (100 mg/L) was added to all tubes followed by 250 µL of deionised water and 500µL acetone. Samples were vortexed for 0.5-1 minute until a homogeneous suspension was formed, and then 5 mL of cyclohexane added before placing the tubes in a tube shaker for 10min and finally centrifuging at 2500 rpm. The organic layer was transferred to 5 mL screw cap disposable glass tubes, and the solvent was evaporated to dryness at 30ºC under a nitrogen stream. The samples were vortexed after being resuspended with 500µL of cyclohexane. The samples were then applied to preconditioned aminopropyl-silica solid-phase extraction (SPE) columns with 2 mL cyclohexane, and the sample extract was collected in round-bottom culture tubes. The analyte was eluted with 2 mL cyclohexane and collected in the same tube before evaporating to dryness at 30ºC under a nitrogen stream. The samples were then reconstituted with 120µL of mobile phase and 50µL injected onto the LC system.

Table 6-3: Conversion of FAEEs Concentrations from nmol/g to ng/g

FAEES	Formula weight	ng/g	nmol/g
Ethyl Laurate	229	1	0.0044
Ethyl Myristate	257	1	0.0039
Ethyl Linolenate	307	1	0.0033
Ethyl linoleate	309	1	0.0032
Ethyl Palmitoleate	283	1	0.0035
Ethyl Palmitate	285	1	0.0035
Ethyl Palmitate-D_5	290	1	0.0034
Ethyl Oleate	311	1	0.0032
Ethyl Oleate-D_5	316	1	0.0032
Ethyl Stearate	313	1	0.0032
Ethyl Stearate-D_5	318	1	0.0031
Ethyl Arachidonate	333	1	0.0030

6.3 Instrument Method Optimisation

6.3.1 Optimization of the Fragmentor Voltage and Collision Energy

MS/MS characterization of the compounds of interest was achieved using the triple quadrupole and electrospray ionization in positive ionization mode. Compound specific optimization of MS/MS was performed using the automatic software optimizer (Mass Hunter) and product ion optimization. Analytes were dissolved in mobile phase at a concentration of 10 mg/L and fragmenter ion optimization was carried out to select the highest abundant precursor ions from the first quadrupole.

Collision energy was optimized to get the most abundant product ions at a collision cell pressure of 10–50 psi using nitrogen gas for collision. Optimization results for the two most abundant ion transitions per analyte were selected in the multiple reaction monitoring scan mode (MRM). Similar product ions were generated when the same tuning solutions were injected in another LC/MS/MS using a Thermo Finnigan LCQ DECA XP Plus ion trap instrument.

6.4 Method Validation

6.4.1 Selectivity

Selectivity is 'the ability of an analytical method to differentiate and quantify the analyte in the presence of other potentially interfering substance in the samples'. There are two types of potential interferences: endogenous matrix interferences and exogenous interferences. Exogenous interferences might include structurally related analytes of interest, interferences or non-structurally related interfering substances in biological samples such as endogenous matrix component, metabolites, impurities or matrix components.(169;215;216)

Selectivity evaluation was carried out by checking any interference from endogenous compounds by the analysis of independent replicate blanks of meconium from different sources compared with an unextracted FAEEs standard

mixture of 1 mg/L prepared in mobile phase. There should be no signal or area response at the expected elution time of the analyte in the meconium blank.

In many LC-MS/MS metods, both analytes of interest and isotope-deuterated internal standards are not chromatographically separated; this was in order to see if there is a contribution of the monitored product ions (from analyte to internal standard and vice versa) causing changes to the signal measured. This was assessed by duplicate analysis of blank meconium spiked with internal standard versus blank meconium spiked with FAEEs standard mixture at a concentration of 8000ng/g without adding internal standards. Exogenous interferences with compounds structurally related to FAEEs was investigated by spiking blank meconium with fatty acid methyl esters (FAMEs) in duplicate at a concentration of 8000ng/g with and without adding the analyte of interest (FAEEs) at the same concentration.

6.4.2 Linearity

Using the standard working solutions at concentrations of 1, 10, and 100 mg/L, calibration curves were prepared in meconium at concentrations ranging from 20–16000ng/g. The concentrations of calibrator points were 20, 50, 100, 400, 600, 1,000, 2,000, 4,000, 8,000, 12,000, and 16,000ng/g. The calibrators were prepared fresh before each batch.

The calibration curve was generated by plotting the peak area ratio versus the spiked analyte concentrations, and the correlation of coefficient (R^2) was calculated. The acceptable R^2 should be greater than 0.99.

6.4.3 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ were determined by preparing meconium samples with decreasing concentrations of each analyte within the expected range of the LOD. LOD concentrations were 1, 2, 5,10, 20, 50, 100, and 400ng/g. Regression analysis was undertaken, and the LOD and LOQ were calculated in accordance with Miller and Miller.(217) The calculation for LOD and LOQ is described in chapter 2 section 2.2.9.

6.4.4 Accuracy and Precision

Accuracy is determined by replicate analysis of QC samples at 2 concentrations (400 and 8000ng/g). The accuracy was calculated as a percentage of the measured mean value against corresponding theoretical or nominal value ("true value"). The mean value should not deviate by more than 20% from the true value.

Intra-day precision (within-day precision) was evaluated using five replicates of meconium control samples prepared at 2 concentrations (400 and 8000 ng/g) and extracted and injected on the same day. Inter-day precision (between-day precision) was evaluated using six meconium control samples prepared in duplicate at two concentrations (400 and 8000 ng/g) and then extracted and injected over a time period of 6 days. The percentage of the coefficient of variance (%CV) was calculated, with an acceptable %CV% of <20%.

6.4.5 Recovery study

Recovery was evaluated at two QC levels (400 and 8000ng/g). The mean peak area ratio of 3 meconium blank samples spiked with FAEEs and d_5-FAEEs was compared with the mean peak area ratio obtained for 3 meconium blank samples where d_5-FAEEs standard mixture was added before the evaporation step. (215)

6.4.6 Assessment of Matrix Effects

The matrix effect was assessed using the post-column infusion approach. Mobile phase and three pooled blank meconium (extract) were injected onto the column simultaneously with FAEEs internal standards at a concentration of 10 mg/L, infused at constant rate into the eluate using a post column tee connection. The presence of a negative or positive peak or response would indicate ion suppression or ion enhancement, respectively.

Matrix effects were also assessed by preparing 2 sets of spiked samples in triplicate at two concentrations (400 and 8000ng/g). The first set was prepared by spiking meconium blank samples, and the second set was prepared at the

same concentration without the matrix. The matrix effect was expressed as a percentage of the mean of the peak area ratio of the two sets.

6.4.7 *Carryover*

Carryove as assessed by injecting a mobile phase blank after the sample containing the calibration standard equivalent to the upper limit of quantitation (16,000ng/g).

6.5 Results and Discussion

6.5.1 Optimization of the Fragmentor Voltage and Collision Energy

The fragmentor voltage was in the range of 100–140 for most of the FAEEs and their deuterated analogues, for optimum abundance of the precursor ions as illustrated in Figures 6-1 and 6-2, respectively.

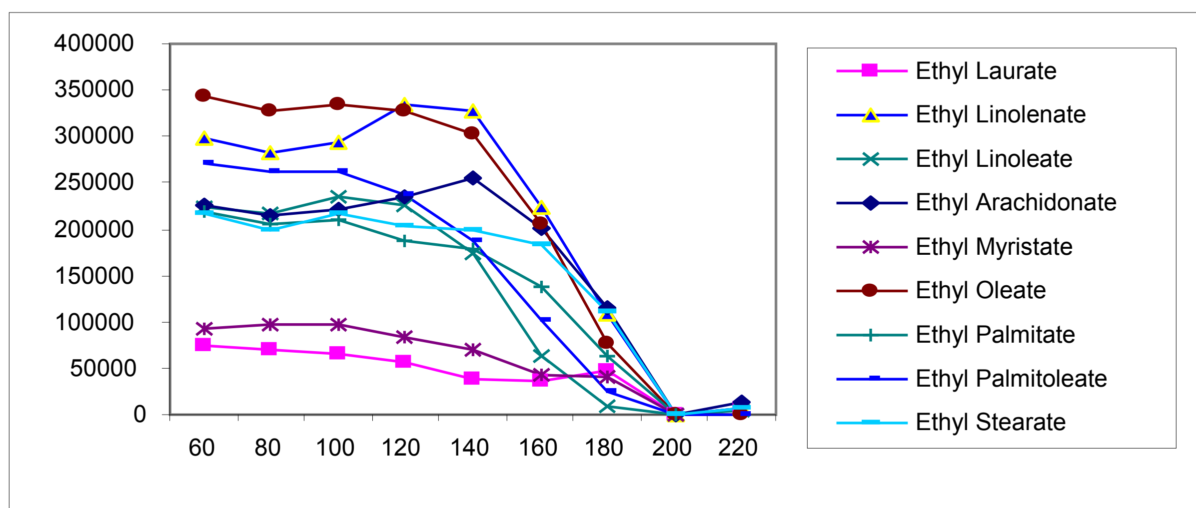


Figure 6-1: Fragmenter Ion Optimization of FAEEs

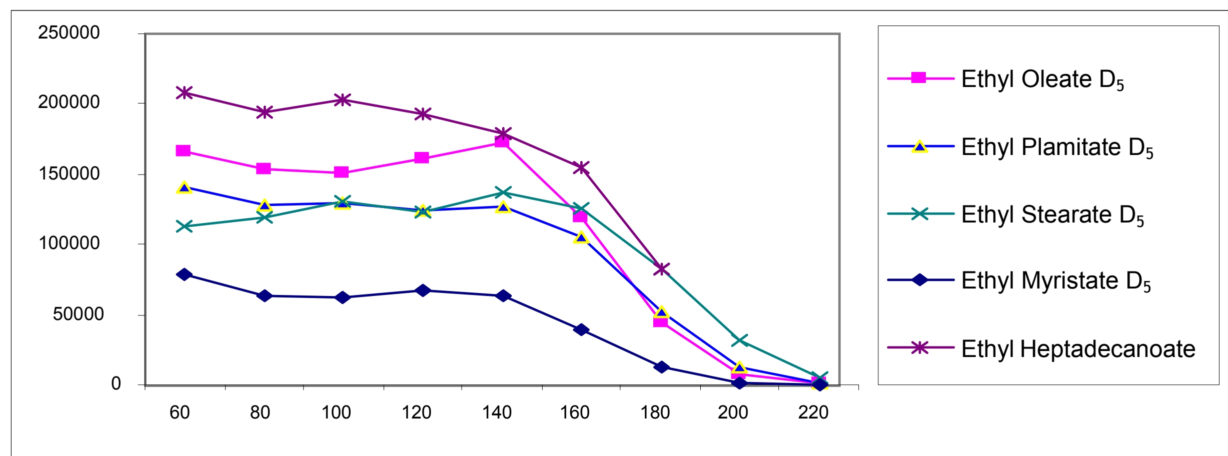


Figure 6-2: Fragmenter Ion Optimization of D5-FAEEs and Ethyl Heptadecanoate

Most analytes had an optimum collision energy of less than 10eV using LC-MS/MS as illustrated in Figure 6-3. However, using the same tuning solution with the ion trap LC/MS/MS, the collision energies were higher (27-42 eV for the same analytes). The collision energy and product ion optimization, using triple quadropole LC-MS/MS was not fully achieved as it was not reproducible. This is most likely due to the low collision energy required to fragment the ions (less than 5ev) which is much lower compared to the Thermo ion trap LC-MS/MS. A summary of the molecular and product ions and optimised collision energy is summarised in Table 6-4.

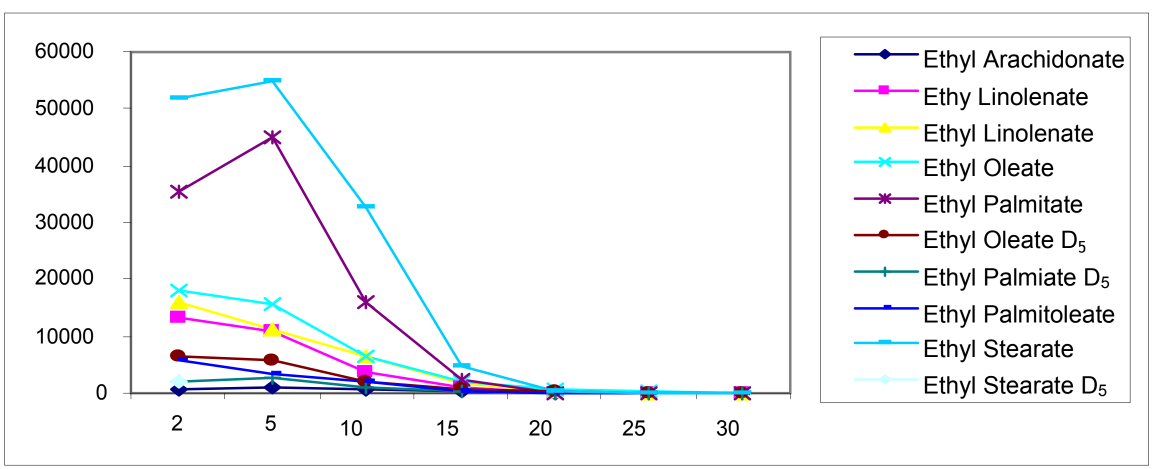


Figure 6-3: Collision Energy Optimization of FAEEs and D5-FAEEs

Multiple Reaction monitoring (MRM) transitions of quantifiers and qualifiers in addition to the fragmenter voltage, collision energy, and retention time are listed in Table 6-5. The FAEEs retention times ranged from 7–21 minutes.

Table 6-4: FAEES and FAEEs-d5 Optimization of Product Ion and Collision Energy (CE)

FAEEs	Molecular Ion	Product Ions	CE Ion Trap	Comment
Ethyl Arachidonate	332.5	287	38	Commercial
Ethyl Heptadecanoate	299	271	36	Commercial
Ethyl Laurate	228.38	201	30	Prepared in house from its acid
Ethyl Lenolenate	306.49	261/243	42	Commercial
Ethyl Linoleate	308.51	263/245	32	Commercial
Ethyl Miristate	256.43	229	32	Commercial
Ethyl Oleate	310.53	265/247	30	Commercial
Ethyl Palmitate	284.48	257	32	Commercial
Ethyl Palmitoleate	283	237	30	Commercial
Ethyl Stearate	312.53	285	34	Prepared in house from its acid
Commercial Deuterated FAEEs-D_5				
Ethyl Miristate-D_5	262	230	30	Commercial
Ethyl Oleate-D_5	316	265	38	Commercial
Ethyl Palmitate-D_5	290	258	30	Commercial
Ethyl Stearate-D_5	318	286	32	Commercial

Table 6-5: MRM (m/z) Transitions for Quantification of FAEEs

FAEEs	MRM Transition (m/Z)	Qualifier product ions	Fragmentor voltage (V)	CE (ev)	Rt (min)
Ethyl Laurate	229→201	-	84	2	7.06
Ethyl Myristate	257→229	-	100	2	9.98
Ethyl Linolenate	307→261	243	135	2	8.52
Ethyl linoleate	309→263	245	120	2	11.16
Ethyl Palmitoleate	283→237	-	108	2	10.44
Ethyl Palmitate	285→257	-	136	6	14.24
Ethyl Palmitate-D_5	290→258	-	136	5	14.03
Ethyl Oleate	311→265	247	135	2	15.23
Ethyl Oleate-D_5	316→265	247	140	2	14.94
Ethyl Stearate	313→285	247	140	5	21.26
Ethyl Stearate-D_5	318→286	-	140	5	20.98
Ethyl Arachidonate	333→287	-	128	5	9.98

6.5.2 Specificity

No endogenous or exogenous interferences were identified at the FAEEs retention times (as summarised in Appendix 6-2) or from structurally-related analytes, as illustrated in Figures 6-4 and 6-5. No contribution from the internal standards to the analytes or vice versa was observed as illustrated in Figure 6-6.

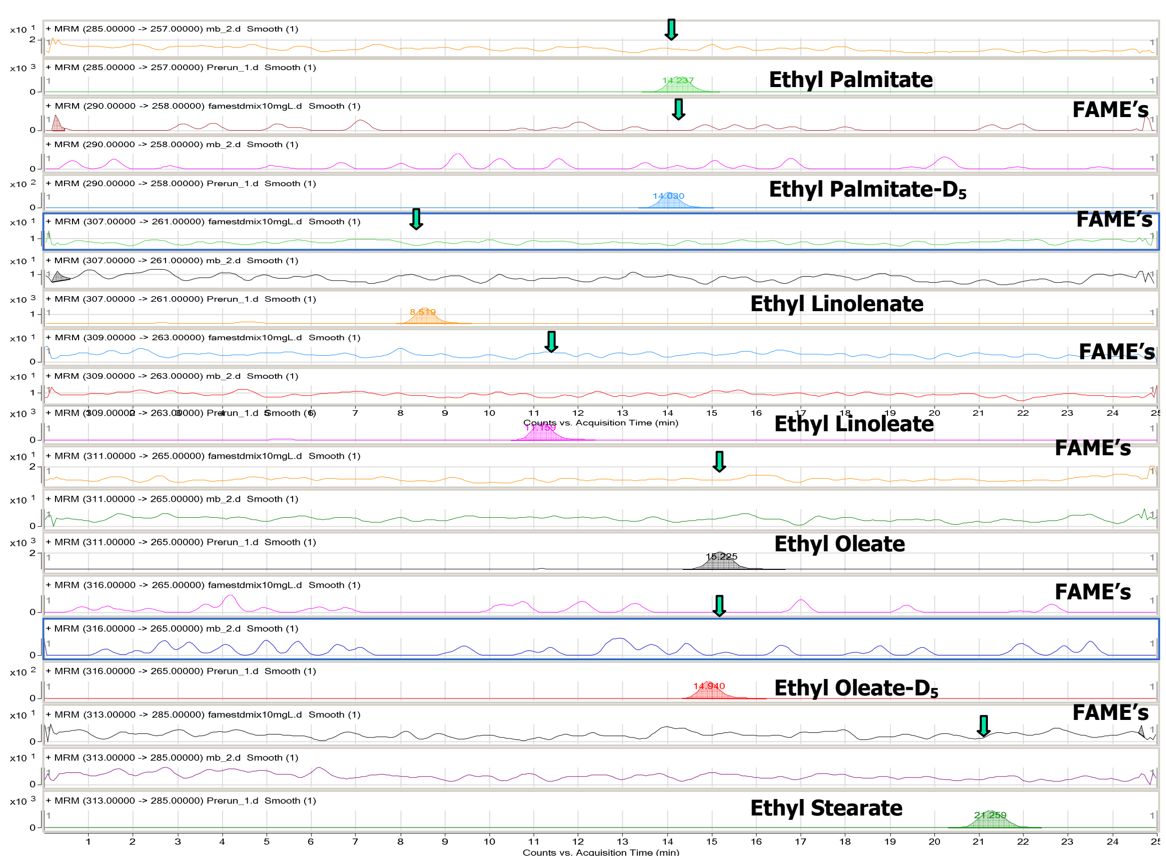


Figure 6-4: Exogenous Specificity Study Spiked Meconium with FAME Mix Standard alone 8000ng/g against Spiked FAEES 8000ng/g plus FAME 8000ng/g Compared with Unextracted FAEES and Unextracted FAME of 10 mg/L. [The arrow indicates no interference].

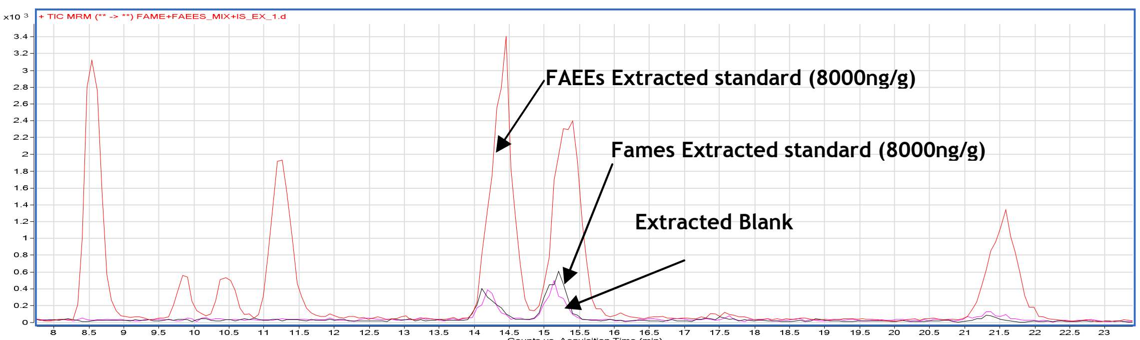


Figure 6-5: Total Ion Chromatogram (TIC) of Extracted FAEEs 8000 ng/g in Meconium Versus Extracted Blank and FAME standard of 8000 ng/g with Internal Standard.

Figure 6-6: Extracted FAAEs With and Without Internal Standard.
[The arrow indicated no contribution from analyte to internal standard]

6.5.3 Validation Results

All regression lines had > 0.995 over the concentration range from LLOQ - 16,000ng/g for each analyte as summarised in Table 6-6. With the exception of ethyl laurate and ethyl arachidonate, the LOD and LOQ were acceptable and are listed in Table 6-6.

Table 6-6: Linearity, LOD, and LOQ of FAEEs in Meconium

FAEES	Correlation Coefficient (R^2) and Regression Equations	LOD (ng/g)	LOQ (ng/g)
Ethyl Laurate	0.998 (y = 0.00002x - 0.0002)	822	2740
Ethyl Myristate	0.996 (y = 0.0001x + 0.02)	25	82
Ethyl Linolenate	0.999 (y = 0.0007x + 0.173)	14	46
Ethyl Linoleate	0.995 (y =0.0007x + 0.1984)	18	60
Ethyl Palmitoleate	0.995 (y = 0.0002x + 0.0867)	14	46
Ethyl Palmitate	0.992 (y = 0.0009x + 0.437)	11	37
Ethyl Oleate	0.995 (y = 0.0007x + 0.2586)	11	38
Ethyl Stearate	0.996 (y = 0.0011x + 0.2214)	11	37
Ethyl Arachidonate	0.998 (y = 0.00002x + 0.0008)	102	339

Intra-day and inter-day precision for all analytes met the criteria for acceptability of <20%. However, the precision for ethyl arachidonate was close to the acceptable limit. The accuracy of all analytes ranged from 75–110%, as detailed in Table 6-7. The mean and QC ranges are also listed in Table 6-7. Ethyl laurate was not reported for the 400ng/g QC as this was below the LOQ.

Table 6-7: Intra-day and Inter-day Precision and Accuracy

FAEES	QC (ng/g)	QC Mean ±2SD (ng/g)	QC Mean (ng/g) n=13	Intraday %CV n=5	Interday %CV n=6	Accuracy %
Ethyl Laurate	400 8000	- LOQ-5219	- 3641	- 11.3	- 12.1	- 76
Ethyl Myristate	400 8000	241-622 3827-9622	432 6725	18.3 10.6	19.3 17.14	81 89
Ethyl Linolenate	400 8000	337-547 5376-9821	442 7598	12.1 9.8	17.1 13.9	80 108
Ethyl Linoleate	400 8000	292-600 5834-8679	446 7256	11.2 9.4	11.2 8.7	90 110
Ethyl Palmitoleate	400 8000	343-744 6562-11880	5439221	9.7 9.5	10.6 15.7	81 88
Ethyl Palmitate	400 8000	219-509 6404-10198	3648301	8.3 4.2	8.2 8.9	110 97
Ethyl Oleate	400 8000	258-598 6884-10413	428 8648	5.3 3.7	17.2 7.3	96 93
Ethyl Stearate	400 8000	283-630 6739-11272	456 9005	9.2 11.1	14.3 15.2	83 89
Ethyl Arachidonate	400 8000	LOQ-705 4883-9817	4837350	9.8 15.5	19.9 18.7	88 85

6.5.4 Recovery and Matrix effect

No ion suppression or enhancement was indicated for the mobile phase, or the three pooled blank meconium samples, as illustrated in Figure 6-7. The recovery for all analytes from meconium ranged from 56% to 138% as summarised in Table 6-8. The percent matrix affect ranged from 54% to 118%. Most analytes (6 out of 9) demonstrated ion suppression and enhancement (1–20%). Three of the analytes (ethyl Laurate, ethyl myristate, and ethyl arachidonate) showed ion suppression or enhancement of > 20%, although the post-column syringe infusion ion suppression study (see Figure 6-7) showed no negative or positive peak in the retention time of the FAEEs. No ion suppression or enhancement was observed.

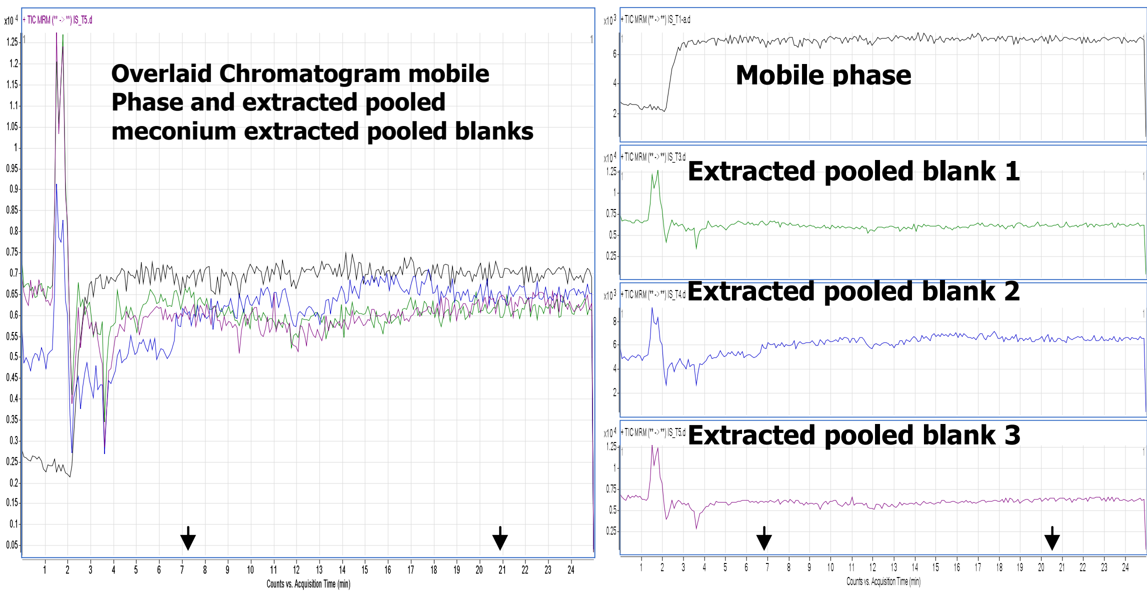


Figure 6-7: Ion Suppression Experiment. Figure (A) Overlaid Chromatograms of Mobile Phase, three pooled meconium blanks showed no ions suppression or enhancement. Figure (B) is same as (A) with separated chromatograms. The expected retention time of FAEEs ranged from 7–21minutes between the black arrows indicated in (A) and (B) expected retention time.

Table 6-8: Recovery and Matrix Effects for FAEEs

FAEES	QC (ng.g)	Recovery (%)	Matrix Effect (%)
Ethyl Laurate	400 8000	- 56	- 54
Ethyl Myristate	400 8000	80 71	153 134
Ethyl Linolenate	400 8000	114 122	93 79
Ethyl Linoleate	400 8000	93 107	102 87
Ethyl Palmitoleate	400 8000	126 123	96 118
Ethyl Palmitate	400 8000	113 97	116 118
Ethyl Oleate	400 8000	87 99	121 108
Ethyl Stearate	400 8000	138 111	99 109
Ethyl Arachidonate	400 8000	108 81	204 157

6.5.5 Carryover

No carryover was observed after injecting mobile phase after the highest calibrator (16,000ng/g) as illustrated in Figures 6-8 and 6-9.

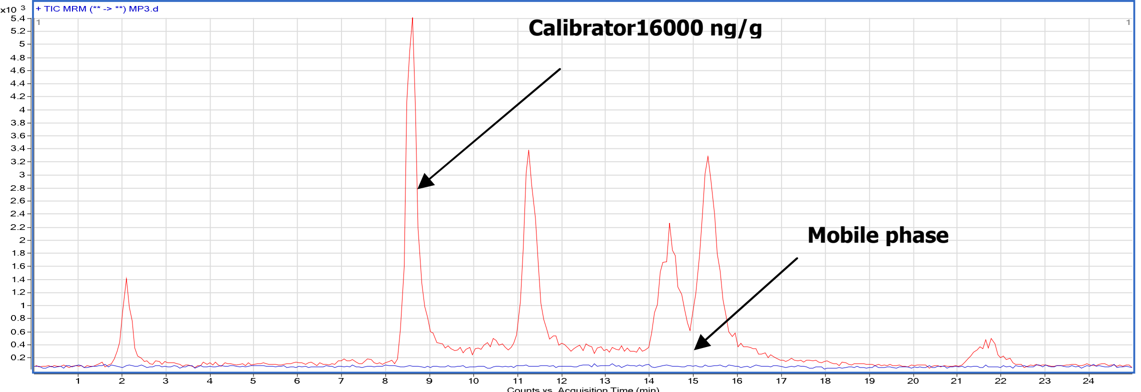


Figure 6-8: Carryover study – Total Ion Chromatogram of Highest Calibrator (16,000ng/g) Versus Blank Mobile Phase.

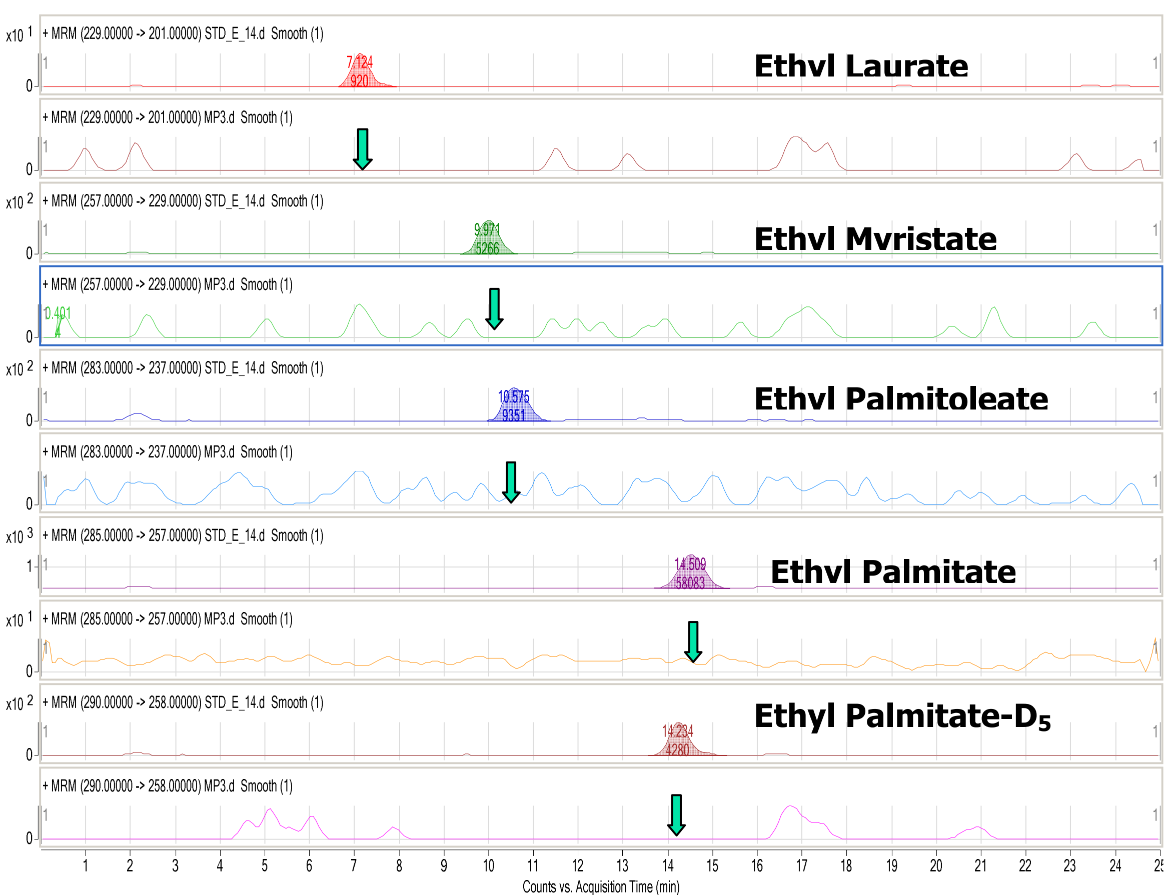


Figure 6-9: Carryover Study – (MRM) Chromatogram of Highest Calibrator (16,000ng/g) Versus Blank Mobile Phase.

6.5.6 FAEEs in Meconium

A number of samples had concentrations of FAEEs exceeding the ULOQ and dilution was not always possible due to insufficient volume of sample remaining. Therefore, all results greater than the ULOQ were reported as >ULOQ, and all results that were less than lower limit of quantification (LLOQ) were reported as <LLOQ. A cut-off point of approximately 500ng/g (equivalent to 2nmol/g) was used to distinguish between positive and negative samples, although for most analytes the LLOQ is less than this value.

A full list of the results for all FAEEs measured in the 84 meconium samples is listed in Appendix 6-3. A total of 51 samples (61%) were positive for at least one FAEE and no FAEEs were detected in 33 samples (39%). Of the 51 positive samples, 49% of these were positive for only one FAEE. A comparison between the positive and negative results for each individual FAEE detected in meconium is illustrated in Figure 6-10.

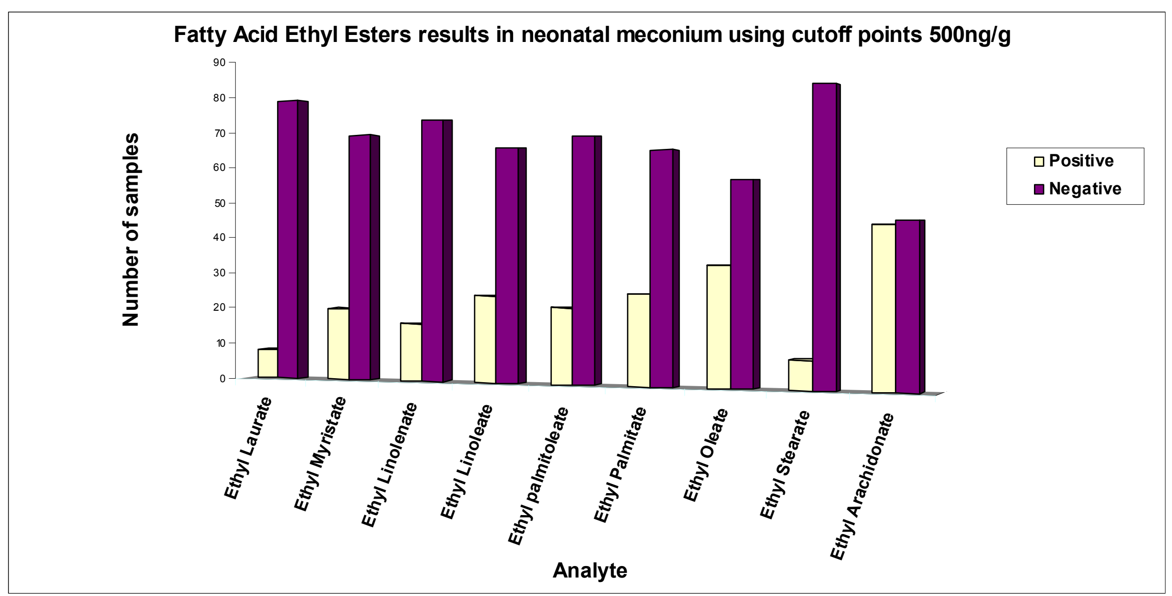


Figure 6-10: Comparison of Positive and Negative Findings for Individual FAEEs in Meconium

Three case samples (case numbers 4, 62, and 101) had a number of the FAEEs concentrations exceeding the ULOQ and are summarised in Table 6-9. It was observed that the concentration of individual FAEEs varies from case to case, as can been seen in case number 4 where ethyl linoleate was >ULOQ while ethyl

palmitate is within the linearity range. However in case #62, ethyl palmitate was >ULOQ and ethyl linoleate was lower than that measured in case # 4.

Table 6-9: Case Samples With Results Exceeding ULOQ for most of FAEEs

Case No.	Ethyl Laurite (ng/g)	Ethyl Myristate (ng/g)	Ethyl Linolenate (ng/g)	Ethyl Linoleate (ng/g)	Ethyl Palmitoleate (ng/g)	Ethyl Palmitate (ng/g)	Ethyl Oleate (ng/g)	Ethyl Stearate (ng/g)	Ethyl Arachidonate (ng/g)
4	>ULOQ (342611)	>ULOQ (124527)	>ULOQ (46627)	>ULOQ (214140)	>ULOQ (56089)	5931	>ULOQ (56421)	906	>ULOQ (4522790)
62	>ULOQ (172912)	>ULOQ (78746)	>ULOQ (25803)	>ULOQ (135051)	>ULOQ (180002)	>ULOQ (64782)	>ULOQ (332134)	4976	>ULOQ (4267963)
101	>ULOQ (194179)	>ULOQ (295840)	>ULOQ (374173	>ULOQ (532002)	>ULOQ (462119)	>ULOQ (23020)	>ULOQ (283700)	4660	>ULOQ (8354465)

The positive 95% confidence interval limit (CI) of all FAEE ranged from 2935–49032ng/g as summarised in Table 6-10. The negative 95% CI limit of all FAEE's ranged from 14–56ng/g. This is comparable to a study by Gareri(152), with the exception of ethyl arachidonate which was 10 times higher in this study.

Table 6-10: The Positive and Negative FAEEs Results and the 95% of Confidence Interval Level

FAEEs	Number of Positive Cases	Positive Mean ng/g n=87	95%(CI) ng/g	Number of Negative Cases	Negative Mean ng/g n=87	95%(CI) ng/g
Ethyl Laurate	10	35154	35153-35155	79	15	14-15
Ethyl Myristate	38	25884	2588-25885	51	53	52-53
Ethyl Linolenate	30	30010	30009-30010	59	50	50-51
Ethyl linoleate	31	49032	49031-49032	58	42	41-42
Ethyl Palmitoleate	26	39255	39254-39256	53	25	24-25
Ethyl Palmitate	30	7128	7127-7129	59	35	34-35
Ethyl Oleate	40	31405	31404-31405	49	32	32-33
Ethyl Stearate	30	2936	2935-2938	59	56	55-56
Ethyl Arachidonate	46	514611	514610-514611	43	26	25-26

Cumulative results for all 9 FAEEs are shown in Figure 6-11. 37% of the samples were negative (< 500ng/g) for cumulative FAEEs and 63% were positive (26% ranged from 500–999.99ng/g and 37% were ≥ 10,000ng/g). By plotting the cases that were < 10,000ng/g versus the concentrations, 4 samples were ≥ 5000ng/g as illustrated in Figure 6-12. In all positive samples, at least 3 analytes were positive.

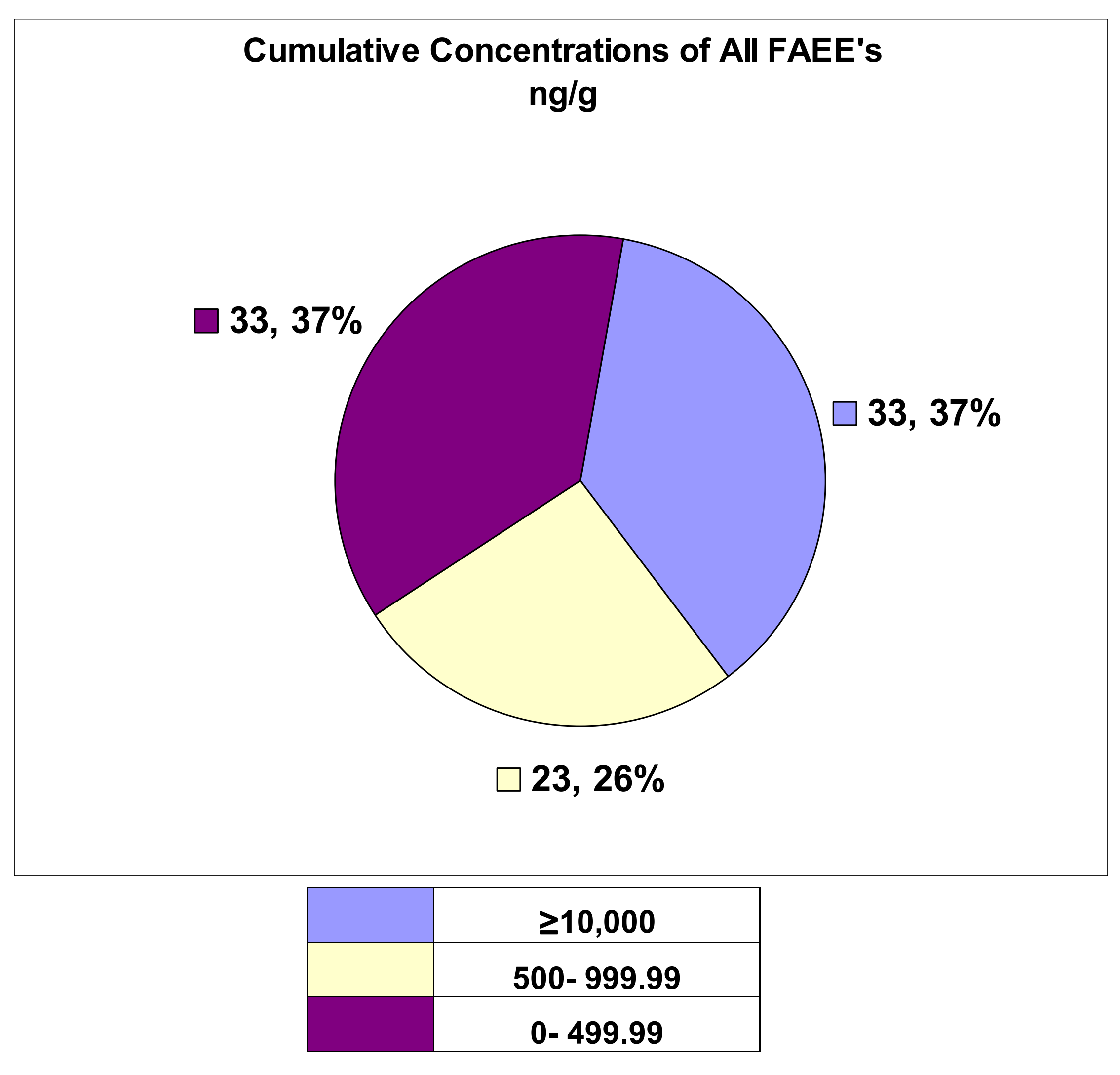


Figure 6-11: Cumulative concentrations of the FAEES in meconium

FAEEs quantified in case # 101 have been summarised in Figure 6-13 which contains an overlay of chromatograms for this case in comparison to the highest standard of the calibration curve (16000 ng/g) with only ethyl stearate being within the ULOQ.

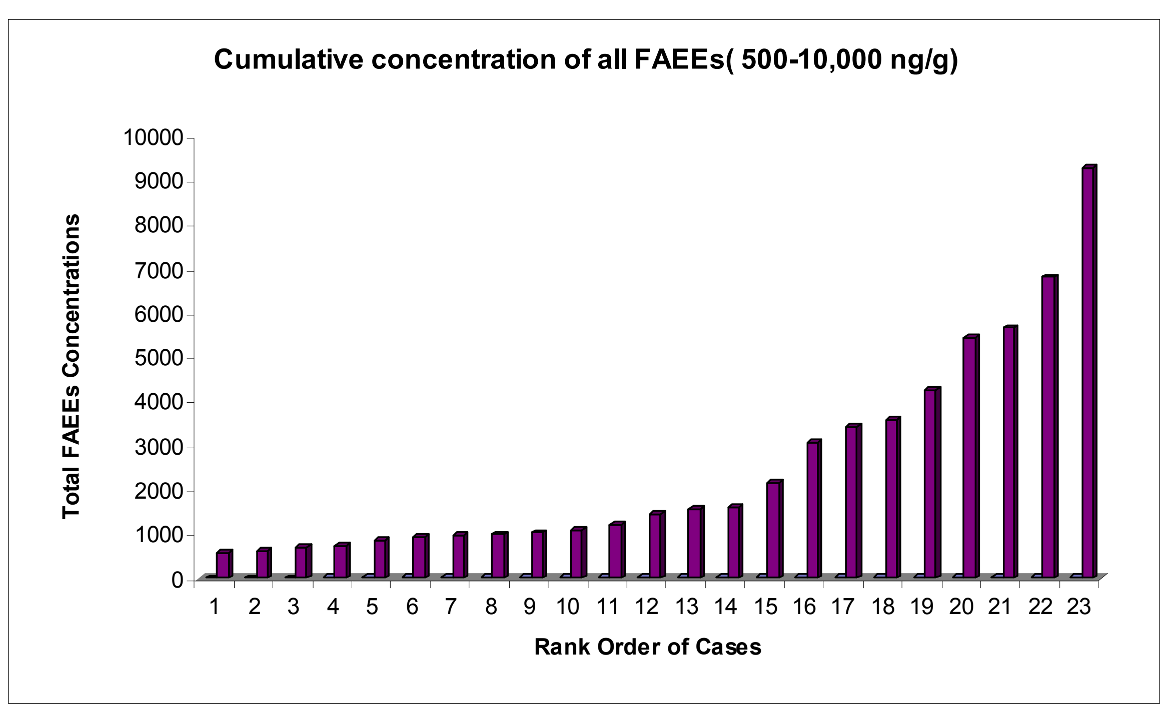


Figure 6-12: Cumulative Concentration of all FAEE Results (500–10,000ng/g)

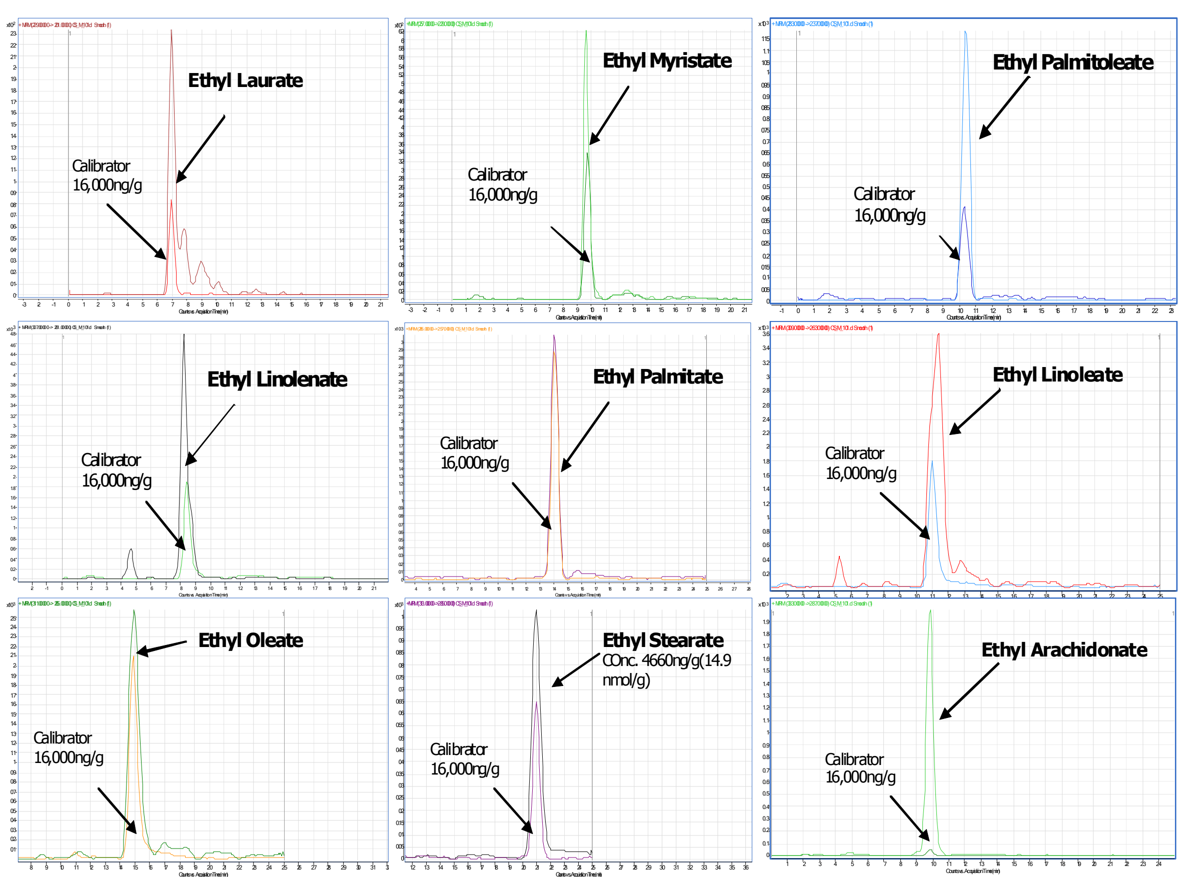


Figure 6-13: Overlaid MRM chromatogram of High-Result FAEE Case samples (101) against the Highest Point of Calibrator curve

Case # 62 (highly positive for most of FAEEs) has been integrated against a negative FAEEs results and illustrated in Figure 6-14. The black arrows highlight where the expected peak of FAEEs should be.

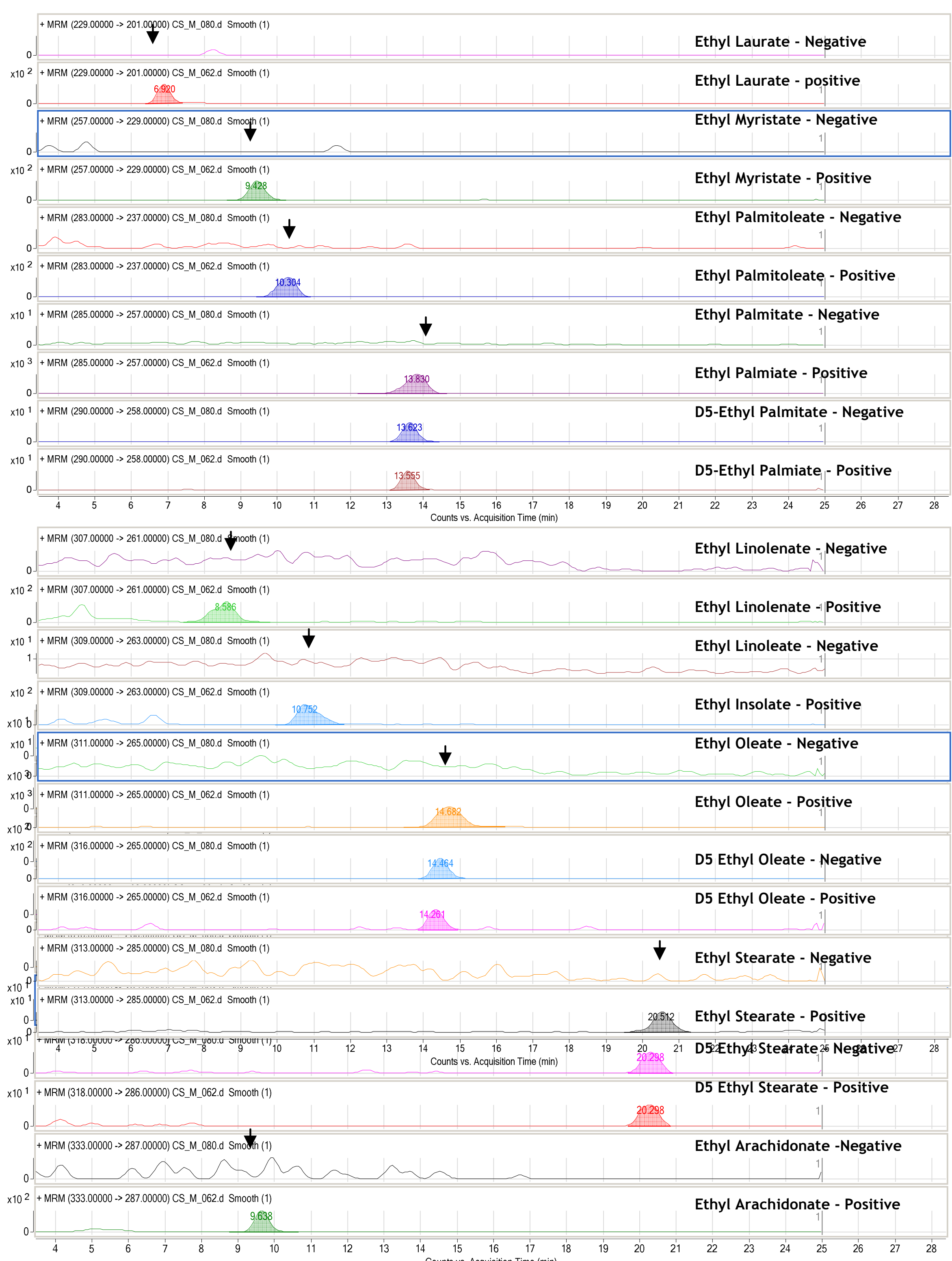


Figure 6-14: MRM Chromatogram of Negative FAEES Case Samples in Meconium versus Positive Samples.

In order to correlate the FAEEs concentrations in meconium to assess maternal gestational alcohol exposure, more information is required in relation to maternal drinking behaviours, history of drug and alcohol abuse, quantity of alcohol consumed, social status, time of drinking during gestation and stage of gestation, and age of the mother.

No correlation between the concentrations of FAEEs in meconium and foetal alcohol exposure could be assessed in this study, as the samples were originally collected for the determination of the incidence of maternal drug use and the questionnaire completed by the mother did not ask specific questions about alcohol use. In any case, self-report is unreliable in these circumstances where the mother will not admit to drinking during her pregnancy for fear of punitive action.

6.6 Conclusion

An LC-MS/MS method has been developed and validated for the determination of FAEEs in meconium. This is the first LC-MS-MS method to use deuterated FAEEs as internal standards for the quantitation of FAEEs in meconium.

The mean concentration in all positive cases was the highest for ethyl arachidonate followed by ethyl linoleate and correlated well with another study. (152;154;155;218) It was not possible to correlate the FAEE concentrations found and gestational alcohol exposure or to indicate the specificity of individual FAEE with minimal or no maternal alcohol abuse history.

Chapter 7 - Conclusion and Future Work

7.1 Conclusion

This project initially involved the development and validation of quantitative methods for the analysis of common volatiles (methanol, ethanol, isopropanol, n-propanol and acetone) and alcohol biomarkers (beta-hydroxybutyrate, BHB and fatty acid ethyl esters, FAEEs) in biological matrices, utilising dual-column HS-GC-FID, GC-MS and LC-MS/MS. This was achieved for each method in accordance with ISO/IEC 17025 and the methods were successfully applied to the analysis of real case samples. The stability of volatiles and BHB in blood was also investigated.

The optimised HS-GC-FID method for volatiles was used to evaluate the use of two internal standards, namely n-propanol and t-butanol, for the quantitative analysis of volatiles in post-mortem samples. Both methods demonstrated excellent linearity, precision and accuracy. n-Ppropanol is an ideal internal standard for use with the dual-column system used in this study and when applied to the analysis of ethanol in blood from living subjects, e.g. road traffic arrests.

Other studies have warned against using n-propanol as an internal standard in putrefied samples due to the production of this volatile post-mortem. A high concentration in the samples would result in an increased response for the internal standard and would correspond to an underestimate of the true concentration of volatiles in the sample. This is not supported with the findings in this study because the majority of samples, selected due to extensive putrefication, had n-propanol concentrations close to the LOQ and therefore had a negligible effect.

The use of ethanol to n-propanol ratio to determine whether ethanol formation has occurred is unlikely to be an effective indicator and other criteria must be considered, including the case history, condition of the specimens, distribution of ethanol in different matrices, and the concentration of ethanol. The internal

standard t-butanol is an effective alternative internal standard for the quantification of volatiles in post-mortem blood, including the ability to measure n-propanol. However, t-butanol co-elutes with acetone using DB-ALC1 or RTX-ALC1 GC capillary columns.

The antioxidant (0.2% sodium metabisulphite) played a significant role in preventing oxidation between volatiles such as isopropanol to acetone, while the use of preservative alone such as (0.2 or 1% sodium fluoride) did not prevent oxidation completely. 1% sodium fluoride in addition to 0.2% (0.2% sodium metabisulphite) as an antioxidant improved the stability of the volatiles. These finding supports the findings of another study.(81) The stability of preserved blood samples, containing fluoride oxalate at 1-2%, was assessed after they were stored at room temperature for a period of between 1 to 5 years, following initial tests for ethanol. Ethanol concentrations measured when compared with the initial test demonstrated recoveries of 50% or more in 85% of the samples tested.

A further study evaluated ethanol stability over a period of six months, with significant loss and formation of ethanol observed in both preserved and unpreserved paired post-mortem blood samples. These cases were submitted to the toxicology laboratories of Forensic Medicine and Science for routine testing and were refrigerated after initial tests. Further losses in ethanol concentration were not significant following an additional storage period of 10 months in the freezer at -22°C.

The analysis of ethanol in bile or vitreous humour provides the forensic toxicologist with an alternative sample to blood in post-mortem cases where this is not available. In addition, lower ethanol concentrations in vitreous humour compared with blood may indicate post-mortem production of ethanol. Urine is not a suitable matrix to estimate ethanol or acetone concentrations in blood.

A robust and sensitive method for the analysis of BHB in post-mortem blood and urine was validated. The analysis of different case samples in this study supports other studies that have suggested BHB is a suitable biomarker for Alcoholic Ketoacidosis (AKA) or Diabetic Ketoacidosis (DKA). This study has highlighted the potential underreporting of the role of AKA in deaths associated with chronic

alcohol abuse in the West of Scotland. Further, AKA does not always equate to low or negative ethanol levels. High levels of ethanol were reported in case samples with blood BHB levels >250 mg/L, which is in agreement to other studies. In all of the case samples with high blood BHB, the acetone concentration was high and as such, acetone can be used as an initial biomarker for AKA but the concentrations measured are not well correlated to BHB.

Acetone is volatile and is unstable in blood, and the production of other volatiles may interfere with it due to putrefaction, therefore, its absence does not preclude pathologically significant ketoacidosis (BHB >250 mg/L). The recommendation following the findings in this study is that all cases associated with a history of chronic alcoholism, should be tested for BHB, even if acetone is low or absent.

The method validation for BHB was successfully applied to post-mortem blood and urine, and plasma and urine from healthy subjects for the analysis of β-hydroxy-β-methylbutyrate (HMB) and γ-hydroxybutyrate (GHB).

The first LC-MS/MS method using commercially available deuterated FAEEs(158;159) as internal standards for the quantitation of FAEEs in meconium was also developed and validated. A third of the meconium samples tested had FAEEs totalling more than 10,000 ng/g which is indicative of foetal exposure to chronic maternal alcohol use. The mean concentration in all positive cases was the highest for ethyl arachidonate followed by ethyl linoleate and this correlates well.

7.2 Future work

A larger study would be required to investigate the prevalence of n-propanol concentrations in putrefied post-mortem blood samples from a range of case types to fully assess the risks associated with the use of this volatile as an internal standard for quantifying volatiles.

The evaluation of different GC capillary columns for better resolution between t-butanol and acetone would be of value, and in addition, to investigate the use

of t-butanol as an internal standard with samples of high fat content as this has been highlighted in other studies as a potential interferent.

Further stability investigations would be of interest to evaluate the effect of anti-coagulant in terms of antioxidation and to investigate the optimum antioxidant concentration required to prevent loss or formation of volatiles. Utilizing human whole blood would also help to evaluate the oxidative effect of enzymes, and extending the volatiles investigated to include, acetaldehyde, propionaldehyde (propanal). Investigation of the stability of volatiles using sodium metabisulfite without adding NaF to evaluate its role as a preservative should also be carried out.

The study investigating the analysis of volatiles in vitreous humour and bile was limited due to the number of samples available for analysis and reanalysis. An extension of the study would provide greater insight into the relationship between femoral blood, vitreous humour and bile and the stability of acetone and ethanol in these alternative matrices.

The evaluation of the stability of BHB and GHB in blood in paired preserved and unpreserved sample when stored in different temperature and concentration of preservative would provide an insight into the presence of these substances at the time of death versus the time of collection.

A further investigation into the use of deuterated internal standards for each of the FAEEs tested would be of interest and then applying this method to investigate FAEE levels in maternal and newborn hair samples using HS-SPME-GC/MS, as recommended by the Society of Hair Testing. A comparison could then be carried out between meconium and hair to identify which sample is better as an indicator of foetal alcohol exposure. The long-term stability of FAEEs in meconium or BHB in postmortem blood has as yet not been fully investigated. Lastly, the role of BHB as a biomarker should be extended to analyze a wide range of case types where individuals have a history of acute and chronic alcohol misuse.

Chapter 8 - Reference list

(1) McAnalley BH. The Chemisrty of Alcoholic Beverages. In: Garriott JC, editor. Medical -Legal Aspects of Alcohol. 2003: 1-22.

(2) Whitfield D. Alcohol and the Bible. http://wooga.drbacchus.com/bible/alcohol.html.The page last cccessed 26 May201.Last modified date . 1996.

(3) Alcohol in Islam. http://www.quranandscience.com/legislative/217-alcohol-in-islam.html.(Accessed 04 June 2011) . 2010.

(4) Alcohol Prohibition. [Interner] From Wipedia the free encyclopedia.Accessed date 08 June 2011, last update date 04 June 2011.Available at http://en.wikipedia.org/wiki/Prohibition. 4-6-2011.

(5) Alcohol Prohibition Poster: Prohibition Failed-Please do something about it. http://www.druglibrary.org/schaffer/history/e1930/prohibitionposter2.htm.Date Acessed 08 June2011 . 2011.

(6) Sadler D. Alcohol & Alcoholism-Lecture Notes. 1-18. 2007. Copyright 2007 Dr. David W. Sadler, Department of Forensic Medicine. University of Dundee.

(7) Tapson F. Alcoholic Drinks, Measures and Units in United Kingdom. Report Version 1.0 , 1-13. 2004.

(8) WHO Global status report on alcohol and health (GISAH). The Global Information System on Alcohol and Health (GISAH).[Map] of Total adult per Capita consumption of pure alcohol(in litres), 2005. 310px200px PNG. -ISBN 978 92 4 156415 1 (NLM classification: WM 274).WHO Library Cataloguing-in-Publication Data. Available at http://gamapserver.who.int/gho/static_graphs/gisah/Global_adult_percapita_consumption_2005.png: 2011.

(9) Alcohol. Fact sheet N°349. 2011. World Health Organization (WHO) , Accessed date 06 June 2011. page last updated date February 2011. Available at http://www.who.int/mediacentre/factsheets/fs349/en/index.html.

(10) Alcohol Statristics in Scotland 2011. Scotland Nsr, editor. Report of 2011, 1-117. 2011. Scotland National Statistics Release© Common Services Agency/Crown Copyright 2010. Available at http://www.alcoholinformation.isdscotland.org.

(11) Corbett J, Dobbie F, Doig M, D'Souza J, Given L, Gray L et al. The Scottish Health Survey 2009: A National Statistics Publication for Scotland. Bromely C, Given L, Ormston R, editors. Volume I : Main Report, 1-337. 20-9-0010. The Scottish Government.St Andrew's House. Edinburgh, the Scottish Government.

(12) Aresu M, Chaudhury E, Diment E, Fuller V, Gordon V, Gunning N et al. Health survey for England 2009. Craig R, HIrani V, editors. Volume 1 health and Life Style, 1-207. 2009. Leeds, The NHS Information Center.

(13) Robinson S, Harris H. Smoking and drinking among adults, General Life style survey. Dunstan S, editor. General Life style survey 2009, 1-84. 2009. South Wales, Office of national Statistics © Crown copyright 2011.

(14) Alcohol and its impact on health -Key Facts. Accessed 06 July 2011.Available at http://www.scotland.gov.uk/Topics/Health/health/Alcohol/health. 6-6-2011.

(15) Alcohol Related Deaths 'falling'. http://news.bbc.co.uk/1/hi/health/7852897.stm, Date accessed 18 May 2011, Page last updated at 14:59 GMT, Tuesday, 27 January 2009 . 27-1-2009. BBC News.

(16) Alcohol Statristics in Scotland 2009. 1-132. 2009. Edinburgh-UK, Scotland National Statistics Release UK © Common Services Agency/Crown Copyright 2009.

(17) Moss L. Scottish alcohol death toll up to six times UK average. http://thescotsman.scotsman.com/scotland/Scottish-alcohol-death-toll-up.5654359.jp date accessed 17 September 2011 . 17-9-2009.

(18) Shaw M, Thomas B, Smith G, Dorling D. The Grim Reapers's road map, An atlas of mortality in Britain. Bristol: Policy Press-2008, University of Bristol, UK, 2011.

(19) National Center for Statistics and Analysis: Traffic Safety Facts- 2008 Data- Alcohol Impaired driving. DOT HS 811 155, 1-6. 2008. New Jersey Avenue SE., Washington,DC, NHTSA's, Available at www.nhtsa.gov.

(20) Wren E. International Blood Alcohol Limits For drunk driving. 1-6. 2003. www.driveandstayalive.com/articles%20and topics /drunk%20driving/artci-drunk-driving-00.

(21) Tackling the Challenge of FASD, The National Organization for Foetal Alcohol Syndrome Training. 16-10-2008. NOFAS UK, © 2007 - 2011 National Organisation on Fetal Alcohol Syndrome - UK. Available at www.nofas-uk.org.

(22) Willian Hogarth 1697-1764 (Gin Lane). http://en.wikipedia.org/wiki/File:William_Hogarth_-_Gin_Lane.jpg.Date accessed 06 June 2011, this page last modified 10 July 2008 . 2011.

(23) Fetal acohol spectrum disorders. A guid for health care professionals. BMA Board of Science , 1-58. 2007. British Medical Association-ISBN1-905545-18-5.

(24) Cook JD. Biochemical markers of alcohol use in pregnant women. Clinical Biochemistry 2003; 36(1):9-19.

(25) Katharine F. Understanding the use of alcohol in pregnancy amongst women in Scotland. Department of Geography, part of faculty of Social and Eviromental Studies at the University of Liverpool, 2008.

(26) Riley P, Infante MA, Warren KR. Fetal Alcohol Spectrum Disorders: An Overview. Neuropsychology Review 2011; 21(2):73-80.

(27) Chan D, Klein J, Karaskov T, Koren G. Fetal exposure to alcohol as evidenced by fatty acid ethyl esters in meconium in the absence of maternal drinking history in pregnancy. Therapeutic Drug Monitoring 2004; 26(5):474-481.

(28) Chan D, Caprara D, Blanchette P, Klein J, Koren G. Recent developments in meconium and hair testing methods for the confirmation of gestational exposures to alcohol and tobacco smoke. Clinical Biochemistry 2004; 37(6):429-438.

(29) Jones L. The Effects of Alcohol on Fetal Development. Birth Defects Research Part C-Embryo Today-Reviews 2011; 93(1):3-11.

(30) National Survey on Drug Use and Health S. Alcohol use Among Pregnant and Nonpregnant Women of Chilbearing Age-2002-2007, The NSDUH Report. NSDUH S, editor. 1-6. 11-9-2008.

(31) Denny CH, Tsai J, Floyd RL, Green PP. Alcohol Use Among Pregnant and Nonpregnant Women of Childbearing Age - United states ,1991-2005. Morbidity & Mortality Weekly Report 58 [19], 529-532. 2009.

(32) Clann AA. Children in Scotland Consultation Response: Changing Scotland's Relationsip with Alcohol. 1-13. 2008. Scotland.

(33) May PA, Gossage JP. Estimating the "prevalence of fetal alcohol syndrome - A summary. Alcohol Research & Health 2001; 25(3):159-167.

(34) May PA, Gossage JP, Kalberg WO, Robinson LK, Buckley D, Manning M et al. Prevalence and Epidemiologic Characteristics of Fasd from Various Research Methods with An Emphasis on Recent In-School Studies. Developmental Disabilities Research Reviews 2009; 15(3):176-192.

(35) Stade BC, Stevens B, Ungar WJ, Beyene J, Koren G. Health-related quality of life of Canadian children and youth prenatally exposed to alcohol. Health and Quality of Life Outcomes 2006; 4(81):1-10.

(36) May PA, Fiorentino D, Gossage JP, Kalberg WO, Hoyme HE, Robinson LK et al. Epidemiology of FASD in a province in Italy: Prevalence and characteristics of children in a random sample of schools. Alcoholism-Clinical and Experimental Research 2006; 30(9):1562-1575.

(37) May PA, Fiorentino D, Coriale G, Kalberg WO, Hoyme HE, Aragon AS et al. Prevalence of Children with Severe Fetal Alcohol Spectrum Disorders in Communities Near Rome, Italy: New Estimated Rates Are Higher than Previous Estimates. International Journal of Environmental Research and Public Health 2011; 8(6):2331-2351.

(38) May PA, Gossage JP, Marais AS, Adnams CM, Hoyme HE, Jones KL et al. The epidemiology of fetal alcohol syndrome and partial FAS in a South African community. Drug and Alcohol Dependence 2007; 88(2-3):259-271.

(39) U.S. Surgeon General Release Advisory on Alcohol Use in Pregnancy. Press release (February, 21, 2005), 1. 21-2-2005. HHS Press, Office.

(40) Pregnant Women with Health Problems Need Care, Not Incarceration, Ob-Gyns Urged to Help Retract Punitive State Legislation. ACOG OaG, editor. The American College of Obstetricians and Gynecologists Committee Opinion #473, "Substance Abuse Reporting in Pregnancy," is published in the January 2011 issue of Obstetrics & Gynecology., 1. 2011. Washington, DC, Copyright © 2011 American Congress of Obstetricians and Gynecologists(ACOG~). All rights reserved.

(41) Carson G, Cox VL, Crane J, Croteau P, Graves L, Kluka S et al. Alcohol Use and Pregnancy Consensus Clinical Guidelines, SOGC Clinical Practice Guidlines . Rowe T, editor. Journal of Obstetrics and Gynaecology Canada No. 245, volume 32, ISSN 1701-2163[8], 1-36. 2010. printed by Dollco Printing, Ottawa, ON, published by the Canadian Psychiatric Association (CPA).

(42) Alcohol and pregnancy message. http://www.dh.gov.uk/en/FreedomOfInformation/Freedomofinformationpublicationschemefeedback/FOIreleases/DH_100220 . 8-6-2009.

(43) Care and support that women should receive during pregnancy. Updated NICE guideline Ref : 2008/022, 1-4. 2010. UK, © National Institute for Health and Clinical Excellence, 2008.

(44) Beeston C, Robinson M, Craig N, Graham L. Monitoring and Evaluating Scotland's Alcohol Strategy. Setting the Scene: Theory of change and baseline picture - Glossary and Appendices. Published by NHS Health Scotland, Edinburgh,March 2011, 1-49. 2011.

(45) Beeston C, Robinson M, Craig N, Graham L. Monitoring and Evaluating Scotland's Alcohol Strategy. Setting the Scene: Theory of change and baseline picture. Published by NHS Health Scotland,Edinburgh, Mar 2011, 1-81. 2011.

(46) Levine B, Caplan Y. Alcohol. In: Levine B, editor. Principles of Forensic Toxicology. Washington, DC: AACC Press, 2003: 89-116.

(47) Jones AW. Disposition and Fate of Ethanol in the body. In: Garriott JC, editor. Medical legal Aspects of Alcohol. Lawyers & Judges Publishing Company, Inc., 2003: 47-112.

(48) Iffland R, Jones AW. Evaluating alleged drinking after driving--the hip-flask defence. Part 1. Double blood samples and urine-to-blood alcohol relationship. Medicine, Science and the Law Journal 2002; 42(3):207-224.

(49) Jones AW. Evidence-based survey of the elimination rates of ethanol from blood with applications in forensic casework. Forensic Science International 2010; 200(1-3):1-20.

(50) Jones AW, Pounder D. Measuring blood alcohol concentration for clinical and forensic purposes. In: Krach S, editor. Drug Abuse Handbook. San Fancisco: CRC Press LLC, 1997: 327-355.

(51) Jones AW. Ultra-rapid rate of ethanol elimination from blood in drunken drivers with extremely high blood-alcohol concentrations. International Journal of Legal Medicine 2008; 122(2):129-134.

(52) Jones AW. Disappearance Rate of Ethanol from the Blood of Human-Subjects - Implications in Forensic Toxicology. Journal of Forensic Sciences 1993; 38(1):104-118.

(53) Boumba VA, Ziavrou KS, Vougiouklakis T. Biochemical pathways generating post-mortem volatile compounds co-detected during forensic ethanol analyses. Forensic Science International 2008; 174(2-3):133-151.

(54) Canfield D, Smith M, Adam H, Houston E. Selection of an Internal Standard for Postmortem Ethanol Analysis. U.S.Department of Transportation Federal Aviation Adminstration DOT/FAA/AM-9815, 1-7. 1998. USA.

(55) Brinkmann B, Fechner G, Karger B, DuChesne A. Ketoacidosis and lactic acidosis - frequent causes of death in chronic alcoholics? International Journal of Legal Medicine 1998; 111(3):115-119.

(56) Caplan YH, Goldberger BA. Blood, Urine and Other Fluid and Tissue specimens for Alcohol analyses. In: Garriott JC, editor. Medical legal Aspects of Alcohol. Lawyers & Judges Publishing Company, Inc., 2003: 149-162.

(57) Mason MF, Dubowski KM. Alcohol, Traffic, and Chemical Testing in United-States - Resume and Some Remaining Problems. Clinical Chemistry 1974; 20(2):126-140.

(58) Payne JP, Hill DW, Wood DGL. Distribution of Ethanol Between Plasma and Erythrocytes in Whole Blood. Nature 1968; 217(5132):963-&.

(59) Jones AW. Distribution of Ethanol Between Saliva and Blood in Man. Clinical and Experimental Pharmacology and Physiology 1979; 6(1):53-59.

(60) Jones AW. Measuring ethanol in saliva with the QED enzymatic test device: comparison of results with blood- and breath-alcohol concentrations. Journal of Analytical Toxicology 1995; 19(3):169-174.

(61) Jones AW. Reference limits for urine/blood ratios of ethanol in two successive voids from drinking drivers. Journal of Analytical Toxicology 2002; 26(6):333-339.

(62) Jones AW, Eklund A, Helander A. Misleading results of ethanol analysis in urine specimens from rape victims suffering from diabetes. Journal of Clinical Forensic Medicine 2000; 7(3):144-146.

(63) Garriott JC. Analysis for Alcohol in Postmortem Specimens. In: Garriott JC, editor. Medical legal Aspects of Alcohol. Lawyers & Judges Publishing Company, Inc., 2003: 163-176.

(64) Cooper GAA, Paterson S, Osselton MD. The United Kingdom and Ireland Association of Forensic Toxicologists Forensic toxicology laboratory guidelines (2010). Science & Justice 2010; 50(4):166-176.

(65) Budd RD. Validity of Post-Mortem Chest Cavity Blood Ethanol Determinations. Journal of chromatography 1988; 449(1):337-340.

(66) Moriya F, Hashimoto Y, Furumiya J, Nishioka S. Effects of perimortem physical factors associated with death on exogenous ethanol concentrations in cardiac blood. Legal Medicine 2005; 7(4):213-216.

(67) Jones AW, Holmgren P. Urine/blood ratios of ethanol in deaths attributed to acute alcohol poisoning and chronic alcoholism. Forensic Science International 2003; 135(3):206-212.

(68) Saady JJ, Poklis A, Dalton HP. Production of urinary ethanol after sample collection. Journal of Forensic Sciences 1993; 38(6):1467-1471.

(69) Jones AW. Ethanol metabolism in patients with liver cirrhosis. Journal of Clinical Forensic Medicine 2000; 7(1):48-51.

(70) Backer RC, Pisano RV, Sopher IM. The Comparison of Alcohol Concentrations in Postmortem Fluids and Tissues. Journal of Forensic Sciences 1980; 25(2):327-331.

(71) Jones AW, Holmgren P. Uncertainty in estimating blood ethanol concentrations by analysis of vitreous humour. Journal of Clinical Pathology 2001; 54(9):699-702.

(72) de Lima IV, Midio AF. Origin of blood ethanol in decomposed bodies. Forensic Science International 1999; 106(3):157-162.

(73) Honey D, Caylor C, Luthi R, Kerrigan S. Comparative alcohol concentrations in blood and vitreous fluid with illustrative case studies. Journal of Analytical Toxicology 2005; 29(5):365-369.

(74) Winek CL, Esposito FM. Comparative study of ethanol levels in blood versus bone marrow, vitreous humor, bile and urine. Forensic Science International 1981; 17(1):27-36.

(75) Winek CL, Henry D, Kirkpatrick L. The Influence of Physical-Properties and Lipid-Content of Bile on the Human-Blood Bile Ethanol Ratio. Forensic Science International 1983; 22(2-3):171-178.

(76) Christop G, Kirch ER, Gearien JE. Determination of Ethanol in Fresh and Putrefied Postmortem Tissues. Journal of chromatography 1973; 87(2):455-472.

(77) Ohshima T, Kondo T, Sato Y, Takayasu T. Postmortem alcohol analysis of the synovial fluid and its availability in medico-legal practices. Forensic Science International 1997; 90(1-2):131-138.

(78) Canfield DV, Kupiec T, Huffine E. Postmortem alcohol production in fatal aircraft accidents. Journal of Forensic Sciences 1993; 38(4):914-917.

(79) Wigmore J, Chow B. Case Report: Detection of neo-formation of ethanol in a postmortem blood sample using n-propanol and a urine sample. Canadian Society of Forensic Science Journal 2000; 33(3):145-149.

(80) Lewis RJ, Johnson RD, Angier MK, Vu NT. Ethanol formation in unadulterated postmortem tissues. Forensic Science International 2004; 146(1):17-24.

(81) Brown GA, Neylan D, Reynolds WJ, Smalldon KW. Stability of Ethanol in Stored Blood .1. Important Variables and Interpretation of Results. Analytica Chimica Acta 1973; 66(2):271-283.

(82) Dick GL, Stone HM. Alcohol loss arising from microbial contamination of drivers' blood specimens. Forensic Science International 1987; 34(1-2):17-27.

(83) O'Neal CL, Poklis A. Postmortem production of ethanol and factors that influence interpretation - A critical review. American Journal of Forensic Medicine and Pathology 1996; 17(1):8-20.

(84) Anderson WH. Collection and Storage of Specimens for Alcohol Analysis. In: Garriott JC, editor. Medical legal Aspects of Alcohol. Lawyers & Judges Publishing Company, Inc., 2003: 237-247.

(85) Dubowski KM, Gadsden RH, Poklis A. The stability of ethanol in human whole blood controls: An interlaboratory evaluation. Journal of Analytical Toxicology 1997; 21(6):486-491.

(86) Yajima D, Motani H, Kamei K, Sato Y, Hayakawa M, Iwase H. Ethanol production by Candida albicans in postmortem human blood samples: effects of blood glucose level and dilution. Forensic Science International 2006; 164(2-3):116-121.

(87) Shan X, Tiscione NB, Alford I, Yeatman DT. A study of blood alcohol stability in forensic antemortem blood samples. Forensic Science International 2011; 211(1-3):47-50.

(88) Lough PS, Fehn R. Efficacy of 1% sodium fluoride as a preservative in urine samples containing glucose and Candida albicans. Journal of Forensic Sciences 1993; 38(2):266-271.

(89) Blume P, Lakatua DJ. The effect of microbial contamination of the blood sample on the determination of ethanol levels in serum. American Journal of Forensic Medicine and Pathology 1973; 60(5):700-702.

(90) Chang J, Kollman SE. The effect of temperature on the formation of ethanol by Candida albicans in blood. Journal of Forensic Sciences 1989; 34(1):105-109.

(91) Jones AW, Hylen L, Svensson E, Helander A. Storage of specimens at 4 degrees C or addition of sodium fluoride (1%) prevents formation of ethanol in urine inoculated with Candida albicans. Journal of Analytical Toxicology 1999; 23(5):333-336.

(92) Smalldon KW, Brown GA. Stability of Ethanol in Stored Blood .2. Mechanism of Ethanol Oxidation. Analytica Chimica Acta 1973; 66(2):285-290.

(93) Parkinson A, Ogilvie B. Biotransforamtion of enobiotics. In: Klaassen C, editor. Casarett and Doull's Toxicology The Basic Science Of Poinsons. Kansas City: Mc Graw-Hill Companies, 2007: 161-304.

(94) Bonventre J, Valanju S, Bastos ML. Evaluation of ethanol analysis on brain and liver by head-space gas chromatography. Forensic Science International 1982; 19(1):75-83.

(95) O'Neal CL, Wolf CE, Levine B, Kunsman G, Poklis A. Gas chromatographic procedures for determination of ethanol in postmortem blood using t-butanol and methyl ethyl ketone as internal standards. Forensic Science International 1996; 83(1):31-38.

(96) David S. Chromatography. In: Barry L, editor. Principles of Forensic Toxicology. Washington, DC: AACC Press, 2003: 89-116.

(97) Moriya F, Hashimoto Y. Postmortem production of ethanol and n-propanol in the brain of drowned persons. American Journal of Forensic Medicine and Pathology 2004; 25(2):131-133.

(98) Kuhlman JJ, Levine B, Smith ML, Hordinsky JR. Toxicological Findings in Federal Aviation Administration General-Aviation Accidents. Journal of Forensic Sciences 1991; 36(4):1121-1128.

(99) Schuberth J. Volatile Compounds Detected in Blood of Drunk Drivers by Headspace Capillary Gas-Chromatography Ion Trap Mass-Spectrometry. Biological Mass Spectrometry 1991; 20(11):699-702.

(100) Saier MH. Multiple mechanisms controlling carbon metabolism in bacteria. Biotechnology and Bioengineering 1998; 58(2-3):170-174.

(101) Pounder D, Jones AW. Measuring Alcohol Postmortem. In: Krach S, editor. DRUG Abuse Handbook. San Francisco: CRC Press LLC, 1997: 356-373.

(102) Winek CL, Wahba WW, Winek CL, Balzer TW. Drug and chemical blood-level data 2001. Forensic Science International 2001; 122(2-3):107-123.

(103) Kinoshita H, Ijiri I, Ameno S, Tanaka N, Kubota T, Tsujinaka M et al. Combined toxicity of methanol and formic acid: two cases of methanol poisoning. International Journal of Legal Medicine 1998; 111(6):334-335.

(104) Iffland R, Berghaus G. Experiences with Volatile Acoholism Indicators (Methanol, Acetone, Isopropanol) in DWI Car Drivers. Alcohol, Drugs and Traffic Safety 1995; 1-2:63-67.

(105) TOXBASE. National Poisons Information Service(Health protection Agency) . 2008.

(106) Helander A, Beck O. Analytical markers of acute and chronic alcohol consumption. In: Bogusz M, editor. Handbook of Analytical Separations. Amsterdam - Oxford: Elsevier, 2008: 567-588.

(107) Helander A, Beck O, Jones AW. Laboratory testing for recent alcohol consumption: Comparison of ethanol, methanol, and 5-hydroxytryptophol. Clinical Chemistry 1996; 42(4):618-624.

(108) Kugelberg FC, Jones AW. Interpreting results of ethanol analysis in postmortem specimens: a review of the literature. Forensic Science International 2007; 165(1):10-29.

(109) Helander A, Jones AW. Biochemical tests for acute and chronic alcohol ingestion. In: Krach S, editor. DRUG Abuse Handbook. San Francisco: CRC Press LLC, 1997: 374-392.

(110) Begleiter H, Reich T, Hesselbrock V, Porjesz B, Begleiter H, Li TK et al. The collaborative study on the genetics of alcoholism. Alcohol Health & Research World 1995; 19(3):228-236.

(111) Fowler JS, Volkow ND, Wang GJ, Pappas N, Logan J, MacGregor R et al. Inhibition of monoamine oxidase B in the brains of smokers. Nature 1996; 379(6567):733-736.

(112) Helander A, Lowenmo C, Johansson M. Distribution of Acetaldehyde in Human Blood - Effects of Ethanol and Treatment with Disulfiram. Alcohol and Alcoholism 1993; 28(4):461-468.

(113) Kroke A, Dierkes J. Urinary Markers of Alcohol Consumption. In: Preedy V, Watson R, editors. Comprehensive Handbook of Alcohol Related Pathology. Oxford: Elsevier Ltd., 2004: 1445-1458.

(114) Dierkes J, Wolfersdorf M, Borucki K, Weinmann W, Wiesbeck G, Beck O et al. Determination of glucuronidated 5-hydroxytryptophol (GTOL), a marker of recent alcohol intake, by ELISA technique. Clinical Biochemistry 2007; 40(1-2):128-131.

(115) Hoiseth G, Karinen R, Christophersen AS, Olsen L, Normann PT, Morland J. A study of ethyl glucuronide in post-mortem blood as a marker of ante-mortem ingestion of alcohol. Forensic Science International 2007; 165(1):41-45.

(116) Anders H, Jones AW. Bichemical Tests For Acute and chronic Alcohol ingestion. In: Steven B.Karch, editor. Drug Abuse Handbook. CRC Press LLC, 1998: 374-394.

(117) Salmela KS, Laitinen K, Nystrom M, Salaspuro M. Carbohydrate-Deficient Transferrin During 3 Weeks Heavy Alcohol-Consumption. Alcoholism-Clinical and Experimental Research 1994; 18(2):228-230.

(118) Hartmann S, Aradottir S, Graf M, Wiesbeck G, Lesch O, Ramskogler K et al. Phosphatidylethanol as a sensitive and specific biomarker-comparison with gamma-glutamyl transpeptidase, mean corpuscular volume and carbohydrate-deficient transferrin. Addiction Biology 2007; 12(1):81-84.

(119) Bruhl A, Faldum A, Loffelholz K. Degradation of phosphatidylethanol counteracts the apparent phospholipase D-mediated formation in heart and other organs. Biochimica et Biophysica Acta-Molecular and Cell Biology of Lipids 2003; 1633(2):84-89.

(120) Aradottir S, Seidl S, Wurst FM, Jonsson BA, Alling C. Phosphatidylethanol in human organs and blood: a study on autopsy material and influences by storage conditions. Alcoholism-Clinical and Experimental Research 2004; 28(11):1718-1723.

(121) Bendroth P, Kronstrand R, Helander A, Greby J, Stephanson N, Krantz P. Comparison of ethyl glucuronide in hair with phosphatidylethanol in whole blood as post-mortem markers of alcohol abuse. Forensic Science International 2008; 176(1):76-81.

(122) Gareri J, Appenzeller B, Walasek P, Koren G. Impact of hair-care products on FAEE hair concentrations in substance abuse monitoring. Analytical and Bioanalytical Chemistry 2011; 400(1):183-188.

(123) Hansson P, Caron M, Johnson G, Gustavsson L, Alling C. Blood phosphatidylethanol as a marker of alcohol abuse: Levels in alcoholic males during withdrawal. Alcoholism-Clinical and Experimental Research 1997; 21(1):108-110.

(124) Borucki K, Schreiner R, Dierkes J, Jachau K, Krause D, Westphal S et al. Detection of recent ethanol intake with new markers: comparison of fatty acid ethyl esters in serum and of ethyl glucuronide and the ratio of 5-hydroxytryptophol to 5-hydroxyindole acetic acid in urine. Alcoholism-Clinical and Experimental Research 2005; 29(5):781-787.

(125) Elliott S, Smith C, Cassidy D. The post-mortem relationship between beta-hydroxybutyrate (BHB), acetone and ethanol in ketoacidosis. Forensic Science International 2010; 198(1-3):53-57.

(126) Felby S, Nielsen E. Determination of ketone bodies in postmortem blood by head-space gas chromatography. Forensic Science International 1994; 64(2-3):83-88.

(127) Iten PX, Meier M. Beta-hydroxybutyric acid - An indicator for an alcoholic ketoacidosis as cause of death in deceased alcohol abusers. Journal of Forensic Sciences 2000; 45(3):624-632.

(128) Thomsen JL. Various mechanisms of death and their possible association with alcoholism. Forensic Science International 1996; 79(3):199-204.

(129) Duffens K, Marx JA. Alcoholic Ketoacidosis A Review. Journal of Emergency Medicine 1987; 5(5):399-406.

(130) Umpierrez GE, DiGirolamo M, Tuvlin JA, Isaacs SD, Bhoola SM, Kokko JP. Differences in metabolic and hormonal milieu in diabetic- and alcohol-induced ketoacidosis. Journal of Critical Care 2000; 15(2):52.

(131) Thomsen JL, Felby S, Theilade P, Nielsen E. Alcoholic ketoacidosis as a cause of death in forensic cases. Forensic Science International 1995; 75(2-3):163-171.

(132) Procedure for the analysis of ethanol in blood, Urine & vitreous Humor. FMS4001[edition No.: 002], 1-6. 3-3-2008. Department of Forensic medicine-University of Glasgow.

(133) Denmark LN. The investigation of beta-hydroxybutyrate as a marker for sudden death due to hypoglycemia in alcoholics. Forensic Science International 1993; 62(3):225-232.

(134) Shima N, Miki A, Kamata T, Katagi M, Tsuchihashi H. Urinary endogenous concentrations of GHB and its isomers in healthy humans and diabetics. Forensic Science International 2005; 149(2-3):171-179.

(135) Osuna E, Vivero G, Conejero J, Abenza JM, Martinez P, Luna A et al. Postmortem vitreous humor beta-hydroxybutyrate: its utility for the postmortem interpretation of diabetes mellitus. Forensic Science International 2005; 153(2-3):189-195.

(136) John C. Mass Spectrometry. In: Barry L, editor. Principles of Forensic Toxicology. Washington, DC: AACC Press, 2003: 139-153.

(137) Holm KMD, Linnet K, Rasmussen BS, Pedersen AJ. Determination of Ketone Bodies in Blood by Headspace Gas Chromatography-Mass Spectrometry. Journal of Analytical Toxicology 2010; 34(9):549-554.

(138) Hassan HMA, Cooper GAA. Determination of beta-Hydroxybutyrate in Blood and Urine Using Gas Chromatography-Mass Spectrometry. Journal of Analytical Toxicology 2009; 33(8):502-507.

(139) Kaufmann E, Alt A. Determination of GHB in urine and serum by LC/MS using a simple one-step derivative. Forensic Science International 2007; 168(2-3):133-137.

(140) Hsu YS, Chen SH. Trace analysis of beta-hydroxybutyrate in human plasma by derivatization and high-performance liquid chromatography. Chromatographia 2003; 57(11-12):735-739.

(141) Miles JM, Schwenk WF, McClean KL, Haymond MW. Determination of ketone body turnover in vivo with stable isotopes, utilizing gas chromatography/mass spectrometry. Analytical Biochemistry 1984; 141(1):110-115.

(142) Heith AM, Morse CR, Tsujita T, Volpacelli SA, Flood JG, Laposata M. Fatty-Acid Ethyl-Ester Synthase Catalyzes the Esterification of Ethanol to Cocaine. Biochemical and Biophysical Research Communications 1995; 208(2):549-554.

(143) Kaphalia BS, Ansari GA. Fatty acid ethyl esters and ethanol-induced pancreatitis. Cell and Molecular Biology 2001; 47 Online Pub:OL173-OL179.

(144) Lange LG. Mechanism of fatty acid ethyl ester formation and biological significance. Annals of The New York Academy 1991; 625:802-805.

(145) Laposata EA, Lange LG. Presence of nonoxidative ethanol metabolism in human organs commonly damaged by ethanol abuse. Science 1986; 231(4737):497-499.

(146) Doyle KM, Bird DA, Alsalihi S, Hallaq Y, CluetteBrown JE, Goss KA et al. Fatty-Acid Ethyl-Esters Are Present in Human Serum After Ethanol Ingestion. Journal of Lipid Research 1994; 35(3):428-437.

(147) Doyle KM, CluetteBrown JE, Dube DM, Bernhardt TG, Morse CR, Laposata M. Fatty acid ethyl esters in the blood as markers for ethanol intake. Jama-Journal of the American Medical Association 1996; 276(14):1152-1156.

(148) Scrimgeour C. Chemistry of Fatty acids. Shahidi F, editor. Sixth Edition, Six Volume Set., 1-44. 2005. Dundee, Scotland, Scottish Crop Research Institute, Bailey's Industrial Oil and Fat Products. Copyright # 2005 John Wiley & Sons, Inc.available at http://media.johnwiley.com.au/product_data/excerpt/22/04713855/0471385522.pdf .

(149) Kaphalia BS, Cai P, Khan MF, Okorodudu AO, Ansari GAS. Fatty acid ethyl esters: markers of alcohol abuse and alcoholism. Alcohol 2004; 34(2-3):151-158.

(150) Refaai MA, Nguyen PN, Steffensen TS, Evans RJ, Cluette-Brown JE, Laposata M. Liver and adipose tissue fatty acid ethyl esters obtained at autopsy are postmortem markers for premortem ethanol intake. Clinical Chemistry 2002; 48(1):77-83.

(151) Goss KA, Alharethi R, Laposata M. Fatty acid ethyl ester synthesis in the preparation of scotch whiskey. Alcohol 1999; 17(3):241-245.

(152) Gareri J, Lynn H, Handley M, Rao C, Koren G. Prevalence of fetal ethanol exposure in a regional population-based sample by meconium analysis of fatty acid, ethyl esters. Therapeutic Drug Monitoring 2008; 30(2):239-245.

(153) Moore C, Jones J, Lewis D, Buchi K. Prevalence of fatty acid ethyl esters in meconium specimens. Clinical Chemistry 2003; 49(1):133-136.

(154) Ostrea EM, Hernandez JD, Bielawski DM, Kan JM, Leonardo GM, Abela MB et al. Fatty acid ethyl esters in meconium: Are they biomarkers of fetal alcohol exposure and effect? Alcoholism-Clinical and Experimental Research 2006; 30(7):1152-1159.

(155) Bearer CF, Lee S, Salvator AE, Minnes S, Swick A, Yamashita T et al. Ethyl linoleate in meconium: A biomarker for prenatal ethanol exposure. Alcoholism-Clinical and Experimental Research 1999; 23(3):487-493.

(156) Kulig CC, Beresford TP, Everson GT. Rapid, accurate, and sensitive fatty acid ethyl ester determination by gas chromatography-mass spectrometry. Journal of Laboratory and Clinical Medicine 2006; 147(3):133-138.

(157) Hutson JR, Aleksa K, Pragst F, Koren G. Detection and quantification of fatty acid ethyl esters in meconium by headspace-solid-phase microextraction and gas chromatography-mass spectrometry. Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences 2009; 877(1-2):8-12.

(158) Kwak HS, Kang YS, Han KO, Moon JT, Chung YC, Choi JS et al. Quantitation of fatty acid ethyl esters in human meconium by an improved liquid chromatography/tandem mass spectrometry. Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences 2010; 878(21):1871-1874.

(159) Pichini S, Pellegrini M, Gareri J, Koren G, Garcia-Algar O, Vall O et al. Liquid chromatography-tandem mass spectrometry for fatty acid ethyl esters in meconium: Assessment of prenatal exposure to alcohol in two European cohorts. Journal of Pharmaceutical and Biomedical Analysis 2008; 48(3):927-933.

(160) Consensus of the Society of Hair Testing on hair testing for chronic excessive alcohol consumption. SOHT , 1-2. 2009.

(161) Triple Quadropole(QQQ) Theory. Customer Education Life Science and Chemical Analysis Agilent 6400 series QQQ LC/MS Technique and Operation Course Number R1893A (Student Manual). 1, 2-27. 2009. UK, Agilent Technologies http://www.agilent.com/Chem/beLABSMART.

(162) Garg U, Dasouki M. Expanded newborn screening of inherited metabolic disorders by tandem mass spectrometry: Clinical and laboratory aspects. Clinical Biochemistry 2006; 39(4):315-332.

(163) Ionization Sources and Jet Stream Technology. Customer Education Life Science &chemical Analysis, Agilent 6400 series QQQ LC/MS Technique and Operation Course Number R1893A (Student Manual). 1, 61-114. 2009. UK, Agilent Technologies http://www.agilent.com/Chem/beLABSMART.

(164) Janathan H. Electrospray Ionization. image] from Wikipedia, the free encyclopedia.Accessed date 09 September 2011 at 02:30.Page last

updated 09 September 2011.Black and White(1,464x1,528 pixles 101MB, jpeg).Available at http://en.wikipedia.org/wiki/File:Electrospray_ionization.jpg . 15-9-2009.

(165) Miller NJ, Miller CJ. Significance tests. In: Miller NJ, Miller CJ, editors. Statistics and chemometrics for Analytical Chemistry. Edinburgh England: Pearson Education Limited, 2005: 39-73.

(166) Guidlines for Blood Transfusion Services in the United Kingdom. 7, 1-331. 2005. London, TSO (the Stationery Office). Available at http://www.offecial-documents.co.uk/document/other/0117033715.asp.

(167) McClelland D. Handbook of transfusion Medicine- United Kingdom Blood services. 4 ed. London: TSO (The Stationary Office) and available Online www. tsoshop.co.uk, 2007.

(168) Miller NJ, Miller CJ. Calibration methods: regression and correlation. In: Miller NJ, Miller CJ, editors. Statistics and chemometrics for Analytical Chemistry. Edinburgh, England: Pearson Education Limited, 2005: 107-149.

(169) Peters FT, Drummer OH, Musshoff F. Validation of new methods. Forensic Science International 2007; 165(2-3):216-224.

(170) Firor R, Meng C. Static Head space Blood Alcohol Analysis with C1888 Network Headspace Sampler, Application. 1-12. 4-6-2009. USA, Agilent Technologies.

(171) Nanikawa R, Kotoku S. Medicolegal Observations on A Dead Body Drawn Up from Sea Bed, with Special Reference to Ethanol and Diatoms. Forensic Science 1974; 3(3):225-232.

(172) Nanikawa R, Ameno K, Hashimoto Y, Hamada K. Medicolegal studies on alcohol detected in dead bodies--alcohol levels in skeletal muscle. Forensic Science International 1982; 20(2):133-140.

(173) Ziavrou K, Boumba VA, Vougiouklakis TG. Insights into the origin of postmortem ethanol. International Journal of Toxicology 2005; 24(2):69-77.

(174) Moriya F, Ishizu H. Can Microorganisms Produce Alcohol in Body Cavities of A Living Person - A Case-Report. Journal of Forensic Sciences 1994; 39(3):883-888.

(175) Kronert K, Kunzel M, Reutter B, Zimmermann C, Liebich HM, Luft D et al. Urinary-Excretion Patterns of Endogenously Produced Alcohols in Type-1 (Iddm) and Type-2 (Niddm) Diabetes-Mellitus Compared with Healthy Control Subjects. Diabetes Research and Clinical Practice 1990; 10(2):161-165.

(176) Blood Ethyl Alcohol Assay Immunalysis Corporation. Version: 2/2005 , 1-3. 2005.

(177) Tsai J. Immunoassay Technologies For Drugs Of Abuse Testing. In: Smith F, editor. Handbook Of Forensic Drugs Analysis. USA: Elsevier Academic Press Publications, 2005: 13-42.

(178) Peters FT. Stability of analytes in biosamples - an important issue in clinical and forensic toxicology? Analytical and Bioanalytical Chemistry 2007; 388(7):1505-1519.

(179) Mathers C. The development of a method for the detection of B-hydroxybutyrate in post-mortem samples . [a presentation of Claire Mathers BSc(cmathers@sgul.ac.uk) at St George's university of London. Presented by Jennifer Button BSc_IATDMCT Nice 2007]. 2007.

(180) Elliott S, Lowe P, Symonds A. The possible influence of micro-organisms and putrefaction in the production of GHB in post-mortem biological fluid. Forensic Science International 2004; 139(2-3):183-190.

(181) Kitchen C, August T, Telepchak M. A solid Phase Extraction Method for Gamma-Hydroxybutyrate(GHB) in Blood,Urine,Vitreous or tissue without Conversion to Gamma - Butyrlactone (GBL). Bristol. USA. patent U.S. Patent Number 6,156,431. 2006 2006.

(182) Telepchak M, August T, Chaney G. Introduction to solid phase Extraction. In: Telepchak M, August T, Chaney G, editors. Forensic and Clinical Applications of Solid Phase Extraction. Totowa,New Jersey: HUMANA PRESS, 2004: 1-39.

(183) Telepchak M, August T, Chaney G. Strategies for the use of Solid Phase Extraction. In: Telepchak M, August T, Chaney G, editors. Forensic and Clinical Applications of Solid Phase Extraction. Totowa,New Jersey: HUMANA PRESS, 2004: 91-108.

(184) Elliott S. The presence of gamma-hydroxybutyric acid (GHB) in postmortem biological fluids. Journal of Analytical Toxicology 2001; 25(2):152.

(185) Marinetti JL. Gamma-Hydroxybutyric Acid and its Analogs, Gamma-Butyrolactone and 1,4-Butanediol. In: Salamone JS, editor. Benzodiazepines and GHB Detection and Pharmacology. Totowa, New Jersey: Humana Press, 2001: 95-127.

(186) Kintz P, Villain M, Cirimele V, Ludes B. GHB in postmortem toxicology: Discrimination between endogenous production from exposure using multiple specimens. Forensic Science International 2004; 143(2-3):177-181.

(187) Misuse of Drugs Act 1971. Chapter 38, 1-42. 2011. UK, UK -gov. avaiable from http://www.legislation.gov.uk/ukpga/1971/38/contents, Accessed Date15 October 2011.

(188) Kugelberg FC, Holmgren A, Eklund A, Jones AW. Forensic toxicology findings in deaths involving gamma-hydroxybutyrate. International Journal of Legal Medicine 2010; 124(1):1-6.

(189) Scott K. The use of hair as a toxicological tool in DFC casework. Journal of Forensic Sciences Society- Science& Justice 2009; 49(4):250-253.

(190) Rahbeeni Z, Ozand PT, Rashed M, Gascon GG, al Nasser M, Al Odaib A et al. 4-Hydroxybutyric aciduria. Brain Development Journal 1994; 16 Supplement:64-71.

(191) Divry P, Baltassat P, Rolland MO, Cotte J, Hermier M, Duran M et al. A new patient with 4-hydroxybutyric aciduria, a possible defect of 4-aminobutyrate metabolism. Clinica Chimica Acta 1983; 129(3):303-309.

(192) Elliott SP. Gamma hydroxybutyric acid (GHB) concentrations in humans and factors affecting endogenous production. Forensic Science International 2003; 133(1-2):9-16.

(193) Stephens BG, Coleman DE, Baselt RC. In vitro stability of endogenous gamma-hydroxybutyrate in postmortem blood. Journal of Forensic Sciences 1999; 44(1):231.

(194) Moriya F, Hashimoto Y. Endogenous gamma-hydroxybutyric acid levels in postmortem blood. Legal Medicine(Tokyo) 2004; 6(1):47-51.

(195) Elian AA. GC-MS determination of gamma-hydroxybutyric acid (GHB) in blood. Forensic Science International 2001; 122(1):43-47.

(196) Berankova K, Mutnanska K, Balikova M. Gamma-hydroxybutyric acid stability and formation in blood and urine. Forensic Science International 2006; 161(2-3):158-162.

(197) Elian AA. Determination of endogenous gamma-hydroxybutyric acid (GHB) levels in antemortem urine and blood. Forensic Science International 2002; 128(3):120-122.

(198) Fieler EL, Coleman DE, Baselt RC. gamma-Hydroxybutyrate concentrations in pre- and postmortem blood and urine. Clinical Chemistry 1998; 44(3):692.

(199) Kavanagh PV, Kenny P, Feely J. The urinary excretion of gamma-hydroxybutyric acid in man. Journal of Pharmacy and Pharmacology 2001; 53(3):399-402.

(200) Jones AW, Holmgren A, Kugelberg FC. Driving under the influence of gamma-hydroxybutyrate (GHB). American Journal of Forensic Medicine and Pathology 2008; 4(4):205-211.

(201) Elliott SP. Further evidence for the presence of GHB in postmortem biological fluid: Implications for the interpretation of findings. Journal of Analytical Toxicology 2004; 28(1):20-26.

(202) LeBeau MA, Montgomery MA, Morris-Kukoski C, Schaff JE, Deakin A. Further evidence of in vitro production of gamma-hydroxybutyrate (GHB) in urine samples. Forensic Sci Int 2007; 169(2-3):152-156.

(203) Slater G, Jenkins D. beta-hydroxy-beta-methylbutyrate (HMB) supplementation and the promotion of muscle growth and strength. Sports Medicine 2000; 30(2):105-116.

(204) Nissen S, Abumrad NM. Iowa State University Research Foundation I, editor. Composition comprising beta-hydroxy-beta-methylbutyric acid and at least one amino acid and methods of use. patent US 6031000. 2004 Nov 2004.

(205) Lee IY, Nissen SL, Rosazza JPN. Conversion of beta-methylbutyric acid to beta-hydroxy-beta-methylbutyric acid by Galactomyces reessii. Applied and Environmental Microbiology 1997; 63(11):4191-4195.

(206) Yu W, Kuhara T, Inoue Y, Matsumoto I, Iwasaki R, Morimoto S. Increased urinary excretion of [beta]-hydroxyisovaleric acid in ketotic and nonketotic type II diabetes mellitus. Clinica Chimica Acta 1990; 188(2):161-168.

(207) Van Koevering M, Nissen S. Oxidation of Leucine and Alpha Ketoisocaproate to Beta Hydroxy-Beta-Methylbutyrate In-Vivo. American Journal of Physiology 1992; 262(1 PART 1):E27-E31.

(208) Landaas S. Increased Urinary-Excretion of 3-Hydroxyisovaleric Acid in Patients with Ketoacidosis. Clinica Chimica Acta 1974; 54(1):39-46.

(209) Eldjarn L, Jellum E, Stokke O, Pande H, Waaler PE. Beta-Hydroxyisovaleric Aciduria and Beta-Methylcrotonylglycinuria - A New Inborn Error of Metabolism. Lancet 1970; 2(7671):521-&.

(210) Gompertz D, Bartlett K, Blair D, Stern CMM. Child with A Defect in Leucine Metabolism Associated with Beta-Hydroxyisovaleric Aciduria and Beta-Methylcrotonylglycinuria. Archives of Disease in Childhood 1973; 48(12):975-977.

(211) Tanaka K, Budd MA, Efron ML, Isselbac KJ. Isovaleric Acidemia - A New Genetic Defect of Leucine Metabolism. Proceedings of the National Academy of Sciences of the United States of America 1966; 56(1):236-&.

(212) Tanaka K, Orr JC, Isselbacher KJ. Identification of [beta]-hydroxyisovaleric acid in the urine of a patient with isovaleric acidemia. Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism 1968; 152(3):638-641.

(213) Landaas S. Accumulation of 3-hydroxyisobutyric acid, 2-methyl-3-hydroxybutyric acid and 3-hydroxyisovaleric acid in ketoacidosis. Clinica Chimica Acta 1975; 64(2):143-154.

(214) Nissen S, Vankoevering M, Webb D. Analysis of Beta-Hydroxy-Beta-Methyl Butyrate in Plasma by Gas-Chromatography and Mass-Spectrometry. Analytical Biochemistry 1990; 188(1):17-19.

(215) Goldberger B, Huestis M, Wilkins D. Commonly practiced quality control and quality assurance procedures for gas chromatography/mass

spectrometry analysis in forensic urine drug-testing laboratories . Forensic Science Review 1997; 9(2):59-80.

(216) Guidance for Industry Bioanalytical Method Validation. 1-22. 2001. USA, U.S. Department of Health and Human Services Food and Drug Adminstration, Center of Drug Evaluation and Research(CDER) Center for veternary Medicine (CVM)Internet at http://www.fda.gov/cder/guidance/index.htm.

(217) Miller NJ, Miller CJ. Statistics and chemometrics for Analytical Chemistry. Fifth edition ed. Edinburgh England: Pearson Education Limited, 2005.

(218) Moore CM, Lewis D. Fatty acid ethyl esters in meconium: biomarkers for the detection of alcohol exposure in neonates. Clinica Chimica Acta 2001; 312(1-2):235-237.

(219) Pragst F, Rothe M, Moench B, Hastedt M, Herre S, Simmert D. Combined use of fatty acid ethyl esters and ethyl glucuronide in hair for diagnosis of alcohol abuse: Interpretation and advantages. Forensic Science International 2010; 196(1-3):101-110.

(220) Pragst F, Auwaerter V, Sporkert F, Spiegel K. Analysis of fatty acid ethyl esters in hair as possible markers of chronically elevated alcohol consumption by headspace solid-phase microextraction (HS-SPME) and gas chromatography-mass spectrometry (GC-MS). Forensic Science International 2001; 121(1-2):76-88.

(221) Zimmermann CM, Jackson GP. Gas Chromatography Tandem Mass Spectrometry for Biomarkers of Alcohol Abuse in Human Hair. Therapeutic Drug Monitoring 2010; 32(2):216-223.

(222) Roehsig M, de Paula DML, Moura S, Diniz EMD, Yonamine M. Determination of eight fatty acid ethyl esters in meconium samples by headspace solid-phase microextraction and gas chromatography-mass spectrometry. Journal of Separation Science 2010; 33(14):2115-2122.

(223) Yegles M, Labarthe A, Auwarter V, Hartwig S, Vater H, Wennig R et al. Comparison of ethyl glucuronide and fatty acid ethyl ester concentrations in hair of alcoholics, social drinkers and teetotallers. Forensic Science International 2004; 145(2-3):167-173.

(224) Chan D, Bar-Oz B, Pellerin B, Paciorek C, Klein J, Kapur B et al. Population baseline of meconium fatty acid ethyl esters among infants of nondrinking women in Jerusalem and Toronto. Therapeutic Drug Monitoring 2003; 25(3):271-278.

(225) De Martinis BS, Martin CC. Automated headspace solid-phase microextraction and capillary gas chromatography analysis of ethanol in postmortem specimens. Forensic Science International 2002; 128(3):115-119.

(226) Sharma VK, Jadhav RK, Rao GJ, Saraf AK, Chandra H. High performance liquid chromatographic determination of alcohols with reference to body

distribution of methanol. Forensic Science International 1991; 50(2):255-261.

(227) Iwasaki Y, Yashiki M, Namera A, Miyazaki T, Kojima T. On the influence of postmortem alcohol diffusion from the stomach contents to the heart blood. Forensic Science International 1998; 94(1-2):111-118.

(228) Chikasue F, Yashiki M, Miyazaki T, Okamoto I, Kojima T. Abnormally high alcohol concentration in the heart blood. Forensic Science International 1988; 39(2):189-195.

(229) Harper DR. A comparative study of the microbiological contamination of postmortem blood and vitreous humour samples taken for ethanol determination. Forensic Science International 1989; 43(1):37-44.

(230) Bogusz M, Guminska M, Markiewicz J. Studies on the formation of endogenous ethanol in blood putrefying in vitro. Journal of Forensic and Legal Medicine 1970; 17(4):156-168.

(231) De Paoli G, Bell S. A rapid GC-MS determination of gamma-hydroxybutyrate in saliva. Journal of Analytical Toxicology 2008; 32(4):298-302.

(232) Kristoffersen L, Karinen R, Stormsvag B, Engstad KM, Olsen KM. "Detemination of GHB in urine by HS-GC-FID". 2008. This poster presentation presented at: The International Association of Forensic Toxicologists(TIAFT) Conference 2-8 June 2008.

(233) Mireault P, Peclet C, Faucher AM, Goudreau N, Cummins D, Laliberté C et al. Prevalence of 4-Hydroxybutanoic Acid (GHB) in Québec". 2008. This poster presentation presented at: The International Association of Forensic Toxicologists(TIAFT) Conference 2-8 June 2008.

(234) Kankaanpaa A, Liukkonen R, Ariniemi K. Determination of gamma-hydroxybutyrate (GHB) and its precursors in blood and urine samples: A salting-out approach. Forensic Science International 2007; 170(2-3):133-138.

(235) Fung HL, Haas E, Raybon J, Xu J, Fung SM. Liquid chromatographic-mass spectrometric determination of endogenous gamma-hydroxybutyrate concentrations in rat brain regions and plasma. Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences 2004; 807(2):287-291.

(236) Wood M, Laloup M, Samyn N, Morris MR, de Bruijn EA, Maes RA et al. Simultaneous analysis of gamma-hydroxybutyric acid and its precursors in urine using liquid chromatography-tandem mass spectrometry. Journal of Chromatography A 2004; 1056(1-2):83-90.

(237) Bortolotti F, De Paoli G, Gottardo R, Trattene M, Tagliaro F. Determination of [gamma]-hydroxybutyric acid in biological fluids by using capillary electrophoresis with indirect detection. Journal of Chromatography B 2004; 800(1-2):239-244.

(238) Villain M, Cirimele V, Ludes B, Kintz P. Ultra-rapid procedure to test for gamma-hydroxybutyric acid in blood and urine by gas chromatography-mass spectrometry. Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences 2003; 792(1):83-87.

(239) Alston WC, Ng K. Rapid colorimetric screening test for [gamma]-hydroxybutyric acid (liquid X) in human urine. Forensic Science International 2002; 126(2):114-117.

Appendix 1: Appendix 1-1 Summary of published techniques for the Determination of Fatty acid ethyl esters (FAEEs) in biological matrices.

References	Name analyte	Internal Standard	Matrix	Instrument	Extraction/Column	Identification	Linearity LOD/LLOQ
(Pragst *et al*. 2010)(219)	FAEEs ethyl myristate ethyl palmitate ethyl oleate ethyl stearate	FAEES- D_5	Hair	HS-SPME-GC/MS-CI	FAEES (220) 30 mg of washed dried hair were cut into about 1mm ieces weighted. Then 0add 0.5mL dimethyl sulfoxide, heptane and FAEEs d5. hepatane layer evaporated	SIM Mode	4-8000pg/mg 2pg/mg 6pg/mg
(Zimmermann & Jackson, 2010) (221)	FAEEs ethyl laurate ethyl myristate ethyl palmitate ethyl oleate ethyl stearate	Ethyl margarate	Hair	CC/MS/MS-CI- HS-SPME SRM Mode	Column RTX-5MS (28 m x 0.25 mm x 0.25 µm)	Laurate 229→201 Myrisate 257→229 Palmitate 285→257 Oleate 311→265 Oleate 311→247 Stearate 313→285 Margarate 299→271	0-2 ng/mg 0.002-0.03 ng/mg
(Roehsig *et al*. 2010)(222)	FAEEs ethyl laurate ethyl myristate ethyl palmitate, ethyl palmitoleate, ethyl linoleate ethyl oleate ethyl stearate ethyl Arachidonate	FAEES-D_5	Hair	HS-SPME-GC/MS-CI	Hair washed with n-heptane Hair extraction with a mixture of dimethylsulfoxide and n-heptane, separation and evaporation of the n-heptane layer	SIM Mode	LOQ-2000 ng/g <100 ng/g <150 ng/g
(Yegles *et al*. 2004)(223)	FAEEs ethyl myristate ethyl palmitate ethyl oleate ethyl stearate	FAEES-D_5	Hair	HS-SPME-GC/MS-NCI	Column RTX-5MS (28 m x 0.25 mm x 0.25 µm) Hair extraction with a mixture of dimethylsulfoxide and n-heptane, separation and evaporation of the n-heptane layer	SIM Mode	0.01 and 0.04ng/mg

Appendix 1-1 continued

References	Name analyte	Internal Standard	Matrix	Instrument	Extraction/Column	Identification	Linearity LOD/LLOQ
(Chan etal. 2003) (224)	FAEES ethyl laurate ethyl myristate ethyl palmitate ethyl linoleate ethyl oleate ethyl stearate	Ethyl Heptadecanoate	Meconium	GC-FID	Liquid liquid extraction Solid Phase Extraction ZB-WAX column (0.50 μm, 0.25 mm × 30 m)	Standard and Retention time	0.16 to 0.22 nmol/g (50 ng/g) 0.32 to 0.44 nmol/g (100 ng/g)
(Moore *et al.* 2003) (153)	ethyl linoleate ethyl palmitate ethyl palmitoleate ethyl oleate ethyl stearate ethyl arachidonate	Ethyl Heptadecanoate	Meconium	GC/MS-CI	Liquid liquid extraction Solid Phase Extraction Bonded fused silica(30m x 0.25 mm i.d.x0.25 μm)	Linoleate 263,245,309 Palmitate 285,286,284 Palmitoleate 283,282,284 Oleate 311,265,247 Stearate 313,312,314 Arachidonate 333,287,268 Heptadecanoate 299,298,300	NIA
(Moore &lewis 2001) (218)	ethyl linoleate ethyl palmitate ethyl palmitoleate ethyl oleate ethyl stearate ethyl arachidonate	Ethyl Heptadecanoate	Meconium	GC/MS/CI	Liquid liquid extraction Solid Phase Extraction Bonded fused silica (30m x 0.25 mm i.d.x0.25 μm)	Linoleate 263,245,309 Palmitate 285,286,284 Palmitoleate 283,282,284 Oleate 311,265,247 Stearate 313,312,314 Arachidonate 332,287,269 Heptadecanoate 299,298,300	25-5000ng/g LOQ50ng/g
(Bearer *et al.*1999) (155)	FAEES ethyl palmitate ethyl palmitoleate ethyl linoleate ethyl linolenate ethyl oleate ethyl stearate ethyl arachidonate	Ethyl Heptadecanoate	Meconium	GC-FID confirmed with GC/MS	Liquid liquid extraction Silica gel chromatography	Standard and Retention time	1.0pmol/g

Appendix 1-1 continued

eferences	Name analyte	Internal Standard	Matrix	Instrument	Extraction/Column	Identification	Linearity LOD/LLOQ
(Kulig *et al*. 2006) (156)	FAEES ethyl Linoleate ethyl palmitate ethyl palmitoleate ethyl oleate ethyl stearate	Ethyl Heptadecanoate	Plasma	GC/MS	Liquid liquid extraction Solid Phase Extraction Polar Carbowax column	Palmitate 88.1, 101.1 Retention time and base ion ratio	5-10nM 60nM
(Kaphalia *et al*.2004) (149)	ethyl Linoleate ethyl palmitate ethyl oleate ethyl stearate	Ethyl Heptadecanoate	Plasma	GC-FID confirmed with GC/MS EI	Liquid liquid extraction Silica gel Thin Layer Chromatography DB 225fused silica	Lenoleate 308 Palmitate 284 Oleate 310 Stearate 312	NIA

Appendix 2-1 Summary of Published Techniques for Volatile analysis

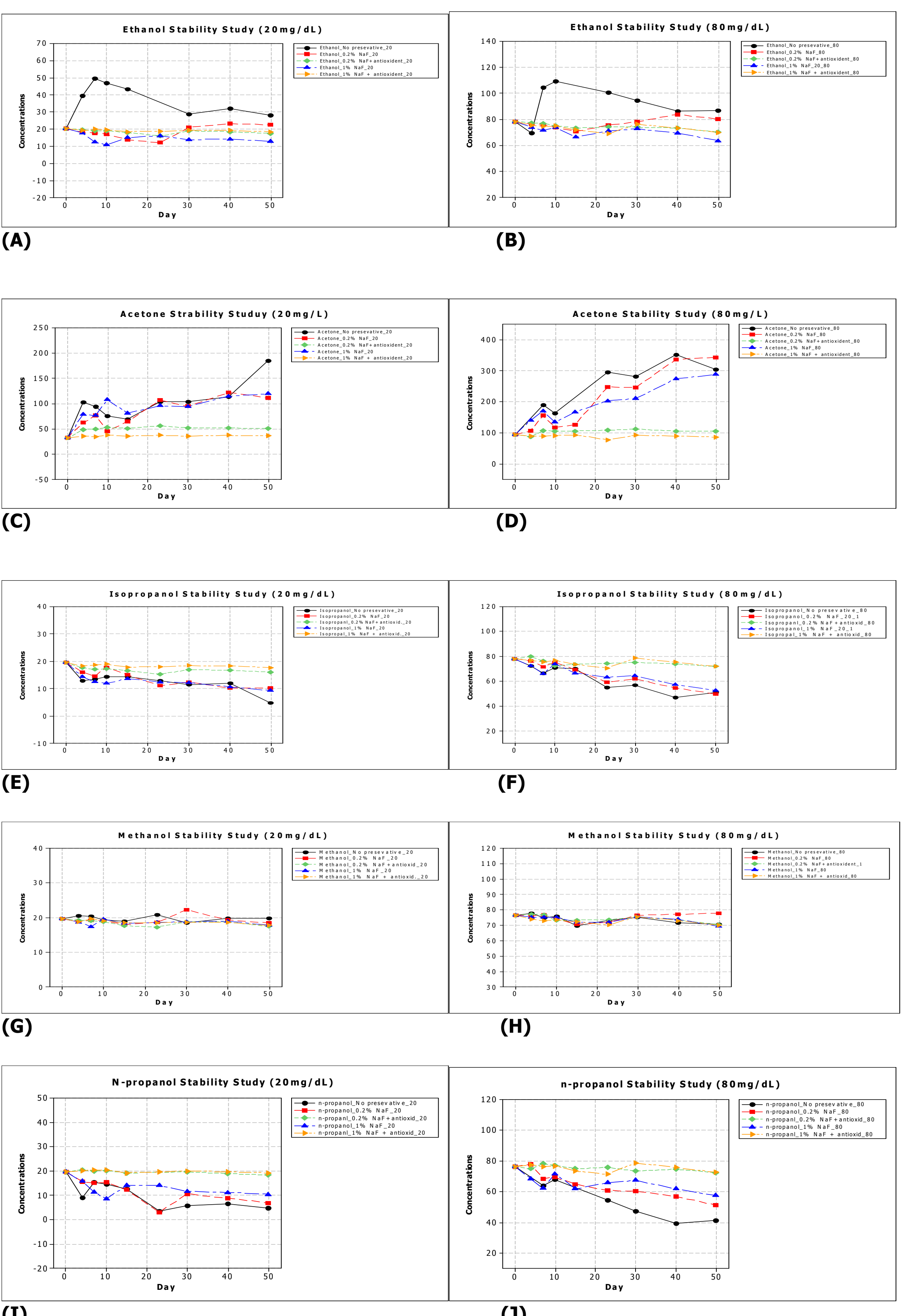
References	Analytes	Internal standard	Matrix and sample preparation	Technique	Column
(225)	Ethanol, methanol, 2-propanol, acetaldehyde, and acetone	Isobutanol	Post-mortem blood, urine, and vitreous humour (no preservative).	HS-GC-FID SMPE	Carbowax fused silica 30mx0.25mmi.d.film thickness 0.25 μm SPME (Polyacrylate 85 μm film thickness)
(73)	Ethanol, methanol, 2-propanol, and acetone	n-propanol t-butanol	Post-mortem blood Vitreous humour	HS-GC-FID	6 foot by l/8 inch stainless steel column packed with 0.2% Carbowax Column
(66)	Ethanol and n-propanol	t-butanol	Post-mortem cardiac Blood: femoral, pulmonary, arterial, venous blood, vitreous humour, urine, pericardial fluid, and small portion of the lung Stomach contents CSF	HS-GC-FID	A 100 by 0.3 cm i.d. glass column, packed with 25% polyethylene glycol 1000 on 80/100 mesh.
(226)	Ethanol, methanol, and n-propanol	*NIP	Lung, liver, spleen, kidney, brain, and heart homogenates, blood, urine and gastric contents	HPLC - refractive index Detector (RID)	A Zorbax ODS (CM; 4.6mm x 25cm stainless steel) column
(77)	Ethanol	*NIP	Synovial fluids, urine and heart blood	Pulse heating HS-GC	1mx2.6 mm packed with Propack Type Q
(80)	Ethanol, acetaldehyde, methanol, 2-propanol, acetone, n-propanol, isobutanol, n-butanol, and sec- butanol	t-butanol	Post-mortem human tissues	HS-GC-FID	Dual-fused silica capillary columns Rtx-BAC1 0.53mmi.d. x 3.0umx 3m Rtx-BAC2 0.53mmi.d. x 2.0um x 30m

***NIP (No Information Provided)**

Appendix 2-1 continued

References	Analytes	Internal standard	Matrix and sample preparation	Technique	Column
(227)	Ethanol	t-butanol, n-propanol,	0.5g of sample and 0.5 mLof internal standard (t-butanol	HS-GC-FID	112.5cm x 0.3cm (id.1 stainless steel tube packed with Porapak (80-100 mesh).
(228)	Ethanol	t-butanol	Heart blood and urine	HS-GC-FID and GC/MS	For GC 112.5 cm x 0.3cm id.1 stainless steel tube packed with Porapak (80-100 mesh). For GC/MS 100cm x 0.26 cm i.d. tube packed with Porapack (80-100 mesh)
(229)	Ethanol	Propan-1-ol	PM blood and vitreous humour	HS-GC-FID	10%w/w polyethylene glycol (PEG) 400 on mesh 100-120 Chromosorb; another column of 0.2% w/w PEG 1500 on mesh 60-80 Carbopack C. stainless steel tubing of 1.22m length and 2.1mm i.d.
(86)	Ethanol, and n-propanol	t-butanol	blood	HS-GC-FID	Glass column 2.1m x 3.2mm, i.d. with Chromosorb W 60/80 AW -DMCS polyethylene glycol 1000, 25%
(72)	Ethanol, 2-propanol n-propanol)	*n*-butanol	Blood, chest fluid, urine, and vitreous humour	HS-GC-FID	The gas chromatographic column, 3-mm i.d. 31.8m long, was packed with Porapak Q
(230)	EtOH, Glucose, lactate, pyruvate acetaldehyde	Isobutanol	Femoral blood subcalvian, heart urine, and vitreous humour	HS-GC-FID	Column temp. is 40C 4 min to 220C for 2 min at rate of 15C/min.

Appendix 3-1 Stability Study of volatiles using t-butanol as an Internal Standard
Room Temperature RT 25°C±2



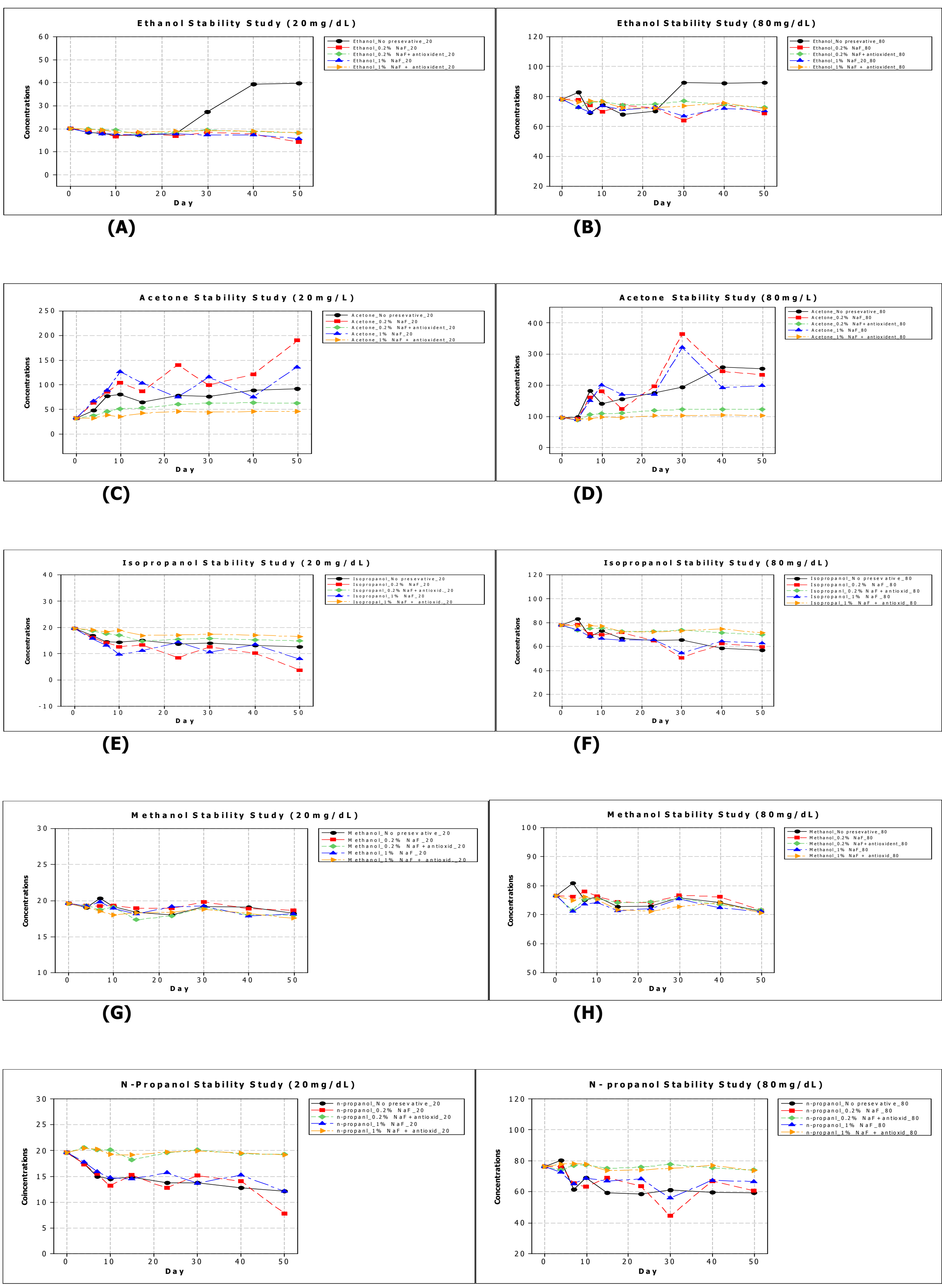
Stability graphs RT 25±2 °Cfor each analyte (20 and 80 mg/dL for all except acetone mg/L) (A) and (B) Ethanol, (C) AND (D) Acetone, (E) and (F) Isopropanol ,(G) and (H) Methanol and ,(I) and (J) n-propanol.

Appendix 3-1 continued

RT 25±2˚C		DAY	20					80				
			ETHANOL	ACETONE	ISOPROPANOL	METHANOL	N_PROPANOL	ETHANOL	ACETONE	ISOPROPANOL	METHANOL	N_PROPANOL
No presertvative	G1	1	21	32	20	20	20	80	91	79	77	78
0.2% Naf	G2	1	20	39	19	20	19	79	99	78	77	76
0.2%Naf +0.2% antioxident	G3	1	20	31	20	20	21	78	99	78	76	75
1% Naf	G4	1	20	28	20	20	20	78	99	78	76	75
1% Naf + 0.2% anti oxidant	G5	1	19	31	19	19	20	76	89	77	76	78
		MEAN	20	32	20	20	20	78	95	78	77	76
		SD	0.75	4.10	0.43	0.46	0.41	1.50	4.74	0.86	0.66	1.47
		2SD	1.50	8.21	0.86	0.91	1.23	2.99	9.47	1.73	1.32	2.94
		u	0.6	1.0	0.4	0.8	0.6	2.3	2.9	1.6	3.1	2.3
		Ue	1.2	1.9	0.8	1.6	1.2	4.7	5.7	3.1	6.1	4.6
		Mean-Ue	**19**	**30**	**19**	**18**	**19**	**74**	**89**	**75**	**70**	**72**
		Mean+Ue	**21**	**34**	**20**	**21**	**21**	**83**	**101**	**81**	**83**	**81**
No presertvative	G1	4	**40**	**102**	**13**	21	**9**	**70**	**354**	**53**	78	**23**
0.2% Naf	G2	4	**18**	**63**	**16**	19	**16**	76	**107**	77	76	78
0.2%Naf +0.2% antioxident	G3	4	20	**49**	18	19	21	77	**87**	80	77	75
1% Naf	G4	4	**18**	**78**	**14**	19	**16**	74	**141**	**72**	75	**68**
1% Naf + 0.2% anti oxidant	G5	4	19	35	18	19	20	76	89	77	74	77
No presertvative	G1	7	**50**	**94**	**13**	20	**15**	**104**	**190**	**66**	75	**64**
0.2% Naf	G2	7	**18**	**77**	**15**	19	**15**	76	**155**	**71**	77	**69**
0.2%Naf +0.2% antioxident	G3	7	19	**50**	**17**	19	20	77	**107**	76	77	78
1% Naf	G4	7	**13**	**76**	**13**	**17**	**11**	**71**	**170**	**66**	75	**62**
1% Naf + 0.2% anti oxidant	G5	7	20	35	19	20	20	75	89	76	73	76
No presertvative	G1	10	**47**	**76**	**14**	19	**15**	**109**	**163**	**71**	76	**68**
0.2% Naf	G2	10	**17**	**45**	18	19	**15**	74	**118**	75	74	**69**
0.2%Naf +0.2% antioxident	G3	10	19	**53**	**17**	19	20	75	**105**	**58**	73	77
1% Naf	G4	10	**11**	**109**	**12**	20	**9**	74	**134**	**74**	75	72
1% Naf + 0.2% anti oxidant	G5	10	20	**38**	19	19	20	75	92	77	73	77
No presertvative	G1	15	**43**	**70**	**14**	19	**13**	**2**	93	**70**	70	**2**
0.2% Naf	G2	15	**14**	**64**	**15**	**18**	**12**	**71**	**127**	**69**	71	**65**
0.2%Naf +0.2% antioxident	G3	15	**18**	**51**	**17**	**18**	19	**73**	**106**	**74**	73	75
1% Naf	G4	15	**15**	**81**	**14**	**18**	**14**	**66**	**167**	**67**	72	**62**
1% Naf + 0.2% anti oxidant	G5	15	**18**	36	18	**18**	19	**72**	93	74	72	73
No presertvative	G1	23	**17**	**104**	**13**	21	**4**	**100**	**295**	**55**	73	**54**
0.2% Naf	G2	23	**12**	**107**	**11**	19	**3**	76	**247**	**59**	72	**61**
0.2%Naf +0.2% antioxident	G3	23	**16**	**56**	**15**	**17**	**15**	74	**108**	**74**	74	76
1% Naf	G4	23	**16**	**96**	**12**	19	**14**	**71**	**202**	**63**	72	**66**
1% Naf + 0.2% anti oxidant	G5	23	19	**37**	18	19	20	**69**	**77**	**71**	70	**71**
No presertvative	G1	30	**29**	**103**	**11**	**18**	**6**	**95**	**281**	**57**	75	**47**
0.2% Naf	G2	30	21	**95**	**12**	**22**	**11**	78	**246**	**62**	77	**60**
0.2%Naf +0.2% antioxident	G3	30	19	**52**	**17**	19	20	74	**112**	75	75	74
1% Naf	G4	30	**14**	**94**	**12**	19	**12**	**73**	**210**	**64**	75	**67**
1% Naf + 0.2% anti oxidant	G5	30	19	36	18	19	20	76	92	79	76	79
No presertvative	G1	40	**32**	**114**	**12**	20	**6**	**86**	**351**	**47**	72	**39**
0.2% Naf	G2	40	**23**	**122**	**10**	19	**9**	**84**	**336**	**54**	77	**57**
0.2%Naf +0.2% antioxident	G3	40	**18**	**52**	**17**	19	19	**73**	**105**	**74**	74	75
1% Naf	G4	40	**14**	**114**	**11**	19	**11**	**69**	**274**	**57**	74	**62**
1% Naf + 0.2% anti oxidant	G5	40	19	**37**	18	19	20	**73**	89	75	73	76
No presertvative	G1	50	**28**	**185**	**5**	20	**5**	**87**	**305**	**51**	71	**41**
0.2% Naf	G2	50	**23**	**110**	**10**	**18**	**7**	80	**343**	**50**	78	**51**
0.2%Naf +0.2% antioxident	G3	50	**17**	**50**	**16**	**17**	**18**	**70**	**106**	**72**	70	72
1% Naf	G4	50	**13**	**120**	**9**	**18**	**10**	**63**	**288**	**52**	**69**	**58**
1% Naf + 0.2% anti oxidant	G5	50	**18**	36	**18**	**18**	19	**70**	**86**	**72**	70	73

Stability results at RT 25±2, °Cof spiked blood. The bolded figures showed deviation from the acceptable range.

Appendix 3-1 continued - **(4± 2°C)**



(I) (J)

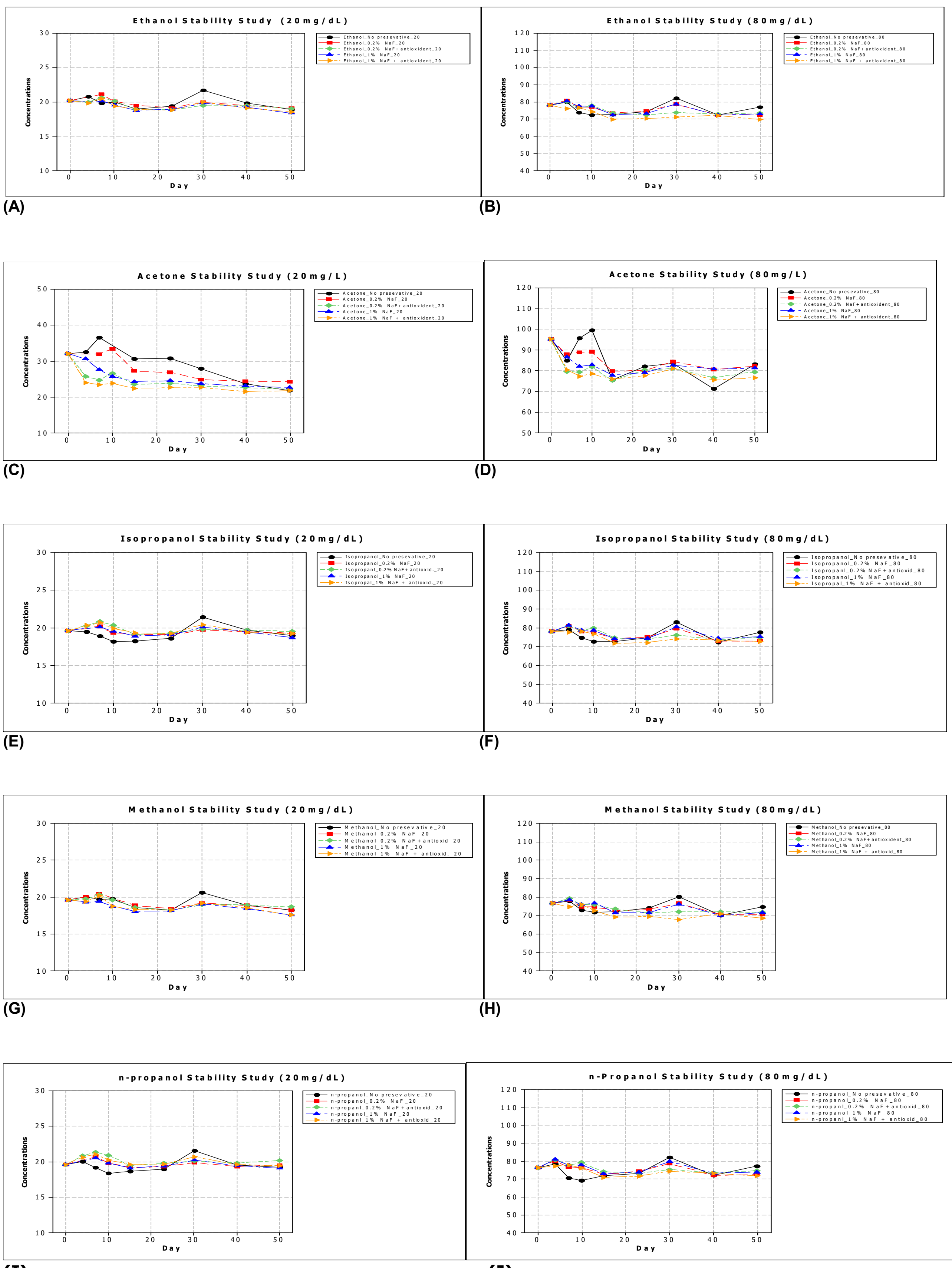
Stability graphs at 4±2°C for each analyte (20 and 80 mg/dL for all except acetone mg/L) (A) and (B) Ethanol, (C) AND (D) Acetone, (E) and (F) Isopropanol ,(G) and (H) Methanol and ,(I) and (J) n-propanol.

Appendix 3-1 continued

4± 2°C		DAY	20					80				
			ETHANOL	ACETONE	ISOPROPANOL	METHANOL	N_PROPANOL	ETHANOL	ACETONE	ISOPROPANOL	METHANOL	N_PROPANOL
No presertvative	G1	1	21	32	20	20	20	80	91	79	77	78
0.2% Naf	G2	1	20	39	19	20	19	79	99	78	77	76
0.2%Naf +0.2% antioxident	G3	1	20	31	20	20	21	78	99	78	76	75
1% Naf	G4	1	20	28	20	20	20	78	99	78	76	75
1% Naf + 0.2% anti oxidant	G5	1	19	31	19	19	20	76	89	77	76	78
		MEAN	20	32	20	20	20	78	95	78	77	76
		SD	0.75	4.10	0.43	0.46	0.41	1.50	4.74	0.86	0.66	1.47
		2SD	1.50	8.21	0.86	0.91	1.23	2.99	9.47	1.73	1.32	2.94
		u	0.6	1.0	0.4	0.8	0.6	2.3	2.9	1.6	3.1	2.3
		Ue	1.2	1.9	0.8	1.6	1.2	4.7	5.7	3.1	6.1	4.6
		Mean-Ue	**19**	**30**	**19**	**18**	**19**	**74**	**89**	**75**	**70**	**72**
		Mean+Ue	**21**	**34**	**20**	**21**	**21**	**83**	**101**	**81**	**83**	**81**
No presertvative	G1	4	19	**48**	**17**	19	**17**	83	98	**83**	81	80
0.2% Naf	G2	4	19	**63**	**16**	19	**17**	78	93	78	76	76
0.2%Naf +0.2% antioxident	G3	4	20	**37**	19	19	21	**73**	88	**73**	71	74
1% Naf	G4	4	**18**	**67**	**16**	19	**18**	**73**	89	74	71	73
1% Naf + 0.2% anti oxidant	G5	4	20	**32**	19	19	20	76	90	77	75	78
No presertvative	G1	7	**18**	**77**	**15**	20	**15**	**69**	**182**	**68**	75	**61**
0.2% Naf	G2	7	**18**	**85**	**14**	19	**15**	74	**161**	**71**	78	**66**
0.2%Naf +0.2% antioxident	G3	7	19	**46**	**18**	19	20	76	**105**	75	75	77
1% Naf	G4	7	**18**	**88**	**13**	20	**16**	**69**	**150**	**68**	74	**65**
1% Naf + 0.2% anti oxidant	G5	7	19	**38**	**18**	19	20	77	92	77	76	78
No presertvative	G1	10	**17**	**80**	**14**	19	**14**	75	**140**	**73**	76	**69**
0.2% Naf	G2	10	**17**	**104**	**13**	19	**13**	**70**	**180**	**70**	76	**63**
0.2%Naf +0.2% antioxident	G3	10	20	**51**	**17**	19	20	77	**109**	75	75	77
1% Naf	G4	10	**18**	**126**	**10**	19	**15**	74	**200**	**67**	74	**69**
1% Naf + 0.2% anti oxidant	G5	10	**18**	**23**	19	18	19	77	98	77	75	78
No presertvative	G1	15	**17**	**65**	**15**	18	**15**	**68**	**155**	**67**	73	**59**
0.2% Naf	G2	15	**18**	**86**	**13**	19	**15**	74	**124**	**72**	74	**69**
0.2%Naf +0.2% antioxident	G3	15	**17**	**53**	**15**	**17**	18	74	**111**	**73**	74	75
1% Naf	G4	15	**17**	**104**	**11**	18	**14**	71	**170**	**65**	71	**67**
1% Naf + 0.2% anti oxidant	G5	15	**18**	**42**	**17**	18	19	**72**	96	**72**	72	74
No presertvative	G1	23	**18**	**79**	**14**	18	**14**	**70**	**175**	**65**	73	**59**
0.2% Naf	G2	23	**17**	**140**	**8**	19	**13**	**72**	**196**	**65**	74	**64**
0.2%Naf +0.2% antioxident	G3	23	19	**60**	**16**	18	20	75	**120**	**73**	74	76
1% Naf	G4	23	**18**	**75**	**14**	19	**16**	**73**	**171**	**66**	72	**69**
1% Naf + 0.2% anti oxidant	G5	23	19	**46**	**17**	18	20	**72**	**102**	**72**	71	74
No presertvative	G1	30	**27**	**76**	**14**	19	**14**	**89**	**193**	**65**	76	**61**
0.2% Naf	G2	30	**18**	**99**	**12**	20	**15**	**64**	**364**	**51**	77	**44**
0.2%Naf +0.2% antioxident	G3	30	19	**63**	**16**	19	20	77	**122**	**74**	76	78
1% Naf	G4	30	**17**	**116**	**11**	19	**14**	**67**	**321**	**54**	75	**56**
1% Naf + 0.2% anti oxidant	G5	30	19	**44**	**17**	19	20	74	**102**	**73**	73	75
No presertvative	G1	40	**39**	**89**	**13**	19	**13**	**89**	**258**	**58**	74	**60**
0.2% Naf	G2	40	**18**	**121**	**10**	19	**14**	75	**244**	**62**	76	**67**
0.2%Naf +0.2% antioxident	G3	40	19	**63**	**15**	18	19	74	**123**	**72**	73	76
1% Naf	G4	40	**17**	**75**	**13**	18	**15**	**72**	**192**	**64**	72	**67**
1% Naf + 0.2% anti oxidant	G5	40	19	**46**	**17**	18	19	76	**104**	75	74	77
No presertvative	G1	50	**40**	**92**	**13**	18	**12**	**89**	**253**	**57**	71	**59**
0.2% Naf	G2	50	**14**	**190**	**4**	19	**8**	**69**	**234**	**60**	72	**61**
0.2%Naf +0.2% antioxident	G3	50	**18**	**62**	**15**	18	19	**73**	**122**	**70**	72	74
1% Naf	G4	50	**16**	**136**	**8**	18	**12**	**70**	**198**	**63**	71	**67**
1% Naf + 0.2% anti oxidant	G5	50	**18**	**46**	**17**	18	19	**72**	**102**	**71**	70	74

Stability results at 4±2°C, of spiked blood. The bolded figures showed deviation from the acceptable range.

Appendix 3-1 continued - (-20 ± 2°C)



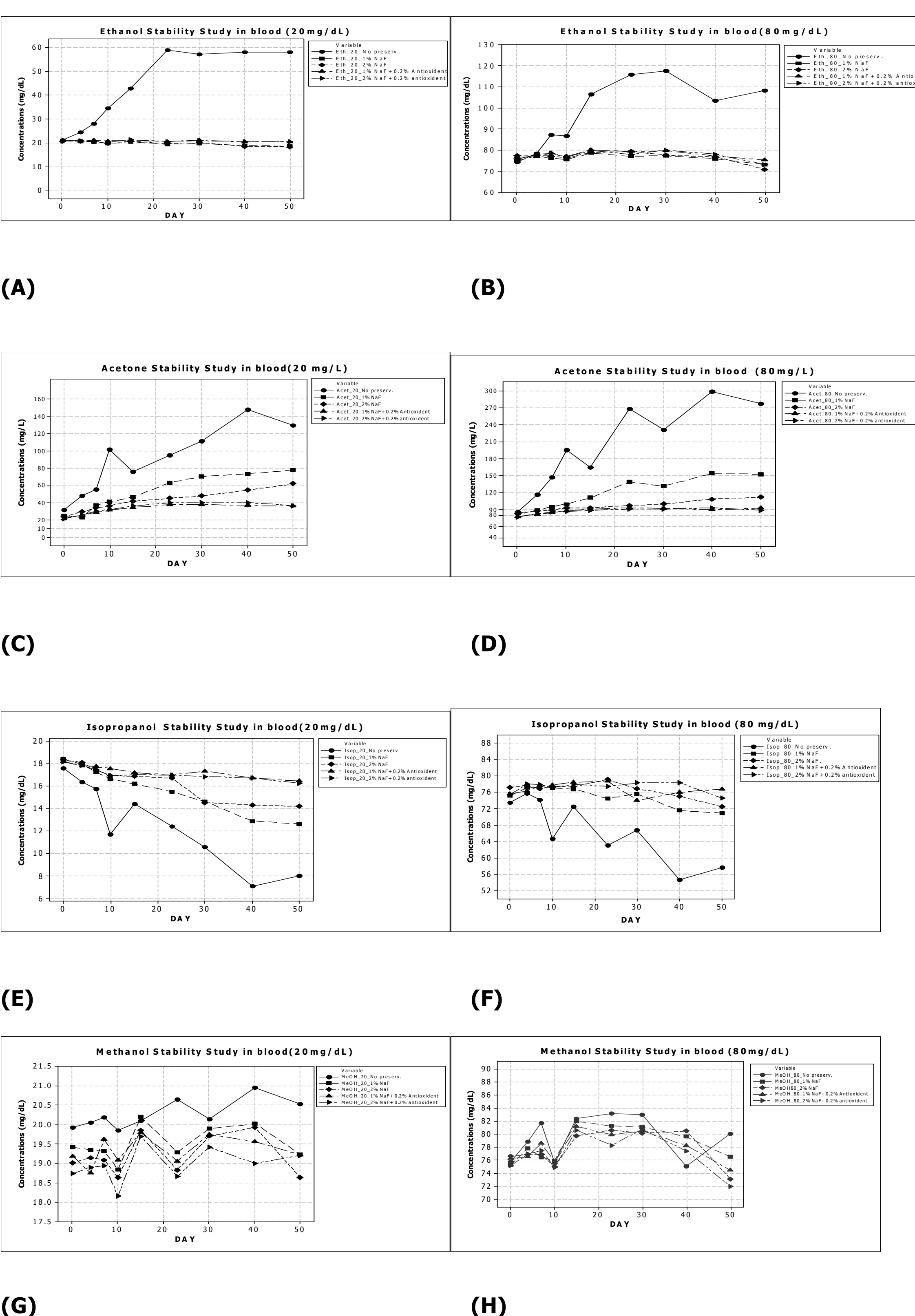
Stability graphs at -22±2°C for each analyte (20 and 80 mg/dL for all except acetone mg/L) (A) and (B) Ethanol, (C) AND (D) Acetone, (E) and (F) Isopropanol ,(G) and (H) Methanol and ,(I) and (J) n-propanol.

Appendix 3-1 continued

-22±2°C		DAY	20					80				
			ETHANOL	ACETONE	ISOPROPANOL	METHANOL	N_PROPANOL	ETHANOL	ACETONE	ISOPROPANOL	METHANOL	N_PROPANOL
No presertvative	G1	1	21	32	20	20	20	80	91	79	77	78
0.2% Naf	G2	1	20	39	19	20	19	79	99	78	77	76
0.2%Naf +0.2% antioxident	G3	1	20	31	20	20	21	78	99	78	76	75
1% Naf	G4	1	20	28	20	20	20	78	99	78	76	75
1% Naf + 0.2% anti oxidant	G5	1	19	31	19	19	20	76	89	77	76	78
		MEAN	20	32	20	20	20	78	95	78	77	76
		SD	0.75	4.10	0.43	0.46	0.41	1.50	4.74	0.86	0.66	1.47
		2SD	1.50	8.21	0.86	0.91	1.23	2.99	9.47	1.73	1.32	2.94
		u	0.6	1.0	0.4	0.8	0.6	2.3	2.9	1.6	3.1	2.3
		Ue	1.2	1.9	0.8	1.6	1.2	4.7	5.7	3.1	6.1	4.6
		Mean-Ue	**19**	**30**	**19**	**18**	**19**	**74**	**89**	**75**	**70**	**72**
		Mean+Ue	**21**	**34**	**20**	**21**	**21**	**83**	**101**	**81**	**83**	**81**
No presertvative	G1	4	21	32	19	20	20	80	85	79	78	78
0.2% Naf	G2	4	**16**	**55**	**18**	20	**14**	81	88	81	78	80
0.2%Naf +0.2% antioxident	G3	4	20	**26**	20	20	21	80	80	81	79	81
1% Naf	G4	4	**17**	**56**	**17**	19	**15**	80	87	81	78	81
1% Naf + 0.2% anti oxidant	G5	4	20	**24**	20	19	21	76	80	78	75	77
No presertvative	G1	7	20	**37**	19	20	19	74	96	75	73	**71**
0.2% Naf	G2	7	21	32	20	20	21	77	89	78	75	77
0.2%Naf +0.2% antioxident	G3	7	21	**25**	21	20	21	77	**79**	78	76	78
1% Naf	G4	7	20	28	20	19	21	77	82	79	76	78
1% Naf + 0.2% anti oxidant	G5	7	20	**23**	21	20	21	77	77	78	76	78
No presertvative	G1	10	20	**51**	**18**	20	**18**	72	100	**73**	72	**69**
0.2% Naf	G2	10	20	33	19	38	20	77	89	78	75	76
0.2%Naf +0.2% antioxident	G3	10	20	**27**	20	20	21	78	**82**	80	76	79
1% Naf	G4	10	20	**26**	20	19	20	78	**83**	79	77	78
1% Naf + 0.2% anti oxidant	G5	10	19	**24**	20	19	20	74	**78**	77	73	76
No presertvative	G1	15	19	31	**18**	19	19	**73**	**76**	**73**	72	72
0.2% Naf	G2	15	19	**27**	19	19	19	74	80	74	73	73
0.2%Naf +0.2% antioxident	G3	15	19	**24**	19	19	19	74	**75**	75	73	74
1% Naf	G4	15	19	**24**	19	**18**	19	**72**	**78**	74	71	73
1% Naf + 0.2% anti oxidant	G5	15	19	22	19	**18**	20	**70**	**76**	**72**	**69**	**71**
No presertvative	G1	23	19	31	19	**18**	19	74	**82**	75	74	73
0.2% Naf	G2	23	19	**27**	19	**18**	19	75	**80**	75	73	74
0.2%Naf +0.2% antioxident	G3	23	19	**24**	19	**18**	20	**72**	**80**	74	71	73
1% Naf	G4	23	19	**25**	19	**18**	19	**73**	**79**	74	72	74
1% Naf + 0.2% anti oxidant	G5	23	19	**23**	19	**18**	20	**70**	**78**	**72**	**69**	72
No presertvative	G1	30	22	**28**	21	21	22	82	84	83	80	82
0.2% Naf	G2	30	20	**25**	20	19	20	79	84	80	77	79
0.2%Naf +0.2% antioxident	G3	30	19	**23**	20	19	20	74	**81**	76	72	75
1% Naf	G4	30	20	**24**	20	19	20	79	**83**	80	76	80
1% Naf + 0.2% anti oxidant	G5	30	20	**23**	20	19	21	**71**	**81**	74	**68**	74
No presertvative	G1	40	20	**24**	20	19	20	**72**	**71**	**72**	70	72
0.2% Naf	G2	40	19	**24**	19	19	19	**72**	**81**	**73**	71	72
0.2%Naf +0.2% antioxident	G3	40	19	**23**	20	19	20	**73**	**77**	74	72	74
1% Naf	G4	40	19	**23**	19	**18**	19	**72**	**81**	74	70	73
1% Naf + 0.2% anti oxidant	G5	40	19	**22**	19	19	20	**72**	**75**	**73**	71	73
No presertvative	G1	50	19	**22**	19	**18**	19	77	**83**	78	75	77
0.2% Naf	G2	50	19	**24**	19	**18**	20	**72**	**82**	73	71	73
0.2%Naf +0.2% antioxident	G3	50	19	**22**	20	19	20	74	**79**	75	72	75
1% Naf	G4	50	18	**23**	19	**18**	19	**73**	**81**	75	71	74
1% Naf + 0.2% anti oxidant	G5	50	19	**22**	19	**15**	19	**70**	**77**	**73**	**68**	72

Study 2 stability results at -22±2°C, of spiked blood. The bolded figures showed deviation from the acceptable range.

Appendix 3-2 Stability Study of volatile using n-propanol Internal standard – (**4± 2°C**)



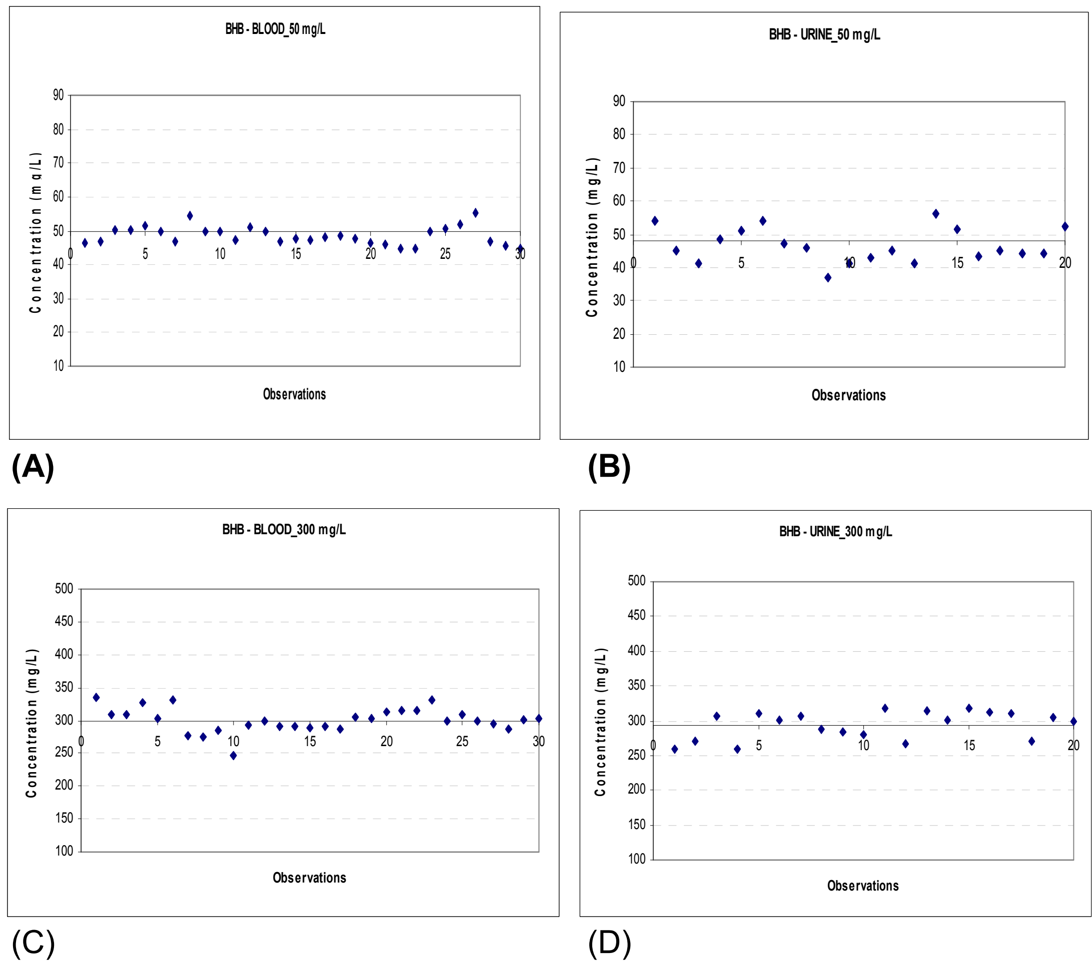
Stability graphs at 4±2˚C for each analyte (20 and 80 mg/dL for all except acetone mg/L) (A) and (B) Ethanol, (C) AND (D) Acetone, (E) and (F) Isopropanol and (G) and (H) Methanol.

4±2°C	Group	Day	20				80			
			ETHANOL	ACETONE	ISOPROPANOL	METHANOL	ETHANOL	ACETONE	ISOPROPANOL	METHANOL
No presertvative	**G1**	1	21	32	18	20	75	86	74	76
1% Naf	**G2**	1	21	25	18	19	75	84	75	75
2%Naf	**G3**	1	21	24	18	19	78	83	77	77
1% Naf + 0.2% anti oxidant	**G4**	1	21	22	18	19	77	78	76	76
2% Naf + 0.2% anti oxidant	**G5**	1	21	22	18	19	76	77	75	75
		MEAN	21	25	18	19	76	82	75	76
		SD	0.14	4.02	0.32	0.45	1.23	3.93	1.30	0.65
		2SD	0.43	12.06	0.97	1.36	3.68	11.80	3.90	1.95
		u	1	1	1	1	2	3	2	3
		Ue	2	2	1	2	5	7	5	6
		Mean-Ue	**19**	**22**	**17**	**18**	**71**	**75**	**71**	**70**
		Mean+Ue	**22**	**27**	**20**	**21**	**80**	**88**	**80**	**82**
No presertvative	**G1**	**4**	**24**	**48**	**16**	20	78	**117**	76	79
1% Naf	**G2**	**4**	21	23	18	19	77	**89**	77	78
2%Naf	**G3**	**4**	21	**30**	18	19	78	88	78	77
1% Naf + 0.2% anti oxidant	**G4**	**4**	21	26	18	19	77	82	78	77
2% Naf + 0.2% anti oxidant	**G5**	**4**	21	26	18	19	78	83	78	77
No presertvative	**G1**	**7**	**28**	**56**	**16**	20	**87**	**148**	74	82
1% Naf	**G2**	**7**	20	**38**	17	19	76	**95**	77	76
2%Naf	**G3**	**7**	20	**34**	17	19	77	**89**	77	77
1% Naf + 0.2% anti oxidant	**G4**	**7**	21	**29**	18	20	79	85	77	79
2% Naf + 0.2% anti oxidant	**G5**	**7**	20	**30**	17	19	78	86	78	78
No presertvative	**G1**	**10**	**35**	**102**	**12**	20	**87**	**196**	**65**	76
1% Naf	**G2**	**10**	20	**41**	17	19	76	**100**	77	76
2%Naf	**G3**	**10**	20	**37**	17	19	76	**93**	77	75
1% Naf + 0.2% anti oxidant	**G4**	**10**	21	**32**	18	19	77	88	77	76
2% Naf + 0.2% anti oxidant	**G5**	**10**	20	**32**	17	18	77	87	77	75
No presertvative	**G1**	**15**	**43**	**76**	**14**	20	**106**	**164**	73	82
1% Naf	**G2**	**15**	20	**47**	**16**	20	79	**111**	77	82
2%Naf	**G3**	**15**	20	**42**	17	20	79	**93**	77	80
1% Naf + 0.2% anti oxidant	**G4**	**15**	21	**35**	17	20	80	**91**	77	81
2% Naf + 0.2% anti oxidant	**G5**	**15**	21	**36**	17	20	80	**89**	78	81
No presertvative	**G1**	**23**	**59**	**95**	**12**	21	**116**	**268**	**63**	**83**
1% Naf	**G2**	**23**	19	**63**	**16**	19	77	**139**	75	81
2%Naf	**G3**	**23**	20	**45**	17	19	79	**97**	79	81
1% Naf + 0.2% anti oxidant	**G4**	**23**	20	**38**	17	19	79	**93**	79	80
2% Naf + 0.2% anti oxidant	**G5**	**23**	20	**40**	17	19	78	**91**	77	78
No presertvative	**G1**	**30**	**57**	**112**	11	20	**118**	**232**	**67**	**83**
1% Naf	**G2**	**30**	20	**70**	**15**	20	77	**132**	76	81
2%Naf	**G3**	**30**	20	**48**	**15**	20	78	**100**	77	80
1% Naf + 0.2% anti oxidant	**G4**	**30**	21	**38**	17	20	80	**92**	77	81
2% Naf + 0.2% anti oxidant	**G5**	**30**	21	**40**	17	19	80	**91**	78	81
No presertvative	**G1**	**40**	**58**	**148**	**7**	21	**103**	**299**	**55**	75
1% Naf	**G2**	**40**	19	**74**	**13**	20	76	**154**	72	80
2%Naf	**G3**	**40**	18	**55**	**14**	20	77	**109**	75	80
1% Naf + 0.2% anti oxidant	**G4**	**40**	20	**37**	17	20	77	**90**	75	78
2% Naf + 0.2% anti oxidant	**G5**	**40**	20	**40**	17	19	78	**93**	78	77
No presertvative	**G1**	**50**	**58**	**130**	**8**	21	**108**	**277**	**58**	80
1% Naf	**G2**	**50**	19	**78**	**13**	19	73	**153**	71	77
2%Naf	**G3**	**50**	**18**	**62**	**14**	19	71	**112**	73	73
1% Naf + 0.2% anti oxidant	**G4**	**50**	20	**36**	**16**	19	75	**93**	73	74
2% Naf + 0.2% anti oxidant	**G5**	**50**	20	**37**	**16**	19	73	**89**	75	72

Appendix 3-2 **Stability results at 4±2 °Cof spiked blood. The bolded figures showed deviation from the acceptable range.**

Appendix 4-1 BHB QC Charts

	Quality Control ranges			
	Blood		Urine	
	50 mg/L	300 mg/L	50 mg/L	300 mg/L
Average	49	301	48	294
Standard Deviation (SD)	2.72	17.95	6.40	20.34
CV%	**5.6**	**6.0**	**13.2**	**6.9**
Mean ± 2SD	43-54	265-336	36-61	253-335



BHB Quality control charts at 50 mg/L (A and B), 300 mg/L (C and D) in blood and urine, respectively

Appendix 4-2 BHB, Acetone and Ethanol Concentrations in Post-mortem blood and urine

(Shaded boxes information is not available)

Case #	Gender	Age	BHB mg//L		Acetone mg//L		Ethanol mg/dL		cause of death
			Blood	Urine	Blood	Urine	Blood	Urine	
1	M	55	135		146		79		1a- unascertained
2	F	67	14	NEG	NEG	NEG	330	440	1a- head injury(haemorrhage from scalp laceration) presumed fall 2a- Acute alcohol intoxication pulmonary thromboembolism
3	F	41	NEG		NEG		NEG		1a- Stab wound of heart
4	M	66	NEG	27	NEG	NEG	NEG	NEG	1a- Coronary artery atheroma; 1b- Ischaemic heart disease
5	F	44	>500	>500	167	224	NEG	16	1a- Fatty degeneration of the liver 1b- Chronic alcoholic
6	F	78	142	<10	NEG	NEG	232	225	1a- Inhalation of smoke and fire gases in a house fire and hypertensive and valvular heart disease
7	F	29	30		NEG		193		1a- Codeine and alcohol intoxication
8	M	33	22	NEG	NEG	NEG	NEG	98	1a- Heroin and ketamine intoxication
9	F	58	89	86	NEG	NEG	NEG	NEG	1a- Suspected alcohol related death
10	M	54	13	NEG	NEG	NEG	325	393	1a- Chest injury; 1b Road traffic collision (driver)
11	M	29	NEG	NEG	NEG	NEG	NEG	NEG	1a- Hanging
12	M	29	NEG	NEG	NEG	NEG	NEG	NEG	1a- Heroin, methadone, diazepam intoxication
13	M	39	23	NEG	NEG	NEG	NEG	NEG	1a- Bronchopneumonia and intoxication by heroin and methadone
14	M	31	NEG		NEG		11		1a- Hypoxia brain damage 1b- Cardiac arrest; 1c- Cardiac enlargement
15	F	33	19		NEG		60		1a- Hypoxia -Ischaemic brain injury b- Cardiac arrest ; 1c- Heroin and alcohol intoxication- Cardiac enlargement
16	M	73	195		50	56	NEG	NEG	1a- Ischaemic heart disease 1b- Coronary artery atheroma
17	F	18	>500	>500	>400	>400	NEG	NEG	1a- Diabetic ketoacidosis; 1b. Insulin dependent diabetic mellitus
18	M	33	86	79	NEG	NEG	83	114	1a- Heroin intoxication
19	M	19	10	NEG	NEG	NEG	NEG	NEG	1a- Head and chest injury 1b- Road traffic collision (driver)
20	F	28	10	NEG	NEG	NEG	NEG	33	1a- Bronchopneumonia; 1b- Morphine intoxication
21	M	44	NEG		NEG		NEG		1a- Cardiac enlargement
22	F	30	21	97	NEG	NEG	16	NEG	1a- Incised wound of arm
23	F	36	85	>500	NEG	332	NEG	NEG	1a- Viral pneumonia with secondary bacterial infection
24	F	34	20	NEG	NEG	NEG	174	266	1a- Heroin and alcohol intoxication
25	F	38	29	NEG	NEG	NEG	NEG	13	1a- Head injury
26	M	81	73	47	NEG	NEG	15	18	1a- Acute pylonephritis; 2a- Coronary artery atheroma

Appendix 4-2 continued

27	F	47	NEG	NEG	NEG	NEG	19	NEG	1a-Ischaemic heart disease and bronchopneumonia
28	F	38	57	NEG	NEG	NEG	NEG	NEG	1a- Acute exacerlation of chronic obstructive airways disease
29	M	71	NEG	NEG	NEG	NEG	205	164	1a- Multiple injuries; 1b- Road Traffic accident (pedestrian)
30	F	66	237	452	15	26	NEG	NEG	1a- Acute hepatic necrosis; 1b- paracetamol Toxicity
31	M	62	19	NEG	NEG	NEG	NEG	NEG	1a- Hanging
32	M	62	28		NEG	NEG	14	NEG	1a- Probable sepsis, source uncertain
33	F	44	15	NEG	NEG	NEG	NEG	NEG	1a- Ischaemic heart disease 1b- Coronary artery atheroma
34	F	53	42	NEG	NEG	NEG	NEG	NEG	1a- Multiple injuries; 1b- Fall from height
35	F	55	26		NEG		NEG		1a- Upper gastrointestinal haemorrhage; 1b- Duodenal ulcer
36	M	34	16	NEG	NEG	NEG	21	31	1a- Gamma- hydroxybutyrate (GHB) intoxication
37	F	43	22	NEG	NEG	NEG	140	208	1a- unascertained
38	M	67	14		NEG	NEG	NEG	NEG	1a- Ischaemic heart disease; 1b- Coronary artery atheroma
39	F	48	69		NEG	NEG	NEG	10	1a- Head injury
40	M	52	42	93	NEG	NEG	227	373	1a- Bronchopneumonia; 1b- Chronic alcohol abuse
41	M	40	99	NEG	NEG	NEG	329	442	1a- Acute alcohol intoxication
42	M	57	10	NEG	NEG	NEG	NEG	NEG	1a- Hanging
43	F	44	NEG		NEG		58		1a- Hypoxic brain damage; 1b. Cardiorespiratory arrest; 1c. Acute severe asthma
44	M	23	NEG	NEG	NEG	NEG	NEG	NEG	1a- tramadol and methadone intoxication
45	M	48	111		31	67	19	31	1a- Alcoholic ketoacidosis; 1b- Chronic alcohol abuse
46	M	42	23	NEG	NEG	NEG	10	16	1a- Myocardial fibrosis and ischaemia - cause unascertained
47	F	37	NEG	NEG	NEG	NEG	NEG	15	1a- Heroin and methadone intoxication
48	F	33	14	NEG	NEG	NEG	NEG	NEG	1a- Heroin intoxication
49	M	59	18	NEG	NEG	NEG	56	79	1a- Hanging
50	M	24	25	NEG	NEG	NEG	155	204	1a- Hanging
51	M	40	NEG	NEG	NEG	NEG	NEG	NEG	1a-Bronchopneumonia; 1b- Chronic obstructive airways disease 2a- Methadone intoxication
52	M	64	76		NEG		NEG		1a- Cardiac enlargement
53	M	61	135	74	NEG	NEG	15	12	1a-Bronchopneumonia; 1b- Chronic obstructive pulmonary disease
54	M	63	38	NEG	NEG	NEG	NEG	NEG	1a- Head Injury; 1b. Presumed fall
55	F	44	57	NEG	NEG	NEG	10	NEG	1a- Bronchopneumonia
56	M	42	19	NEG	NEG	NEG	83	117	1a- Heroin intoxication

Appendix 4-2 continued

57	F	34	NEG		NEG		NEG		1a- Multiorgan failure; 1b. Hypothermia and drug intoxication
58	F	45	NEG	NEG	NEG	NEG	NEG	NEG	1a- Clozapine intoxication 2a- Resolving Bronchopneumonia
59	M	57	NEG	NEG	NEG	NEG	NEG	NEG	1a- Ischaemic heart disease; 1b- Coronary artery atheroma
60	M	41	54	NEG	NEG	NEG	59	103	1a- Drowning
61	M	62	NEG		NEG		65		1a- Ischaemic heart disease and Bronchopneumonia
62	F	41	NEG	NEG	NEG	NEG	NEG	19	1a- Methadone and dehydrocodone intoxication; 1b- Coronary artery atheroma
63	M	63	17	NEG	NEG	NEG	235	260	1a- Hanging
64	M	61	NEG	NEG	NEG	NEG	73	10	1a- Hanging
65	F	42	NEG	NEG	NEG	NEG	34	NEG	1a- Multiple injuries; 1b- Fall from height
66	F	26	NEG	NEG	NEG	NEG	33	98	1a- Heroin intoxication
67	M	25	25	NEG	NEG	NEG	146	272	1a- Heroin, methadone and alcohol intoxication
68	M	49	NEG		NEG		NEG		1a. Hed and chest injuries; 1b. Road traffic accident
69	M	44	NEG	NEG	NEG	NEG	NEG	NEG	1a- Heroin intoxication
70	M	33	NEG		NEG		NEG		1a- Hanging
71	M	32	27	45	NEG	NEG	NEG	NEG	1a- Fatty degeneration of the liver; 1b. Chronic alcohol abuse
72	M	44	16	NEG	NEG	NEG	241	287	1a- Fatty degeneration of the liver 1b- Chronic alcoholic abuse
73	M	71	208	308	67	84	NEG	NEG	1a- Acute puelonephritis; 1b- Possible hypothermia
74	M	35	145	335	83	183	NEG	NEG	1a- Fatty degeneration and cirrhosis of the liver 1b- Chronic alcoholic abuse
75				53	NEG	NEG	59(liver blood)	NEG	External case
76			18		NEG		NEG		External case
77			19	NEG	NEG	NEG	204	266	External case
78			32		NEG		NEG		External case
79	M	33	NEG	NEG	NEG	NEG	NEG	27	1a- Cocaine Related Cardiac Arrythmia
80	M	24	11	NEG	NEG	NEG	243	287	1a- head and chest injury 1b- Fall from height
81	F	40	249	498	121	145	NEG	66	1a- Fatty degeneration of the liver 1b- Chronic alcoholic abuse
82	M	51	NEG	NEG	NEG	NEG	NEG	NEG	1a- Ischaemic heart disease; 1b- Coronary artery atheroma
83	M	49	13	NEG	NEG	NEG	NEG	25	1a- Ischaemic heart disease; 1b- Coronary artery atheroma 2a- Hypoglycaemia due to insulin dependents diabetes mellitus
84	M	36	13	NEG	NEG	NEG	NEG	NEG	1a- Coronary artery atheroma
85	M	42	12	NEG	NEG	NEG	131	143	1a- Hanging
86	F	35	33		NEG		NEG		1a- unascertained
87	M	42	NEG	NEG	NEG	NEG	11	NEG	1a- Heroin and Alcohol Intoxication
88	M	48	29	NEG	NEG	NEG	190	267	1a- Heroin and alcohol intoxication
89	M	34	>500	>500	297	>400	NEG	NEG	1a- Drowning; 1b. Diabetic ketoacidosis; Insulin dependent diabetic mellitus
90	M	45	16	NEG	NEG	NEG	124	169	1a- Sharp force injury of neck

Appendix 4-2 continued

91	M	37	NEG	NEG	NEG	NEG	196	198	1a- Stab wound of chest
92	F	29	17		NEG		168		1a- Hanging
93	F	24	46	348	NEG	226	NEG	NEG	1a- Hanging
94	M	41	16		NEG		NEG		1a- Hanging
95	M	46	18	NEG	NEG	NEG	12	75	1a- Sudden unexpected death in epilepsy; 1b- Old head injury
96	F	70	16		NEG		17		1a- Drowning
97	M	65	16	NEG	NEG	NEG	247	315	1a- Chest injury; 1b- Road Traffic accident (driver)
98	M	67	383	358	72	85	338	392	1a. Alcohol related death
99	M	38		158	45	58	NEG	18	1a. Undetermined
100	M	80	215	497	82	111	23	14	1a. Hypothermia
101	M	25	24	NEG	NEG	NEG	38	84	1a. Unascertained
102	M	36	11		NEG		NEG		1a. Unascertained
103			10						External case
104	M	61	263	>500	42	147	193	286	1a. Ischaemic heart disease and gastro intestinal haemorrhage from erosive oesophagitis. 2a. Fatty degeneration of the liver
105	F	56	396		177		34		1a. Fatty degeneration of the liver due to; 1b. chronic alcohol abuse.
106			31						External case
107	M	25	14	23	NEG		NEG		1a. Methadone, heroin and cocaine Toxicity.
108			0						External case
109	M	39	19		NEG	NEG	NEG	NEG	1a. Methadone and alcohol Intoxication
110	F	57	>500	>500	399	620	NEG	NEG	1a-Alcoholic Stetosis and Ischaemic heart disease; 1a Fatty degeneration of the liver; 1b- Chronic Alcoholic
111			>500	>500	419	448	85	NEG	External case (Expected Ketoacidosis)
112	M	42	>500		469		30	NEG	1a-Ischaemic heart disease 1b- Coronary artery atheroma 2a- Insulin dependent diabetes mellitus
113	F	36	165		40		NEG		Fatty degeneration of the Liver Chronic Alcohol Abuse
114			420						Suspected Alcoholic ketoacidosis -BHB requested (External Case)
115	M	36	24		14		314		1a. Fatty degeneration of the Liver; 1b. Chronic Alcohol Abuse
116	M	43	11	14	NEG	NEG	376	476	1a. Acute alcohol intoxication; 1b. Fatty degeneration of the liver due to chronic alcohol abuse
117	F	60	44		NEG		55		1a. Hepatic Failure,; 1b. Fatty degeneration of the Liver; 1c. Chronic Alcohol Abuse; 2a. Fracture of the humerus from fall
118			44						Suspected GHB intoxication (External Case)
119	M	64	51		NEG		112		1a- unascertained Chronic Alcohol Abuse
120			372		57	71	257	305	Fatty degeneration of the Liver; Chronic Alcohol Abuse (External Case)
121	M	76	48		NEG		NEG		1a. Perforation of oesophagus; 1b. Chronic stricture
122	M	43	>500		169	247	NEG	NEG	1a. Fatty degeneration of the Liver Chronic Alcohol Abuse

Appendix 5-1 Summary table of techniques used for detection of GHB and related compounds

Ref.	Analyte(s)	Internal standard	Matrix	Separation Technique	Detection	Identification	Linearity	LLOQ
(125)	BHB	D6-GHB	Urine Blood Vitreous Humour Plasma	GC/MS-EI Column: DB-5 Zebron: ZB-5MS (30m x 0.25mm i.d. x 0.25 μm)	Full Scan	233 BHB and 239 IS	31.25-500 mg/L	1 mg/L LOD and 31.25 mg/L LOQ
(138)	BHB	D6-GHB	Urine, Blood	GC/MS-EI: Column:DB-5 (30m x 0.25mm i.d. x 0.25 μm)	Full Scan	233,191,117 BHB and 239,240,241 IS	50-500 mg/L	2-7 mg/L (Blood) 2-6 mg/L (Urine)
(231)	GHB	D_6-GHB	Saliva	GC-MS	SIM	GHB 233 and 234, D_6-GHB239 and 240	0.50-50.0mg/L	0.50 mg/L
(232)	GHB	Alpha methylene-Gamma-butyrolactone	Urine	GC-FID-HS Column: Rtx-BAC1(30m x 0.32mm i.d. x 1.8 μm)			10-400 mg/L	

ACN: acetonitrile; B: blood; BSTFA: N,O-bis (trimethylsilyl) trifluoroacetamide; GC: gas chromatography; LC: liquid chromatography; LLOQ: lower limit of quantification; LOD: limit of detection; MeOH: methanol; Sr: serum; SIM: selected ion monitoring; SPE: solid-phase extraction; SPME solid phase micro extraction: SRM: selected reaction monitoring; U: urine. EDTA ethylene diamine tetraaceitic acid: MAMP methamphetamine: MDMA

Appendix 5-1 continued

Ref.	Analyte(s)	Internal standard	Matrix	Separation technique	Detection	Identification	Linearity	LLOQ
(233)	GHB	D6-GHB	Urine, Blood	GC-MS Column: DB5-5MS (15 m x 0.25 mm i.d. x 0.25 µm)	Full Scan			
(231)	GHB	D6-GHB	Saliva	GC-MS- SIM Column:Rtx-5Ms Restech (30m x 0.25mm i.d. x 0.5 µm)	SIM	233 and 234 for GHB; 239, 240, and 241 for GHB-d(6); and 55, 73, and 97 for 1,7-heptanediol.	0.5-50.0 mg/L	LLOQ: 0.5 mg/L
(233)	GHB,Ket, MAMP and MDMA	D4-Ket, D5-MAMP,D5-MDMA and D6-GHB	Urine	SPME-GC-MS Column: (30m x 0.25mm i.d. x 0.10 µm), Fibers 100 µm PDMS	Full Scan	following: 180 Keta 87 GHB, 186 and 102 for MAMP and MDMA.	Ket,GHB, MDMA 0.1ug/ mL MAMP 0.05 µg/mL	Ket,GHB, MDMA 0.1-20 µg/mL MAMP 0.05-20 µg/mL
(234)	GHB,GBL,BD	Benzyl Alcohol	Urine, Blood	GC-EI-MS for GHB and BD column is DB-5MS 30m x 0.32mm i.d. x 1.0 µm) GC-FID for GBL column 30m x 0.53 i.d. x 1.0 µm)	SIM	233,117 and 147 GHB, 177,166 and 147 BD. 165 and 135 IS	=0.99 or better	

Appendix 5-1 continued

Ref.	Analyte(s)	Internal standard	Matrix	Separation Technique	Detection	Identification	Linearity	LLOQ
(139)	GHB	D6-GHB	Urine, Serum	LC/MS ion trap. Column: (C18 Reverse Phase 2.1mm x 30mm x 3.5 µm) mobile phase A ammonium formate and acetonitrile	MS/MS	MS, 161,93 and 149 GHB. 167 IS	2-100 µg/ mL	2.4 µg/mL (Urine) 0.6 µg/mL (Serum)
(179)	BHB	D6-GHB	Blood	GC-MS EI mode Column: HP-5MS (30m x 0.25mm i.d. x 0.25 µm	SIM	BHB-TMS: 147, 117, 191 and 91. GHB-D6-TMS: 239, 73 and 240.	50-500 mg/L	
(134)	GHB, AHB, SA & BHB	2-Hydroxycaproic acid(HCA)	Urine	GC/MS-EI Column: DB-17 fused silica, 30m x 0.32mm i.d. x 0.25 µm).	Standard addition method Full scan	Quantified was 233 for GHB, AHB and BHB. Qualifier ions were selected in SIM Mode: 147 and 117 for GHB, 147 and 131 for AHB, 191 and 147 for BHB, 147 and 73,147 for IS.	0.03-30 µg/ mL GHB, 0.01-30 µg/ mL ABH, 1-300 µg/mL BHB	0.03 ng/mL in full scan mode and 0.01 ng/mL in SIM mode (LOD)

Appendix 5-1 continued

Ref.	Analyte(s)	Internal standard	Matrix	Separation Technique	Detection	Identification	Linearity	LLOQ
(235)	GHB	D6-GHB	Tissue, Plasma	LCMS-Triple quadropole/APCI: Column: C18 Aqua mobile phase 90% nM formic acid and Acetonitrile	MRM	1 transition (103 → 57) GHB (109 → 61) IS		0.1 µg/mL
(236)	GHB,GBL and BD	D6-GHB and D6-GBL	Urine	LC Tandem MS-EI mode Column: 3mm x 100mm x 5 µm)	MRM	(105 → 87) GHB (111 → 93) IS (87 → 45) GBL (93 → 49) IS (91 → 73) BD	GHB and BD 1-80 mg/l and GBL is 1-50mg/L	1 mg/L
(186)	GHB	D6-GHB	Blood Bile Vitreous Humour	GC/MS column (HP5,30m x 0.25mm i.d. x 0.25 µm)		m/z 233,204 and 147 for GHB and 239 for GHB-D6	1-200 mg/L	1mg/L
(237)	GHB,GBL and BD	Alpha-Hydroxybutyric acid	Urine	Capillary length 40cm to detector, 75 µm i.d.	Indirect UV detector at 214nm		25-500 µg/ mL	2.0 µg/mL
(194)	GHB	Methanol	Blood	GC-FID Head space, Column is DB624 (30m x 0.545mm i.d. x 3 µm)	7.88 the retention time of GHB and 8.61 for IS		0.5-83 µg/ mL	0.5 µg/mL

Appendix 5-1 continued

Ref.	Analyte(s)	Internal standard	Matrix	Separation Technique	Detection	Identification	Linearity	LLOQ
(180)	GHB	D6-GHB	Blood microbial broth	GC/MS DB5-MS5 (30m x 0.32mm i.d.)	Full Scan between 6.2-7.0min	m/z 233 for Derivatized GHB and 239 for GHB-D6		1 mg/L
(235)	GHB	D6-GHB	Tissue and Plasma	LCMS triple quadropole/APCI: C18 Aqua column	MRM	1 transition (103 → 57) GHB (109 → 61) IS		0.1 µg/mL
(140)	BHB	2,2-dinitrobiphenyl	Plasma	HPLC-UV detector, Column (55mm x 4.0mm i.d. x 3 µm particle	UV detection at 320nm			5 nmol/mL, LOQ 50nmol/mL
(238)	GHB	D6-GHB	Urine, Blood	GC/MS-EI: HP5 MS 30-m Column (30mm x 0.25mm i.d. x 0.25 µm)	Full Scan extracted ion monitoring	233,204 AND 147 GHB and 239 IS	1-200 mg/L	1 mg/L
(197)	GHB	D6-GHB	Urine, Blood	GC/MS-EI: HP5 Column (15mm x 0.25mm i.d. x 0.25 µm)	SIM	233,234,235GHB and 239,240,241 IS		

Appendix 5-1 continued

Ref.	Analyte(s)	Internal standard	Matrix	Separation Technique	Detection	Identification	Linearity	LLOQ
(239)	GHB		Urine	Colorimetric method, the ferric hydroxymate test for ester detection	Purple colour indicate presence of GHB			0.5-1 mg/mL
(195)	GHB	D6-GHB	Blood	GC/MS-EI: HP1 Column (12mm x 0.2mm i.d. x 0.33 µm)	SIM	233,234,235GHB and 239,240,241 IS	0.1-20 mg/dl	0.1 mg/dL
(126)	Ketone bodies(Acetone, BHB and Acetoacetate)		Blood	GC-FID Head space, 30m long DBWAX megabore column, film thickness 1 um,	Acetone peak heights		Acetone and acetoacetate 0-100umol/L BHB 0-300 umol/L	
(141)	Ketone body(Acetoacetate and BHB)		Blood	GCMS EI mode, column. 4m x 6mm packed	SIM	Peak height of 275 and 277m/z for BHB and peak height of 273 and 275 m/z for acetoacetate		

Appendix 5-2 GHB concentrations in blood and urine postmortem case samples

(Shaded boxes information is not available)

Case #	Gender	Age	GHB mg/L		Cause of Death
			Blood	Urine	
1	M	55	NEG		1a- unacertained
2	F	67	20	NEG	1a- head injury (haemorrhage from scalp laceration) 1b- presumed fall 2a- Acute alcohol intoxication pulmonary thromboembolism
3	F	41	NEG		1a- Stab wound of heart
4	M	66	11		1a- Coronary artery atheroma 1b- Ischaemic heart disease
5	F	44	22 *	NEG	1a- Fatty degeneration of the liver 1b- Chronic alcoholic
6	F	78	13 *	14	1a- Inhalation of smoke and fire gases in a house fire and hypertensive and valvular heart disease
7	F	29	NEG		1a- Codeine and alcohol intoxication
8	M	33	13	NEG	1a- Heroin and ketamine intoxication
9	F	58	10	12	1a- Suspected alcohol related death
10	M	54	27	NEG	1a- Chest injury 1b Road traffic collision (driver)
11	M	29	15	NEG	1a- Hanging
12	M	29	11	NEG	1a- Heroin, methadone, diazepam intoxication
13	M	39	NEG	NEG	1a- Bronchopneumonia and intoxication by heroin and methadone
14	M	31	13		1a- Hypoxia brain damage 1b- Cardiac arrest 1c- Cardiac enlargement
15	F	33	25		1a- Hypoxia -Ischaemic brain injury 1b- Cardiac arrest 1c- Heroin and alcohol intoxication- Cardiac enlargement
16	M	73	NEG	NEG	1a- Ischaemic heart disease 1b- Coronary artery atheroma
17	F	18	NEG		1a- unacertained (Pending further investigation)
18	M	33	NEG	NEG	1a- Heroin intoxication
19	M	19	23	NEG	1a- Head and chest injury 1b- Road traffic collision (driver)
20	F	28	27	NEG	1a- Bronchopneumonia 1b- Morphine intoxication
21	M	44	16		1a- Cardiac enlargement
22	F	30	17	NEG	1a- Incised wound of arm
23	F	36	NEG	NEG	1a- Viral pneumonia with secondary bacterial infection
24	F	34	21	NEG	1a- Heroin and alcohol intoxication

Appendix 5-2 continued

25	F	38	17	NEG	1a- Head injury (Pending further investigation)
26	M	81	28	23	1a- Acute pylonephritis 2a- Coronary artery atheroma
27	F	47	13	NEG	1a-Ischaemic heart disease and bronchopneumonia
28	F	38	17		1a- Acute exucerlation of chronic obstructive airways disease
29	M	71	13	NEG	1a- Multiple injuries 1b- Road Traffic accident (pedestrian)
30	F	66	NEG	NEG	1a- Acute hepatic necrosis 1b- paracetamol Toxicity
31	M	62	14	NEG	1a- Hanging
32	M	62	NEG		1a- Probable sepsis, source uncertain
33	F	44	14		1a- Ischaemic heart disease 1b- Coronary artery atheroma
34	F	53	16		1a- Multiple injuries 1b- Fall from height
35	F	55	14		1a- Upper gastrointestinal haemorrhage 1b- Duodenal ulcer
36	M	34	**>500**	**>500**	1a- Gamma- hydroxy butyrate (GHB) intoxication
37	F	43	22	NEG	1a- unacertained
38	M	67	19		1a- Ischaemic heart disease 1b- Coronary artery atheroma
39	F	48	NEG	NEG	1a- Head injury
40	M	52	20		1a- Bronchopneumonia 1b- Chronic alcohol abuse
41	M	40	23		1a- Acute alcohol intoxication
42	M	57	17		1a- Hanging
43	F	44	18		1a- unacertained (Pending further investigation)
44	M	23	15		1a- tramadol and methadone intoxication
45	M	48	27		1a- Alcoholic ketoacidosis 1b- Chronic alcohol abuse
46	M	42	14		1a- Myocardial fibrosis and ischaemia - cause unacertained
47	F	37	14		1a- Heroin and methadone intoxication
48	F	33	20		1a- Heroin intoxication
49	M	59	26		1a- Hanging
50	M	24	16		1a- Hanging

Appendix 5-2 continued

51	M	40	23		1a- Bronchopneumonia 1b- Chronic obstructive airways disease 2a- Methadone intoxication
52	M	64	<10		1a- Cardiac enlargement
53	M	61	25		1a- Bronchopneumonia 1b- Chronic obstructive pulmonary disease
54	M	63	23	15	1a- unacertained (Pending further investigation)
55	F	44	22	NEG	1a- Bronchopneumonia
56	M	42	10	NEG	1a- Heroin intoxication
57	F	34	12		1a- Multiorgan failure (Pending further investigation)
58	F	45	19		1a- Clozapine intoxication 2a- Resolving Bronchopneumonia
59	M	57	17	NEG	1a- Ischaemic heart disease 1b- Coronary artery atheroma
60	M	41	NEG		1a- Drowning
61	M	62	29		1a- Ischaemic heart disease and Bronchopneumonia
62	F	41	14	19	1a- Methadone and dehydrocodone intoxication 1b- Coronary artery atheroma
63	M	63	30	NEG	1a- Hanging
64	M	61	22	18	1a- Hanging
65	F	42	19	NEG	1a- Multiple injuries 1b- Fall from height
66	F	26	22	NEG	1a- Heroin intoxication
67	M	25	26	NEG	1a- Heroin, methadone and alcohol intoxication
68	M	49	21		
69	M	44	27	NEG	1a- unacertained (Pending further investigation)
70	M	33	22		1a- Hanging
71	M	32	23	NEG	1a- Suspected seizure-related death (Pending further investigation)
72	M	44	NEG	21	1a- Fatty degeneration of the liver 1b- Chronic alcoholic abuse
73	M	71	18	NEG	1a- Acute puelonephritis 1b- Possible hypothermia

Appendix 5-2 continued

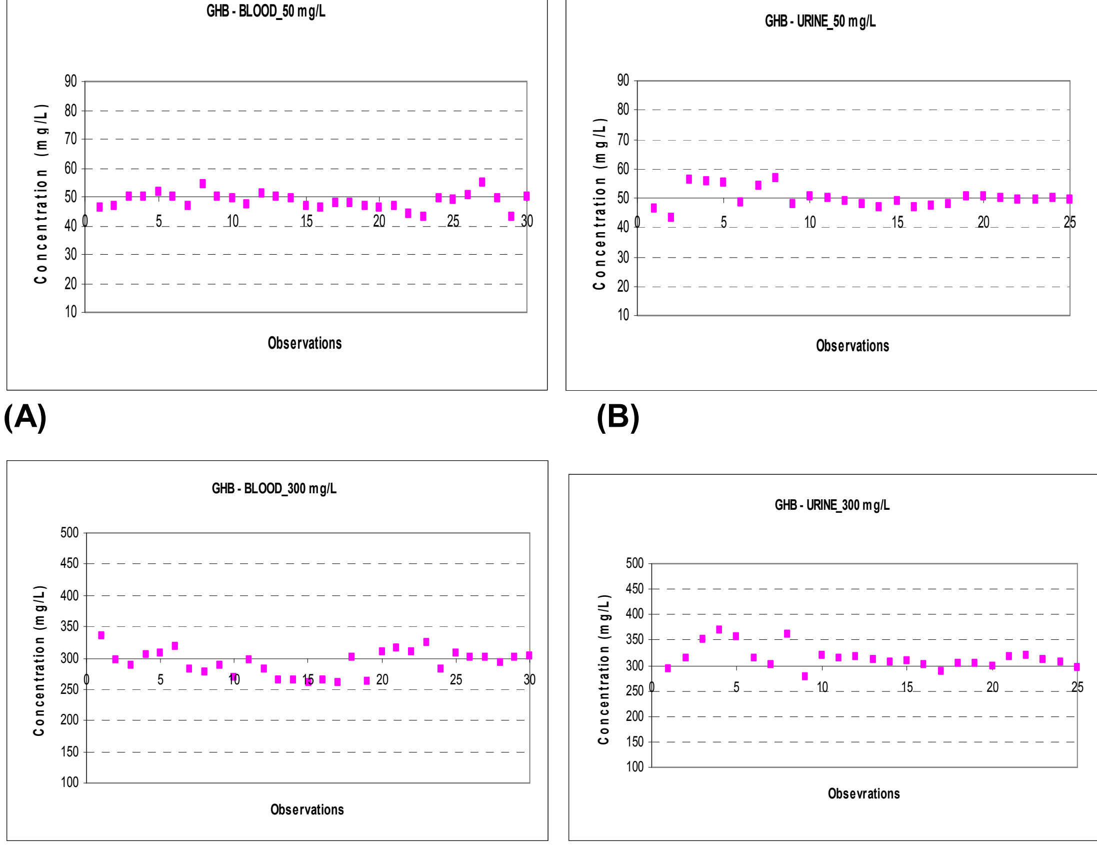
74	M	35	NEG	NEG	1a- Fatty degeneration and cirrhosis of the liver 1b- Chronic alcoholic abuse
75				60	External case
76			23		External case
77			29	NEG	External case
78			50		External case
79	M	33	32	NEG	1a- unacertained (Pending further investigation)
80	M	24	19	NEG	1a- head and chest injury 1b- Fall from height
81	F	40	NEG	NEG	1a- Fatty degeneration of the liver 1b- Chronic alcoholic abuse
82	M	51	NEG	NEG	1a- Ischaemic heart disease 1b- Coronary artery atheroma
83	M	49	18	NEG	1a- Ischaemic heart disease 1b- Coronary artery atheroma 2a- Hypoglycaemia due to insulin dependents diabetes mellitus
84	M	36	17	NEG	1a- Coronary artery atheroma
85	M	42	46	NEG	1a- Hanging
86	F	35	25		1a- unacertained (Pending further investigation)
87	M	42	NEG	NEG	1a- Cardiomegaly (Pending further investigation
88	M	48	14	NEG	1a- Heroin and alcohol intoxication
89	M	34	NEG	NEG	1a- Drowning (Pending further investigation)
90	M	45	14	NEG	1a- Sharp force injury of neck
91	M	37	12		1a- Stab wound of chest
92	F	29	20		1a- Hanging
93	F	24	29	NEG	1a- Hanging
94	M	41	15		1a- Hanging
95	M	46	27		1a- Sudden unexpected death in epilepsy 1b- Old head injury
96	F	70	21		1a- Drowning
97	M	65	22		1a- Chest injury 1b- Road Traffic accident (driver)
98	M	67	38	25	Alcohol related death

Appendix 5-2 continued

99	M	38		38	Undetermined
100	M	80	14	18	Hypothermia
101	M	25	54	NEG	Unascertained
102	M	36	65		Unascertained
103			**369**		Suspected GHB/GBL Intoxication
104	M	61	26	22	Ischaemic heart disease and gastro intestinal haemorrhage from erosive oesophagitis. Fatty degeneration of the liver due to chronic alcohol abuse
105	F	56	25		Fatty degeneration of the liver due to chronic alcohol abuse.
106			**300**		Suspected GHB/GBL Intoxication
107	M	25	30	2.00	Methadone, heroin and cocaine Toxicity.
108			**>500**	>500	Suspected GHB/GBL Intoxication
109	M	39	56		Methadone and alcohol Intoxication
110	F	57	12.00	8.00	1a-Alcoholic Stetosis and Ischaemic heart disease , 1a Fatty generation of the liver 1b- Chronic Alcoholic
111			11	9	External case (Expected Ketoacidosis)
112	M	42	82		1a-Ischaemic heart disease 1b- Coronary artery atheroma 2a- Insulin dependent diabetes mellitus
113	F	36	30		Fatty degeneration of the Liver Chronic Alcohol Abuse
114	M		27		Suspected Alcoholic ketoacidosis -BHB requested
115	M		45		Fatty degeneration of the Liver Chronic Alcohol Abuse
116	M		32	17	Very Fatty Liver , Cardiac enlargement
117	M		17		Hepatic Failure, Fatty degeneration of the Liver Chronic Alcohol Abuse
118	M		**264**		Suspected GHB intoxication
119	M		44		1a- unacertained Chronic Alcohol Abuse , Fatty Liver
120	M		59		Fatty degeneration of the Liver Chronic Alcohol Abuse

Appendix 5-3 GHB QC Charts

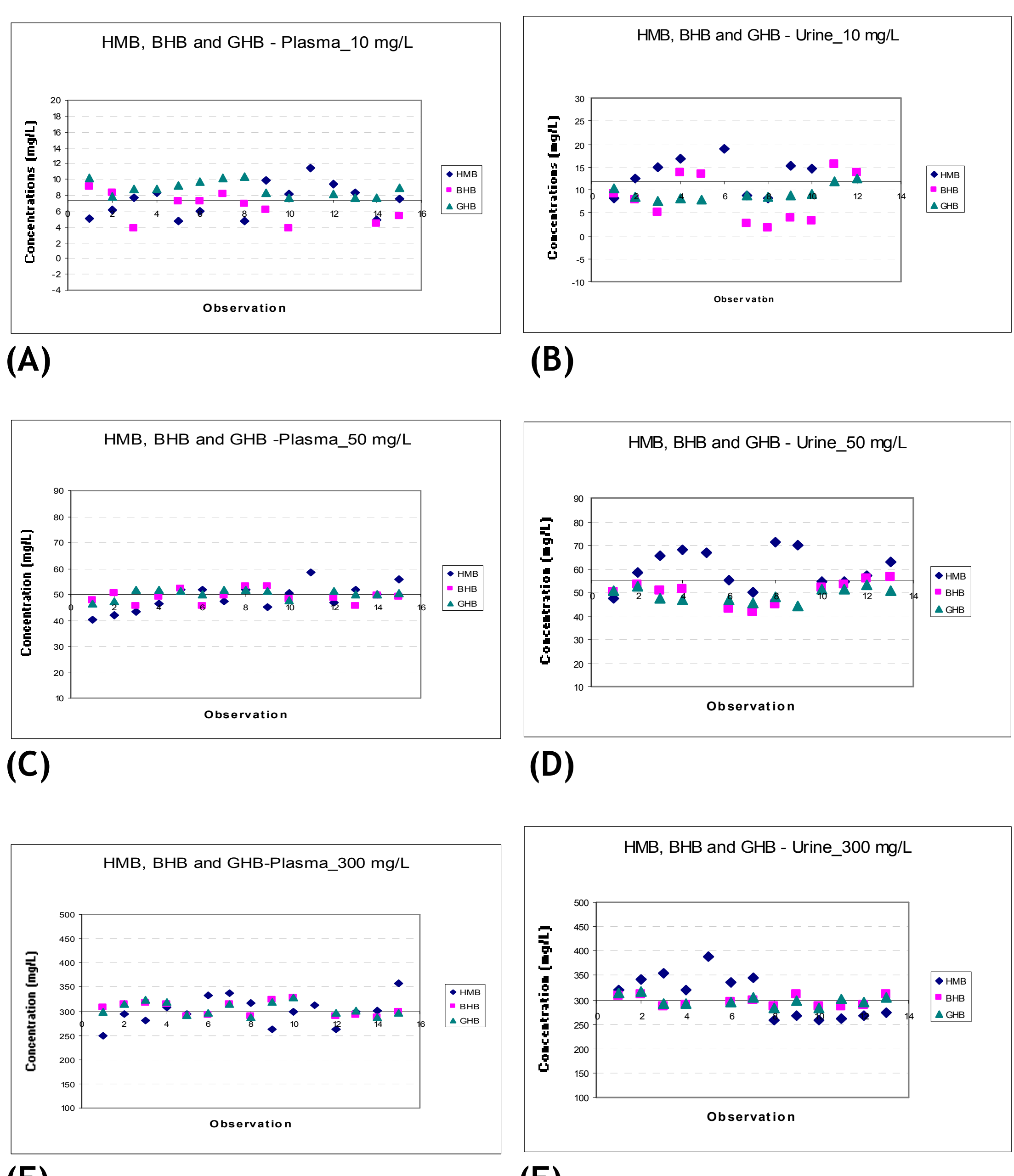
	QC Ranges			
	Blood		Urine	
	50 mg/L	300 mg/L	50 mg/L	300 mg/L
Average	49	293	50	314
Standard Deviation (SD)	2.61	20.11	3.12	22.42
CV%	5.4	6.7	6.3	7.1
Mean ± 2SD	43-54	253-333	44-56	270-359



(C) (D)

GHB quality control charts at 50 mg/L (A and B), 300 mg/L (C and D) in blood and urine, respectively.

Appendix 5-4 HMB/BHB and GHB QC Charts



Quality control charts for HMB, BHB and GHB in plasma and urine. at 10 mg/L (A and B), 50 mg/L (C and D) and 300 mg/L (E and F)

	PLASMA									URINE								
	HMB			GHB			BHB			HMB			GHB			BHB		
	10 mg/L	50 mg/L	300 mg/L	10 mg/L	50 mg/L	300 mg/L	10 mg/L	50 mg/L	300 mg/L	10 mg/L	50 mg/L	300 mg/L	10 mg/L	50 mg/L	300 mg/L	10 mg/L	50 mg/L	300 mg/L
AVERAVE	7.29	50	304	8.84	50	305	6.39	49	304	13	60	307	9.19	49	299	8.15	50	296
STDEV	2.14	4.26	26.96	0.97	1.78	13.71	1.62	2.54	14.26	3.95	8.14	44.53	1.61	2.96	10.34	5.16	5.03	10.97
2 SD	4.27	8.52	53.93	1.94	3.55	27.43	3.24	5.08	28.51	7.90	16.28	89.07	3.23	5.93	20.68	10.32	10.06	21.93
Mean + 2SD	**12**	**59**	**358**	**11**	**54**	**333**	**10**	**54**	**333**	**21**	**76**	**396**	**12**	**55**	**319**	**18**	**60**	**318**
Mean - 2SD	**3**	**42**	**250**	**7**	**47**	**278**	**3**	**44**	**276**	**5**	**44**	**218**	**6**	**43**	**278**	**0**	**40**	**274**

Appendix 6-1 Preparation and optimization of in house deuterated internal standard

Materials used for preparation in-house deuterated standards were ethanol Deuterated (D6) D, 99 % Anhydrous, from Cambridge Isotope Laboratories.Inc. (CIL) USA. Ethanol 100 AR 99.9% Joseph Miller (Denaturants), Liverpool. Thionyl Chloride from Sigma Aldrich Company Ltd.Dorset,UK

Fatty acids standards (Arachidonic acid Approx. 99% GC grade, Heptadecanoic acid 98%, Linoleic Acid minimum ≥ 99%, Linolenic ≥ 99%. Lauric acid (Dodecanoic acid) 98%, Myristic acid 99-100%., Oleic Acid ≥ 99% GC grade, Palmitic acid minimum 99% GC grade, Palmitoleic Acid Approx. 99% GC grade, Stearic acid Grade I 99% GC grade) were obtained from Sigma Aldrich Company Ltd.Dorset,UK

FAEEs deuterated internal standard was prepared according to Pragst, *et al.* (220)10 mg of each FAEEs added with ethanol-D6 Place in dry ice at -78ºC add 10uL thionyl chloride Heat at 40ºC for 2 hours. Cyclohexane was added after evaporation at 10 psi, then followed by vortex another evaporation steps. This later step was repeated two times and then bring it up with cyclohexane to have 2 mg/mL final concentrations and stored at-20ºC.

R–C(=O)OH + $SOCl_2$ → R–C(=O)Cl + Hcl

Fatty Acid | Thionyl Chloride | Acyl Chloride

R–C(=O)Cl + CD_3CD_2OD → R–C(=O)O–CD_2CD_3 + Hcl

Acyl Chloride | Ethanol-D6 | Fatty Acid Ethyl Ester-D5

Chemical reaction during preparation of deuterated FAEES

Appendix 6-1 continued

Purity of in house deuterated internal standard

Deuterated in-house internal standards were prepared at concentration of 50 mg/L to carry out the purity study. From stock solution (2 mg/ mL) a working standard of a concentrations of 50 mg/L was prepared. A 100 µl of 50 mg/L was added to a labelled vial. Cyclohexane was evaporated to dryness. 100 µL methanol was added to give final concentration was of 50 mg/L. All standards were stored at -20ºC

Intrumentation and conditions

Gas chromatography-mass spectrometry (GC-MS) was utilised using an Agilent GC-MS 7890A GC system, a 5975 mass spectrometry detector (MSD), and ChemStation software (Agilent, UK). A DB-5+ DG capillary column (30m x 0.25 mm I.D., 0.25 µm film thickness), purchased from Agilent Technologies Ltd., UK, was used. Additional instruments included a nitrogen evaporator, micro-centrifuge, heater block, and vortex.

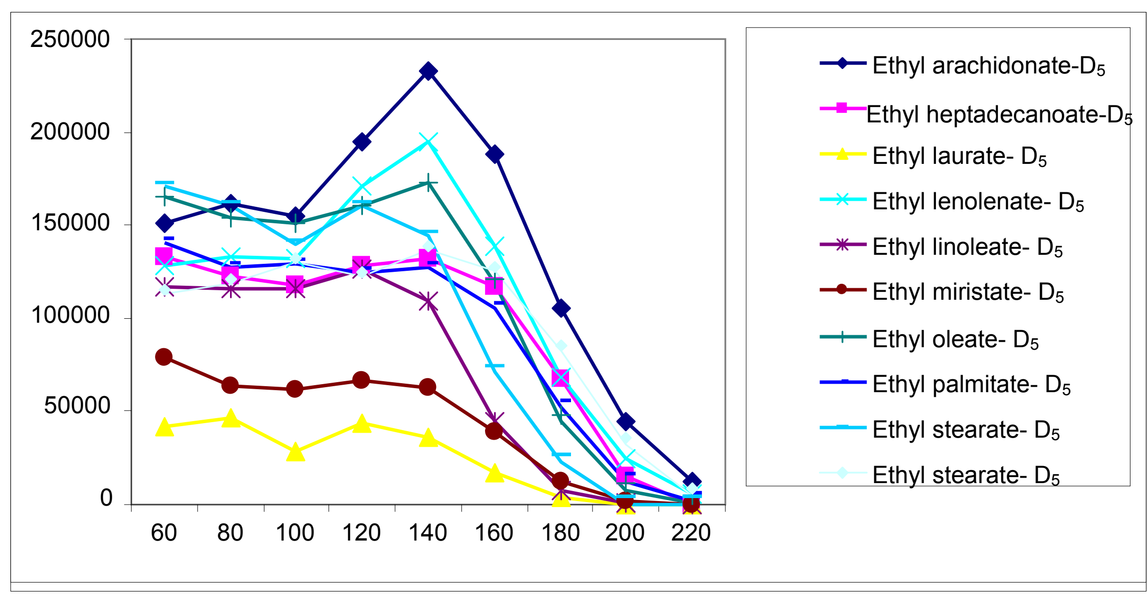
The GC Program conditions of 50°C for 1 minutes then 10°C /min to 250°C for 60 minutes, total run time was 27 minutes using splitless mode. The ions for corresponding analytes were extracted includes(D5-ethyl arachidonate 338, D5-Ethyl Laurate 234, D5-ethyl myristate 262, D5-ethyl linolenate 312, D5 ethyl linoleate 314, D5-ethyl Palmitoleate 288, D5-ethyl palmitate, 290, D5-ethyl oleate 316 and D5-ethyl stearate 318, D5ethyl heptadecanoate 304).

Area percent report was generated and area peak percent of analyte in relation of the total peak areas present in the same chromatogram were for non derivatized ranging from 32-89%. Intensity report was also printed and the intensity for the area of (m/z+2), (m/z+3), (m/z+4), (m/z+5) were listed in the same excel sheet. purity was calculated as the percentage of the area intensity (m/z+5) for all internal standards versus the sum intensities of m/z+2 plus m/z+3 plus m/z+4 plus m/z+5. The purity ranged from 81- 98 % for as indicated in

Appendix 6-1 continued

Purity of in-house deuterated standards

Internal Standard	Molecular ions from GC/MS	RT	(Area) M/z+2	(Area) M/z +3	(Area) M/z+4	(Area) M/z+5	Total Intensities	Purity %
Ethyl arachidonate-D5	337.4	22.302	4790	4171	5200	59872	74033	**81**
Ethyl heptadecanoate-D5	303.4	20.188	1280	4875	55632	3229696	3291483	**98**
Ethyl laurate-D5	233.3	14.997	225	676	3274	118720	122895	**97**
Ethyl lenolenate-D5	311.4	20.799	3103	3023	6547	647616	660289	**98**
Ethyl linoleate-D5	313.4	20.751	708	5633	14801	851072	872214	**98**
Ethyl miristate-D5	261	17.212	917	3097	53936	1796608	1854558	**97**
Ethyl oleate-D5	315.4	20.828	3091	5366	13177	389376	411010	**95**
Ethyl palmitate-D5	289.4	19.234	606	4703	96920	3643392	3745621	**97**
Ethyl palmitoleate-D5	287.4	19.013	453	4318	6252	552064	563087	**98**
Ethyl stearate-D5	317.5	21.083	1016	4730	23400	1598464	1627610	**98**



Fragmenter ion optimization of In house d_5-FAEEs and ethyl heptadecanoate

Most analytes have a collision energy of less than 10ev using LC/MS/MS triple quadrupole However, using the same tuning solution with the ion trap LC/MS/MS, the collision energies were higher (27-42 eV for the same analytes)

Appendix 6-1 continued

Fragmentor ion optimization of in house internal standard was carried out and the range of optimum fragmentor voltage was between 100 and140

FAEES and FAEEs-d_5 Optimization of product ion and Collision Energy (CE)

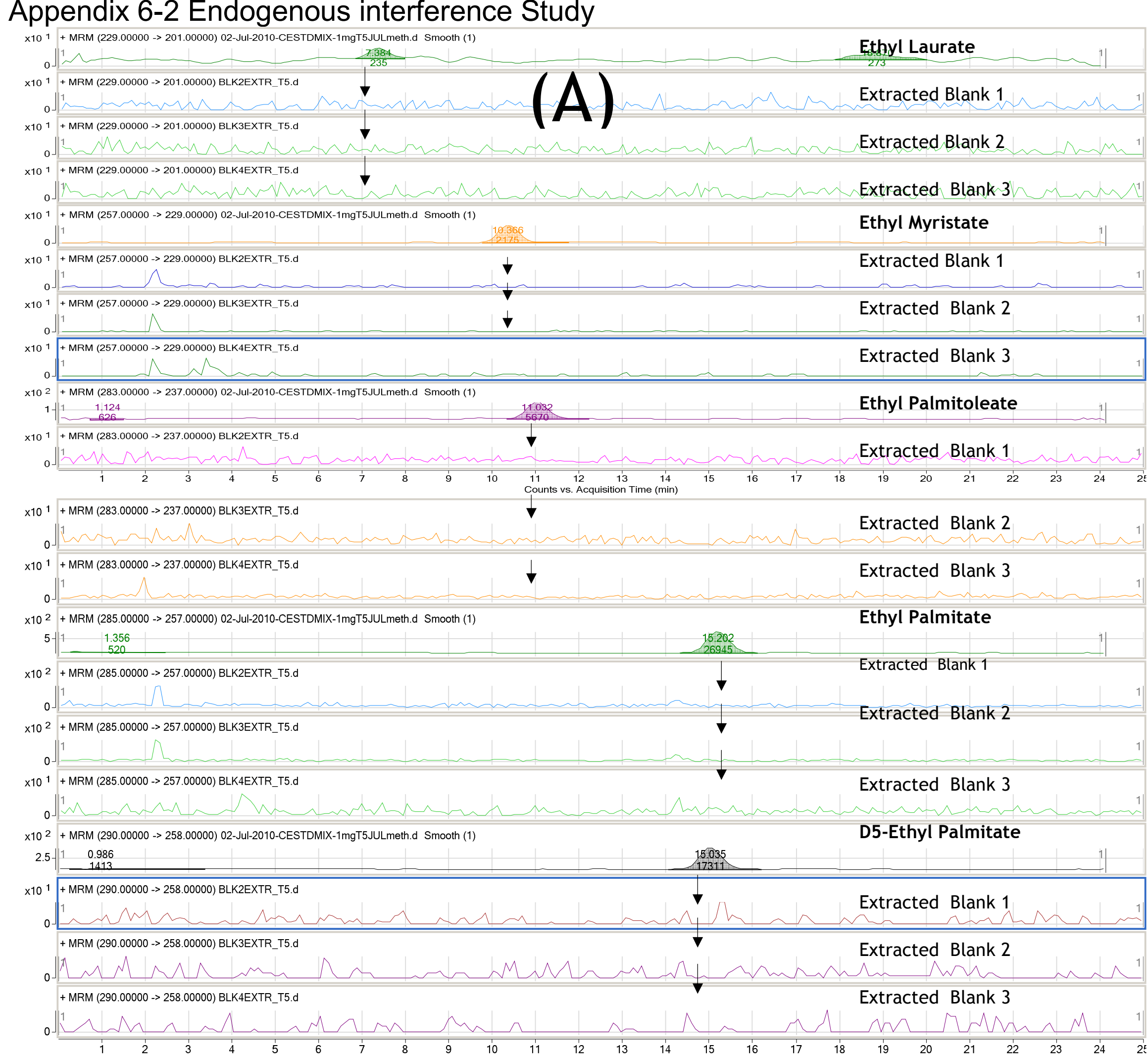
Name	Molecular Ion	Product Ions	Collision Energy By Deca LC/MS/MS	Comment
		FAEEs		
Ethyl Arachidonate	332.5	287	38	Commercial
Ethyl heptadecanoate	299	271	36	Commercial
Ethyl Laurate	228.38	201	30	Prepared in house from its acid
Ethyl Lenolenate	306.49	261/243	42	Commercial
Ethyl Linoleate	308.51	263/245	32	Commercial
Ethyl Miristate	256.43	229	32	Commercial
Ethyl Oleate	310.53	265/247	30	Commercial
Ethyl plamitate	284.48	257	32	Commercial
Ethyl Plamitoleate	283	237	30	Commercial
Ethyl Stearate	312.53	285	34	Prepared in house from its acid
Ethyl heptadecanoate	299	271	36	Commercial
Ethyl Plamitoleate	283	237	30	Commercial
		Commercial Deuterated FAEEs- d_5		
Ethyl Miristate_d_5	262	230	30	Commercial
Ethyl Oleate_d_5	316	265	38	Commercial
Ethyl plamitate_d_5	290	258	30	Commercial
Ethyl Stearate_d_5	318	286	32	Commercial
		In house Deuterated FAEEs- d_5		
Ethyl Arachidonate_d_5	338	287	30	Prepared in house from its acid
Ethyl heptadecanoate_d_5	304	272	40	Prepared in house from its acid
Ethyl Laurate_d^5	234	202	27	Prepared in house from its acid
Ethyl Lenolenate_d_5	312	261/243	30	Prepared in house from its acid
Ethyl Linoleate_d5	314	263/245	32	Prepared in house from its acid
Ethyl Miristate_d_5	262	230	30	Prepared in house from its acid
Ethyl Oleate_d_5	316	265	38	Prepared in house from its acid
Ethyl plamitate_d_5	290	258	30	Prepared in house from its acid
Ethyl Plamitoleate_d_5	288	237	30	Prepared in house from its acid
Ethyl Stearate_d_5	318	286	32	Prepared in house from its acid

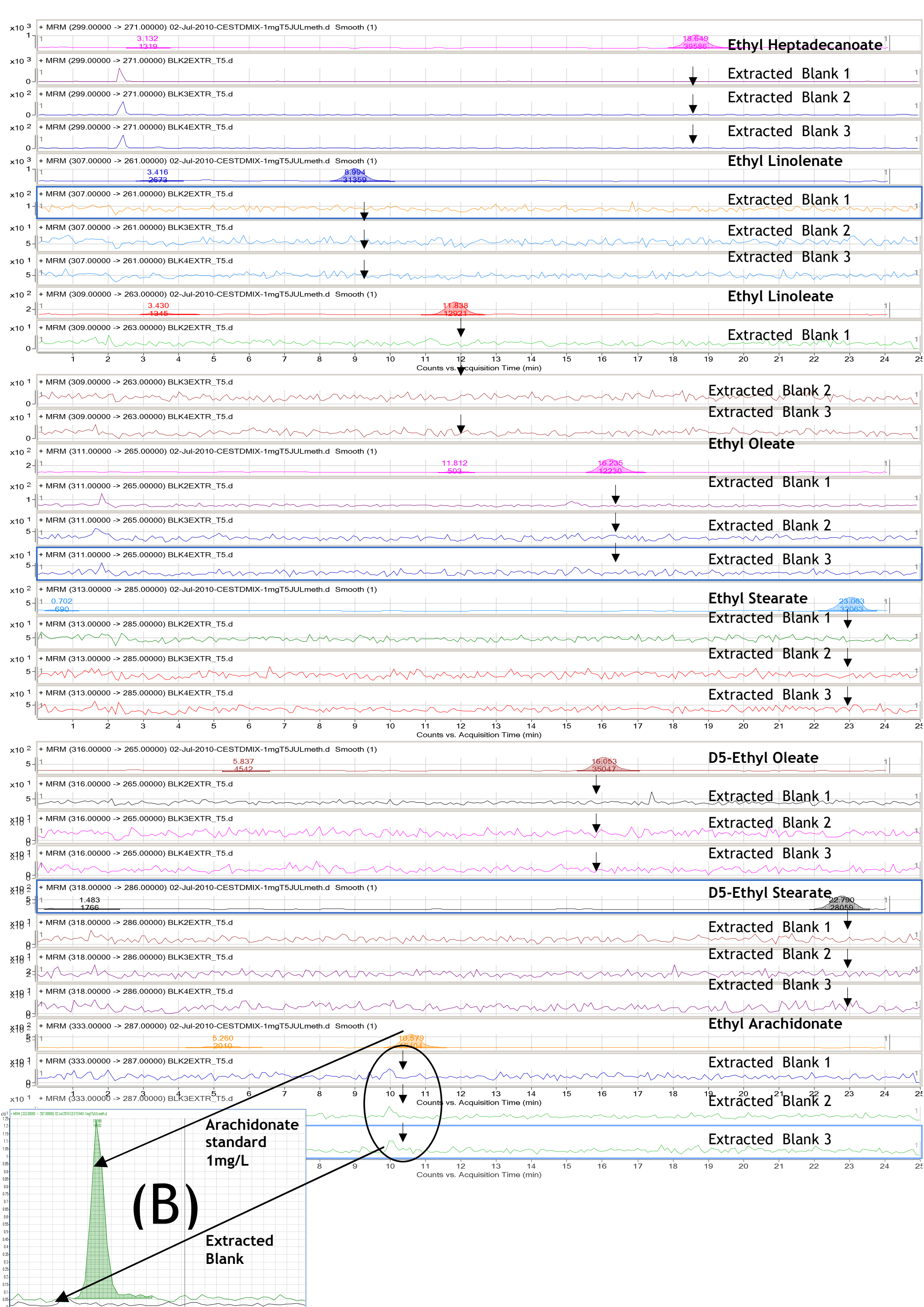
Appendix 6-1 continued

Collision energy and product ions optimization using triple quadropole LC/MS/MS were not fully achieved it was not reproducible ,this could be because very small CE was needed to fragment those ion (less than 5ev) which is too low compared to ion trap LC/MS/MS.

Collision energy of FAEEs analytes, including both commercial and In house internal standards, were lower than 10ev in a range of 2–6 (table 5-2); this made the optimization for some analytes more difficult, possibly due to the fragility of some compounds at this low collision energy. The optimum published collision energy of FAEEs ranged between 10 and 30 using triple quadrupole LC/MS/MS.(159) Collision energy was in a range of 27-42 ev when it was optimized by other ion trap LC/MS/MS in the same laboratory. This could be another reason not to have another ion transition for all analytes to be used as qualifiers

Appendix 6-2 Endogenous interference Study





(A) EXTRACTED pooled meconium blank samples with no internal standard versus unextracted FAEEs at concentration of 1 mg/L. (B) magnified peak of ethyl arachidonate overlaid with extracted meconium blank. The arrow indicated no endogenous interference from analyte in extracted blanks.

Appendix 6-2 continued

Appendix 6-3 FAEEs results in meconium

Meconium Case No.	Ethyl Laurate (ng/g)	Ethyl Myristate (ng/g)	Ethyl Linolenate (ng/g)	Ethyl Linoleate (ng/g)	Ethyl Palmitoleate (ng/g)	Ethyl Palmitate (ng/g)	Ethyl Oleate (ng/g)	Ethyl Stearate (ng/g)	Ethyl Arachidonate (ng/g)
Case_002	ND	<LLOQ(42)	ND	ND	ND	ND	**137**	ND	<LLOQ(99)
Case_003	ND	ND	ND	ND	ND	ND	ND	ND	ND
Case_004	**>ULOQ(342611)**	**>ULOQ(124527)**	**>ULOQ (46627)**	**>ULOQ(214140)**	**>ULOQ(56089)**	**5931**	**>ULOQ(56421)**	**906**	**>ULOQ(4522790)**
Case_005	ND	**162**	ND	ND	ND	ND	**>ULOQ(28773)**	ND	**2444**
Case_006	ND	**104**	ND	ND	ND	ND	ND	ND	ND
Case_007	<LLOQ (1404)	**202**	ND	ND	ND	ND	ND	ND	ND
Case_008	<LLOQ(692)	ND	ND	ND	ND	ND	ND	ND	ND
Case_009	ND	ND	ND	ND	ND	ND	ND	ND	ND
Case_010	ND	ND	**2315**	ND	ND	ND	**11756.0**	ND	ND
Case_011	ND	ND	ND	ND	ND	ND	ND	ND	ND
Case_012A	ND	ND	ND	ND	ND	ND	ND	ND	ND
Case_012B	ND	ND	ND	ND	ND	ND	ND	ND	ND
Case_013_1	ND	ND	ND	ND	ND	ND	ND	ND	ND
Case_013_2	ND	ND	**105**	ND	ND	ND	ND	ND	ND
Case_014	<LLOQ(115)	**423**	**190**	**1157**	ND	**201**	**2248**	ND	**>ULOQ(19434)**
Case_015	ND	ND	ND	ND	ND	**278**	**497**	**63**	**>ULOQ(22326)**
Case_M_016A*	**>ULOQ(22491)**	**12502**	**7194**	**>ULOQ(43792)**	**>ULOQ(44880)**	**12696**	**>ULOQ(42064)**	**2099**	**>ULOQ(1293142)**
Case_M_016B*	**12186**	**13705**	**7680**	**>ULOQ(24194)**	**>ULOQ(36722)**	**12578**	**>ULOQ(37002)**	**3362**	**>ULOQ(1091539)**
Case_M_017	<LLOQ(777)	**249**	**354**	**1531**	ND	**87**	**1914**	ND	**>ULOQ(31939)**
Case_M_018	**7045**	**4798**	**1924**	**>ULOQ(19580)**	**7911**	**2395**	**>ULOQ(19764)**	ND	**>ULOQ(254981)**
Case_M_019	**6889**	**5605**	**2330**	**>ULOQ(17849)**	**5034**	**2251**	**15335**	**88**	**>ULOQ(504873)**
Case_M_020	ND	ND	ND	ND	ND	ND	ND	ND	ND
Case_M_022	<LLOQ (990)	**2728**	**2297**	**>ULOQ(16879)**	**13843**	**11063**	**>ULOQ(22697)**	**3173**	**>ULOQ(452925)**
Case_M_023*	ND	**848**	ND	ND	ND	ND	ND	ND	ND
Case_M_024_1	ND	ND	ND	ND	ND	ND	ND	ND	ND
Case_M_024_2	ND	ND	ND	ND	ND	ND	ND	ND	ND
Case_M_025	ND	ND	ND	ND	ND	ND	ND	ND	ND
Case_M_026*	ND	**1100**	**740**	**14990**	**6540**	**1340**	**15840**	ND	**>ULOQ(171232)**
Case_M_027*	ND	ND	ND	ND	ND	ND	ND	ND	ND
Case_M_028*	ND	**95**	ND	ND	ND	ND	ND	ND	ND
Case_M_030*	ND	ND	**392**	ND	ND	ND	ND	ND	ND
Case_M_031*	ND	ND	ND	ND	ND	ND	ND	ND	ND
Case_M_033	ND	ND	**127**	**2473**	**985**	**810**	**1819**	ND	**>ULOQ(160207)**

Appendix 6-3 continued

Meconium Case No.	Ethyl Laurite (ng/g)	Ethyl Myristate (ng/g)	Ethyl Linolenate (ng/g)	Ethyl Linoleate (ng/g)	Ethyl Palmitoleate (ng/g)	Ethyl Palmitate (ng/g)	Ethyl Oleate (ng/g)	Ethyl Stearate (ng/g)	Ethyl Arachidonate (ng/g)
Case_M_034	ND	ND	**89**	ND	ND	ND	**141**	ND	ND
Case_M_038	ND	**162**	ND	ND	ND	ND	ND	ND	ND
Case_M_039	<LLOQ (2581)	**4683**	ND	**5816**	**2503**	**1495**	**5603**	ND	**>ULOQ(150522)**
Case_M_038	ND	**162**	ND	ND	ND	ND	ND	ND	ND
Case_M_039	<LLOQ (2581)	**4683**	ND	**5816**	**2503**	**1495**	**5603**	ND	**>ULOQ(150522)**
Case_M_038	ND	**162**	ND	ND	ND	ND	ND	ND	ND
Case_M_039	<LLOQ (2581)	**4683**	ND	**5816**	**2503**	**1495**	**5603**	ND	**>ULOQ(150522)**
Case_M_041A	ND	**1545**	**605**	ND	ND	ND	ND	ND	ND
Case_M_041B	ND	**208**	ND	ND	ND	ND	ND	ND	**3370**
Case_M_043A	<LLOQ(1192)	**825**	**160**	**182**	**171**	**1018**	**825**	ND	**>ULOQ(50079)**
Case_M_043B	<LLOQ(998)	**330**	**214**	**1080**	ND	**1312**	**2240**	**156**	**11879**
Case_M_044	**>ULOQ(30189)**	**5143**	**1173**	**11404**	**518**	**611**	**14990**	ND	**>ULOQ(53868)**
Case_M_045	<LLOQ(550)	**372**	ND	**348**	ND	**567**	**1378**	ND	**6049**
Case_M_046	ND	**89**	ND	**385**	ND	**311**	**1758**	ND	**11336**
Case_M_048	ND	ND	ND	ND	ND	ND	ND	ND	ND
Case_M_049	ND	ND	ND	ND	ND	ND	ND	ND	**5445**
Case_M_050	**2751.0**	**3083**	**1544**	**21925**	**1127**	**2052**	**>ULOQ(22432)**	**274**	**>ULOQ(504209)**
Case_M_051	ND	ND	ND	ND	ND	ND	ND	**232**	ND
Case_M_052	ND	ND	ND	ND	ND	ND	ND	ND	**10357**
Case_M_053_1	ND	ND	ND	ND	ND	ND	ND	ND	ND
Case_M_053_2	ND	ND	<LOQ(15)	ND	ND	ND	ND	ND	**115**
Case_M_054	<LOQ(983)	**2311**	**456**	**5320**	**10578**	**5045**	**6518**	<LOQ(42)	**>ULOQ(122928.5)**
Case_M_056	ND	ND	ND	**430**	ND	ND	ND	ND	ND
Case_M_057	ND	**207**	ND	ND	ND	ND	**127**	ND	5315
Case_M_058	<LLOQ(971)	**1110**	**505**	**5211**	**1268**	**3388**	**10149**	**478**	**>ULOQ(59993.82)**
Case_M_059	ND	**217**	ND	**33**	**512**	**547**	**1085**	**155**	**9653**
Case_M_060	<LLOQ(189)	**2226**	**235**	**5202**	**13081**	**604**	**13178**	ND	**>ULOQ(35774)**
Case_M_062	**>ULOQ(172912)**	**>ULOQ(78746)**	**>ULOQ(25803)**	**>ULOQ(135051)**	**>ULOQ(180002)**	**>ULOQ(64782)**	**>ULOQ(332134)**	**4976**	**>ULOQ(4267963)**
Case_M_063	ND	**359**	ND	ND	ND	ND	ND	**396**	**3482**
Case_M_064*	ND	ND	ND	**492**	ND	ND	**433**	ND	ND
Case_M_066*	ND	ND	ND	ND	ND	ND	ND	ND	ND
Case_M_067*	<LLOQ(619)	**1361**	**1142**	**11773**	**5631**	**1690**	**>ULOQ(20761)**	**157**	**>ULOQ(67863)**
Case_M_072*	<LLOQ(799)	ND	ND	ND	ND	ND	ND	ND	<LLOQ(222)
Case_M_073*	<LLOQ(146)	**374**	ND	**665**	**220**	**825**	**2042**	ND	**10516**

Appendix 6-3 continued

Meconium Case No.	Ethyl Laurite (ng/g)	Ethyl Myristate (ng/g)	Ethyl Linolenate (ng/g)	Ethyl Linoleate (ng/g)	Ethyl Palmitoleate (ng/g)	Ethyl Palmitate (ng/g)	Ethyl Oleate (ng/g)	Ethyl Stearate (ng/g)	Ethyl Arachidonate (ng/g)
Case_M_074*	<LLOQ(1228)	**1178**	**853**	**9814**	**3950**	**1482**	**>ULOQ(20252)**	**228**	**>ULOQ(48773)**
Case_M_075	ND	ND	ND	ND	ND	ND	ND	**144**	ND
Case_M_076	ND	**205**	ND	**312**	**692**	**906**	**2538**	ND	**>ULOQ17860)**
Case_M_077*	ND	ND	ND	ND	ND	ND	ND	ND	ND
Case_M_080	ND	ND	ND	ND	ND	ND	ND	ND	**1084**
Case_M_081	ND	ND	ND	ND	ND	ND	ND	ND	ND
Case_M_083*	ND	ND	ND	ND	ND	ND	ND	ND	ND
Case_M_084	ND	ND	ND	ND	ND	ND	ND	ND	ND
Case_M_085*	ND	ND	ND	ND	ND	ND	ND	**525**	**6268**
Case_M_086*	ND	ND	ND	ND	ND	ND	ND	ND	**564**
Case_M_087A*	ND	ND	ND	ND	915	ND	ND	**302**	ND
Case_M_087B*	ND	ND	ND	ND	ND	ND	ND	ND	ND
Case_M_088*	ND	ND	ND	ND	ND	ND	ND	**370**	ND
Case_M_089*	**3201**	**3290**	**1849**	**>ULOQ(17963)**	**8350**	**6642**	**>ULOQ(31188)**	**1377**	**>ULOQ(578490)**
Case_M_090*	ND	ND	ND	ND	**815**	<LLOQ(20)	**1646**	ND	**29400**
Case_M_093	ND	ND	ND	ND	ND	ND	ND	ND	**1441**
Case_M_094*	ND	ND	ND	ND	ND	ND	ND	**268**	ND
Case_M_095*	ND	ND	ND	118	ND	ND	**140**	**79**	<LOQ(266)
Case_M_096_1	ND	ND	ND	ND	ND	ND	ND	**192**	ND
Case_M_096_2	ND	ND	ND	ND	ND	ND	ND	ND	ND
Case_M_097	ND	ND	ND	ND	ND	ND	ND	ND	ND
Case_M_098	<LLOQ(58)	ND	ND	ND	ND	ND	ND	**5**	ND
Case_M_099	ND	ND	ND	ND	ND	ND	ND	ND	**726**
Case_M_101	**>ULOQ (194179)**	**>ULOQ(295840)**	**>ULOQ(374173)**	**>ULOQ(532002)**	**>ULOQ(462119)**	**>ULOQ(23020)**	**>ULOQ(283700)**	**4660**	**>ULOQ(8354465)**
Case_M_102	ND	ND	**2507**	ND	ND	ND	**486**	**70**	ND
Case_M_103	ND	ND	**91**	ND	ND	ND	ND	ND	**870**
Case_M_104	<LLOQ (851)	**2297**	**377**	**8579**	**358**	**449**	**5338**	**206**	**>ULOQ(192301)**
Case_M_105	ND	ND	ND	ND	**574**	ND	**973**	ND	ND
Case_M_106	ND	ND	ND	ND	ND	ND	ND	**<LOQ (19)**	**963**
Case_M_107	ND	ND	ND	ND	ND	ND	ND	**205**	**3203**

Appendix 7 Papers and Awards in Support of this thesis

Publications

- Huda M. Hassan and Gail A. A. Cooper. 2009. Determination of Beta-hydroxybutyrate in Blood and Urine using Gas Chromatography-Mass Spectrometry. ***J. Anal. Toxicol. 33:502 - Abstract Only***
  The work presented at the Society of Forensic Toxicology (SOFT) annual meeting, **OKlahoma, USA, October 2009.**

- Maciegj J.Bogusz and Huda M. Hassan, Role of Accreditation Procedures in Maintaining Quality, In: ***"Quality Assurance in the Pathology Laboratory: Forensic, Technical and Ethical Aspects", (Chapter 7) J.B.Maciej, ed., CRC Press, (2011).139-204***

Publication Under Progress and Oral Presentation

- Huda M. Hassan and Gail A. A. Cooper: Method for Quantification of Gamma-Hydroxybutyrate (GHB) in Postmortem Blood by using Gas Chromatography Mass Spectrometry. Presented and in the proceeding of the Fourth Saudi International Conference 2010, SIC10 Held in University of **Manchester**, **UK. July 2010**

- Huda M. Hassan and Gail A. A. Cooper: The Effect of preservative and anti-oxidant on the volatiles in aged reconstituted Ante-mortem blood after short term storage. Abstract and manuscripts were submitted for JAT Special issues for year 2011. ***In progress***
  The work presented at the Society of Forensic Toxicology (SOFT) annual meeting, **Virginia, USA, October 2010.**

- Huda M. Hassan and Gail A. A. Cooper: Simultaneous determination of of ß-hydroxy-ß-methylbutyrate (HMB), beta-hydroxybutyrate (BHB) and gamma-hydroxybutyrate (GHB) in urine and plasma using gas chromatography mass spectrometry (GC-MS**)**. Abstract and manuscripts were submitted for **JAT Special issues for year 2011.** ***In Progress***

- Huda M. Hassan and Gail A. A. Cooper: A Liquid Chromatography Tandem Mass Spectrometry Method for the quantification of FAEE's in Meconium, Presented and in the proceeding of UK & Ireland Association of Forensic Toxicologists (UKIAFT), **Glasgow, UK.September 2010**

- Huda M. Hassan and Gail A. A. Cooper: Evaluation of Internal Standards for the Determination of Common Volatiles by Headspace Gas Chromatography Flame Ionisation Detection**.** ***In Progress*** **for publication**

- Pamela Cabarcos Fernández, Pamela's supervisor,Huda M. Hassan, Karen Scott: Determination of Ethyl Glucuronide in hair using LC/MS/MS**.** ***In Progress*** **for publication**

Appendix 7 continued

Posters

- *Huda M. Hassan and Gail A. A. Cooper: Determination of β-Hydroxybutyrate (BHB) in Blood and Urine using, Gas-Chromatography-Mass Spectrometry (GC-MS),* **IUPAC Glasgow UK, August 2009.**

- *Huda M. Hassan and Gail A. A. Cooper: Evaluation of the Immunalysis Enzymatic Assay for the Quantitative Determination of Ethanol in Post-Mortem Blood,* ***Saudi International Conference - University of Surry June 2009***.

- *Huda M. Hassan and Gail A. A. Cooper: Comparison Study of Ethanol and Acetone Levels in Post-Mortem Blood, Vitreous Humour, Bile and Urine,* ***Saudi International Conference (SIC) - University of Surry June 2009***.

- *Huda M. Hassan and Gail A. A. Cooper: Stability of Ethanol, Acetone, Iso-propanol and Methanol in Human Blood,* ***50 the Years Anniversary of Forensic Toxicology department – University of Glasgow March 2009***.

Awards

- **Educational Research Award (ERA) -** Society of Forensic Toxicology(SOFT) annual meeting, Oklahoma City, USA, October 2009.
  Huda M. Hassan and Gail A. A. Cooper (*200)9. Determination of BHB in Blood and Urine using GC-MS*

- **Silver Award (Best Research and Scientific paper)** – 4th Saudi International Conference 2010. SIC2010 University of Manchester 30-31 July 2010.
  Huda M. Hassan and Gail A. A. Cooper: Method for Quantification of GHB in Postmortem Blood using GC-MS. (2010)

- **Distinguished Researcher** – 4th Saudi International Conference 2010 University of Manchester 30-31 July 2010

- **Best Poster (UKIAFT)** – UKIAFT AGM, Aberdeen 27-28 July 2011
  Claire Parks, Huda M. A. Hassan and Gail A. A. Cooper (2011). Correlation of Volatile Concentrations in Biological Matrices.

Journal of Analytical Toxicology, Vol. 33, October 2009

Determination of β-Hydroxybutyrate in Blood and Urine Using Gas Chromatography–Mass Spectrometry

Huda M.A. Hassan* and **Gail A.A. Cooper**
Forensic Medicine and Science, University of Glasgow, Glasgow G12 8QQ, Scotland, United Kingdom

Abstract

β-Hydroxybutyrate (BHB) is considered a potential biomarker for alcoholic ketoacidosis (AKA). A robust and sensitive method was developed and validated for the quantitative determination of BHB in postmortem blood and urine using deuterated γ-hydroxybutyrate as an internal standard. Samples were analyzed by gas chromatography–mass spectrometry following liquid–liquid extraction and silyl derivatization. The limits of detection and lower limits of quantification in blood and urine were 2 and 7 mg/L and 2 and 6 mg/L, respectively. The interday and intraday precision was measured by coefficients of variation for blood and urine and ranged from 1.0 to 12.4% for quality control samples spiked at 50 and 300 mg/L. The linear range of 50–500 mg/L resulted in an average correlation of $R^2 > 0.99$, and the average extraction recoveries in blood and urine were ≥ 82% and ≥ 59%, respectively. BHB remains stable in blood spiked at a concentration of 300 mg/L for 15 days when stored within a refrigerator (2–5°C). Postmortem blood and urine samples were analyzed using the validated method for cases where the deceased had a history of chronic alcohol abuse to establish the use of BHB as a potential marker of AKA.

Introduction

Alcohol biomarkers have the potential to be indicators for chronic and acute alcohol consumption and can be detected in the body long after the elimination of ethanol, which decreases rapidly over time (1). β-Hydroxybutyrate (BHB) is one of the ketone bodies and is considered a potential biomarker for alcoholic ketoacidosis (AKA) (2–4).

Ketone bodies are created at moderate levels in human bodies. A high concentration of ketones reduces the pH of the body and causes ketoacidosis. Ketone bodies are formed by the breakdown of fatty acids and deamination of amino acids; the common ketone bodies produced in the body are acetone, acetoacetate, and BHB as illustrated in Figure 1. Ketoacidosis can occur in starvation, diabetic ketoacidosis (DKA), and AKA.

In many sudden deaths among individuals with a history of alcohol abuse, the cause of death is undetermined following the postmortem examination and associated investigations (e.g., histology, microbiology, and toxicology). Low or absent blood ethanol along with elevated levels of acetone do not in themselves determine the cause of death, but these findings in combination with a history of chronic alcohol abuse suggest AKA (5).

DKA is associated with Type 1 diabetes and is related to insulin deficiency and hyperglycemia. Insulin controls the level of glucose in the body; if it is low, it causes a glucose overload, dehydration, and then acidosis. AKA is associated with chronic ethanol abuse and decreased food intake. AKA can lead to hypoglycemia, and in some cases, hyperglycemia due to glucosuria and low food intake in chronic alcoholics may occur (4). Other factors that differentiate between AKA and DKA are that the BHB/acetoacetate ratio is higher in AKA versus DKA, 7:1 versus 3:1, respectively, and the ratio of lactate/pyruvate is 19:1 versus 11:1 (6).

Analytical methods originally utilized for the analysis of γ-hydroxybutyrate (GHB) (7) and BHB (8) were modified and optimized for the analysis of BHB in postmortem blood and urine. The aims of this study were to develop a sensitive and

A B C
$HO-CD_2-CD_2-CD_2-C(=O)-O^-Na^+$
D

Figure 1. Ketone bodies acetone (A), acetoacetate (B), BHB (C), and GHB-d_6 (D).

* Author to whom correspondence should be addressed.
E-mail: Huda.Hassan@formed.gla.ac.uk.

Reproduction (photocopying) of editorial content of this journal is prohibited without publisher's permission.

Journal of Analytical Toxicology, Vol. 33, October 2009

robust method for determination of BHB in blood and urine, to investigate the potential of BHB as a biomarker for AKA in medicolegal cases, and to investigate the stability of BHB in blood.

Materials and Methods

Materials

The internal standard used was deuterated GHB (GHB-d_6, 1 mg/mL) as illustrated in Figure 1 and was purchased from Cerilliant (Round Rock, TX). BHB and *O*-bis (trimethylsilyl) trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) were sourced from Sigma Aldrich (Basingstoke, England). Acetonitrile, methanol, and ethyl acetate, all HPLC-grade, and 98% sulfuric acid were all obtained from VWR International (West Chester, PA). Expired packed red blood cell pouches were obtained from the Western Infirmary Blood Bank (Glasgow, Scotland), and urine was obtained from drug-free volunteers.

Preparation of blank blood, working standards, and quality controls

Saline solution was prepared by weighing 9.5 g of sodium chloride into a volumetric flask and dissolving in 1 L of deionized water. The volume of packed red blood cells was measured and diluted 1:1 with saline solution. An intermediate standard was prepared in water at a concentration of 500 mg/L from a stock standard of BHB prepared at a concentration of 2.0 g/L in water. The internal standard solution was prepared at a concentration of 10 mg/L in methanol. Quality control material was prepared by spiking blood and urine at concentrations of 50 and 300 mg/L, respectively.

Specimens

Postmortem blood ($n = 13$) and urine ($n = 12$) were selected from 14 cases where the cause of death was not known and there was a history of alcohol abuse (Table I). Preserved samples were analyzed in preference to unpreserved samples, but these were not available for all cases. The samples collected with preservative (0.2% sodium fluoride) are noted in the table. All unpreserved and preserved samples were stored at 4°C ± 1°C until analysis.

Instrumentation

Gas chromatography–mass spectrometry (GC–MS) was utilized using a Thermoquest Trace GC with X-calibur software (Thermo Fisher Scientific, Leicestershire, U.K.). The column used was a DB-5 capillary column (5% phenyl/95% methylsiloxane, 30 m × 0.25-mm i.d., 0.25-mm film thickness) pur-

Table I. BHB Summary of Case Results

Case	BHB mg/L			Acetone mg/L			Ethanol mg/dL			
No.	Blood	Urine	VH	Blood	Urine	VH	Blood	Urine	VH	History/Cause of Death
1	205	> 500		145*	431	152	19*	81	30	Alcoholic/fatty liver, cause of death undermined
2	343	> 500		35*	452	41	NEG*	NEG	NEG	Fatty liver
3	239	429		694*	780		30*	NEG		Diabetic/possible ketoacidosis or drug/alcohol-related
4		> 500			201	325		12	25	Known alcohol abuser
5	<50	> 500		20*	164		12*	58		Chronic alcohol abuse
6	203	> 500		40*	54		NEG*	NEG		Fatty liver
7	328			387*			NEG*			Diabetic/possible ketoacidosis or drug/alcohol related
8	214*	> 500		19*	89		NEG*	NEG		Alcoholic/fatty liver, cause of death undetermined
9	198*	432		194*	342		12*	NEG		Alcohol abuse
10	138*	323	341	76*	103	71	67*	88	72	Undetermined cause of death, fibrosis
11	233	307		157*	225		340*	538		Liver failure, known alcoholic, smell of alcohol, micronodular cirrhosis, heart disease
12	211			311*			NEG*			Suspected drug-related death/ alcohol abuse but no smell of alcohol
13	246	252		50*	76		297*	347		Found dead in his room
14	267*	> 500		250*	314		NEG*	NEG		Fatty degeneration of the liver, chronic alcohol abuse, fatty liver

* Preserved samples.

chased from Agilent Technologies (Berks, U.K.).

GC–MS conditions

The initial oven temperature was held at 60°C for 2 min, ramped to 180°C at 20°C/min, then ramped to 250°C at 50°C/min, and held for a further 1 min. Total run time was 10.3 min. The source temperature was 200°C; the transfer line temperature was 250°C; the injector base temperature was 250°C using splitless mode, and the carrier gas flow rate was 1.2 mL/min. The mass detector multiplier voltage was 500 V, dwell time was 0.25, and scan range was 40–300.

Data were collected in full scan mode, and the ions monitored were *m/z* 191, and 117 for BHB and *m/z* and 240 for the internal standard GHB-d_6. The ions used for quantification were *m/z* 233 for BHB and *m/z* 239 for GHB-d_6.

Experimental

Blood extraction method

One hundred microliters of blood was transferred to a 2-mL snap top polypropylene microcentrifuge tube, and 100 µL of internal standard (10 mg/L) was added to all samples in addition to 500 µL acetonitrile as the extraction solvent. Samples were vortex mixed for 30 s and then centrifuged at 1500 rpm for 15 min. The upper layer was collected, transferred to a clean vial, and evaporated at 45°C ± 1°C to dryness with nitrogen. For the derivatization step, 75 µL of BSFTA + 1% TMCS was added to all samples, mixed, and heated at 90°C for 10 min. Samples were transferred to GC vials and 2 µL injected on column. Figure 2 illustrates a typical chromatogram and spectra for BHB and the internal standard GHB-d_6 extracted from blood.

Urine extraction method

One-hundred microliters of urine was transferred to a 2-mL snap-top polypropylene microcentrifuge tube. One hundred microliters of internal standard (10 mg/L) and 100 µL of 0.025 M sulfuric acid were added to all samples in addition to 1 mL ethyl acetate as the extraction solvent. Samples were vortex mixed for 30 s and then centrifuged at 1500 rpm for 15 min. The upper layer was collected, transferred to a clean vial, and evaporated at room temperature to dryness with nitrogen. For the derivatization step, 75 µL of BSFTA + 1% TMCS and 25 µL of ethyl acetate were added to all samples, mixed, and heated at 70°C for 30 min. Samples were transferred to GC vials and 2 µL injected on column. Figure 3 illustrates a typical chromatogram and spectra for BHB and the internal standard GHB-d_6 extracted from urine.

Method Validation

Linearity

Calibration standards were prepared by spiking blank blood and urine with BHB at concentrations ranging from 50 to 500 mg/L. The calibration standards were extracted with the postmortem samples and quality control samples. The peak-area ratio of the BHB to the GHB-d_6 was calculated, and the cali-

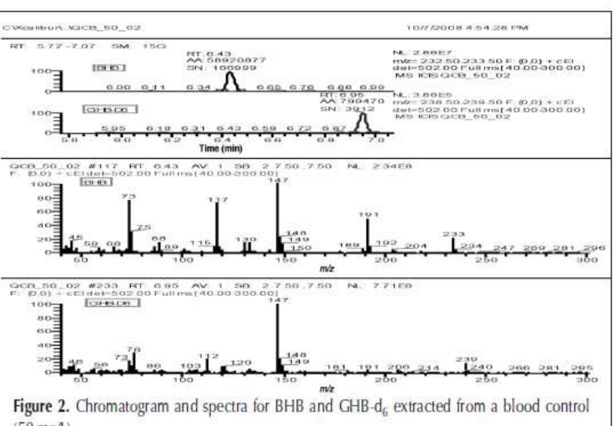


Figure 2. Chromatogram and spectra for BHB and GHB-d_6 extracted from a blood control (50 mg/L).

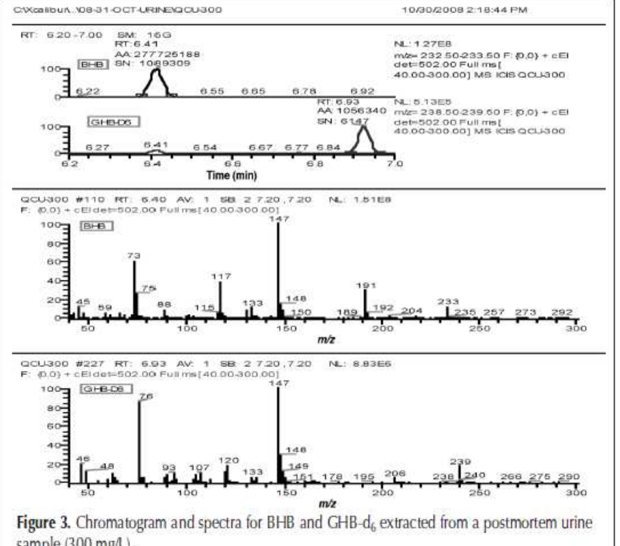


Figure 3. Chromatogram and spectra for BHB and GHB-d_6 extracted from a postmortem urine sample (300 mg/L).

Journal of Analytical Toxicology, Vol. 33, October 2009

bration curve was generated by plotting the area ratio against the concentration (Figure 4). The correlation of coefficient (R^2) should be greater than 0.99.

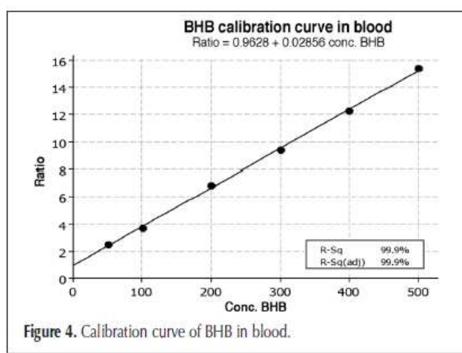


Figure 4. Calibration curve of BHB in blood.

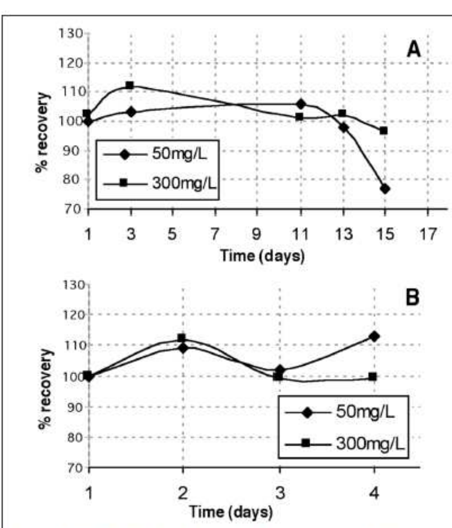


Figure 5. Stability study of spiked blood stored at 2–5°C for 15 days (A) and processed blood samples stored at room temperature for 4 days (B).

Table II. BHB Validation Results

Method	Intraday Precision (CV%)		Interday Precision (CV%)		LOD and LLOQ (mg/L)		Relative Recovery %		Absolute Recovery %	
	50 mg/L	300 mg/L	50 mg/L	300 mg/L	LOD	LLOQ	50 mg/L	300 mg/L	50 mg/L	300 mg/L
Urine	7 (n = 6)	5 (n = 6)	12 (n = 8)	6 (n = 8)	2	6	149	191	73	59
Blood	5 (n = 8)	1 (n = 8)	12 (n = 8)	7 (n = 8)	2	7	98	69	86	82

Limits of detection (LOD) and quantification (LOQ)

The LOD and lower LOQ (LLOQ) were determined by diluting the spiked samples with decreasing concentrations of BHB within the expected range of the LODs (1.25–25 mg/L). Regression analysis was undertaken, and the LOD and LLOQ were calculated in accordance with Miller and Miller (9).

Case samples that are positive for BHB at concentrations that are above or below the linear range (50–500 mg/L) of this method, which is routinely used in the laboratory, are reported as less than the LOQ (< 50 mg/L) or greater than the highest calibrator (> 500 mg/L).

Recovery and matrix effect

The absolute recovery of BHB from blood and urine was assessed by preparing spiked blood and urine controls at concentrations of 50 and 300 mg/L. The controls were extracted as described previously; however, 100 µL of the internal standard at a concentration of 10 mg/L was added prior to evaporation. The absolute recovery was determined by comparing the extracted BHB/GHB-d_6 ratio to that of the unextracted standards at the same concentration. All analyses were carried out in triplicate and the average calculated.

The relative recovery was obtained by extracting water spiked at concentrations of 50 and 300 mg/L, calculating the BHB/GHB-d_6 ratio and comparing that to the ratio calculated for blood and urine, which is expressed as a percentage. All analyses were carried out in triplicate and the average calculated.

The purpose of assessing the absolute recovery was to check the overall efficiency of the system. The relative recovery was carried out to check the matrix effect (10).

Method precision

Intraday precision (within-day precision). Urine (n = 6) and blood (n = 8) spiked controls were prepared at two different concentrations (50 and 300 mg/L) and were then extracted in a batch with case samples and injected on the same day.

Interday precision (between-day precision). Urine and blood spiked controls were prepared at two different concentrations (50 and 300 mg/L) and were then extracted in batches (n = 8) with case samples on different days. The percentage of the coefficient of variation (CV%) was calculated, and the acceptable CV% was < 20%.

Stability studies

Two small studies were carried out to assess the stability of the derivatized extracts and to assess the stability of BHB in blood. The stability of the derivatized extract was evaluated by repeatedly injecting the same two levels (50 and 300 mg/L) of processed samples for four days following derivatization and storage at room temperature (22°C ± 2°C).

The stability of BHB in blood was assessed by extracting replicate spiked samples (50 and 300 mg/L) from the same quality control lot stored at 2–5°C and analyzed at time intervals of day 1, 3, 11, 13, and 15. The percentage re-

covery was calculated from the mean concentration of QC samples injected in day 3, 11, 13, and 15 and compared as a percentage to day one (normalized as 100%). The 95% confidence interval (CI) from the mean concentration ($n = 6$) for day one was calculated, and stability was assumed when the percentage of the mean of the QC samples was within 80–120% as illustrated in Figure 5 (10).

Results and Discussion

Method validation results

Table II summaries the validation data for BHB in urine and blood. The LOD and the LLOQ in blood and urine were 2 and 7 mg/L and 2 and 6 mg/L, respectively, for BHB. The interday and intraday precision was measured by CV% for blood and urine and ranged from 1.0 to 12.4% for quality control samples spiked at 50 and 300 mg/L. The linear range of 50–500 mg/L resulted in an average correlation of $R^2 > 0.99$ (Figure 4). The absolute recovery of BHB from blood was ≥ 82%, and the relative recoveries were 98% and 69% at concentrations of 50 and 300 mg/L, respectively. The recovery of BHB from blood using the described method is not adversely affected by the matrix.

The absolute recovery of BHB from urine was ≥ 59%, and the relative recoveries were 149% and 191% at concentrations of 50 and 300 mg/L, respectively. The recovery of BHB using the described method was significantly lower from water than urine.

The absolute recovery of BHB at the higher concentration of 300 mg/L was lower for both blood and urine raising questions in relation to the efficiency of the method. However, both methods demonstrated excellent linearity over an extended calibration of 50–500 mg/L.

Table III. Stability Data Spiked Blood Stored at 2–5°C for 15 Days

Day	Recovery %	
	50 mg/L	300 mg/L
1	100	100
3	103	117
11	106	105
13	98	106
15	77	100

Table IV. Processed Samples Stored at Room Temperature for 4 Days

Day	Recovery %	
	50 mg/L	300 mg/L
1	100	100
2	109	112
3	102	99
4	113	99

Stability of BHB

Table III and IV summarize the stability data for BHB in blood and BHB following derivatization. The initial study to investigate the stability of BHB in blood was conducted over a period of 15 days. Although there was some variation in the measured concentrations, BHB remained stable in blood spiked at a concentration of 300 mg/L for 15 days when stored within a refrigerator (2–5°C) as illustrated in Figure 5A. There was some loss of BHB in the control samples spiked at 50 mg/L on day 15, and a longer stability study is required to investigate this further. BHB remains stable for approximately four days at room temperature after derivatization (Figure 5B).

Case studies

Fourteen cases (Table I) were investigated for BHB with blood ($n = 13$) and urine ($n = 12$) samples predominantly available for analysis, but one vitreous humor sample was also available and analyzed. The cases were selected where levels of acetone were high, and the cause of death was either undetermined or the deceased had a history of chronic alcohol abuse. Two of the 14 cases were known diabetics (Cases 3 and 7), but in all cases, the deceased had a history of alcohol abuse, and postmortem findings included fatty degeneration or fibrosis of the liver.

BHB levels in postmortem blood ≥ 260 mg/L have been associated with fatalities attributed to AKA (4). BHB was elevated within the toxic or fatal ranges in blood for all the cases investigated except one (Case 5) where the BHB level was normal < 50 mg/L. The range of BHB in urine in all 12 cases was between 252 and > 500 mg/L.

Blood acetone levels were elevated in all cases, although in five cases acetone levels were ≤ 50 mg/L. Ethanol levels in all case samples were low or less than the LOD, except for samples from Cases 11 and 13 where the ethanol level for both urine and blood samples was high.

Conclusions

A robust and sensitive method for the analysis of BHB in postmortem blood and urine was validated. The method was utilized to investigate the stability of BHB in blood and the potential of BHB as a biomarker for AKA. BHB was found to be stable in blood for 15 days when stored in the refrigerator, and initial findings support the use of BHB as a biomarker for AKA. The presence of BHB in postmortem blood could provide supporting evidence in deaths associated with chronic alcohol consumption, but further work is required to fully investigate the significance of these findings.

References

1. A. Helander, O. Beck, and A.W. Jones. Laboratory testing for recent alcohol consumption: comparison of ethanol, methanol, and 5-hydroxytryptophol. *Clin. Chem.* **42:** 618–624 (1996).
2. S. Felby and E. Nielsen. Determination of ketone-bodies in post-

mortem blood by head-space gas chromatography. *Forensic Sci. Int.* **64:** 83–88 (1994).
3. J.L. Thomsen. Various mechanisms of death and their possible association with alcoholism. *Forensic Sci. Int.* **79:** 199–204 (1996).
4. P.X. Iten and M. Meier. Beta-hydroxybutyric acid—an indicator for an alcoholic ketoacidosis as cause of death in deceased alcohol abusers. *J. Forensic Sci.* **45:** 624–632 (2000).
5. F.C. Kugelberg and A.W. Jones. Interpreting results of ethanol analysis in postmortem specimens: a review of the literature. *Forensic Sci. Int.* **165:** 10–29 (2007).
6. G.E. Umpierrez, M. DiGirolamo, J.A. Tuvlin, S.D. Isaacs, S.M. Bhoola, and J.P. Kokko. Differences in metabolic and hormonal milieu in diabetic- and alcohol-induced ketoacidosis. *J. Crit. Care* **15:** 52–59 (2000).
7. S. Elliott, P. Lowe, and A. Symonds. The possible influence of micro-organisms and putrefaction in the production of GHB in post-mortem biological fluid. *Forensic Sci. Int.* **139:** 183–190 (2004).
8. C. Mathers. St. George's University of London. The development of a method for the detection of β-hydroxybutyrate in postmortem samples, 2007.
9. J.N. Miller and J.C. Miller. Calibration methods in instrumental analysis: regression and correlation. In *Statistics and Chemometrics for Analytical Chemistry*, 4th ed. Prentice Hall, New York, NY, 2000, pp 107–150.
10. F.T. Peters, O.H. Drummer, and F. Musshoff. Validation of new methods. *Forensic Sci. Int.* **165:** 216–224 (2007).

Role of Accreditation Procedures in Maintaining Quality

7

MACIEJ J. BOGUSZ AND HUDA HASSAN

Contents

Abbreviations 140
7.1 Definitions of Accreditation 141
7.2 Role and Aims of Accreditation Components in Pathology and Laboratory Medicine 143
  7.2.1 Role of Proficiency Testing 143
  7.2.2 Role of Inspection 147
  7.2.3 Role of Quality Management 149
    7.2.3.1 Quality Management Plan 149
    7.2.3.2 Requirements of CAP, ISO 15189, and ISO/EC 17025 Regarding QM 150
7.3 Legal Frames, Regulations, and Accreditation Bodies in Pathology, Laboratory Medicine, and Related Areas 153
  7.3.1 International Standards of ISO 153
    7.3.1.1 Development of ISO 17025 and ISO 15189 156
    7.3.1.2 Relationship between ISO 9001, ISO 17025, and ISO 15189 156
    7.3.1.3 Management Requirements of ISO 15189 157
    7.3.1.4 Technical Requirements of the ISO 15189 162
    7.3.1.5 Technical Requirements of the ISO/IEC 17025:2005 166
    7.3.1.6 Accreditation of Testing Laboratories and Medical Laboratories According to ISO 17025 and/or ISO 15189 168
  7.3.2 International Accreditation Organizations 172
    7.3.2.1 International Laboratory Accreditation Cooperation 172
    7.3.2.2 International Accreditation Forum 173

The Effect of Preservative and Anti-oxidant Reagents in Aged Reconstituted Ante-mortem Blood after Short- Term Storage

Huda M. Hassan and Gail A. A. Cooper
Forensic Medicine and Science, School of Medicine,
University of Glasgow, G12 8QQ, Scotland
e.mail:Huda.Hassan@formed.gla.ac.uk:
web.mail: hassanh11@googlemail.com

Abstract

The stability of common volatiles was investigated under controlled conditions over a period of 50 days. Blood was spiked with ethanol, methanol, isopropanol and n-propanol at 20 and 80 mg/dL and acetone at 20 and 80 mg/L. Aliquots were stored at 25, 4, and -22 ºC with or without a preservative (sodium fluoride) or antioxidant (sodium metabisulphite). Samples were analysed using headspace gas chromatography with a flame ionisation detector (HS-GC-FID and utlising t-butanol as internal standard. Acetone levels increased while isopropanol and n-propanol levels decreased in both preserved and unpreserved samples at room temperature and when refrigerated. Methanol remained stable in the presence of a preservative and antioxidant in all conditions. Formation of ethanol was observed in samples stored at room temperature or refrigerated without preservative. All volatiles were stable when stored in the freezer.

An evaluation of the stability of ethanol in real case samples was also undertaken. Blood samples collected under Section 5 of the Road Traffic Act 1988 are stored in vials containing both preservative and anticoagulant. A total of 219 blood samples received within Forensic Medicine and Science at the University of Glasgow over a period of 5 years were initially analysed and refrigerated until the case was heard in court. Following completion the samples were removed from the refrigerator and stored at room temperature for varying periods of time spanning 5 years. A loss of on average 30% of the ethanol content was observed when the samples were re-analysed.

Finally, seventy-one paired preserved and unpreserved post-mortem blood samples stored in the refrigerator for six months were re-analyzed. Loss of ethanol was observed in approximately 50% of the 71 preserved samples and 40% of unpreserved samples. An increase in ethanol concentration of between 34 to 55% was observed in approximately 7% of the preserved samples and 25% of unpreserved samples after 6 months of storage in a refrigerator.

Key words: volatiles, stability, preservative

Simultaneous determination of of ß-hydroxy-ß-methylbutyrate (HMB), beta-hydroxybutyrate (BHB) and gamma-hydroxybutyrate (GHB) in urine and plasma using gas chromatography mass spectrometry (GC-MS)

Huda M. Hassan and Gail A. A. Cooper
Forensic Medicine and Science, School of Medicine,
University of Glasgow, G12 8QQ, Scotland
e.mail:Huda.Hassan@formed.gla.ac.uk:
web.mail: hassanh11@googlemail.com

Abstract

β-hydroxy-β-methylbutyrate (HMB) or β-hydroxyisovaleric acid (β-HIV) is available as a nutritional supplement and is taken to increase strength and muscle mass in humans involved in resistance training programmes. Although in 1999 the International Olympic Committee's medical advisory board categorized HMB as a legal substance the potential for misuse is considerable.

The aims of this study were, to develop and validate a method for the determination of HMB in plasma and urine and to simultaneously determine other structurally related compounds, such as BHB and GHB and apply this method to analyse real case samples.

A GC-MS method was developed for the simultaneous determination of HMB, BHB and GHB in plasma and urine using deuterated GHB-d_6 as an internal standard. The method was linear over a range of 10-500 mg/L with a correlation coefficient (R^2) greater than 0.99. The inter-day and intra-day precision was measured in both matrices at less than 10%. The recovery and accuracy ranged between 42-77% and 98-102% respectively for all analytes in both plasma and urine. The limits of detection (LOD) for HMB, GHB and BHB were 2.3, 1.0 and 2.2 mg/L in urine respectively, and 2.3, 1.5 and 0.9 mg/L in plasma. The lower limits of quantification (LLOQ) were 7.7, 3.3 and 7.3 mg/L and 7.8, 5.1 and 2.9 mg/L respectively, in urine in plasma.

Keywords: β-hydroxy-β-methylbutyrate (HMB) or β-hydroxyisovaleric acid (β-HIV); β-hydroxybutyrate (BHB); gamma-hydroxybutyrate (GHB); Plasma; Urine

A Liquid Chromatography Tandem Mass Spectrometry Method for the Quantification of Fatty Acid Ethyl Esters in Meconium.

Huda M. Hassan and Gail A. A. Cooper
Forensic Medicine and Science, School of Medicine,
University of Glasgow, G12 8QQ, Scotland
e.mail:Huda.Hassan@formed.gla.ac.uk:
web.mail: hassanh11@googlemail.com

Abstract

Babies born from mothers who consume alcohol during pregnancy are at elevated risk to have Neonatal alcohol related birth defects (ARBDs), Foetal Alcohol syndrome (FAS) and alcohol-related neurodevelopment disorders (ARNDs). To facilitate earlier diagnosis and treatment and to prevent undesirable outcomes, screening for fatty acid ethyl esters as alcohol biomarkers could confirm maternal alcohol consumption. Foetal exposure of alcohol can cause central nervous system dysfunction, pre- and postnatal growth problems, cardiac defects in neonates and attention deficit disorders may leads to mental retardations.

A Liquid Chromatography Mass spectrometry tandem MS (LC/MS/MS) method have been developed and validated for detection and quantification of fatty acids ethyl esters in meconium includes: ethyl lenolenate (E18:3), ethyl linoleate (E18:2), ethyl palmitoleate (E16:1), ethyl palmitate (E16:0), ethyl oleate (E18:1), ethyl stearate (E18:0) and ethyl arachidonoate (E20:4). The internal standards used were deuterated includes ethyl oleate-D5, ethyl steareate-D5 and ethyl palmitate-D5.

The method have been applied to eighty four real neonatal meconium case samples collected from Glasgow Royal Infirmary in order to investigate the neonatal exposure to alcohol in the absence of maternal drinking history. Results have been compared with the published data to identify the most specific and sensitive fatty acid ethyl ester biomarker. The cut-off point to distinguish the positive from negative was also evaluated

Key Words: Meconium, Fatty acid ethyl esters, Alcohol, LC/MS/M

Internal Standards for the Determination of Common Volatiles by Headspace Gas Chromatography Flame Ionisation Detection.

Huda M. Hassan and Gail A. A. Cooper
Forensic Medicine and Science, School of Medicine,
University of Glasgow, G12 8QQ, Scotland
e.mail:Huda.Hassan@formed.gla.ac.uk:
web.mail: hassanh11@googlemail.com

Abstract

The most common method for sensitive and precise analysis of ethanol and other volatile in biological fluids and tissues is Gas Chromatography. This methods can also distinguish between ethanol and other volatiles and by using dual column system and it is fitted with dual column capability and provides two sets of data that can used in combination with increased confidence to identify analytes of interest, since it reduces the number of false positive results due to other post-mortem volatiles that may be eluting in the same retention time. . Head Space - Gas Chromatography copelled with Flame Ionization Detector (HS-GC-FID) technique is common in forensic and toxicology laboratories for medico-legal investigations and the interpretation of toxicology results.

A wide range of volatile compounds may be produced in post-mortem specimens. n-propanol is amongst these and is considered to be an indicator of putrefaction. Several studies have therefore recommend that n-propanol should not be used as an internal standard for quantification of alcohol by (HS-GC-FID). A comparison between two validated procedures and evalauation of dual column system used have been carried out using n-propanol and t- butanol as an internal standards. The two validated methods used were robust, sensitive, and fully validated using GC-FID-HS. The correlation coefficients (R^2) were >0.999 over the linear range of 10–500 mg/dL (and mg/L for acetone). The inter-day and intra-day precision was between 1-5% for all analytes in all methods used.

One hundred and twenty one putrefied post-mortem blood samples received within laboratories of Forensic Medicine and Science were analysed using both methods. Evaluation of other volatile levels mainly the amount of n-propanol and it is relation with ethanol level in post-mortem blood have been evaluated. The putrefied post-mortem blood samples produced several results in which n-propanol was present at a level that could be viewed as a result of putrefaction. It might tend to underestimate the concentration of other volatiles if n-propanol is used as an internal standard in post-mortem samples that are positive for n-propanol.

Key words: volatiles, n-propanol, ethanol, putrefaction

Quatification of Gamma-Hydroxybutyrate (GHB) in Postmortem Blood by using Gas Chromatography Mass Spectrometry.

Huda M. Hassan and Gail A. A. Cooper
Forensic Medicine and Science, School of Medicine,
University of Glasgow, G12 8QQ, Scotland
e.mail:Huda.Hassan@formed.gla.ac.uk:
web.mail: hassanh11@googlemail.com

Abstract

Gamma–hydroxybutyric acid (GHB) and its precursors, gamma butyrolactone (GBL) and 1, 4 butanediol (DB), are increasingly popular drugs of abuse. GHB misuse and accidental overdosing may lead to death and GHB has been detected in post-mortem blood in concentrations ranging from 3.2 – 168 mg/L in cases with no previous history of GHB use (Fieler, Coleman and Baselt, 1998). A sensitive and specific method for the determination of GHB in blood and urine was modified from a method previously validated and published (Hassan *et al.* 2009) using GC-MS. The method has been applied to identify GHB-related fatalities in postmortem cases and to evaluate the levels in postmortem cases with no known history of GHB intoxication.

Keywords: GHB; GC-MS; Postmortem; Blood; Urine

Saudi International Conference
SIC 04

Saudi minds in action

CERTIFICATE OF WINNING

This Certificate is Awarded to :

Huda Hassan

Silver Paper Award

in Health & Medical Science

At The Fourth Saudi International Conference 2010, SIC04

Held at **University of Manchester**
Great Manchester, the United Kingdom.

30th and 31st of July 2010

Turki Binshalhoub
SIC04 Conference Chair

Prof. Ghazi AlMakky
Saudi Arabian Cultural Attaché

Saudi International Conference
SIC 04

Saudi minds in action

ACKNOWLEDGMENT

This Certificate is Awarded to :

Huda Hassan

Distinguished Researcher

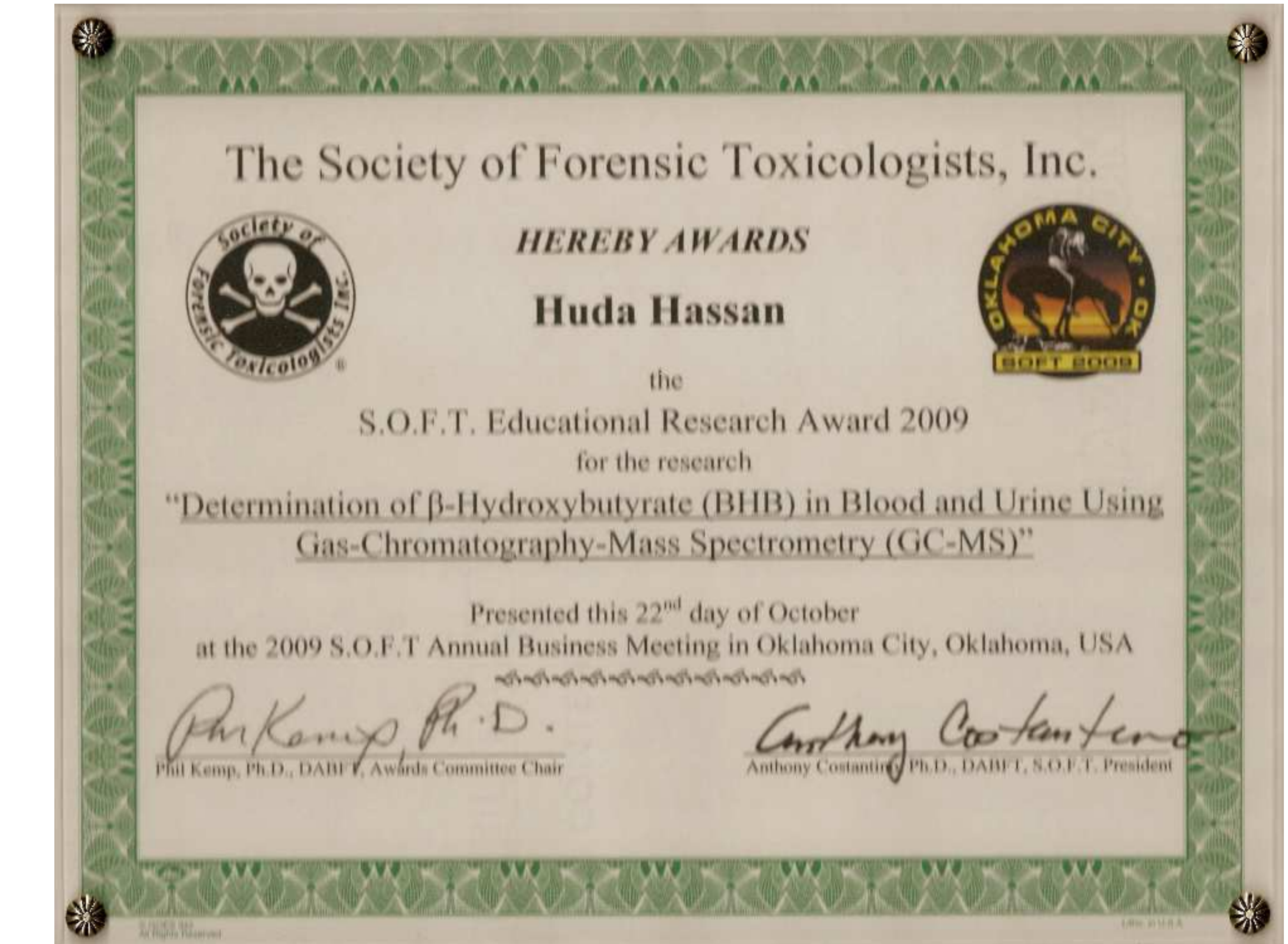
In Recognition for Your
Valuable contribution at
The Fourth Saudi International Conference
2010, SIC04

Held at **University of Manchester**
Great Manchester, the United Kingdom.

30th and 31st of July 2010

Turki Binshalhoub
SIC04 Conference Chair

Prof. Ghazi AlMakky
Saudi Arabian Cultural Attaché



The Society of Forensic Toxicologists, Inc.

HEREBY AWARDS

Huda Hassan

the

S.O.F.T. Educational Research Award 2009

for the research

"Determination of β-Hydroxybutyrate (BHB) in Blood and Urine Using Gas-Chromatography-Mass Spectrometry (GC-MS)"

Presented this 22nd day of October
at the 2009 S.O.F.T Annual Business Meeting in Oklahoma City, Oklahoma, USA

Phil Kemp, Ph.D., DABFT, Awards Committee Chair

Anthony Costantino, Ph.D., DABFT, S.O.F.T. President