Pure

Bond University

MASTER'S THESIS

Amphetamine-like compounds in pre-workout supplements

Koh, Andy

Award date:
2017

Link to publication

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal.

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 09. Oct. 2026

Amphetamine-like Compounds in Pre-Workout Supplements

By

Andy Hsien Wei Koh

Submitted in total fulfilment of requirements of the degree of

Masters of Science by Research (Health Sciences)

January 2017

Faculty of Health Sciences and Medicine
Bond University,
Queensland, Australia

Assistant Professor Anna Lohning and Professor Russ Chess-Williams

Thesis Summary

Pre-workout supplements (PWS), like most nutritional supplements, are classified by the Therapeutic Goods Administration (TGA) in Australia as complementary medicines and are therefore subject to much less stringent regulation compared to pharmaceuticals. Complementary medicines (also known as 'traditional' or 'alternative' medicines) include vitamin, mineral, herbal, aromatherapy and homoeopathic products.

PWS comprise a group of sports supplements purported to provide consumers with a boost in athletic performance facilitated by the inclusion of stimulatory compounds usually of plant origin. Typically, PWS are multi-component in nature generally containing caffeine, an amine-based central nervous system (CNS) stimulant, taurine, β-Alanine and creatine (Eudy et al., 2013). The stimulants cause cardiovascular stress when combined with exercise (Haller et. al, 2002). The increase in PWS-related adverse effect reports correlates with the increased use/prevalence in the community.

The overall goal of this thesis was to determine the amounts of stimulants in PWS, both the biogenic amines derived from *Citrus aurantium* (CA) extracts as well as caffeine and its dimethylxanthine (DMX) derivatives. Research questions have been put forward relating to the components within PWS and whether they comply with any guidelines or regulatory limits and whether differences are evident between PWS made in Australia compared to overseas. To address these questions, the aims were to develop two HPLC protocols for the quantitation of 1) adrenergic amines present in CA-containing PWS and 2) caffeine and DMXs in PWS. These were the first protocols to implement a single quadrupole mass detector (QDa) for rapid mass confirmation in-line with UV-Vis detection on a reverse phase-high performance chromatography (RP-HPLC) system for detection of these compounds.

It was hypothesised that the caffeine content in overseas-manufactured PWS would be higher than locally-produced PWS. This was found not to be the case and, in fact, variations in caffeine content were minimal across the sample set. Significant discrepancies were found, however, between determined levels of caffeine in some products and that stated on their label.

A highlight of this study was the development of a RP-HPLC protocol able to resolve the isobaric isomers, theophylline (TP) and paraxanthine (PX), not normally demonstrated in other similar reported methods. PX, though not a constituent of CA, was found in one of the overseas PWS. Conclusive answers to the research questions were limited by the small sample size of CA-containing PWS in this study. For example, no significant differences in synephrine levels between locally and internationally manufactured PWS were observed in this pilot study. Furthermore, when the active compounds were labelled as a 'proprietary blend' this posed a challenge to accurately compare the quantities of the key ingredients. Future research into the quantification of these amines and other synthetic stimulants on a hydrophilic interaction liquid ion chromatography (HILIC) could result in greater sensitivity of the methods especially when coupled to a mass detector.

Declaration of Authorship

This thesis is submitted to Bond University in fulfilment of the requirements of the degree of Masters of Science (Research). This thesis represents my own original work towards this research degree and contains no material that has previously been submitted for a degree or diploma at this University or any other institution, except where due acknowledgement is made.

…….

Andy Hsien Wei, Koh

January 2017

Research Output

Journal Article Publications

Koh A.H.W., Chess-Williams R., Lohning A. E. (2016) A Rapid High-Performance Liquid Chromatography-Mass Detection Assay for Caffeine and Dimethylxanthines in Pre-workout Supplements, Journal of Chromatography B (in submission)

Ryan P., **Koh A.H.W.**, Lohning A.E., Rudrawar S. (2017) Solid-Phase O-Glycosylation with Glucosamine derivative for the Synthesis of Glycopeptide, New Journal of Chemistry (in submission)

Koh A.H.W., Chess-Williams R., Lohning A.E. (2017) HPLC-UV/MS detection of Adrenergic Amines in *Citrus aurantium* containing Pre-workout Supplements , Journal of Chromatography B (in submission)

Conference Proceedings

Koh A., Chess-Williams R., Lohning A. (2016) A Rapid HPLC-MS Assay for Caffeine and Dimethylxanthines in Pre-workout Supplements, ACROSS International Symposium on Advances in Separation Science, TAS (30/11/2016)

Koh A., Chess-Williams R., Lohning A. (2016) A Rapid HPLC-MS Assay for Caffeine and Dimethylxanthines in Pre-workout Supplements, Australian Society for Medical Research Symposium, QLD (13/11/2016)

Koh A., Chess-Williams R., Lohning A. (2016) Development and Validation of a Rapid HPLC-UV/MS Assay for Determining Caffeine in Pre-workout supplements, Bond University Higher Degree Research Conference, Bond University, QLD (12/10/2016)

Koh A., Chess-Williams R., Lohning A. (2015) Caffeine Content in Ephedra Free Pre-workout supplements, Gold Coast Health and Medical Research Conference, QLD (7/12/2015)

Koh A., Chess-Williams R., Lohning A. (2015) Caffeine Content in Ephedra Free Pre-workout supplements, Bond University Higher Degree Research Conference, Bond University, QLD (18/11/2015)

Acknowledgements

Words cannot describe how I feel to reflect on these past two years. I started this journey with the youth and enthusiasm of any 20-year-old. Throughout my candidature, I have gained many skills and have grown not only as a student, but as a person. It was a memorable experience that cannot be complete without the following acknowledgements.

First and foremost, I would like to thank my supervisors, Dr. Anna Lohning and Professor Russ Chess Williams. I am grateful for your time, efforts and patience with me. Anna's everlasting enthusiasm and kindness will always be cherished. Russ's calmness and wisdom was the one of the many keys to this project's success. Thank you to the both of you for being my muse to stride further into the tough world of research.

Next, I would like to acknowledge the team from Waters Australia, Adam Rhode, Martin Hinton and Jo Ford. Thank you so much for guiding me through the inner workings of the HPLC. You have opened my eyes to the world of chromatography and I will never take it for granted.

To my loving parents, thank you so much for supporting me through my education since my undergraduate days at Bond University. To my mother, I hope that by the time you read this you will be in the best picture of health. Your strength to endure so much while I am away had inspired me to be the best I can. To my father, I hope I made you proud! To my brothers, thank you for support from many kilometres away.

I'd like to acknowledge the many friends that played many parts of my time here at Bond. It was an amazing opportunity to work with many of you. To the other PhD students in room 5_1_25, I will never forget you nor the words of kindness you had given me to go through this process. Kepada kawan-kawanku, guru-guruku dan keluargaku di Malaysia, terima kasih kepadamu. Tanpa sokongan anda, saya tidak boleh mencapaikan impian ini.

阿姨: 谢谢您在我学习上给我的支持。我永远不会忘记您为我做的每一顿饭。您做的饭是最好的，我非常感激您对我的帮助。谢谢！

Finally, I would like to acknowledge my girlfriend, Jessie, for being there for me when I need you the most. Through my moments of joy, to the long nights of writing and editing, you have been there from the very start. Thank you for believing in me.

"Behind every great man, is a woman rolling her eyes."

-Jim Carrey

Table of Contents

Thesis Summary I
Declaration of Authorship III
Research Output V
Acknowledgements VII
Table of Contents IX
List of Figures XIII
List of Tables XV
List of Abbreviations XVII
Chapter 1 Introduction 1
1.1 Pre-workout Supplements – Background, Use and Regulation 3
1.1.1 Common Herbal Extracts Found in PWS 7
1.1.1.1 *Ephedra Sinica* (ES) 7
1.1.1.2 *Acacia rigidula* (AR) 8
1.1.1.3 *Citrus aurantium* (CA) 8
1.1.2 Current Regulations of PWS in Australia (TGA) Compared to Overseas . 9
1.2 Structural and Chemical Properties of Key Stimulants in PWS 14
1.2.1 Amphetamine-like Compounds 14
1.2.2 Caffeine and Dimethylxanthines 16
1.3 Pharmacological Properties of Key Stimulants in PWS. 17
1.3.1 Pharmacodynamics of The Adrenergic Amines in CA 17
1.3.2 Pharmacokinetics of Adrenergic Amines in CA 20
1.3.3 Pharmacology of Caffeine and Dimethylxanthine Derivatives 22
1.4 Quantitative Analysis of Components in PWS 25
1.4.1 HPLC Analysis of Active Components of CA in Dietary Supplements .. 29
1.4.2 HPLC Analysis of Caffeine & Derivatives 33
1.5 Research Questions 34
1.6 Hypotheses 34
1.7 Aims 34
Chapter 2 General Methods 35
2.1 General Principles of Chromatography 37
2.2 Developing a Systematic Work Flow 42
2.2.1 Sample Preparation 43
2.2.1.1 Liquid- Liquid Extraction (LLE) 44
2.2.1.2 Solid Phase Extraction (SPE) 45

2.2.2 Stationary Phase Selection 46
2.2.3 Mobile Phase Selection 47
2.2.4 pH Modifiers in Mobile Phase 47
2.2.5 Apparatus and Materials 48
2.3 Method Validation 49
2.3.1 Selectivity 49
2.3.2 Precision 49
2.3.3 Accuracy 50
2.3.4 Linearity 50
2.3.5 Limit of Detection and Limit of Quantification 50
2.3.6 S/N Determination Within the Empower 3 Software. 51
2.3.7 The Theory of Internal Standard Use 53
Chapter 3 Development of a Rapid HPLC-MS Protocol for Synephrine, Octopamine and Tyramine in CA Extracts in Pre-workout Supplements 55
3.1 Background 57
3.2 Experimental 59
3.2.1 Chemicals and Reagents 59
3.2.2 Standard and Sample Preparation 59
3.3 Method Development 60
3.3.1 Effect of Column Chemistry 60
3.3.2 Effect of pH 64
3.3.3 Effect of Organic Modifier 67
3.3.4 Effect of Temperature 69
3.3.5 Optimisation of UV-Vis Spectra 70
3.3.6 Optimisation of QDa Mass Detection 72
3.3.7 Optimised HPLC Conditions for CA Separation 78
3.4 Analytical Validation of CA by HPLC/UV 79
3.4.1 Linearity, LOD, LOQ 79
3.4.2 Accuracy & Precision 80
3.4.3 Recovery 81
3.5 Quantification of Adrenergic Amines in CA-containing PWS 82
3.6 Conclusion 83
Chapter 4 A Rapid HPLC-MS Protocol for Caffeine and Dimethylxanthines in Pre-workout Supplements 85
4.1 Background 87
4.2 Experimental 89

4.2.1 Chemicals and Reagents 89
4.2.2 Standard Preparation 89
4.3 Method Development 90
4.3.1 Effect of Column Chemistries 90
4.3.2 Effect of pH 93
4.3.3 Effect of Sampling Rate 94
4.3.4 Effect of Organic Modifier 95
4.3.5 Effect of Temperature 96
4.3.6 Optimisation of the UV-Vis Detector 97
4.3.7 Representative Chromatograms 97
4.3.8 Optimised HPLC/UV-MS Procedure 100
4.4 Method Validation 102
4.4.1 Linearity 102
4.4.2 Detection and Quantification Limits 103
4.4.3 Precision 104
4.4.4 Accuracy 105
4.5 Determination of Caffeine and DMX in PWS samples 106
4.6 Application and Usability of the Method 108
4.7 Conclusion 109
Chapter 5 Discussions, Future Directions and Conclusion 111
5.1 General Discussion 113
5.1.1 Adrenergic Amines Found in CA-containing PWS 115
5.1.2 Caffeine and DMX Found in PWS 119
5.2 Limitations 123
5.3 Future Directions for the Analysis of Stimulants in PWS 124
5.4 Concluding Remarks 127
References 129
Appendices 145

List of Figures

Figure 1 Photograph of Ephedra Sinica 7
Figure 2 Photograph of Citrus aurantium 9
Figure 3 Scheduling of medicines and poisons implemented by the TGA of Australia. Comprehensive breakdown and explanation is shown in Appendix 9. 10
Figure 4 The degradation pathway of trace amines. MAO represents the monoamine oxidases; ADH represents the aldehyde dehydrogenase; PNMT represents the phenylethanolamine-N-methyl transferase 21
Figure 5 (Left) adenosine receptor binding to adenosine which causes nervous activity to slow down. (Right) presence of caffeine that acts as an adenosine antagonist that result in an increased nervous activity (Pang & Ko, 2006) 23
Figure 6 Schematic of a generic HPLC set up 26
Figure 7 Diagrammatic representation of a chromatogram depicting the relationship between retention time (t_R) and the column dead time (t_o) 38
Figure 8 Diagram of method development workflow 42
Figure 9 Example of a typical PWS supplement label 43
Figure 10 Diagrammatic representation of the LLE method used 44
Figure 11 Representative diagram of the SPE procedure with the Oasis HLB cartridge 45
Figure 12 Representative Alliance e2695 set up paired to a UV/Vis detector and a QDa detector 48
Figure 13 Empower 3 software window of noise and drift calculation 51
Figure 14 Empower 3 software of custom field window 52
Figure 15 Comparison of column chemistry: (A) Xselect CSH phenylhexyl (3.5 µm, 4.6 x 100 mm, i.d.) and (B) Xbridge C18 (2.5 µm, 3.0 x 100 mm, i.d.). The standard compounds are represented by: (a) Octopamine; (b) Synephrine; (c) Tyramine; (d) Phenylephrine 60
Figure 16 Eddy diffusion effects can result in peak broadening (sourced: CrawfordScientific) 62
Figure 17 Diagram of analyte interaction on stationary phase pore (sourced: Crawford Scientific) 63
Figure 18 Chromatographs achieved from the three pH conditions: (A) 0.1% Formic acid adjusted to pH 3; (B) (Red) 0.1% Ammonium hydroxide adjusted to pH 10 (Black) 0.15% Ammonium hydroxide adjusted to pH 11. 65
Figure 19 Chromatographs achieved from the two organic modifiers: (A) Acetonitrile as an organic modifier and (B) Methanol as the organic modifier. The standard compounds are represented by: (a) Octopamine; (b) Synephrine; (c) Tyramine; (d) Phenylephrine 68
Figure 20 Chromatogram of octopamine peak at different temperatures on Xbridge column at 242 nm 69
Figure 21 Representative diagram of the λ_{max} determined for synephrine, where λ_{max} =290 nm and 236 nm 70
Figure 22 UV detection on a PDA at pH 11 and 35°C where λ_{max} = 242nm for all amines 71
Figure 23 Chromatograph of matrix blank and standards in a matrix blank with the elution order of Octopamine (t = 1.5 mins), Synephrine (t = 2.5 mins), Phenylephrine (t= 4.0 mins) and Tyramine (t = 4.4 mins). 72

Figure 24 3D chromatographs of synephrine at different cone voltages; 1 V (a); 5 V (b); 15 V (c); 20 V (d); 30 V (e). m/z= 150 (front)and m/z= 168 (back) were observed..... 73
Figure 25 Mass spectra of the detected standards on a QDa with observed fragmentation occurring..... 74
Figure 26 3D plot of the standards present in the spiked matrix blank with the elution order of octopamine (t = 1.5 mins), synephrine (t = 2.5 mins), phenylephrine (t= 4.0mins) and tyramine (t = 4.4 mins) 75
Figure 27 (A) Chromatograph of standards in a CA-containing PWS sample with the elution order of Octopamine (t = 1.5 mins), Synephrine (t = 2.5 mins), Phenylephrine (t= 4.0mins) and Tyramine (t = 4.4 mins). (B) 3D plot of the standards present in the matrix blank. (C) Mass fragments of sample at 15 V..... 77
Figure 28 Standard Curves for synephrine, octopamine and tyramine. Ax = area of response of analyte; AIS= area of IS response; [IS]= concentration of IS..... 80
Figure 29 Comparison of columns: (A) Xselect CSH phenylhexyl (3.5µm, 4.6 x 100mm, i.d.) and (B) T3 HSS C18 (5 µm, 3.0 x 150 mm, i.d.). The standard compounds are represented by: (a) TB; (b) PX; (c) TP; (d) caffeine 91
Figure 30 Chromatographs of caffeine and dimethylxanthine adjusted to pH 3 (0.1% formic acid) and pH 2 (0.1% TFA). The standard compounds are represented by: (a) TB; (b) PX; (c) TP; (d) caffeine 93
Figure 31 Chromatographs of caffeine and DMX at 1 point/sec and 40 point/sec 94
Figure 32 Chromatographs achieved from the two organic modifiers: (A) Acetonitrile as an organic modifier and (B) methanol as the organic modifier. The standard compounds are represented by: (a) TB; (b) PX; (c) TP; (d) caffeine;(e) etofylline..... 95
Figure 33 Chromatogram of the effect of temperature on the separation of caffeine on phenyl-hexyl column 96
Figure 34 Representative chromatograms or caffeine and DMX standards at high concentrations of (100 µg/mL) (top); chromatogram of caffeine present in pre-workout supplement A (middle); and chromatogram of caffeine, TP and PX present The approximate retention times: TB= 3.7 min, PX= 4.6 min, TP= 4.9 min, etofylline (I.S.) = 5.3 min and caffeine= 6.2 min. On phenyl-hexyl column at λ=272 nm 97
Figure 35 3D chromatogram/ mass plot. X-axis =time (min), Y-axis =Intensity of m/z signal, Z-axis = mass to charge ratio (m/z). Caffeine (red); TB, PX, and TP (green); etofylline (blue) is present in standards (left). Whereas only caffeine and etofylline is present in the spiked pre-workout sample A (right)..... 98
Figure 36 Mass detected at individual peaks of compounds, TB, PX, TP, etofylline, and caffeine 99
Figure 37 Chromatogram of standards (top) coupled with the Total Ion Chromatogram (TIC) scanning from a range of 100 Da to 600 Da (middle) and Single Ion recording (SIR) of respective compounds 99
Figure 38 Chromatograph of caffeine and 3 DMX standards at 8 different concentrations..... 102
Figure 39 Intra-day 8 point standard curve for TB. Points represent average of 3 replicates..... 103
Figure 40 Structural simiarities between DMAA and amphetamine 125

List of Tables

Table 1 ingredient formulation of Ephedra-free dietary supplements..... 5
Table 2 Chemical properties of different amphetamine-like compounds 15
Table 3 Chemical properties of caffeine and DMX 16
Table 4 Comparison of HPLC analytical methods for CA analysis..... 30
Table 5 Comparison of chemical properties of synephrine, octopamine and tyramine . 31
Table 6 Comparison of methods for caffeine analysis 33
Table 7 Comparison of column chemistry and dimensions 46
Table 8 Summary of effects of column chemistry on CA amines..... 61
Table 9 Summary of pH conditions respective to their modifier 64
Table 10 Summary of optimised HPLC conditions 78
Table 11 HPLC separation gradient used for UV-MS analysis of caffeine and derivatives..... 78
Table 12 Method calibration data for linearity of synephrine, octopamine and tyramine 79
Table 13 Intra- and Inter-day precision, accuracy and analytical recovery of standards 81
Table 14 Synephrine, Octopamine andTtyramine content in PWS (mg/g) (mean ± RSD %) 83
Table 15 Summary of effects of column chemistry on Caffeine and DMX..... 91
Table 16 Summary of optimised HPLC conditions 100
Table 17 HPLC separation gradient used for UV-MS analysis of caffeine and derivatives..... 101
Table 18 LOD/ LOQ for caffeine and derivatives..... 103
Table 19 Intra-day precision assay of standard and analytical recovery of IS in samples 104
Table 20 Analytical recovery of etofylline (IS) spiking in PWS 105
Table 21 Caffeine and methylxanthine content (mean± S.D., n= 3) in dietary supplements 106
Table 22 Amount of caffeine present compared to respective label contents 107
Table 23 Synephrine, octopamine and tyramine content in PWS and SRM 3258(mg/g) 116
Table 24 Amount of amines found calculated to the serving size of the PWS (mg/serve) 117
Table 25 Caffeine and methylxanthine content in PWS (mean +/- SD) (mg/g PWS) . 120
Table 26 Amount of caffeine present compared to its PWS label..... 121

List of Abbreviations

µg	Microgram
ACN	Acetonitrile
AOAC	Association of Analytical Chemists
AR	*Acacia rigidula*
BfR	The Federal Institute for Risk Assessment (Germany)
CA	*Citrus aurantium*
CAF	Caffeine
CE	Capillary Electrophoresis
CNS	Central Nervous System
DMAA	1,3-Dimethylamine
DMX	Dimethylxanthine(s)
ESI	Electron Spray Ionisation
FA	Formic Acid
FDA	U.S. Food and Drug Administration
FSANZ	Food Standards Australia and New Zealand
GC	Gas Chromatography
HETP	Van Deemter equation
HILIC	Hydrophilic Interaction Liquid Ion Chromatography
HPLC	High Pressure Liquid Chromatography
HRA	Health Risk Assessment (report)
ICH	The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
IPR	Ion-pair Reagent
IS	Internal Standard
K	Retention factor
LC	Liquid Chromatography
LLE	Liquid-Liquid extraction
MAO	Monoamine Oxidase
MAOI	Monoamine Oxidase Inhibitor
MeOH	Methanol

mg	Milligram
mL	Millilitre
MS/MS	Triple quadrupole tandem MS
MS^n	Ion Trap MS
N	Efficiency
NA	Noradrenaline
ng	nanogram
NH_4OH	Ammonium Hydroxide
OCT	Octopamine
OPA	o-Phthadialdehyde
PDA	Photo-Diode Array
PEA	Phenethylamine
pg	picogram
PWS	Pre-Workout Supplement(s)
PX	Paraxanthine
QDa	Quadrupole Diode array
R	Resolution
RDD	Recommended Daily Dose
RP-HPLC	Reverse phase-High Pressure Liquid Chromatography
RSD	Relative Standard Deviation
RT	Retention Time
S/N	Signal-to-Noise
SDS	Sodium Dodecyl Sulphate
SPE	Solid Phase Extraction
SRM	Standard Reference Material
SYN	Synephrine
TAAR-1	Trace Amine Associated Receptors
TB	Theobromine
TGA	Therapeutic Goods Administration
THF	Tetrahydrofuran

TP	Theophylline
TYT	Tyramine
UPLC	Ultra-High Pressure Liquid Chromatography
UV	Ultraviolet
WADA	World Anti-Doping Agency
α	Selectivity
αAR	α- Adrenoceptor
βAR	β- Adrenoceptor
λ_{max}	Maximum Wavelength

Chapter 1 Introduction

1.1 Pre-workout Supplements – Background, Use and Regulation

Pre-workout supplements (PWS) are multi-ingredient sports supplements used not only by individuals to improve performance, but have been playing a more prominent role in the general population to improve alertness (Hoffman et al., 2009). *Ephedra sinica* (ES) extracts contain sympathomimetic, ephedrine and was one of the most popular CNS stimulant before it was banned in 2004- after it was correlated with adverse cardiovascular effects (Gurley, Steelman, & Thomas, 2015). *Acacia rigidula* (AR) was another popular stimulant that was banned in 2014, due to its active ingredient's structural similarities to methamphetamine (Pawar et al., 2014). *Citrus aurantium* (CA) is also used in dietary weight-loss supplements due to the purported thermogenic activity of its key component, synephrine (Bell et al., 2004). For example a U.S. population study found that CA-containing supplements have been gaining popularity as a method of boosting energy and improving health alongside multi-vitamins in the adolescent and young adult population (Bailey, 2014). Disturbingly however, increased numbers of reports of adverse effects to the U.S. Food and Drug Administration (FDA) have raised concerns about the use and abuse of PWS consumption. The most notable case report was reported by Eliason et al. (2012) where two U.S. military soldiers died from cardiac arrest after taking a PWS containing 1,3-dimethylamine (DMAA). Unlike pharmaceuticals, these products do not require manufacturers to provide unequivocal evidence for the efficacy of their product and are often perceived to be safe for consumption (Hung et al., 2011).

PWS usually contain a blend of amino acids and stimulants from plant extracts represented with the key composition set out in Table 1. Appendices 1-8 provide examples of PWS labels selected for analysis in this study. Difficulties in obtaining an accurate picture of PWS ingredients stems from relaxed legal requirements for labelling of ingredients for complimentary medicines as well as in the inclusion of components as 'proprietary blends'. For example, the ingredient *Panax notoginseng*, labelled in Gold standard Preworkout (Optimum Nutrition), was identified as *American ginseng extract* in Muscle Prime (Allmax Nutrition). While Table 1 includes ingredients from only 8 selected PWS there are literally hundreds of PWS formulations available, some made in Australia and others from overseas sources where manufacturing standards may be less stringent. Many PWS contain phytochemicals with distinctive CNS or cardiovascular stimulant properties. Although some

of the plant extracts from which these derive may have been tested individually, the safety and/or efficacy of the combination of plant extracts has not been established.

β-Alanine was found to be a common ingredient in many PWS. β-Alanine is a nonessential amino acid produced in the liver and obtained from protein rich foods in the diet. The basis for its use in PWS is that it is a substrate, along with histidine, for carnitine formation in muscle cells (Caruso et al., 2012). Carnitine's main role is to facilitate the transport of medium to long chain fatty acids into the mitochondria however, due to the basicity of the imidazole ring; it also provides intramuscular buffering thereby preventing acidosis during prolonged muscle contractions. Another common ingredient found in PWS is creatine, another amino acid-derived compound produced by the kidneys and liver to serve as a high energy phosphate reservoir for skeletal muscle cells.

Table 1 ingredient formulation of Ephedra-free dietary supplements

Ingredients	360 Rage™	Beast Mode™	DyNO™	Ergo Blast™	Gold standard PW™	Kardio Fire™	Muscle Prime™	Pump HD™
BCAA		✓	✓		✓	✓	✓	
B alanine	✓	✓			✓	✓		✓
L-tyrosine		✓		✓		✓	✓	
Arginine		✓					✓	
Citrulline Malate	✓	✓	✓		✓		✓	✓
Alpha Lipoic Acid							✓	
N-Acetyl-Cysteine		✓			✓		✓	
Creatine				✓	✓		✓	✓
Taurine							✓	
Caffeine anhydrous	✓		✓	✓	✓	✓	✓	✓
Grape Seed extract	✓							
Maritime Pine Tree Bark extract	✓							
Toothed Clubmoss extract	✓			✓				
Higenamine HCl			✓	✓				
Raspberry ketone						✓		
Olumbago zeylanica extract								✓
Echinacea purpurea extract								✓
Rhodiola root extract			✓					✓
Astragalus membranaceus extract					✓			
Panax notoginseng extract					✓		✓	
CA extract	✓		✓	✓	✓	✓	✓	✓
Co-enzyme Q 10				✓			✓	
Cordyceps							✓	✓
Hawthorne Berry extract							✓	
Ginko Biloba Extract		✓		✓			✓	✓
Selenium							✓	
Niacin (Vit. B3)		✓	✓		✓		✓	✓
Vitamin B6		✓	✓		✓		✓	✓
Vitamin B12		✓	✓		✓		✓	✓
Vitamin C	✓	✓	✓			✓	✓	
Maltodextrin	✓	✓			✓		✓	
Natural and Artificial flavourings	✓	✓	✓	✓	✓	✓	✓	✓

Earlier this decade, reports of PWS consumption by individuals outside the athletic performance arena surfaced. For example, workers engaged in occupations involving long (or late-night) shifts were taking advantage of the stimulatory effects of PWS to prolong alertness (Simmons, 2013). Management of the Bowen Basin mine in Western Australia banned the use of PWS, "JACK3D", because of the perceived risks involved in concurrently operating heavy machinery and/or reduced decision-making ability (Duffy, 2012).

For a large segment of the general population, the desire to lose weight via consumption of weight-loss or 'fat-burning' supplements have expanded the sale of CA-containing supplements into this very lucrative market. Many of the individuals in this segment of the community may be overweight or obese; currently taking prescription medication; or are of an older age and already predisposed to cardiovascular diseases. These factors heightened the risk and thus a need for reliable and unbiased research into the safety and efficacy of the individual compounds in PWS was warranted. There needs to be a greater focus on the potential interactions between PWS additives and/or medications.

1.1.1 Common Herbal Extracts Found in PWS

1.1.1.1 *Ephedra Sinica* (ES)

Ephedra sinica has been used in traditional Chinese medicine for many centuries and is the botanical source of ephedrine alkaloids, pseudoephedrine, norpseudoephedrine and norephedrine. These CNS-active compounds resemble epinephrine (as well as amphetamine) in structure and this is likely responsible for their similarity in pharmacological effects.

Ephedra was an ingredient in PWS for around 10 years (1994-2004) and during this time experienced considerable controversy. A full historical review of ephedra use in the USA has been documented (Palamar, 2011). Despite the purported claims for ergogenic and health benefits of ephedra-containing PWS, a concerning number of adverse effects, led to its prohibition in 2004 by the FDA (Gurley, Steelman, & Thomas, 2015). The range of reported adverse events included cardiac arrhythmias (Dwyer, Allison, & Coates, 2005; Haller, 2000), myocardial infarction (Haller, 2000; Smith et al., 2014), strokes (Bouchard et al., 2005; Haller, Jacob, & Benowitz, 2002; Holmes & Tavee, 2008), seizures (Kockler, McCarthy, & Lawson, 2001; Moawad, Hartzell, Biega, & Lettieri, 2006) and rhabdomyolysis (Rhidian, 2011; Stahl, Borlongan, Szerlip, & Szerlip, 2006). Due to the complex nature of the PWS formulations, it was difficult to directly link ephedra to these adverse effects, although an increasing number of emergency medicine cases reported were strongly correlated with use of ephedra-containing PWS.

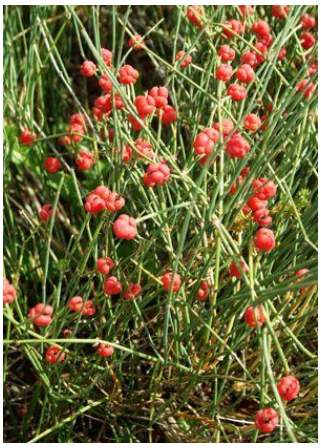


Figure 1 Photograph of Ephedra Sinica

1.1.1.2 *Acacia rigidula* (AR)

The *Acacia* genus contains over 50 different species containing various amines and alkaloids. *Acacia rigidula* (AR) grows predominantly in the southern regions of Texas, USA. Based on the current literature, there have not been any reports of its use in traditional medicine. A study in 1998 has reported the presence of a phenethylamine-type compound, *N*-methyl-β-phenethylamine (*N*-MePEA) and *N*-methyltyramine (*N*-MeTYR) (Clement, Goff, & Forbes, 1998).

In recent years, AR extracts have been commonly listed on PWS labels as stimulatory ingredients. The increased use and lack of information on AR extracts in PWS led the U.S. FDA to establish a method for the quantitative determination of biogenic amines, phenethylamine (PEA), tyramine and tryptamine derivatives (Pawar, Grundel, Fardin-Kia, & Rader, 2014). The study conducted by Pawar et al. on AR analysis contrasted their findings with those of Clement et al. and found that 20 of the 21 supplements tested had high amounts of PEA. Importantly, PEA does occur naturally in AR or extracts thereof. Worryingly, β-methylphenylethylamine (BMPEA), a positional isomer of methamphetamine was found in 9 of the 21 samples with amounts ranging from 963 µg/g to 60,500 µg/g and it was likely to have been misidentified as amphetamine in 1998. There is no safety data on the biological effect of the BMPEA in humans (Pawar et al., 2014). Since this study was concluded, AR-containing products were banned in 2015 by both the FDA and TGA on the basis that products containing AR have not previously been consumed as a food and lacked substantial safety evidence (Upadhyay, 2014).

1.1.1.3 *Citrus aurantium* (CA)

Citrus aurantium (CA) is known common as names such as bitter orange, sour orange, Seville orange, chongcao, and neroli. The plant has its origins in tropical Asia and has been used in traditional Chinese medicines. The main volatile components in the flowers and peel of CA are the flavonoids, hesperidin and naringin whereas the non-volatile alkaloid components include synephrine, octopamine and tyramine (Pellati, Benvenuti, Melegari, & Firenzuoli, 2002). Synephrine acts as an α-adrenergic agonist, with some β_3-adrenergic agonist properties (Astrup, 2000). β3- receptors are found mainly on adipose tissue, and regulate lipolysis and thermogenesis (Ferrer-Lorente, Cabot, Fernández-López, & Alemany, 2005). This was the basis for the purported CA efficacy as a weight-loss agent. Animal studies have shown that

synephrine significantly reduced food intake in rats (Verpeut, Walters, & Bello, 2013). On the other hand, pilot studies conducted by Greenway et al. failed to demonstrate a significant difference between the treatment and control groups for food intake, appetite ratings or body composition (Greenway et al., 2006). To date there is a lack of evidence to support the use of CA in appetite control and weight loss in humans (Onakpoya, Davies, & Ernst, 2011). The pharmacological effects will be further discussed later in this chapter.

Figure 2 Photograph of Citrus aurantium

1.1.2 Current Regulations of PWS in Australia (TGA) Compared to Overseas

Pre-workout supplements are considered complementary medicines, under the Therapeutic Goods Act of 1989 as it contains herbal material (i.e. plant extracts) (Therapeutic Goods Administration, 2013). The Therapeutic Goods Administration (TGA) is a sub-group of the Australian Government Department of Health responsible for regulating the supply and export of therapeutic goods. These goods include prescription medicines, complementary medicines, vaccines, vitamins and minerals (including supplements) medical devices, blood and blood products. A complementary medicine is defined by the TGA as a therapeutic good consisting wholly or principally of 1 or more designated active ingredients, each of which has a clearly established identity and a traditional use. The TGA employs a risk-based (watch and wait) approach to regulation of complementary medicine whereby products containing herbal materials that present a higher risk, must be registered with the Australian Register of Therapeutic Goods (ARTG). Lower-risk substances may be found on the TGA's registry of 'Substances which can be used as a Listed Medicine'. These may be classified as active, component or excipient and may have further restrictions on their use such as that mentioned for caffeine and synephrine.

The TGA also conducts post-market regulatory monitoring of products to ensure safety for consumption via a two-tiered system whereby registered medicines are tested by the TGA for quality, safety & efficacy whereas listed ingredients are only tested for quality and safety not efficacy. Where an Australian-made product produces unexpected or undesirable side effects, the product may be reported to the TGA. When the TGA receives a report from a consumer of an adverse effect following consumption of a PWS, for example, they will conduct an investigation. This includes sending a sample to a laboratory for a quantitative component analysis (TGA, 2016b) which, depending on their workload, can take up to 6-12 months. The TGA may, following their investigations, consider enforcing the discontinuation of the product (that is, if the company and or product still exists). It is estimated that only a fraction of adverse effects are ever reported and often reports are made directly to the manufacturer. In the latter case, there is a disincentive for the company to relay the report to the TGA.

Since the TGA also regulates the importation of PWS and ingredients therein, products entering Australia are subject to scrutiny upon arrival into the country. In reality, due to limitations on staff and/or time, only the larger import quantities are usually checked. However, with the increased popularity of 'online stores' consumers may not necessarily know the origin of the product. TGA scrutiny can be side-stepped by having small product orders mailed directly to consumers. The TGA regularly posts updates on adulterated and discontinued products (TGA, 2016a). However, this method of reporting may be inefficient as general consumers do not necessarily know where to look for such information.

In Australia, medicines, poisons and therapeutic goods are regulated under the Therapeutic Goods Act (1989), and in particular, the Poisons Standard (November 2016) being the legislative instrument. Within this framework substances are classified into schedules which help determine how medicines and poisons are made available to the public *(https://www.legislation.gov.au/Details/F2016L00036)* (Gill, 2016). Schedules are ranked according to the level of regulatory control required such that Schedule 1 (medicines that are not in use), Schedule 2 (pharmacy medicines) to Schedule 10 (dangerous substances that are prohibited for sale) (Figure 3).

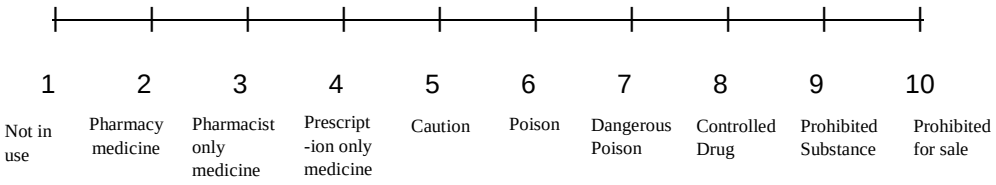


Figure 3 Scheduling of medicines and poisons implemented by the TGA of Australia. Comprehensive breakdown and explanation is shown in Appendix 9.

Amphetamine is classified as a Schedule 8 substance (drugs of addiction) while ephedrine, for example, a Schedule 4 substance (restricted prescribed substance) (Australian Government Department of Health, 2015) (Gill, 2016).

Synephrine is classed as a Schedule 4 substance with a recommended daily dose (RDD) of 30 mg. A therapeutic dose in treatment of hypotension, as defined by the Poison Standard of Australia was about 300 mg daily and there was little evidence of harmful effects at 30 mg in a daily dose (Gill, 2015). The scheduling of synephrine was considered in 2002 when CA extracts were first advertised with manufacturer's claims of enhanced metabolite support and weight loss (National Drugs and Poisons Schedule Committee, 2003). At that time approximately 1000 products listed in the ARTG containing synephrine ranging from 2 μg to over 31 mg.

Octopamine was banned as an ergogenic aid in competitive sports by the World Anti-Doping Agency (WADA) in 2002 however, despite this; octopamine has yet to be considered for scheduling due to a lack of reported adverse effects. Octopamine is present in lower concentrations compared to synephrine in CA extracts. Octopamine is been shown to act as a monoamine neurotransmitter in invertebrates, but its role in vertebrates is uncertain (Roeder, 1999). Many dietary supplements (including PWS) that contain octopamine have been shown to raise blood pressure (Haller, Benowitz, & Jacob, 2005). Tyramine, on the other hand, has not been subject to a ban by WADA nor any other sporting associations. There are currently no legislative restrictions relating to either tyramine or octopamine.

In 2011, Health Canada conducted a health risk assessment (HRA) report on the use of synephrine, octopamine and caffeine as well as the peel of CA. Like the TGA, Health Canada also employs a risk-based approach in evaluating safety and efficacy of products. Evidence for the results derived from a single six-week study which concluded that at the maximum RDD of 320 mg caffeine and 40 mg synephrine participants did not show harmful effects (Colker, Klalman, Torina, & Perlis, 1999). The study involved 20 subjects with a body-mass index of >25 kg/m^2 where they were sub-divided 3 groups: group A, ingested 975 mg CA extract, 528 mg caffeine and 900 mg of St. John's wort; group B was a placebo; and group C served as a control without any supplement intervention. The diets and exercise sessions were controlled and the amount of body weight change was measured. This study had a few limitations, one, was the small sample size of overall patients per group. 20 individuals is a miniscule representation of the human population. Besides that, there was a statement on

conducting laboratory tests within the results of their study but there was no further discussion or description on the vital signs of their patients during the study. Apart from that, there were no discussions on the variability of the health of the individuals apart from a body-mass index of >25 kg/m^2. The study would have been improved had specific exclusion criteria for their patients been included. Based on this study however, the HRA report concluded that a dose of up to 50 mg/day *p*-synephrine was not likely to trigger a cardiovascular event. In addition, they concluded that, for healthy individuals, the use of less than 40 mg/day of synephrine in conjunction with 320 mg/day of caffeine was classified as low risk. It should be noted that this health risk assessment was funded by the company and conducted by Stohs, a known advocate and senior scientist for AdvantraZ® (CA extract) (Bloomberg Business, 2015; Stohs, et al., 2011; Stohs, Preuss, & Shara, 2011, 2012).

In contrast to Canada's safety assessment of PWS, in 2013, the Federal Institute for Risk Assessment (BfR) of Germany assessed the risks of PWS and weight-loss supplements containing both synephrine and caffeine. The BfR looked at the quantities of synephrine and caffeine contained therein and noted a high level of variability in suggested doses between products. As both these constituents are known to affect the cardiovascular system, their combined consumption posed a higher risk to consumers. The German BfR established a maximum safe dosage of synephrine of 25.7 mg (The Federal Insitute for Risk Assessment, 2013). Since CA extracts also contain other stimulants aside from synephrine, such as octopamine and tyramine, further cardiovascular effects such as increased heart rate and blood pressure would be expected to occur.

According to the BfR, many of the products assessed contained more than the maximum safe dosage of 25.7 mg thereby breaching the regulation. On this basis, the BfR classified sports supplements, such as PWS as unsafe for human consumption. Moreover, since the target market was likely individuals undergoing physical exertion, the added strain on the cardiovascular system was higher. Moreover, adverse effects from the use of weight-loss supplements containing CA would be amplified and pose a greater risk for an overweight, ageing population with likely pre-existing cardiovascular problems (The Federal Insitute for Risk Assessment, 2013).

Caffeine, another prominent inclusion in PWS, is the only methylxanthine subject to specific product labelling requirements – the details of which vary depending on country of manufacturing origin. In the US, for example, only added caffeine must be listed as an ingredient while in Australia products containing caffeine are required to state the amount and

also carry a warning. Currently no recognised health-based guide for acceptable caffeine intake exists, although Food Standards Australia and New Zealand (FSANZ) recommends a maximum daily caffeine limit in caffeinated foods or beverages of 95 mg/day for children (aged 5-12) and 210 mg/day for adults (approximately 3 cups of coffee per day) (Smith et al., 2000), In Australia however, the composition of PWS and other dietary supplements, classified as complementary medicines, is regulated by the Therapeutic Goods Administration.

A recent review on caffeine toxicity suggested that undeclared herbal components in most supplements could expose consumers to an increased risk of caffeine toxicity (Musgrave, Farrington, Hoban, & Byard, 2016). Since there is a degree of overlap in the stimulatory effects of caffeine and dimethylxanthines (DMX), the total effect is likely additive (Fredholm, 2011). These combined circumstances make it difficult for individuals to keep track of their caffeine intake which may lead to potential adverse effects from overconsumption. Such increased PWS consumption beyond athletes and fitness enthusiasts into recreational use by the general population (Gibson, 2014), and concomitant the increased adverse health reports may result in more stringent caffeine content/ labelling requirements leading to an increased demand for analysis of products.

1.2 Structural and Chemical Properties of Key Stimulants in PWS

The two key stimulant groups within PWS of interest for this research are the amphetamine-like compounds within CA, synephrine, octopamine and tyramine; and caffeine with its dimethyl derivatives.

1.2.1 Amphetamine-like Compounds

Table 2 summarises the key physicochemical properties of our compounds of interest and compares their similarity to amphetamine and ephedrine. The compounds mentioned in the table can be classed as substituted phenethylamines where the variations in molecular bonding results in different chemical and physical properties. Like amphetamine and ephedrine, synephrine is a phenethylamine however distinctly containing one phenol hydroxyl group absent in the banned compounds. In fact, they are structurally in between the natural catecholamine and the banned amphetamine compounds. Synephrine contains a total of two hydroxyl groups, positioned in B (ring) and W (side chain), as well as a methyl group on position Y. This correlates with its high degree of polar surface area. Octopamine has similarly substituted hydroxyl groups at position B and W but lacks the methyl group on position Y (on the amine nitrogen). Tyramine has a hydroxyl group substituted only on position B. As expected all the compounds have basic pKa values (between 8.55 for and 10.4 for tyramine).

The similarity in structure of these compounds have led many researchers to hypothesise similar pharmacological outcomes (Broadley, 2010; Chen et al., 1981; Clement et al., 1998; Gibbons, 2012; Liles et al., 2006; Oberlender & Nichols, 1991; Pawar et al., 2014). Synephrine and octopamine, predominantly exists as the naturally occurring *p*-synephrine and *p*-octopamine, with the hydroxyl group located on the para-position on the benzene ring of the molecule (Mattoli et al., 2005; Pellati, et al., 2005; Rossato et al., 2010).

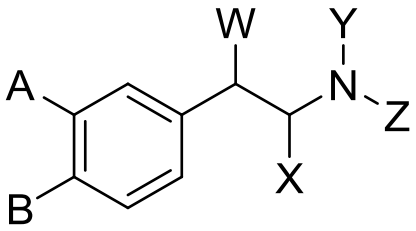


Table 2 Chemical properties of different amphetamine-like compounds

Compound	Formula	MW	A	B	W	X	Y	Z	logP	pKa	Polar surface area (PSA) (Å)^2	Rotatable bonds	Hydrogen bond donors	Hydrogen bond acceptors	Stereo-centres
Synephrine	$C_9H_{13}NO_2$	168.2	H	OH	OH	H	CH_3	H	1.98	9.46	40.46	2	2	2	1
Tyramine	$C_8H_{11}NO$	137.2	H	OH	H	H	H	H	0.86	10.41	46.20	2	3	3	1
Octopamine	$C_8H_{11}NO_2$	153.2	H	OH	OH	H	H	H	-0.59	9.64	66.48	2	3	3	1
Epinephrine	$C_9H_{13}NO_3$	183.2	OH	OH	OH	H	H	H	-0.54	8.55	72.72	3	4	4	1
Nor-epinephrine	$C_9H_{13}NO_3$	169.2	OH	OH	OH	H	H	H	-1.26	8.58	86.70	2	4	4	1
Amphetamine	$C_9H_{13}N$	135.2	H	H	H	CH_3	H	H	1.76	9.90	26.02	2	1	1	1
Ephedrine	$C_{110}H_{15}NO$	165.2	H	H	OH	CH_3	H	CH_3	1.13	9.65	32.3	3	2	2	2

1.2.2 **Caffeine and Dimethylxanthines**

Methylxanthines such as, caffeine (1,3,7-trimethylxanthine), theobromine (3,7-dimethylxanthine), and theophylline (1,3-dimethyxanthine) are distinguished by variations in the number and position of methyl groups around the xanthine ring (coupled pyrimidinedione and imidazole ring structure) (Talik, Krzek, & Ekiert, 2012) shown in Table 3**.** While many methylxanthines are of plant origin and found in foods and beverages such as coffee, teas, yerba mate and cocoa, paraxanthine (1,7-dimethylxanthine), is a metabolic product of caffeine degradation in humans and is not found in plants (Orru et al., 2013).

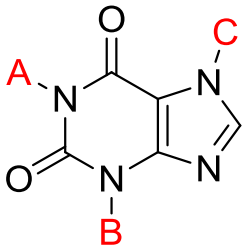


Table 3 Chemical properties of caffeine and DMX

Compound	Formula	MW	A	B	C	log P	pKa	Polar Surface area (PSA) $(Å)^2$
Caffeine	$C_8H_{10}N_4O_2$	194.2	CH_3	CH_3	CH_3	-0.1	14	58.4
Theobromine	$C_7H_8N_4O_2$	180.2	H	CH_3	CH_3	-0.8	9.9	67.2
Paraxanthine	$C_7H_8N_4O_2$	180.2	CH_3	H	CH_3	-0.2	10.76	67.2
Theophylline	$C_7H_8N_4O_2$	180.2	CH_3	CH_3	H	0	8.81	69.3

1.3 Pharmacological Properties of Key Stimulants in PWS.

1.3.1 Pharmacodynamics of The Adrenergic Amines in CA

Synephrine is commonly identified as the active component of CA and acts primarily as an α-adrenergic receptor (αARs) agonist (de Oliveira et al., 2013; Pellati et al., 2002). Functional studies showed that synephrine had agonist activity specific on the α_{1A}-AR subtype but showed some antagonism for the pre-synaptic α_{2A} and α_{2C}-ARs present in nerve terminals (Ma et al., 2010). Other studies that tested the binding affinities of synephrine reported that *p*-synephrine had overall lower affinity for αARs compared to *m*-synephrine (Brown et al., 1988; Hibino, Yuzurihara, Kase, & Takeda, 2009; Hwa & Perez, 1996). The presence of hydroxyl groups at the β-carbon (position W in Table 2 Chemical properties of different amphetamine-like compounds) increases its polarity and thus reduces its ability to cross the blood brain barrier (BBB). This would decrease the likelihood of synephrine affecting central nervous system (CNS) activity (Brunton, Lazo, & Parker, 2005)**.**

Synephrine, like ephedrine, is also known to act as a β-adrenoceptor (βAR) agonist, specifically on β_3-AR (Arch, 2002; Carpene et al., 1999) which can result in increased lipolysis and basal metabolic rate producing the net effect of weight loss (Haaz et al., 2006). To date there is little clinical evidence to support synephrine's thermogenic properties. A study in mice, conducted in 2008, demonstrated that neither CA extracts (5 g/kg and 10 g/kg standardised to contain 2.5% synephrine) nor an orally-administered dose of *p*-synephrine (300 mg/kg) significantly increased body temperature (Arbo et al., 2008). Arbo et al. (2009) also showed that there was a lower weight gain in rats that were administered 30 and 300 mg/kg of *p*-synephrine for 28 consecutive days compared to the controls. However, these results may not be necessarily transferable to humans as a double blind, placebo controlled trial in humans involving a dietary supplement containing 21 mg of synephrine and 304 mg of caffeine coupled with an exercise regime did not significantly increase thermogenic effect during exercise (Haller et al., 2005).

Synephrine is a chiral molecule and it exists in a *S* (+)-form and a *R* (-)-form. A study by Jordan et al., determined that *S* (+)-synephrine was 1 to 2 orders of magnitude less active than the *R* (-)-synephrine on β1-adrenoceptors in guinea-pig atria (Jordan, Midgley, Thonoor, & Williams, 1987). Brown et al., described the level of activity of *S* (+)-synephrine to be less active than the *R* (-)-synephrine on the α1-adrenoceptors on a rat aorta. It should be noted that these studies may not appropriately represent the receptor binding properties of synephrine enantiomers in humans. The determination of the enantiomeric composition of pharmaceuticals and nutraceuticals is important to understand the pharmacology of the synephrine component of CA-containing PWS. There are no current studies published that determine the specific synephrine enantiomers present in CA extracts or CA-containing PWS.

Octopamine was first identified in the posterior salivary glands of an octopus (*Octopus vulgaris)* from which its name derives. It is also present in plants, invertebrates and vertebrates (David & Coulon, 1985) as well as plants such as the fruit of CA. It plays a significant role in neurotransmitter activity for invertebrates similar to the physiological activity of adrenaline in mammals (Roeder, 1999). Non-naturally occurring *m*-octopamine is the most potent amongst its isomers followed by *p*-octopamine, which is 10-fold less potent than *m*-octopamine

Octopamine was found to act as a β_1-adrenergic receptor agonist (EC_{50} of 3.1 ± 0.4μM (Kleinau et al., 2011)) as well as a β_3-adrenergic receptor agonist (present in adipocytes (Carpene et al., 1999)). Moreover, there was no apparent β2-receptor binding up to a concentration of 6.7 μM while allosterically inhibiting other β_2 agonists such as isoprenaline. *m*-Octopamine, in particular, is also an α-adrenergic receptor agonist that is suggested to be one hundredth of the potency of noradrenaline to increase blood pressure (Fregly, Kelleher, & Williams, 1979) due to its higher α_1 adrenergic receptor affinity (6-fold less than noradrenaline) compared to α_2-adrenergic receptor affinity (150-fold less potent than noradrenaline) (Brown et al., 1988).

Tyramine can be naturally found in common dietary sources such as chocolate, aged cheese, aged meat and certain fruits and vegetables as well as in as alcoholic beverages. (Ziegleder et al.,1992). Similar to noradrenaline, tyramine indirectly acts as a sympathomimetic amine. These amines have weak actions on adrenoceptors, but are transported by the neuronal noradrenaline transporter (NET). In the nerve terminal, the amines are taken up into synaptic vesicles by the vesicular monoamine transporter (VMAT) to release noradrenaline into the cytosol.

The established mechanism for vascular effects of trace amines is that they behave by indirectly acting as sympathomimetic amines by stimulating the release of noradrenaline from sympathetic neurons (Broadley et al., 2009). To support the indirect mechanism of action of tyramine and other trace amines, reserpine treatment was used on isolated tissue and in vivo. Reserpine is a drug used for the control of high blood pressure by reducing the amount of noradrenaline from vesicular stores of sympathetic neurons. The treatment reduces the responses to sympathetic nerve stimulation and the indirect effects of trace amines (Burn & Rand, 1958). In rats, pressor effects due to ephedrine were caused by a direct effect on α-adrenoceptors, whereas tyramine showed indirect stimulation (Liles et al., 2006). The effects of directly acting sympathomimetic amines, such as noradrenaline and isoprenaline, are increased by chronic treatment with reserpine, due to the up regulation of the adrenoceptors (Chess-Williams, Grassby, Broadley, & Sheridan, 1987). Reserpine causes the release and breakdown of noradrenaline (NA) as well as depleting neurons of NA. Thus, no response to the indirectly acting sympathomimetics after reserpine could be obtained. Reserpine depletes the neurones of noradrenaline which results in no downregulation of receptors. Hence, the increase in receptor activity would increase the response of directly acting drugs (Chess-Williams et al., 1987).

Synephrine, octopamine and tyramine can be described as trace amines which interact with trace amine associated receptors (TAARs). The initial discovery of a new family of G protein-coupled receptors (GPCRs) was based on the pharmacological profile of its prototypical receptor, trace amine-associated receptors (TAAR1), this set of GPCRs was later termed TAARs (Borowsky et al., 2001). These receptors are expressed in both rats and humans, with only slight variations in response to drugs, due to their low homology (Wainscott et al., 2007). The receptors are intracellular in contrast to adrenergic receptors, which are located on the cell surface membrane (Xie et al., 2008).

The TAAR1 receptor has structural similarities to rhodopsin/ β-adrenergic receptor family that responds to tyramine and β-phenylethylamine (Barak et al., 2008). The amount of data on the receptor binding properties of synephrine and octopamine are currently limited.

1.3.2 Pharmacokinetics of Adrenergic Amines in CA

Interestingly trace amounts of synephrine are produced in humans, possibly as a degradation product of the catecholamines – to which they share structural similarity. (Watson et al., 1990). A portion of cytosolic noradrenaline is deaminated by monoamine oxidases (MAO) whereas some is released into synaptic cleft to act on post-synaptic receptors. MAO inhibitors (MAOI) prevent inactivation of the transmitter displaced from the vesicles within the terminal. MAO inhibition enhances the action of tyramine as it is normally degraded by gastrointestinal tract and liver MAO before entering the systemic circulation. The occurrence of dietary tyramine-provoked hypertensive crisis is a well-known interaction associated with irreversible MAO-A inhibition (Chen , Swope, & Dashtipour, 2007).

Referring to metabolic pathways, synephrine is not degraded by catechol-o-methyltransferases. The substitutions on the α-carbon block oxidation by MAO. However, synephrine lacks an α-carbon ligand resulting in degradation via MAO. The oxidation of synephrine and octopamine by MAO results in the production of *p*- and *m*-hydroxymandelic acid as well as hydrogen peroxide which could result in oxidative stress (Grandy, 2007) (Figure 4). The plasma half-life of *m*-synephrine is two to three hours (Haaz et al., 2006). Experiments show synephrine undergoes phase 2 metabolism, glucuronidation and sulfation (Ibrahim, Couch, Williams, Fregly, & Midgely, 1985). A study in 1983 that investigated the composition of urine after oral ingestion of synephrine found that 47% sulphated synephrine, 30% hydroxymandelic acid, 12% conjugated synephrine and 6% hydroxyphenylglycol sulphate was produced (Ibrahim et al., 1985). Another study, investigating enantiomeric differences for synephrine showed that 20-50% of synephrine ingested was excreted in conjugated R (-)-synephrine form, and 10% as *S*(+)-synephrine (Kusu, Matsumoto, Arai, & Takamura, 1996).

In-vitro studies have shown that both *m*- and *p*-synephrine isomers are taken up by Caco-2 cells, suggesting that they can be absorbed by the gastrointestinal tract at an equal rate (Rossato et al., 2010)**.** However, poor bioavailability of synephrine has been observed in a human study involving the oral ingestion of 5.5 mg of synephrine. The peak plasma concentration of the subjects was less than 1 ng/mL (Haller et al., 2005).

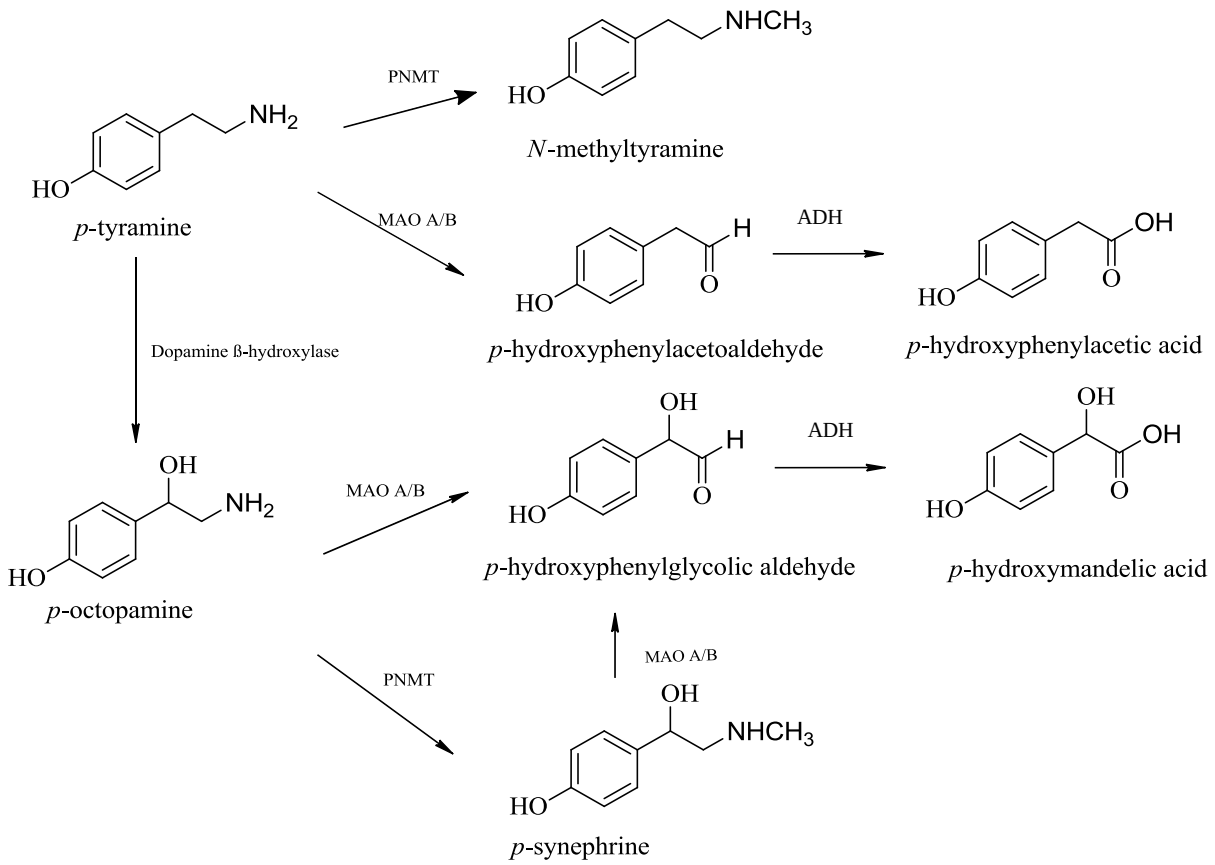


Figure 4 The degradation pathway of trace amines. MAO represents the monoamine oxidases; ADH represents the aldehyde dehydrogenase; PNMT represents the phenylethanolamine-N-methyl transferase

CA extracts contain flavonoids, specifically 6', 7'-di-hydroxy-bergamotin, a P4503A4 selective antagonist (Fugh-Berman & Myers, 2004). As cytochrome P4503A4 metabolises a majority of existing drugs, for example, MAOI antidepressants, it is not advisable to also take CA-containing PWS (Fugh-Berman & Myers, 2004). The use of MAOI would impair the metabolism of synephrine and potentially cause hypertension due to its prolonged vasoconstriction (Grandy, 2007).

1.3.3 Pharmacology of Caffeine and Dimethylxanthine Derivatives

Reports that caffeine increases endurance performance and/or recovery (Hodgson Randell, & Jeukendrup, 2013; Leveritt et al., 2012; Santos et al., 2014; Stadheim et al., 2013), likely gave further impetus to caffeine being marketed as an ergogenic aid in PWS. Caffeine consumption and the increased risk of cardiovascular diseases depend on a number of factors such as the variability of population, dose, confounding factors and/or low statistical power of the studies (Zulli et al., 2016). Since there is overlap in the stimulatory effects of caffeine and DMX, the total effect is likely additive (Fredholm, 2011). These combined effects make it difficult for individuals to keep track of their total stimulant intake which may lead to potential adverse effects from stimulant overconsumption.

Caffeine has a dose-dependent effect on the CNS which includes mood enhancement, wakefulness, insomnia, seizures and anxiety. These CNS effects derive from the antagonism of the adenosine receptors that are present in the brain. However, caffeine's haemodynamic effects are attributed to the antagonism of the adenosine receptors, A1 and A2 (Figure 5). This includes increased heart rate, elevated blood pressure and peripheral and coronary vasoconstriction (Benowitz, 1990). Caffeine is also known to inhibit cAMP-mediated phosphodiesterase which leads to the accumulation of the second messenger; cyclic adenosine monophosphate (cAMP) enhances the effect of biogenic amine stimulation at adrenergic receptors. The mobilization of intracellular calcium by activation of γ-aminobutyric acid (GABA) neurotransmission (Riksen, Rongen, & Smits, 2009).

Caffeine has been shown to decrease blood flow to the cerebral, mesenteric and hepatic system. It also produces a diuretic effect via inhibiting antidiuretic hormone (ADH) which increasing glomerular filtration which results in higher water and sodium excretion (Benowitz, 1990). Higher doses of 400 mg/day caffeine would cause bronchodilation, hypokalaemia from the movement of intracellular calcium, hyperglycaemia and lipolysis (Monteiro, Alves, Oliveira, & Silva, 2016). Chronic use of caffeine with daily doses exceeding 300 mg can lead to a dose-dependent tolerance, which increases the risk of hypokalaemia which may cause ventricular arrthymias (Shi et al, 1990; Goldfarb, 2014).

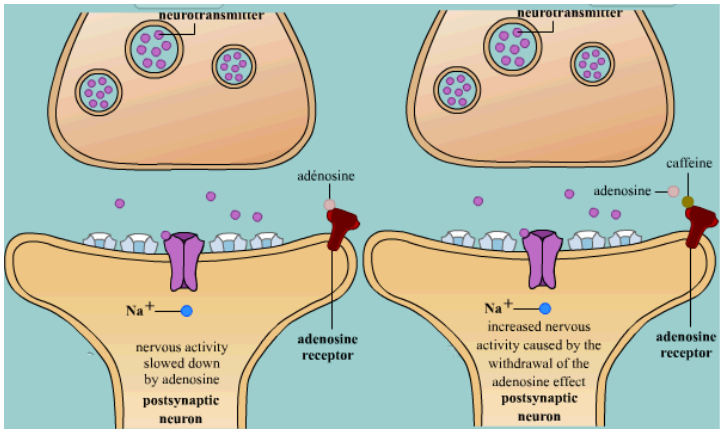


Figure 5 (Left) adenosine receptor binding to adenosine which causes nervous activity to slow down. (Right) presence of caffeine that acts as an adenosine antagonist that result in an increased nervous activity (Pang & Ko, 2006)

Caffeine is recognised to potentiate the cardiovascular and CNS effects of other stimulants which contain plant-derived α- and β-adrenergic agonists such as those from the *Ephedra species* (ephedrine, pseudoephedrine, norephedrine and methylephedrine) (Brown , Porter, Ryder, & Branch, 1991; Haller, Jacob, & Benowitz, 2004; Lake, Rosenberg, & Quirk, 1990).

TP and TB are present in coffee, tea and cola beverages albeit in lower concentrations than caffeine. However, PX is the main metabolite of caffeine in humans and is not naturally found in plants (Orru et al., 2013). TP is often used as a bronchodilator but its use is restricted by a narrow therapeutic range of 5-20 µg/mL (Mitenko & Ogilvie, 1973), where adverse effects are known to occur above 20 µg/mL. TB has fewer CNS effects than caffeine and TP, but has been reported to be a potent cardiovascular stimulant (Monteiro et al., 2016).

The physiological effects of PX are not as well explored but it may increase skeletal muscle contraction (Hawke, Allen, & Lindinger, 2000). Orru et al., showed that in rats, PX has a stronger locomotor activating effect than caffeine or the two other main metabolites of caffeine, TP and TB. As previously described for caffeine, the locomotor activating doses of PX more efficiently counteract the locomotor depressant effects of an adenosine A (1) than an adenosine A (2A) receptor agonist. Ex vivo experiments demonstrated that PX, but not caffeine, can induce cGMP accumulation in the rat striatum. These findings suggest that the inhibition of the cGMP-preferring phosphodiesterase (PDE) is involved in the locomotor activating effects following the

acute administration of PX (Orru et al., 2013). There was a considerably lower amount of attention given to PX for efficacy research compared to caffeine.

The challenge associated with PWS quantitation include content inconsistency, labelling inadequacies, product adulteration and contamination, combination of plant extracts with little evidence of safety. A study in 2013 found that caffeine quantity varies considerably between brands of ephedra-free dietary supplements sold in the USA (Kole & Barnhill, 2013). Furthermore, some products fail to provide an adequate indication of the exact quantity of caffeine in the product (Foster et al., 2013). This would potentially be harmful to users that metabolise caffeine at a slower rate or who may be prescribed stimulants (i.e. Adderall® and Ritalin®) or MAOI.

1.4 Quantitative Analysis of Components in PWS

Chromatography is a physical method of separation which exploits a compound's polarity. Analytes of interest partition between two phases, stationary or mobile, according to their affinity for that phase (Heftmann, 2004). Chromatography can be categorised by either the type of physical chromatographic bed (column or planar); the choice of mobile phase (either gas or liquid); or the separation mechanism (ion-exchange, size-exclusion, solubility etc.). Gas chromatography (GC), pioneered by Martin & Synge in 1941, is suitable for separating complex mixtures of volatile components, however derivatisation is often necessary to volatilise non-volatile analytes. In addition, highly polar compounds (such as amines) are not easily analysed with GC. Liquid chromatography (LC), on the other hand, has no sample volatility issues, is suitable for polar compounds and faster analyses are possible with generally shorter columns.

High Pressure Liquid Chromatography (HPLC) instruments are capable of pumping solvent to a limit of around 5,000 psi depending on system robustness. HPLC, was introduced in the 1980s, is now one of the most widely used analytical instrumental techniques (Heftmann, 2004). Ultra-high Pressure LC (or UPLC) systems are capable of achieving higher pressures (up to around 15,000 psi) resulting in fast run times and higher resolution, However UPLC systems are understandably costlier. In LC analyses, normal-phase separation uses a polar stationary phase chemistry, whilst reverse-phase employs a packing material coated usually with a non-polar, hydrocarbon substance such as phenylhexane or octadecane (C18). The latter is preferable for a wider range of polarity compounds and is less expensive in terms of solvent usage creating less organic waste. Therefore, for the separation of the analytes of interest in this study – a set of charged amines as well as non-polar components, reversed-phase (RP-HPLC), was selected as the ideal choice keeping in mind instrumentation limitations.

Figure 6 depicts a typical HPLC system consisting of mobile phase pump, a degasser which removes gasses from solvent, an injector, the column and a detector.

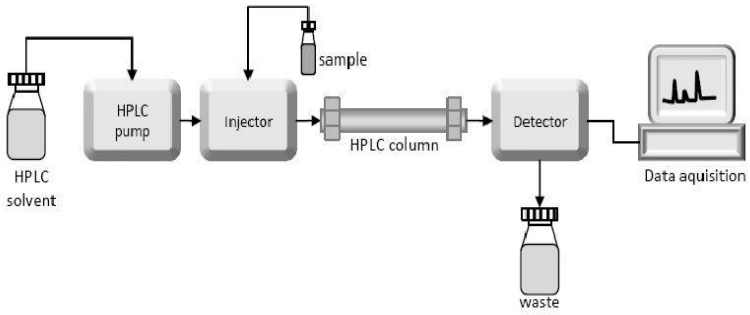


Figure 6 Schematic of a generic HPLC set up

The most common mobile phase for modern HPLC is generally a mixture of water, methanol or acetonitrile. The percentage of these components is held constant throughout the entire run is termed an isocratic elution protocol. When the composition of the mobile phase changes over the course of the run, however, it is called a gradient elution profile. Increasing the organic component generally increases the elution strength of the mobile phase. Additives to the mobile phase such as buffers or tetrahydrofuran (THF) are often required to improve the analyses. A more detailed description of these and the theory of chromatography will be presented in Chapter 2.

Once separated, analytes exit the column and enter the detector. In the case of a UV-absorbing analyte, such as amphetamine-like compounds and xanthines in this study, a UV detector is employed. Selecting the most suitable wavelength for analysis is not always as simple as determining the maximum wavelength (λ_{max}) especially in multi-component analyses due to co-elution issues. There are many different types of detectors available for HPLC analysis – the choice of which may depend on availability, although certain detectors are more suitable for some purposes over others. Electrochemical detectors are highly sensitive and based on detecting electrical currents generated from a reduction/oxidation of the analyte. They are often employed in catecholamine and neurotransmitter analyses. UV detectors generally require a chromophore for analyte detection such as an aromatic ring or conjugated double bond system. Photodiode array detectors (PDA) are UV-based detectors but differ from the simpler UV detectors by the position of the beam splitter. By placing the splitter after the sample, all wavelengths can be detected simultaneously which provides a wealth of spectral data. This is advantageous especially if the ideal wavelengths are not known.

The detectors mentioned so far are considered non-destructive in nature, thus samples may be collected for further analysis. Mass spectrometry (MS), though a destructive technique is used to determine the mass of the analyte (up to 4 decimal places in some instances) and can unambiguously confirm structure. In MS, analytes are volatilised on entry to the detector, followed by ionisation and fragmentation. Charged ions then travel towards the mass detector via a magnetic field which deflects the ions such that the lighter species travel on a different trajectory to the heavier ones thus forming the basis of separation. A single set of magnets is termed a 'single quadrupole' instrument. Combining multiple sets of magnets in series (for example, a 'triple-quadrupole' instrument) facilitates vast improvements in analytical power. The key to structure determination in this technique lies in the fragmentation of parent sample ions. Unique fragmentation patterns for individual compounds become like fingerprints then as a means of structure confirmation. A comprehensive review of HPLC detectors is beyond the scope of this thesis. Scott presents an excellent though not recent review on chromatographic detection techniques (Scott, 1996). Thus the combination of LC-MS is a powerful tool for both separation and detection of analytes in a sample matrix.

A typical chromatogram is a plot of an intensity of absorption versus retention time. Ideally each analyte peak should be sharp and symmetrical and completely resolved to baseline with no obvious co-elution issues. Achieving this however involves a considerable amount of trial and error. Optimisation refers to the process of determining the most ideal conditions that produce the best separation and is often a balance in terms of time and cost efficiency. When developing HPLC methods from scratch, many factors contribute to the final optimised method including column chemistry/parameters, mobile phase selection, pH, column temperature, flow rate, gradient optimisation, purity of samples, sample matrix etc. Additional chemicals, or modifiers, are often included in HPLC analyses and enhance retention and/or separation. The type of solvent or modifier used can greatly influence the selectivity and retention factor for the analyte in question (Dolan, 2008)

Sample preparation is often required to remove contaminants in complex matrices such as PWS. The two sample preparation methods that will be explored in Chapter 2 are liquid-liquid extraction (LLE) and solid phase extraction (SPE). LLE involves the phase separation of an analyte between two immiscible liquids. These typically comprise an organic solvent, such as dichloromethane or ethyl acetate and an aqueous phase. For basic components, such as the amines in this analysis, the polarity of the molecules is manipulated by adjusting the pH of the aqueous phase to firstly pKa +2 to force them into the organic phase and later pKa -2 to allow phase transfer back into the aqueous phase for further analysis. SPE is a separation process by which the compounds of interest in a liquid mixture are immobilized on a silica-based packing material before being eluted off. SPE has the advantage of reducing the amount of sample required for analysis as well as the amount of solvent used in the process. SPE has a large variety of sorbent material, which allows better matching of the phase for the analytes. Therefore it was possible to develop a procedure isolating a larger range of compounds.

1.4.1 HPLC Analysis of Active Components of CA in Dietary Supplements

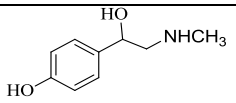
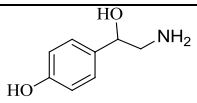
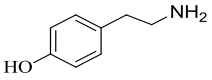
Several studies have been conducted on the determination of phenethylamine alkaloids present in CA-containing dietary supplements. Those which related to the chromatographic determination of phenethylamine alkaloids were reviewed by Pellati & Benvenuti (2007). In this review, however, they focused on the detection of *citrus* alkaloids that are present in dietary supplements rather than in common foods, drinks and plant material. These methods included GC and GC-mass spectrometry (GC-MS) (Cabezas et al., 2013; Marchei, Pichini, Pacifici, Pellegrini, & Zuccaro, 2006; Rossato et al., 2010); capillary electrophoresis (CE) (Avula, Upparapalli, & Khan, 2005; Chizzali, Nischang, & Ganzera, 2011; Liu, Cao, Zheng, & Chen, 2008; Luo, Tan, Xu, Yang, & Yang, 2008); thin layer chromatography (TLC) (Bagatela, Lopes, Cabral, Perazzo, & Ifa, 2015; Shawky, 2014); ion exchange liquid chromatography (Niemann & Gay, 2003; Tang et al., 2006; Thevis, Koch, Sigmund, Thomas, & Schanzer, 2012); and UPLC (Kim, Kwak, Ahn, & Park, 2014). The most common method for adrenergic amine analysis in CA was on RP-HPLC (Table 4) (Beyer, Peters, Kraemer, & Maurer, 2007; Di Lorenzo et al., 2014; Viana et al., 2013; Viana et al., 2015; Wang & Zhao, 2015). Further background of these methods will be discussed in Chapter 4. However, the above mentioned approaches to adrenergic amine determination in CA-containing PWS involved extensive sample preparation, long run times and/or the use of non-volatile mobile phases which were incompatible with mass spectral detection.

Table 4 Comparison of HPLC analytical methods for CA analysis

Compound analysed	Sample preparation	Stationary phase	Mobile phase	Detection	Accuracy	Limits of Quantitation & Detection	Author
synephrine, octopamine, tyramine, *N*-methyltyramine, hordenine	Sonication with 0.1% H_3PO_4	Luna C18 column (150 mm x 3.0 mm, 5 µm)	ACN/20mM borate buffer (pH 8.8) (20:80, v/v), with 10mM sodium 1-hexane sulfonate	UV, 224 nm	97.7-104.0% (syn), 98.9-99.6% (oct), 95.9-117% (tyr), 90.7-103% (nmt), 99.1-107% (hor)	LOQ: 300 µg/g in raw plant material	(Roman et al., 2007)
synephrine, octopamine, tyramine, *N*-methyltyramine, hordenine	sonication with methanol/DMSO (1:1)	Synergi Hydro-RP column (250 mm x 4.6 mm, 4 µm)	0.1 Sodium acetate buffer (pH 5.5)/ACN	UV, 280 nm	98.0-103%	LOD: 0.03 µg/mL (syn), 0.02 µg/mL (oct),0.02 µg/mL (tyr), 0.05 µg/mL (nmt), 0.05 µg/mL (hor)	(Avula, Upparapalli, Navarrete, & Khan, 2005)
synephrine, octopamine, tyramine	sonication with 0.1M HCl	Spherigel C18 column (250 mm x 4.6 mm, 4 µm)	Aqueous solution of 32mM 1-ethyl-3-methyllimidazolium tetrafluroborate [EMIM][BF4]	UV, 273 nm	95.3-101.3% (syn), 74.4-103.2% (oct), 80.0-101.5% (tyr)	LOD: 0.2µg/mL (syn), 0.1 µg/mL (oct), 0.1 µg/mL (tyr),	(Tang et al., 2006)
synephrine, octopamine, tyramine, *N*-methyltyramine, hordenine	sonication with water	pre-column derivatization(A): Luna C18 column (250 mmx 4.6 mm, 5 µm), Direct RPLC	A: methanol/water (55:45, v/v) gradient.	A:fluorescence detector λem = 455 nm , λex = 340 nm. B:with	A: 99.5-101.3%;RSD=0.8-1.2% . B:98.7-102.6%;RSD=1.1-2.3%	LOD: A= 0.05-0.07 pmol, B= 0.21-1.04 pmol. LOQ: A=0.17-0.23 pmol, B=0.7-3.47 pmol	(Gatti & Lotti, 2011)
synephrine, octopamine, tyramine, *N*-methyltyramine, hordenine	magnetic stirring sonication with	LiChrospher RP-18 column (250 mm x 4 mm, 5 µm)	Aqeous SDS:SDS in ACN (62:38, v/v)	UV, 224 nm.	Average RSD values <15%	LOD: 1.8-7.5 ng/mL LOQ: 6.0-25 ng/mL	(Di Lorenzo et al., 2014)
synephrine	ACN/water (80:20, v/v)	Xterra RP18 column (150 mm x 4.6 mm, 5 µm)	Water (5 mM SDS)/ACN gradient	UV, 210 nm	101.10%	LOD: 0.01 µg/mL	(Schaneberg & Khan, 2004)
synephrine, octopamine, tyramine	sonication with 0.37% HCl	HyperClone C18 BDS 1 column (100 mm x 4.6, 3 µm)	water (3 mM SDS): ACN/methanol (3 mM SDS) gradient	UV 210 nm	97.5-102.0%	LOD: 0.23 µg/mL (syn),0.46µg/mL (oct), 0.23 µg/mL (tyr). LOQ: 0.8 µg/mL (syn), 0.62 µg/mL (oct), 0.8 µg/mL (tyr)	(Ganzera et al., 2005)
Synephrine	magnetic stirring with dilute mobile phase	YMC phenyl column (250 mmx 3.0 mm, 5 µm)	0.1M sodium acetate/acetic acid (pH 4.8) with triethylamine and 2% CAN	UV, 255 nm	85-102%		(Hurlbut et al., 1998)
Synephrine	magnetic stirring with acidified acetone/water (4:1, v/v)	Zorbax 300-SCX column (250 mm x 4.6 mm, 5 µm)	0.4M Sodium phosphate buffer (pH 3.0/water/ACN (50:35:15, v/v/v)	UV: 205,210 and 225 nm	93.2-97.5%	LOQ: 0.016 µg	(Niemann & Gay, 2003)
synephrine enantiomers	sonication at ambient temperature	LiChrospher RP-18 column (75mm x 4mm, 5µm) connected to Sumichiral OA-5000 column (250 mm x 4.6 mm, 5 µm)	aqeous 1 mM copper (II) acetate and 20 mM ammonium acetate (pH 6.4)/ methanol (99:1, v/v)	MS, 1.0V	selectivity: 1.23 Resolution: 1.09	-	(Kusu, Matsumoto, & Takamura, 1995)
synephrine, octopamine, tyramine, *N*-methyltyramine, hordenine	PFE and sonication techniques	Discovery HS F5 (200 mm x 4.6 mm, 5 µm)	water (10 mM SDS) (pH 2.5) / acetonitrile (72:28, v/v)	UV, 220 nm	-	LOD: 4.2 ng (syn), 3.5 ng (oct), 5.9 ng (tyr),5.5 ng (nmt)	(Putzbach et al., 2007)
positional isomers of synephrine	sonication in water	Discovery HS F5 (150mm x 4.6 mm, 5 µm)	10 mM ammonium acetate in water: ammonium acetate in metanol (70:30, v/v)	UV, 220 nm	-	LOD: 11 ng (*p*-synephrine), 10 ng (*m*-synephrine). LOQ: 33 (*p*-synephrine), 30 (*m*-synephrine)	(Santana et al., 2008)

For the analysis of charged, basic species such as synephrine, octopamine and tyramine (Table 5), with relatively high pKa's ranging from 8.9 to 10.5, conditions must be in place to either neutralise them or introduce a modifier such as an ion pair reagent (IPR) in order to achieve good peak shape. Unlike dissociated acids, which, due to their negative charge, elute rapidly, protonated bases would often have long retention times and poor peak shape. This retention behaviour is due to the interaction of the positively charged amine with residual silanol groups on the silica surface of the stationary phase not coated with the non-polar adsorbed material. One method of suppressing silanol ion formation is to use IPR such as sodium dodecyl sulphate (SDS) which binds to the silanol groups resulting in improved peak shape and retention behaviour. A number of studies incorporated IPRs for the detection of Citrus amines (Ganzera, Lanser, & Stuppner, 2005; Penzak et al., 2001; Putzbach, Rimmer, Sharpless, & Sander, 2007; Roman, Betz, & Hildreth, 2007; Tang et al., 2006). Ganzera et al. reported a method for the simultaneous detection of alkaloids in CA and ES. The samples were extracted with 0.37% hydrochloric acid via sonication. The column used was a HyperClone C_{18} BDS 1 column (100 mm x 4.6 mm, 3 µm), with a gradient elution of mobile phase A, 3 mM aqueous SDS (pH 4.0); B, 0.1% phosphoric acid containing 3 mM SDS; C, acetonitrile/methanol (2:1, v/v) at a flow rate of 1 mL/min. The UV detection was at 210 nm. The dietary supplements containing CA contained 0.08-3.05% of synephrine whilst octopamine and tyramine were found to be below the limits of detection (LOD). This method was sensitive but the use of the IPR hindered the use of mass detection.

Table 5 Comparison of chemical properties of synephrine, octopamine and tyramine

			
Compounds	Synephrine	Octopamine	Tyramine
Molecular Formula	$C_9H_{13}NO_2$	$C_8H_{11}NO_2$,	$C_8H_{11}NO$,
Molecular Mass (g/mol)	167.2	153.2	137.2
pKa	9.3	8.9	10.5

Use of IPRs, however, is usually prohibitive with MS detectors since they lower the selectivity and decrease reproducibility. An alternative is to derivatise the sample used by Gatti et al. (2012) for the analysis of primary phenethylamines in supplements using pre-column derivatization with fluorescence detection and in contrast to direct RP-LC with fluorescence detection. The chromatographic separation after pre-derivatization with O-phthadialdehyde (OPA) was performed with a Phenomenex Luna 5 µm C18 column (250 mm x 4.6 mm) using methanol and sodium acetate buffer (pH 5.5) by gradient elution fluorescence detection, the total run time was 55 minutes. The study concluded that the derivatization method yielded recovery ranging from 99.5-101.3% with RSD ranging from 0.8 to 1.2% (Gatti et al., 2012; Liu et al., 2009). However, derivatization protocols are time consuming, subject to introduced additional impurities and/or cause ion-suppression for mass detection (Qi et al., 2014). However, these methods also used IPRs for amine retention. HPLC-quadrupole MS is a selective and sensitive method, but is more suited to quantify known compounds that are available as pure reference standards. HPLC coupled to triple quadrupole tandem MS (MS/MS) or HPLC coupled to an ion trap MS (MS^n) is a better set up for unknown compounds. HPLC-MS/MS has been applied to the detection of ephedrine alkaloids and synephrine in dietary supplements (Gay, Niemann, & Musser, 2006; Putzbach et al., 2007; Santana, Sharpless, & Nelson, 2008).

1.4.2 HPLC Analysis of Caffeine & Derivatives

Several analytical methods have been used for the analysis and quantification of caffeine and DMX. These methods include gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS) (Shrivas & Wu, 2007), capillary electrophoresis (CE) (Chen, et al., 2010; Meinhart et al., 2010; Uysal et al., 2009), thin layer chromatography (TLC) (Aranda & Morlock, 2007; Ford et al., 2005), and UPLC (Vaclaviket al., 2014; Venhuis et al., 2014; Zacharis et al., 2013). Based on a recent review (Monteiro et al., 2016), RP-HPLC is the most common analytical method used for caffeine and DMX analysis. Table 6 shows a small representation of the current methods available for caffeine analysis in supplements and biological fluids.

Most methods of DMX separation in biological fluids and foods do not separate PX and TP (Bispo et al., 2002; Hasegawa et al., 2009; Kanazawa et al., 2000; Martinez-Lopez et al., 2014; Thomas et al.,2004). It has been noted that the three DMX isomers are difficult to separate in RP-HPLC (Randon et al., 2010). The three DMX are configurational isomers, have identical masses and are challenging to differentiate analytically without derivatization. Further developments for a HPLC/UV-MS method used in our studies will be discussed in chapter 4.

Table 6 Comparison of methods for caffeine analysis

Compound analysed	Sample preparation	Stationary phase	Mobile phase	Detection	Accuracy	Author
Caffeine, TB, TP	Filtered using filter paper and Soxhlet extraction	Bondesil C18	methanol-water-acetiic acid	UV 273 nm	N/A	(Bispo et al., 2002)
Caffeine, TB, TP	Dissolve in water and filtered with filter paper	Li-Chrospher 100 RP-18 (244 mm x 4.4 i.d., 5 µm)	water, ethanol, ecitic acid	UV 273 nm	94% to 106%	(de Aragao, Veloso, Bispo, Ferreira, & de Andrade, 2005)
Caffeine, TB, TP	N/A	UPLC C18 BEH (50 mm × 2.1 mm, 1.7 µm)	0.1 vol% formic acid/CH_3OH (92.5:7.5, v/v)	UV 272 nm	90% and 108%	(Zacharis et al., 2013)
Caffeine, Taurine	extracted with 4 mL of hexane/isopropanol vortexed and	Phenomenex Luna C18 (150 mm × 4.6 mm, 3 µm)	water/methanol/acetic acid (75:20:5, v/v/v)	Mass spectrometry	70.1 and 94.4%	(Marchei, Pellegrini, Pacifici, Palmi, & Pichini, 2005)
Caffeine, TB, TP	centrifuged (10 mins, 10,500 rpm)	Kinetex C18 (4.6 × 250 mm, 5 µm)	water/ methanol +0.1% formic acid	UV 272 nm	95 and 104.5%	(Martinez-Lopez et al., 2014)

1.5 Research Questions

1. Do CA-containing PWS comply with the 30 mg per dose limit of synephrine set by the TGA of Australia?
2. Is there a discrepancy in synephrine concentrations between overseas sourced and locally manufactured PWS?
3. Are the levels of caffeine measured in a range of CA-containing PWS products, consistent with that listed on their labels?

1.6 Hypotheses

1. It is hypothesised that pre-workout supplements containing CA as a labelled ingredient contain levels of synephrine higher than the 30 mg/dose limit set by the TGA,
2. It is hypothesized that pre-workout supplements sourced from overseas will have higher amounts of synephrine compared to locally available PWS.
3. It is hypothesized that levels of caffeine in pre-workout supplements labelled to contain CA are higher than that listed on their labels.

1.7 Aims

The primary aim of this thesis was to quantitatively determine the amounts of synephrine, octopamine, and tyramine in range of CA-containing PWS by HPLC with UV-MS detection. A secondary aim was to determine the amounts of caffeine and DMX present in PWS with HPLC-UV/MS.

Chapter 2 General Methods

2.1 General Principles of Chromatography

Successful chromatography requires that parameters which affect the passage of molecules through a column be measured, known as elution. The distribution of analytes in chromatography can be expressed in terms of the equilibrium of the analyte between two phases whereby:

$$[A]_{mobile} \leftrightarrow [A]_{stationary}$$

Where the equilibrium constant, K_{eq} can be expressed as

$$K_{eq} = \frac{[A]_{stationary}}{[A]_{mobile}}$$

Such that, at low concentrations of A, the concentration of A in the mobile phase is directly proportional to that in the stationary phase. These quantities, however, are difficult to measure. (Note overloading the column leads to a disproportionate amount of A in one phase and decreased column efficiency). Alternatively the capacity factor, K'_{eq} (the ratio of the moles of A in each phase) provides a means of incorporating volume and also time into an equation developed for determining the amount of time an analyte spends in the stationary phase or retention factor, k (see below). These quantities, including void volume/time and retention volume/time are more easily determined from the chromatography. A full mathematical derivation was expanded by Mikes (2011).

The key goal in most chromatographic analysis is obtaining optimum resolution of each analyte of interest to facilitate accurate quantification. Achieving this requires understanding of the factors that influence resolution. Resolution (R) can be represented by the following equation which relates resolution to other key metrics in chromatography such as efficiency (N), selectivity (α) and retention factor (k) (Heftmann, 2004). Several equations for resolution in LC have been described, varying in their degree of complexity and discussed here. A simplified equation is presented in Equation 1 which assumes that the value of k (and α) for both analytes are similar:

$$R = \frac{\sqrt{N}}{4}.(a-1).\frac{k_1}{1+k_{avg}} \qquad \text{(Equation 1)}$$

The k and α of the column are influenced by pH and in the strength of the mobile phase. Efficiency and resolution depend on the variations of column size, packing material and internal dimensions and flow rate. k_1 is the retention factor of the first product and k_{avg} is the average retention factor of the first product multiple sample set.

The retention factor (k) is a unitless measure of a compound's retention on a chromatographic system such that:

$$k = \frac{V_R - V_0}{V_0} = \frac{t_R - t_0}{t_0} \qquad \text{(Equation 1.1)}$$

where V_R is the anaylte's retention volume, V_0 is the volume of the mobile phase in the chromatographic system. t_R represents the retention time of the analyte, and t_0 is the elution time of an unretained component, known as the column dead time. (t_0 can also be described as t_M (according to International Union of Pure and Applied Chemistry, IUPAC, terminology). Changes in k result in the largest effect on resolution since R is proportional to k (R ∝ k/ (1-k), see Eq. 1).

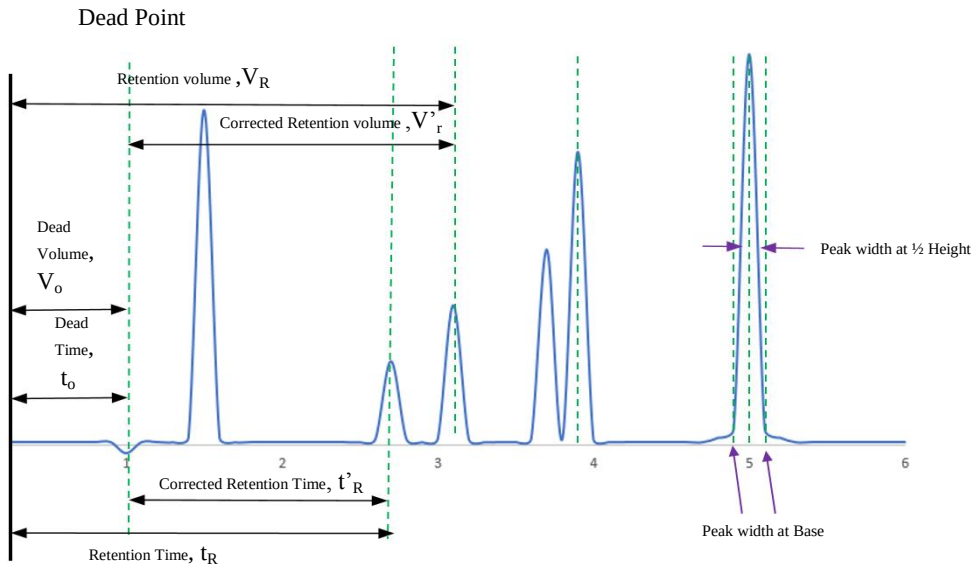


Figure 7 Diagrammatic representation of a chromatogram depicting the relationship between retention time (t_R) and the column dead time (t_o).

Unreliable chromatography ensues with retention factors of less than 1 since analytes are unlikely to be retained. Conversely, k values over 10 means longer run times and lower peak heights. Thus an optimum value for k is around 5, although k values within the range of between 1 to 10 are satisfactory. k can easily be determined from the t_R of each analyte knowing the dead time of the system. One of the easiest ways to modify the retention factor is to alter the mobile composition by increasing the organic component. This coaxes the hydrophobic component of the analyte back into the mobile phase from the stationary phase thereby eluting earlier. Since k is not influenced by small changes in flow rate nor column dimensions, this is often one of the first parameters to optimise and useful for modifying a method developed on a different system (Dorsey, Cooper, Wheeler, Barth, & Foley, 1994).

Selectivity (α) is the ability of the chromatographic system to discriminate between two different analytes. It is defined as the ratio of corresponding retention factors, expressed as:

$$\alpha = \frac{k_1}{k_2} \qquad \text{(Equation 1.2)}$$

Where k_2 and k_1 are the retention factors of two analytes.

At a value of 1 the two analytes are co-eluting thus, by definition, selectivity values are always greater than 1 and should be as high as possible. The higher the value the greater the distance between the apices of the two peaks.

Selectivity is a factor of the chemistry of analyte, mobile and stationary phases, so modifications in all these factors can be performed to optimise selectivity. For example, changing the organic solvent from methanol to acetonitrile, changing the pH of the mobile phase, changing the column chemistry or altering the column temperature.

Efficiency is a measure of the degree of peak broadening produced by a particular column. Peak broadening is caused by dispersion forces on the analytes as they transverse the system resulting in their typical Gaussian shape. It can be expressed mathematically as in Equation 1.3:

$$N = 16\left(\frac{t_R}{w}\right)^2 \text{ or } N = 5.55\left(\frac{(t_R)^2}{\left(w_{1/2}\right)^2}\right) \qquad \text{(Equation 1.3)}$$

where t_R is the retention time, w is the peak width of the baseline, $w_{1/2}$ is the width of peak at half height of peak.

Efficiency is expressed as the number of theoretical plates (*N*) (Heftmann, 2004). Theoretical plates was a concept originating from fractional distillation and applied to chromatography. It was a concept whereby the entire column can be thought of as consisting of discrete sections, whereby the analytes can equilibrate. In lay terms, the higher the number of plates, the greater the equilibration and the narrower peak shape. *N* depends on column dimensions, but for a typical column of 4.6µm diameter of 100 mm length acceptable values of N would be between 5000 - 8000. *N* is inversely proportional to the space between the plates or plate height. Note that the latter is often referred to as the Height Equivalent or Theoretical Plate or HETP (Dorsey et al., 1994). Values of efficiency are often given for quoting the efficiency of a particular column. Predetermined values may be in place in a quality control setting such that when the column efficiency passes below a set value, the column use is terminated.

Band broadening is a phenomenon that decreases the efficiency of the separation, which results in poor peak resolution and overall chromatographic performance. The Van Deemter equation was described to understand the main factors that result in a loss of column efficiency. The equation relates the HETP of the column to the various kinetic parameters during mobile phase flow. It was described as the following:

$$HETP = A + \frac{B}{u} + Cu \qquad \text{(Equation 2)}$$

Where A is the Eddy-diffusion parameter, B is the diffusion coefficient of eluting particles, C is the mass transfer coefficient of the analyte between mobile and stationary phase, and u is the linear velocity of the mobile phase.

Eddy diffusion describes the variations in mobile phase flow or analyte flow path within the column. The variations in an analyte flow ‘path’ are caused by the lack of homogeneity in the column packing material and particle size. A well-packed column with smaller stationary particle size is ideal to minimise Eddy Diffusion.

The diffusion coefficient can determine the extent of band broadening resulting from analyte molecules contained in the injection solvent dispersing within the tubing of the HPLC. The amount of analyte dispersion is influenced by the length and internal diameter of the tubing as well as the internal volume of the detector flow cell. Increasing the flow rate of the method will reduce the effect of the diffusion coefficient.

Mass transfer occurs due to stagnation of mobile phase within the porous surfaces of the packing material of the stationary phase. Band broadening also occurs when the analyte molecules adsorb to the pores of the stationary phase at varying rates. These effects may be reduced by decreasing the size of the packing material and lowering the flow of the mobile phase

2.2 Developing a Systematic Work Flow

Chromatographic method development can often be time-consuming and difficult whereby the typical approach is to select a combination of solvents, pH, buffer and column as a starting point, making adjustment to the composition until a suitable method is selected. The systematic workflow outlined in Figure 8 below, was used to develop the methods set out in Chapters 3 and 4.

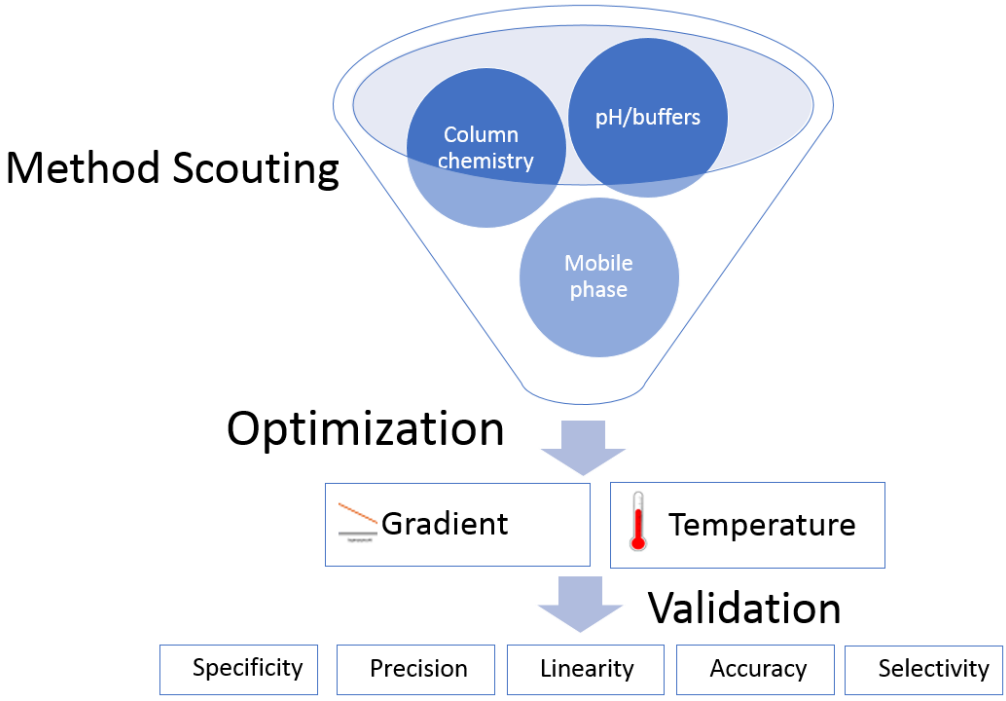


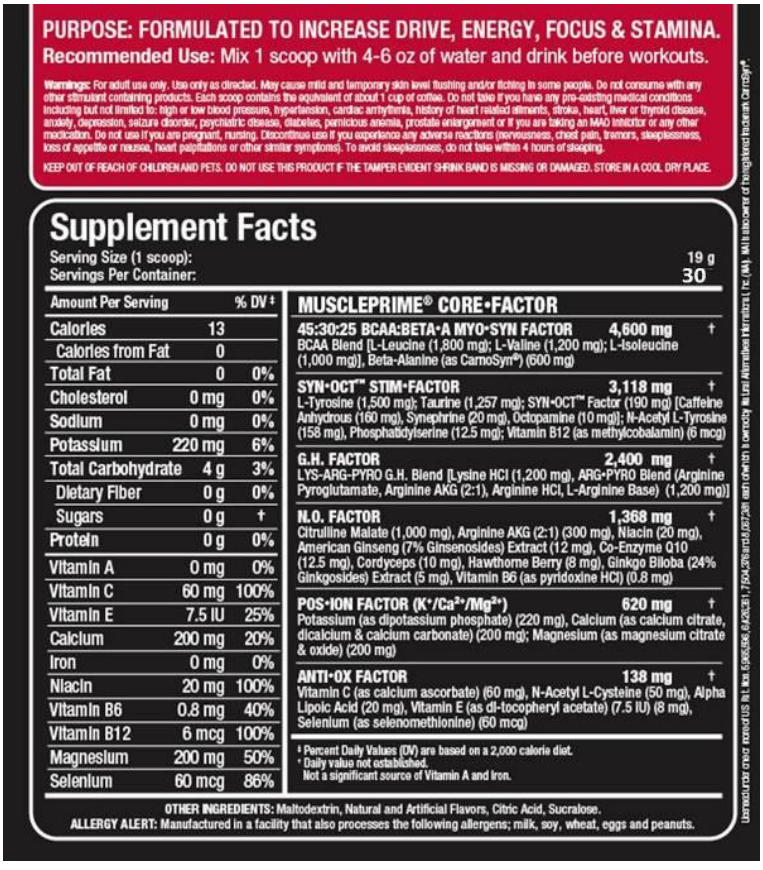
Figure 8 Diagram of method development workflow

Factors such column chemistry, strength of organic solvents, temperature and pH were evaluated to determine the most effective experimental parameters to achieve resolution. The best conditions producing optimum values for these parameters were used to refine the chromatography.

Finally, a single-laboratory validation was conducted. The following method section describes experimental procedures which apply equally to both analytical experiments, such as solid phase extraction of samples (sample clean-up). In cases where specific methodology applicable to one or the other experiments apply, these more specific protocols may be found within their respective chapters, for example, Chapter 3 - HPLC detection of amphetamine-like compounds and Chapter 4 - HPLC detection of caffeine.

2.2.1 Sample Preparation

An essential aspect for obtaining good chromatography is to ensure the sample injected into the HPLC system is free from unwanted contaminants that may interfere with the chromatography. The challenge of sample preparation for PWS is the complexity of its matrix. Most PWS contain a mixture of ingredients in a 'proprietary blend'. A problem with proprietary blends is that the FDA does not monitor the PWS labels to guarantee specific amounts of ingredients are present. Common ingredients generally found in PWS are caffeine, creatine, arginine, taurine, β-Alanine, and phosphate (Eudy et al., 2013). In addition, other non-active ingredients include colouring, flavourings and bulk powders with a wide range of physicochemical properties. Figure 9 shows an example of a typical label of a PWS supplement containing CA. Two sample preparation techniques were explored based on their conventional use and straight forward approach.



PURPOSE: FORMULATED TO INCREASE DRIVE, ENERGY, FOCUS & STAMINA.

Recommended Use: Mix 1 scoop with 4-6 oz of water and drink before workouts.

Warnings: For adult use only. Use only as directed. May cause mild and temporary skin level flushing and/or itching in some people. Do not consume with any other stimulant containing products. Each scoop contains the equivalent of about 1 cup of coffee. Do not take if you have any pre-existing medical conditions including but not limited to: high or low blood pressure, hypertension, cardiac arrhythmia, history of heart related ailments, stroke, heart, liver or thyroid disease, anxiety, depression, seizure disorder, psychiatric disease, diabetes, pernicious anemia, prostate enlargement or if you are taking an MAO inhibitor or any other medication. Do not use if you are pregnant, nursing. Discontinue use if you experience any adverse reactions (nervousness, chest pain, tremors, sleeplessness, loss of appetite or nausea, heart palpitations or other similar symptoms). To avoid sleeplessness, do not take within 4 hours of sleeping.

KEEP OUT OF REACH OF CHILDREN AND PETS. DO NOT USE THIS PRODUCT IF THE TAMPER EVIDENT SHRINK BAND IS MISSING OR DAMAGED. STORE IN A COOL DRY PLACE.

Supplement Facts

Serving Size (1 scoop): 19 g
Servings Per Container: 30

Amount Per Serving		% DV‡
Calories	13	
Calories from Fat	0	
Total Fat	0	0%
Cholesterol	0 mg	0%
Sodium	0 mg	0%
Potassium	220 mg	6%
Total Carbohydrate	4 g	3%
Dietary Fiber	0 g	0%
Sugars	0 g	†
Protein	0 g	0%
Vitamin A	0 mg	0%
Vitamin C	60 mg	100%
Vitamin E	7.5 IU	25%
Calcium	200 mg	20%
Iron	0 mg	0%
Niacin	20 mg	100%
Vitamin B6	0.8 mg	40%
Vitamin B12	6 mcg	100%
Magnesium	200 mg	50%
Selenium	60 mcg	86%

MUSCLEPRIME® CORE•FACTOR

45:30:25 BCAA:BETA•A MYO•SYN FACTOR 4,600 mg †
BCAA Blend [L-Leucine (1,800 mg); L-Valine (1,200 mg); L-Isoleucine (1,000 mg)], Beta-Alanine (as CarnoSyn®) (600 mg)

SYN•OCT™ STIM•FACTOR 3,118 mg †
L-Tyrosine (1,500 mg); Taurine (1,257 mg); SYN•OCT™ Factor (190 mg) [Caffeine Anhydrous (160 mg), Synephrine (20 mg), Octopamine (10 mg)]; N-Acetyl L-Tyrosine (158 mg), Phosphatidylserine (12.5 mg); Vitamin B12 (as methylcobalamin) (6 mcg)

G.H. FACTOR 2,400 mg †
LYS-ARG-PYRO G.H. Blend [Lysine HCl (1,200 mg), ARG-PYRO Blend (Arginine Pyroglutamate, Arginine AKG (2:1), Arginine HCl, L-Arginine Base) (1,200 mg)]

N.O. FACTOR 1,368 mg †
Citrulline Malate (1,000 mg), Arginine AKG (2:1) (300 mg), Niacin (20 mg), American Ginseng (7% Ginsenosides) Extract (12 mg), Co-Enzyme Q10 (12.5 mg), Cordyceps (10 mg), Hawthorne Berry (8 mg), Ginkgo Biloba (24% Ginkgosides) Extract (5 mg), Vitamin B6 (as pyridoxine HCl) (0.8 mg)

POS•ION FACTOR (K^+/Ca^{2+}/Mg^{2+}) 620 mg †
Potassium (as dipotassium phosphate) (220 mg), Calcium (as calcium citrate, dicalcium & calcium carbonate) (200 mg); Magnesium (as magnesium citrate & oxide) (200 mg)

ANTI•OX FACTOR 138 mg †
Vitamin C (as calcium ascorbate) (60 mg), N-Acetyl L-Cysteine (50 mg), Alpha Lipoic Acid (20 mg), Vitamin E (as dl-tocopheryl acetate) (7.5 IU) (8 mg), Selenium (as selenomethionine) (60 mcg)

‡ Percent Daily Values (DV) are based on a 2,000 calorie diet.
† Daily value not established.
Not a significant source of Vitamin A and Iron.

OTHER INGREDIENTS: Maltodextrin, Natural and Artificial Flavors, Citric Acid, Sucralose.
ALLERGY ALERT: Manufactured in a facility that also processes the following allergens; milk, soy, wheat, eggs and peanuts.

Figure 9 Example of a typical PWS supplement label

2.2.1.1 Liquid- Liquid Extraction (LLE)

LLE was first trialled, during the early stages of project method development, as it was a relatively cheap and simple technique (Figure 10)

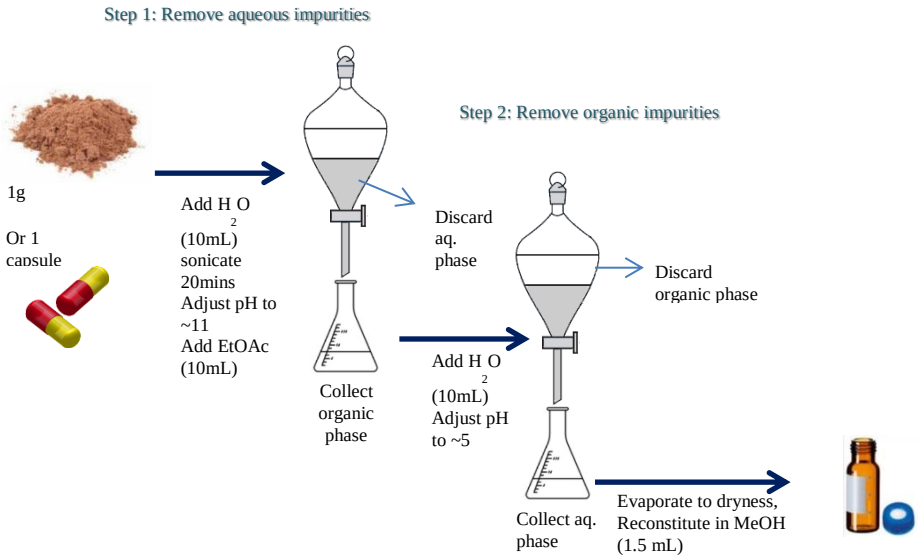


Figure 10 Diagrammatic representation of the LLE method used

Powdered PWS sample (1 g) was dissolved in a total of 10 mL of MilliQ water and 5M sodium hydroxide (NaOH), where the pH was adjusted to 12. This ensured the trace amines were in a neutral state. The mixture was sonicated for 30 minutes to completely dissolve the components. The solution was then placed into a separating funnel. Ethyl acetate (10 mL) was used to extract the amines. The aqueous layer was discarded and the remaining organic phase was kept. Then, 1 mL of 1M hydrochloric acid and 9mL of water was added to the solution to protonate the retained amines. Upon separation of solvent layers, the organic layer was discarded and the aqueous layer was evaporated to dryness at room temperature (24°C). The dried residue was then reconstituted with 1.5 mL methanol and aliquoted for HPLC analysis.

The LLE method was subject to poor repeatability and reproducibility and this, coupled with long sample preparation time, limitations to the selectivity and difficulties with emulsions, made a rapid analysis of PWS difficult. For this reason, it was decided to turn to a much more consistent and robust method of sample preparation such as solid phase extraction.

2.2.1.2 Solid Phase Extraction (SPE)

The Oasis PRIME HLB (3 mL, 60 mg cartridge, Waters, Ireland) cartridges were selected because they were especially suitable for basic compound analysis such as the amphetamine like compounds of interest in CA-containing supplements. These cartridges were also suitable for the extraction of xanthines. The workflow is set out in Figure 11.

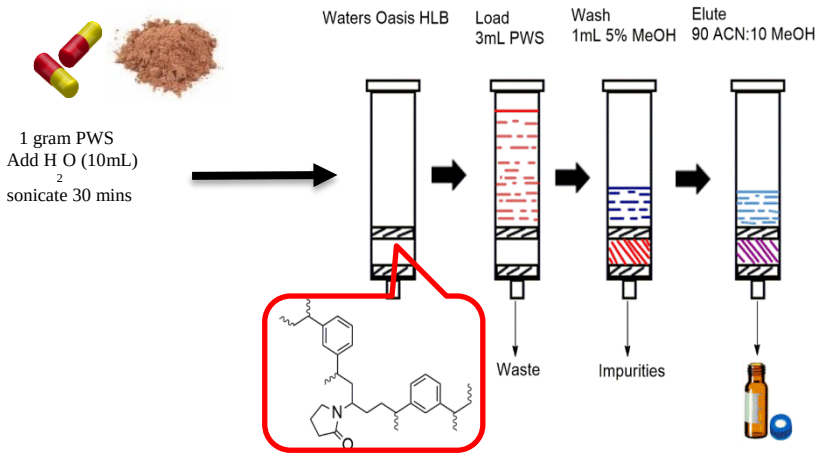


Figure 11 Representative diagram of the SPE procedure with the Oasis HLB cartridge

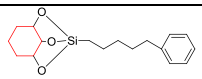
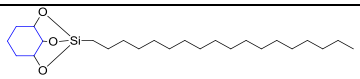
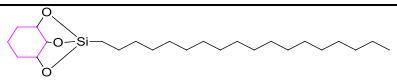
CA-containing PWS (1 g) was weighed and 10 mL of MilliQ water was added. The mixture was sonicated under ambient temperature for 30 minutes. MilliQ water was used to wash polar impurities away whilst an acetonitrile: methanol mixture (90:10, v/v) was used to elute the non-polar components within PWS into 2 mL HPLC vial. Samples were evaporated to dryness prior to reconstitution in mobile phase and stored at 4°C before subsequent HPLC analysis. Each supplement sample was prepared in triplicate. This method of sample clean-up was found to have a high degree of specificity with the SPE sorbent, producing a more consistent yield, and using less solvent per sample compared to LLE.

2.2.2 Stationary Phase Selection

The column selection was made based on the specific analyte being considered. For example, columns best able to retain charged amines, such as the amphetamine-like compounds, must be stable at high pH and able to maximise aromatic interactions between the analyte and the stationary phase particle coating. During method development, access to a wide range of columns can be a limiting factor with budget constraints. In these cases, developing skills in optimising the parameters such as those discussed in section 2.1 is essential.

The choices of columns available for this study was narrowed down to a Xselect CSH phenyl-hexyl (3.5 µm, 4.6 x 100 mm, i.d., Waters, Ireland), Xselect HSS T3 (5 µm, 3.0 x 150 mm, i.d., Waters, Ireland) and Xbridge BEH C18 (2.5 µm, 3.0 x 100 mm, i.d., Waters, Ireland).

Table 7 Comparison of column chemistry and dimensions

Ligand and Particle Type	 CSH Phenyl- Hexyl	 BEH C18	 HSS T3
Particle Size (µm)	3.5	2.5	5.0
pH range	1-11	1-12	2-8
Temperature limit (°C)	45- 60	45-80	45
Description	General purpose alternative selectivity ligand that provides π-π interactions with polyaromatic compounds, increased loading capacity for basic compound	General purpose column ideally suited for method development due to extreme pH stability and applicability to the broadest range of compound classes.	Aqueous mobile-phase compatible column designed for exceptional polar compound retention.

The column packing materials listed in Table 7 for each column consisted of a Charged Surface Hybrid (CSH), Ethylene Bridged Hybrid (BEH), and High Strength Silica (HSS) proprietary to Waters (Waters, 2016). The HSS and BEH columns chemistry were C18 although with different pH and temperature resistances due to their respective end-capping. However, the phenyl-hexyl chemistry of the CSH column has advantages in aromatic compounds retention such as an increased π-π interaction (Gibson & Fowler, 2014). Further specific developments of the column chromatography will be discussed in Chapter 3 and 4.

2.2.3 Mobile Phase Selection

The organic mobile phases trialled during method development, acetonitrile and methanol, are commonly used in LC. Acetonitrile is a stronger solvent compared to methanol and this fact can be exploited during the optimisation process. Both solvents have low absorbance at typical analysis wavelengths for UV detection with acetonitrile having a UV cut-off wavelength of 200 nm while that for methanol is 240 nm. An increase in the organic content of the mobile phase of 10% will decrease the retention factor, k, for each analyte by a factor of 2 to 3. In addition, organic modifiers have different viscosities. A less viscous mobile phase is desirable as it reduces the backpressure of the chromatographic system. Further discussions on the impact of mobile phase selection will be detailed in their respective results section (Chapter 3 & 4).

2.2.4 pH Modifiers in Mobile Phase

As mentioned, many RP-HPLC experiments are maintained at low pH (around 2-3) to minimise silanol ionisation. Unfortunately, at such low pH values, amphetamine-like compounds (pKa > 9), would be positively charged and interact with the negatively charged silanol groups on the stationary phase increasing retention and causing peak tailing. Acidic modifiers such as trifluroacetic acid (TFA) and formic acid (FA) were used to decrease the pH of the mobile phases reducing charged silanol group interactions. However, the acidity of TFA is greater than formic acid. The impact of acid as a pH modifier will be explored further in Chapter 4.

Retention of basic analytes is still possible using a method such as ion-pair reagents which overcomes these unwanted silanol interactions. It was important to identify pH modifiers that would overcome these interactions while being compatible with QDa mass detection. As a result, ammonium hydroxide was selected for the separation of our amphetamine-like compounds discussed in Chapter 3.

2.2.5 Apparatus and Materials

All experiments were carried out using an Alliance e2695 (Waters, Ireland) separations module with a quaternary solvent system, equipped with auto-sampler, 100 µL injection loop, column oven, model 2489 UV/Vis Detector (Waters, Ireland) in series with an Acquity quadrupole diode array (QDa) mass detector (Figure 12). Empower V3 software was used for data acquisition and processing. Samples were filtered through a 0.45-micron filter prior to solid phase extraction (Oasis PRIME HLB, 3 mL, 60 mg cartridge, Waters, Ireland). Synephrine, octopamine, tyramine, phenylephrine, caffeine, TP, TB, PX and etofylline were obtained from Sigma-Aldrich (Sydney, Australia). HPLC grade Optima methanol, Optima acetonitrile and Optima grade formic acid were from Fisher Scientific (Geel, Belgium). HPLC grade water from a Milli-Q system (Millipore, Bedford, MA, USA) was used. All chemicals were of analytical grade suitable for LC-MS.

Figure 12 Representative Alliance e2695 set up paired to a UV/Vis detector and a QDa detector

2.3 Method Validation

The validation process aims to demonstrate that the analytical method being developed is fit for purpose. In a quality assurance setting, there are specific regulatory requirements that must be demonstrated for a method to be considered valid (ICH Expert Working Group, 1994). A complete method validation involves inter-laboratory testing with more than one laboratory. However, access to other laboratories is often not possible and certainly beyond the scope of this study. Validation involves a demonstration of reliable results and an acceptable degree of repeatability. The validation parameters are set by the International Conference On Harmonisation Of Technical Requirements For Registration Of Pharmaceuticals For Human Use (ICH Expert Working Group, 1994) was the chosen guideline for this study.
The validation parameters determined were as follows:

2.3.1 Selectivity

Selectivity is the ability to distinguish between the analyte being measured and other substances present. Selectivity was confirmed by the use of UV-detection and QDa-MS by incorporating a starting condition blank as well as a dietary supplement blank (not containing any of the analytes being measured).

2.3.2 Precision

Intra-day and inter-day assay precision, reported as relative standard deviation (RSD %) of standard peak areas and were calculated based on the equation:

$$RSD\ \% = Standard\ deviation/\ mean \times 100$$

Intra-assay precision was determined by analysing three different concentrations (5, 50, and 100 µg/mL) of the internal standard (IS) from each method in triplicate on the same day. Inter-day precision was determined based on analysing these concentrations over three separate days.

2.3.3 Accuracy

Accuracy was determined by evaluating spiked PWS with 3 concentrations of standards calculating RSD % between the mean values.

2.3.4 Linearity

For each run, 6 standard concentrations were prepared and analysed with three independent injections. Concentrations ranged between 5 µg/mL – 100 µg/mL. Linear regressions were generated on 3 separate days using the standards to IS peak area ratios by weighted ($1/x^2$) least-squares linear regression. Calibration curve equation and correlation coefficients (r^2) were calculated. The acceptance criteria for acceptable calibration curve were r^2 of 0.99 or better.

2.3.5 Limit of Detection and Limit of Quantification

Determination of the limits of detection (LOD), limits of quantitation (LOQ), intra-assay and inter-assay precision were performed according to outlined in the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Q2 (R1), 2005). The calibration curves were obtained by plotting the area of base peak of extracted chromatogram against the internal standard. Limits of detection (LOD) and limits of quantitation (LOQ) were determined based on their respective signal-to-noise ratio (S/N) where the desired S/N for the LOD is between 3 or 2:1, whereas the desired S/N for the LOQ is 10:1.

2.3.6 S/N Determination Within the Empower 3 Software.

In 2009, the United States Pharmacopeia (USP) defined Signal-to-Noise (S/N) as:

$$S/N = \frac{2h}{hn} \qquad \text{(Equation 3)}$$

Where *h* is the height of the peak corresponding to the component and *hn* is the difference between the largest and smallest noise values observed over a distance equal to at least 5 times the width at half-height of the peak. This approach does not compensate for local systematic drift. Basically, if the noise within this system increases, the S/N decreases which could result in larger LOD and LOQ values. Therefore, it is important to use the most representative noise value possible. To reduce the effects of drift, peak-to-peak noise calculation was done on the Empower 3 software. The peak-peak noise determines the best-fit line regression line to the noise and calculates the residual amount for each data point.

Peak-to-Peak noise was determined by the inclusion of a custom field (see below) in the 'Noise and Drift' tab of the processing method. A noise interval of 60 points ensures it is at least a minimum of five times the width at half height of the peak of interest (Figure 13). The noise was determined in a peak-free region of the chromatogram to abide to the USP S/N time range.

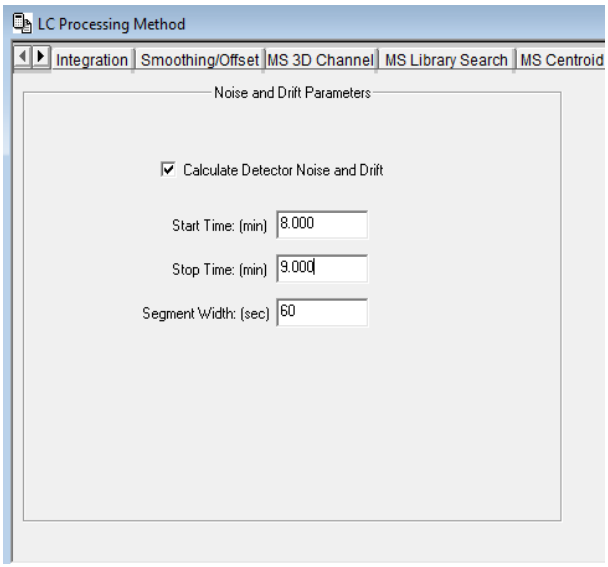


Figure 13 Empower 3 software window of noise and drift calculation

To determine the appropriate S/N within the method, a custom field was defined to determine the USP Signal-to-Noise. The custom field formula was:

$$\frac{S}{N} = 2 * Height * \frac{Scale\ to\ \mu V}{Peak\ to\ peak\ noise}$$

Figure 14 shows this formula and the appropriate custom field parameters in the Empower 3 Edit Custom Field window. The S/N was calculated for each individual analyte peak found with each subsequent analysis.

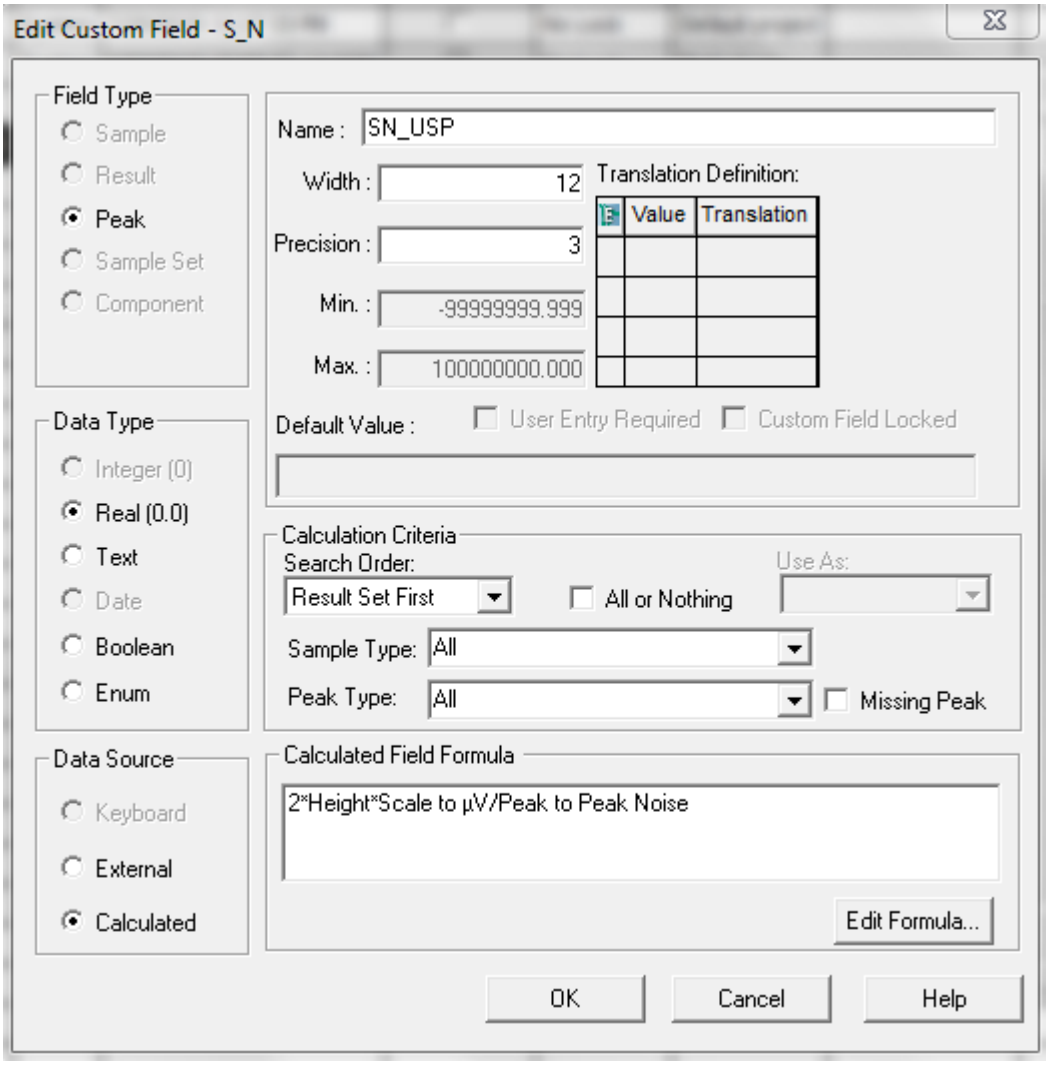


Figure 14 Empower 3 software of custom field window

2.3.7 The Theory of Internal Standard Use

An internal standard (IS) is a known amount of a compound (different from the analyte) that is added to the unknown sample. The signal of the analyte is compared with signals from the IS to find out how much analyte is present. An ideal IS should be chemically similar to the analytes of interest, yet should have a chromatographic peak well separated from the analytes. This is so that uncontrolled matrix effects such as a change in analyte signal might have a similar effect on the IS.

To use an IS, we prepared a known mixture of standard and IS to measure the relative response of the two compounds. The area *(A)* under each peak is proportional to the concentration (*C*) of the compounds injected. However, the detector generally has a different intensity to each component of the same injected concentrations of the analyte (x) and the IS. The magnitude of difference of area found is known as the response factor (F). This is shown in equation 2.

$$\frac{A_x}{C_x} = F \, . \, \frac{A_{IS}}{C_{IS}} \qquad \text{Equation 3}$$

The equations above use a single mixture to find the response factor. To reduce the effects of experimental error, a multipoint calibration curve is preferred. Equation 3 is rearranged so that the variables of the equation can be plotted on a graph, where the gradient of the graph will be the response factor (Equation 3.1).

$$\frac{A_x}{A_{IS}} = F \, . \, \frac{C_x}{C_{IS}} \qquad \text{Equation 3.1}$$

Further application of the internal standard method will be discussed in Chapter 3, where phenylephrine is used as an internal standard to the method.

Chapter 3 Development of a Rapid HPLC-MS Protocol for Synephrine, Octopamine and Tyramine in CA Extracts in Pre-workout Supplements

3.1 Background

Concomitant with the increased popularity of CA-containing PWS in the community is the need for assessing the safety and efficacy of the components therein, especially in light of reports of their adverse effects. PWS are, by nature, complex matrices which require a selective and sensitive means of quantifying the active constituents. A review of techniques for the separation, subsequent detection and finally quantification of the amphetamine-like compounds in PWS has already been discussed in Chapter 1**.**

The most common method for separation of the adrenergic amines in CA was found to be RP-HPLC paired to a UV detector (Beyer et al., 2007; Di Lorenzo et al., 2014; Viana et al., 2013; Viana et al., 2015; X. L. Wang & Zhao, 2015). HPLC stationary phases are made of C18 bounded to silica, where the basic amines could interact with the silanol ions, resulting in poor peak shape and retention (Neue et al., 2004). HPLC methods of *CA* amine separation with ion-pair reagents were used to improve retention of charged amines and improve peak shape (Di Lorenzo et al., 2014; Ganzera et al., 2005; Penzak et al., 2001; Putzbach et al., 2007; Roman et al., 2007).

Di Lorenzo et al., developed a method to measure the amount of active amines in dietary supplements containing CA. HPLC analysis was carried out with a LiChrospher RP-18 column (250 mm x 4 mm, 5 µm), with a 30 minute gradient elution at 1 mL/min with mobile phases: A, 2.9 g/L sodium dodecyl sulphate (SDS) in water at pH 4.2; B, 2.9 g/L SDS: ACN (62:38, v/v). UV detection was set at 224 nm. The analytical recovery of the amines ranged from 85.9% to 108.4% and the LOD: 1.8-7.5 ng/mL LOQ: 6.0-25 ng/mL. The use of the SDS IPR prevented the potential use of MS.

Santana et al. (2008) developed two LC methods for the baseline separation and quantitative determination of *p*-synephrine and *m*-synephrine. The incorporation of MS assisted in confirming the identity of the resolved isomers. The chromatography involved a Supelco Discovery HS-F5 (pentaflurophenyl) column (150 mm x 4.6 mm, 5 µm); mobile phase A= 10 mmol/L ammonium acetate in water; B= ammonium acetate in methanol; A/B= 70/30; at a flow rate of 1 mL/min. UV detection was set at 220 nm. The method resulted in a linear range of 0.001-11.318 µg of *p*-synephrine with LOD of 11 ng. The tandem MS set up had gradient conditions as above but with a flow rate of 0.5 mL/min with the detection and quantification was carried out with the use of ESI-MRM/MS/MS. The linear range for this set up was 0.005- 10 ng with an LOD of 0.005 ng. The results showed that *m*-synephrine was not detected in any of the tested dietary supplements. The study concluded that the LC/UV method was a more convenient method to use as a general screening method, while the LC/MS/MS method was more suited to a confirmatory method for verifying the presence of particular synephrine structural isomers in dietary supplements.

The aforementioned approaches to adrenergic amine determination in CA-containing PWS either involved extensive sample preparation, long run times and/or the use of non-volatile mobile phases. Concern for the increasing consumption of stimulant-containing dietary supplements in the general population (Eichner & Tygart, 2016) drives the need of an analytical method that is rapid, sensitive and cost effective.

The aim of this study was to develop a rapid, accurate and simple HPLC-UV/MS method for the determination and quantification of the synephrine, octopamine and tyramine in CA-containing PWS.

3.2 Experimental

3.2.1 Chemicals and Reagents

The chemicals and reagents used in this chapter are detailed in General Methods (Chapter 2). The five PWS selected were MusclePrime (AllMax Nutrition) (A); Gold Standard Preworkout (OptimumNutrition) (B); 360Rage (360°Cut Performance Supplements) (C); ErgoBlast (ErgoGenix) (D) and PumpHD (BPISports) (E). The matrix blank was a PWS without CA, Beast Mode (Beast Sport's Nutrition). The PWS samples A, C and D were purchased online (manufactured in the USA) while samples B and E were sourced from a local health store in South-East Queensland, Australia

3.2.2 Standard and Sample Preparation

Stock solutions (1 mg/mL) were prepared for all standards as well as internal standard (IS) and stored at 4°C until analysis. Phenylephrine (*m*-synephrine) was selected as IS since it is a positional isomer of synephrine and not naturally found in CA extracts (Pellati & Benvenuti, 2007). A linear dilution was prepared for each standard between 5 µg/mL to 100 µg/mL for all standards. The results of concentrations outside this linear range will be discussed in section 3.4.1. The PWS samples were prepared using the SPE method detailed in Chapter 2.

3.3 Method Development

According to the method development process depicted in Figure 8 (Chapter 2), by changing various parameters such as different column chemistries, mobile phase composition, temperature and gradient combinations, an optimised method for quantifying the adrenergic stimulants in PWS was achieved. The details of this process are outlined in the following sections to attain the best conditions for separation.

3.3.1 Effect of Column Chemistry

Two chromatographic columns were compared; an Xbridge C18 (2.5 µm, 3.0 mm x 100 mm, i.d., Waters, Ireland) and an Xselect CSH phenyl-hexyl (3.5µm, 4.6 mm x 100mm, i.d., Waters, Ireland)**.** These were selected based on their ability to interact with aromatic amines.

To compare the effect on analyte retention of the two columns a series of 100 µg/mL standard mix (including IS) injections were run using an initial mobile phase gradient A: H_2O + 0.1% NH_4OH: 95 – 0% and B: ACN + 0.1% NH_4OH: 5 – 100% over a period of 20 minutes with a flow rate of 1 mL/min at 30°C.

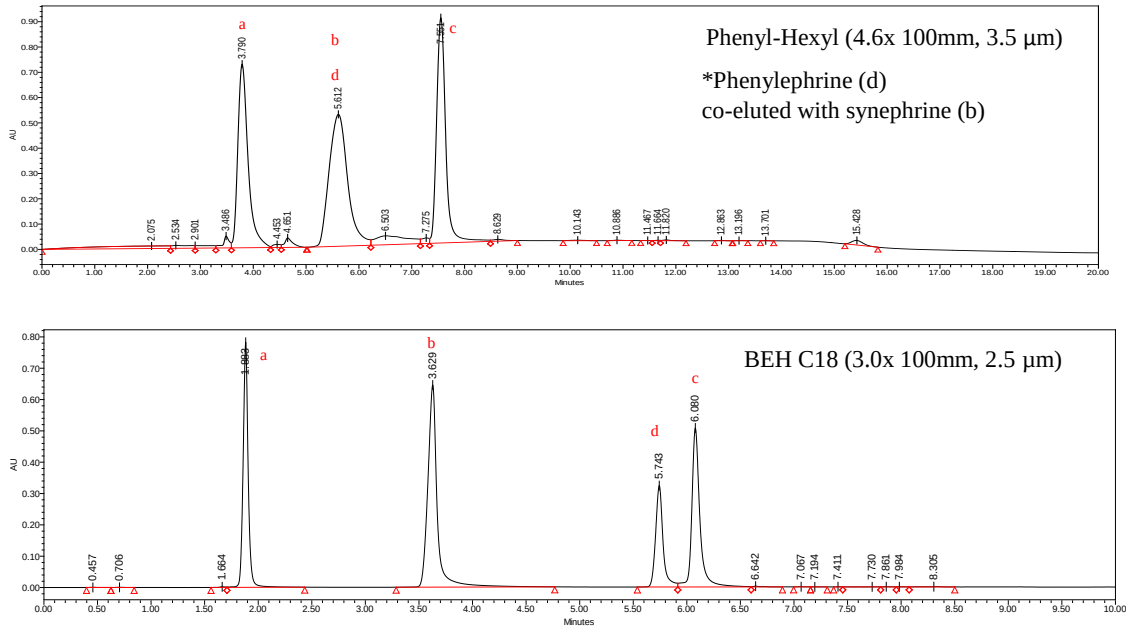


Figure 15 Comparison of column chemistry: (A) Xselect CSH phenylhexyl (3.5 µm, 4.6 x 100 mm, i.d.) and (B) Xbridge C18 (2.5 µm, 3.0 x 100 mm, i.d.). The standard compounds are represented by: (a) Octopamine; (b) Synephrine; (c) Tyramine; (d) Phenylephrine

The elution order of the amines from the two columns was octopamine, followed by synephrine, phenylephrine and finally tyramine (Figure 15). This was as expected from their *clogP* values. The results are summarised in Table 8.

Table 8 Summary of effects of column chemistry on CA amines

Compound	RT on Phenyl-hexyl column (mins)	RT on C18 column (mins)
Octopamine	3.79	1.88
Synephrine	5.61	3.63
Phenylephrine	Not detected*	5.74
Tyramine	7.55	6.06

*May have co-eluted with synephrine

The phenyl functional group of the phenyl-hexyl column introduce the potential for additional molecular interactions such as π-π interactions (Yang, Fazio, Munch, & Drumm, 2005). These effects most likely explain the increased RT for the analytes on the phenyl-hexyl column.

It was noted that the peaks observed on the phenyl-hexyl chromatogram were broader than those seen on the C18 chromatogram. Peak broadening can occur due to factors such as eddy diffusion, longitudinal diffusion and mass transfer, as mentioned in the previous chapter. Since the length of the column and instrument set up are similar, the two factors that would cause the broadening were eddy diffusion and mass transfer. Eddy diffusion is a term used to describe the variations in mobile phase flow and the analyte flow path within the chromatographic column represented in Figure 16. It is often related to the quality of the column packing. The larger particle size of the phenyl-hexyl column could have led to eddy diffusion effects and thus broader peaks observed in the chromatogram.

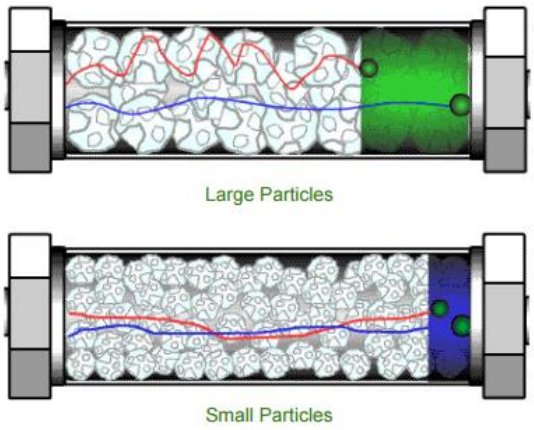


Figure 16 Eddy diffusion effects can result in peak broadening (sourced: CrawfordScientific)

The particle size of the phenyl-hexyl column (3.5µm) is larger than the C18 column (2.5µm). The difference in particle size could affect the rate of adsorption of the amines based on the various flow paths of the amines such that larger particles could cause a slowing down as the analytes traverse the column. If this was the case, one would expect the retention time for analytes on the phenyl hexyl column to be longer. This is indeed the case and therefore eddy diffusion could be an additional factor explaining the longer retention time on the phenyl-hexyl column.

Mass transfer is dependent on the porosity of the stationary phase, where the amines could interact with the stationary phase at different rates of adsorption (Figure 17).

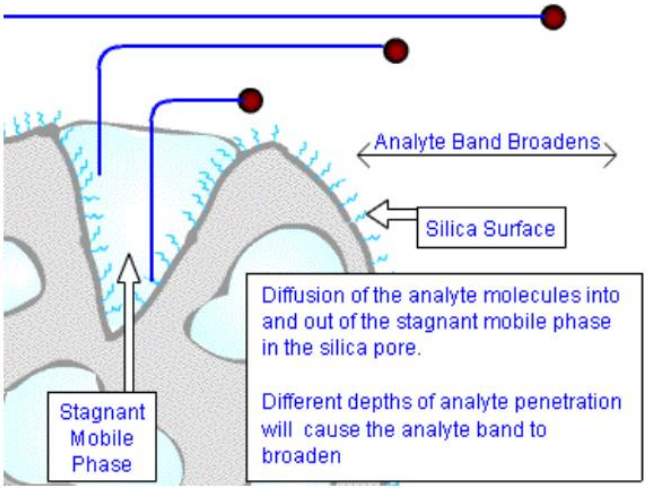


Figure 17 Diagram of analyte interaction on stationary phase pore (sourced: Crawford Scientific)

The pore diameter for both columns was 130Å, but the internal diameter of the phenyl hexyl column (4.6mm) was larger than the C18 column (3.6mm). The smaller diameter of the packing material caused an increase in linear velocity within the column. The increased linear velocity would have reduced the rate of mass transfer in the C18 column, therefore decreasing peak broadening.

Importantly it was noted that a separate peak for phenylephrine was not observed in the phenyl-hexyl chromatogram. Since *p*-synephrine and phenylephrine (*m*-synephrine) are geometric isomers, it was likely that the broad synephrine peak in the phenyl column was a result of the co-elution of phenylephrine and *p*-synephrine.

On the basis of the above observations of greater peak resolution, decreased amount of peak broadening and its ability to separate the two synephrine isomers, the Xbridge BEH C18 was selected as the preferred column for this analysis.

3.3.2 Effect of pH

The question being investigated here was really which effect most influenced the chromatography: either the unwanted silanol interactions incurred at a high pH or that due to having charged amines at a low pH. To answer this question three different pH values were tested as to their effect on chromatography– the details of which are set out in Table 9.

Table 9 Summary of pH conditions respective to their modifier

pH	% Buffer/Modifier
3	0.1% Formic acid
10	0.1% NH_4OH
11	0.15% NH_4OH

Figure 18 shows a direct correlation between an increase in pH with an increased retention as well as peak resolution. It can be seen from the results that the separation of amines by RP-HPLC, is enhanced using a mobile phase pH higher than the amines' pKa. As mentioned in Chapter 1, by maintaining pH above their pKa, the majority of the population of basic amines will be unionised which reduces interactions with the any exposed silanol groups on the stationary phase, reducing peak tailing and asymmetry (Calabuig-Hernandez, Garcia-Alvarez-Coque, & Ruiz-Angel, 2016). Note that many HPLC columns, however, are not stable at high pH. The ethylene bridged hybrid (BEH) particles of the Xbridge enable pH resistance at high pH (Waters, 2016).

In summary, ammonium hydroxide (NH_4OH) was chosen as the most suitable pH modifier.

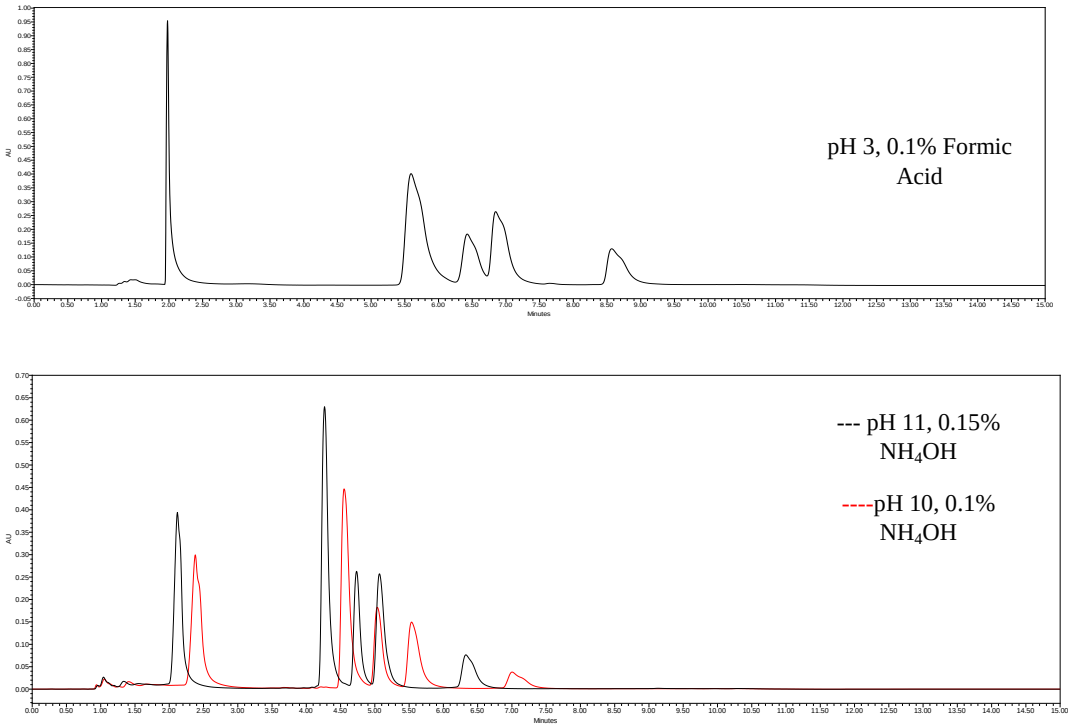


Figure 18 Chromatographs achieved from the three pH conditions: (A) 0.1% Formic acid adjusted to pH 3; (B) (Red) 0.1% Ammonium hydroxide adjusted to pH 10 (Black) 0.15% Ammonium hydroxide adjusted to pH 11.

However, use of ammonium hydroxide must be carefully considered. For example, a study by Wang et al. described studies on column oxidative dimerization of anilines to form azo and hydazo species (Wang et al., 2011). They similarly used an Xbridge C18 column with ammonia as the modifier but could be duplicated on multiple columns capable of higher pH including Gemini NX C18 column, (Phenomenex, Torrance, CA). They concluded that coupling reactions are most likely caused by the oxidative reagents formed by the complexation of unknown elements on the surface of the Xbridge stationary phase and ammonia in the high pH aqueous mobile phase. A paper by Myers proposed a possible mechanism of the on-column reaction of *N*-nitrosation that occurs with analytes containing primary and secondary amines (such as these adrenergic amines) when ammonium hydroxide is used as an organic modifier (Myers et al., 2013). The ammonia was established to be the source of nitroso nitrogen occurring at the stainless steel column frit and the metal ablated from the frit. The process was thought to be initiated by removal of the chromium oxide protective film from the stainless steel by acetonitrile (Myers et al., 2013). It was suggested not to subject the working column to extreme pH of more than 12 as it could lead to shorter column life.

In summary, ammonium hydroxide was selected as the pH modifier, controlled to pH 11 as it was suitable for the determination of amines with minimal peak broadening and was compatible with MS detection due to its volatile nature.

3.3.3 Effect of Organic Modifier

The extent to which the type of organic solvent and concentration thereof had on retention time was investigated. Acetonitrile was compared with methanol to see which produced the best chromatography.

The initial gradient of mobile phases A: H_2O + 0.15% NH_4OH: 95 – 0% and B: Organic Modifier + 0.15% NH_4OH: 5 – 100% over a period of 10 minutes, with a flow rate of 1.5 mL/min at 35°C. Figure 19 shows the different chromatography achieved when changing between acetonitrile and methanol. Acetonitrile was observed to increase peak symmetry compared to methanol. This may be explained by the fact that methanol is protic (Harris, 2016). This could influence the way the mobile phase interacts with the silica surface and target components. Acetonitrile on the other hand, has lower viscosity would reduce the back pressure and often results in slightly better peak shape (Castells & Castells, 1998). The separation between tyramine and phenylephrine however was smaller when using acetonitrile (Figure 19). This is likely due to the higher elution strength of acetonitrile compared to methanol (Castells & Castells, 1998).

Notwithstanding the above, acetonitrile was selected as the organic modifier because of its ability to resolve the compounds with minimal band spread and interference. A 2% (v/v) concentration of acetonitrile at starting conditions and gradient shift to 65% (v/v) was shown to completely resolve the compounds without interference from other components in the supplements.

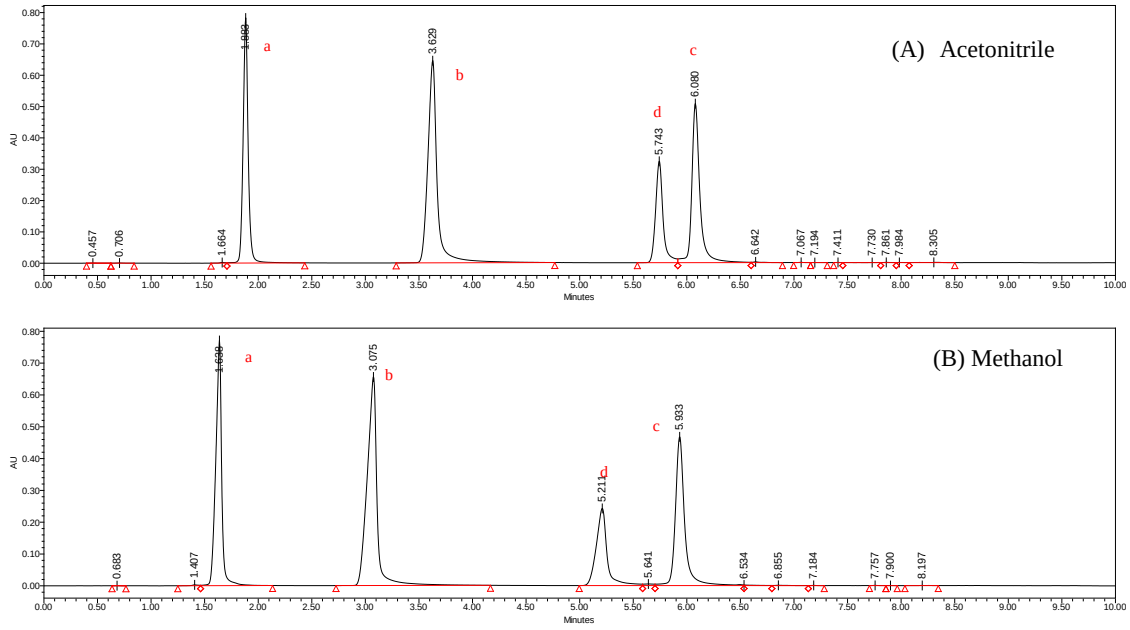


Figure 19 Chromatographs achieved from the two organic modifiers: (A) Acetonitrile as an organic modifier and (B) Methanol as the organic modifier. The standard compounds are represented by: (a) Octopamine; (b) Synephrine; (c) Tyramine; (d) Phenylephrine

3.3.4 Effect of Temperature

Optimising the column temperature is an important step in HPLC method development. The previously selected gradient was trialled with different column temperatures, ranging from 25°C to 40°C. Figure 20 shows a representative diagram of the effects of temperature on the peak formed by octopamine. The retention time of the compounds decreased with the increase in temperature. The higher the temperature resulted in faster exchange of analytes between mobile phase and stationary phase (Harris, 2016). This would likely result in the decreased rate of adsorption between the stationary phase and the amines.

It was observed that when the temperature increased, the peak shape improved. The difference the gradient elution between 35° and 40° were the peak shape. The higher temperature reduces the viscosity of the organic phase, which would allow for a greater flow rate within the column (Harris, 2016). The effect of the 'mass transfer' at 40°C is lower, as the internal flow rate increases, resulting in less peak broadening . The increased peak symmetry and reduced retention times lead to the selection of 40°C as the preferred temperature for the gradient method.

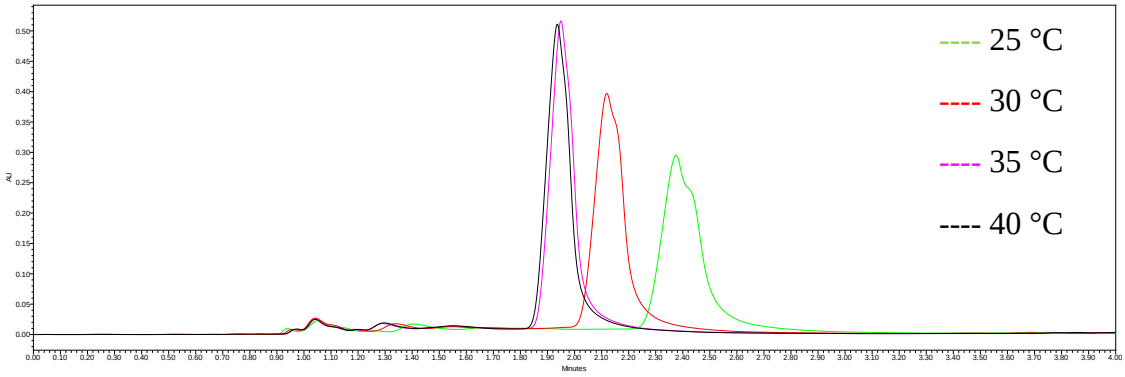


Figure 20 Chromatogram of octopamine peak at different temperatures on Xbridge column at 242 nm

3.3.5 Optimisation of UV-Vis Spectra

Based on the literature, the typical UV wavelength for the amines in CA ranges between 210-255 nm and 273-280 nm as mentioned in Chapter 1. It was determined that the λ_{max} = 236 nm and 290 nm for synephrine on the spectrophotometer. A representative diagram is shown in Figure 21 as the λ_{max} was similar for octopamine and tyramine.

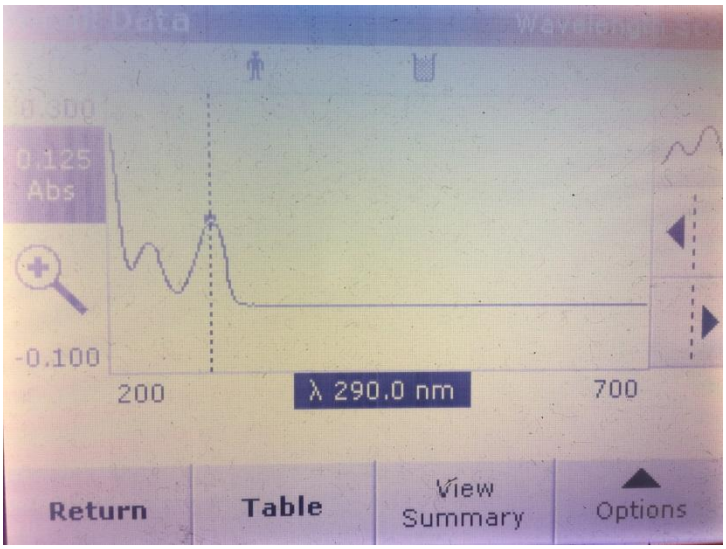


Figure 21 Representative diagram of the λ_{max} determined for synephrine, where λ_{max} =290 nm and 236 nm

However, a Photo-diode array (PDA) was later used to further validate the optimal UV spectra. Contrary to the literature and the initial scan, the optimal UV for the amines was determined to be 242 nm for the amines of interest as shown in Figure 22. A possible inference for this shift in λ_{max} was the different chemical environments of standards in the two detectors. It should be noted that the standards were determined with a gradient elution with acetonitrile and NH_4OH as organic and pH modifiers whilst detected with the PDA. The pH of standards were at pH of 11 whilst being analysed on the PDA, the pH was not recorded but was assumed to be lower during the spectrophotometer analysis. The pH of the sample solution could have caused a shift in the equilibrium of the amines (Babic, Horvat, Pavlovic, & Kastelan-Macan, 2007). The temperature was 35°C on the PDA analysis whereas the temperature of the spectrophotometer was at 25°C. It has been noted that an increase in temperature can cause a decrease in maximum wavelength absorption due to the changes in π-electron transition at different temperatures (Yang et al., 2004).

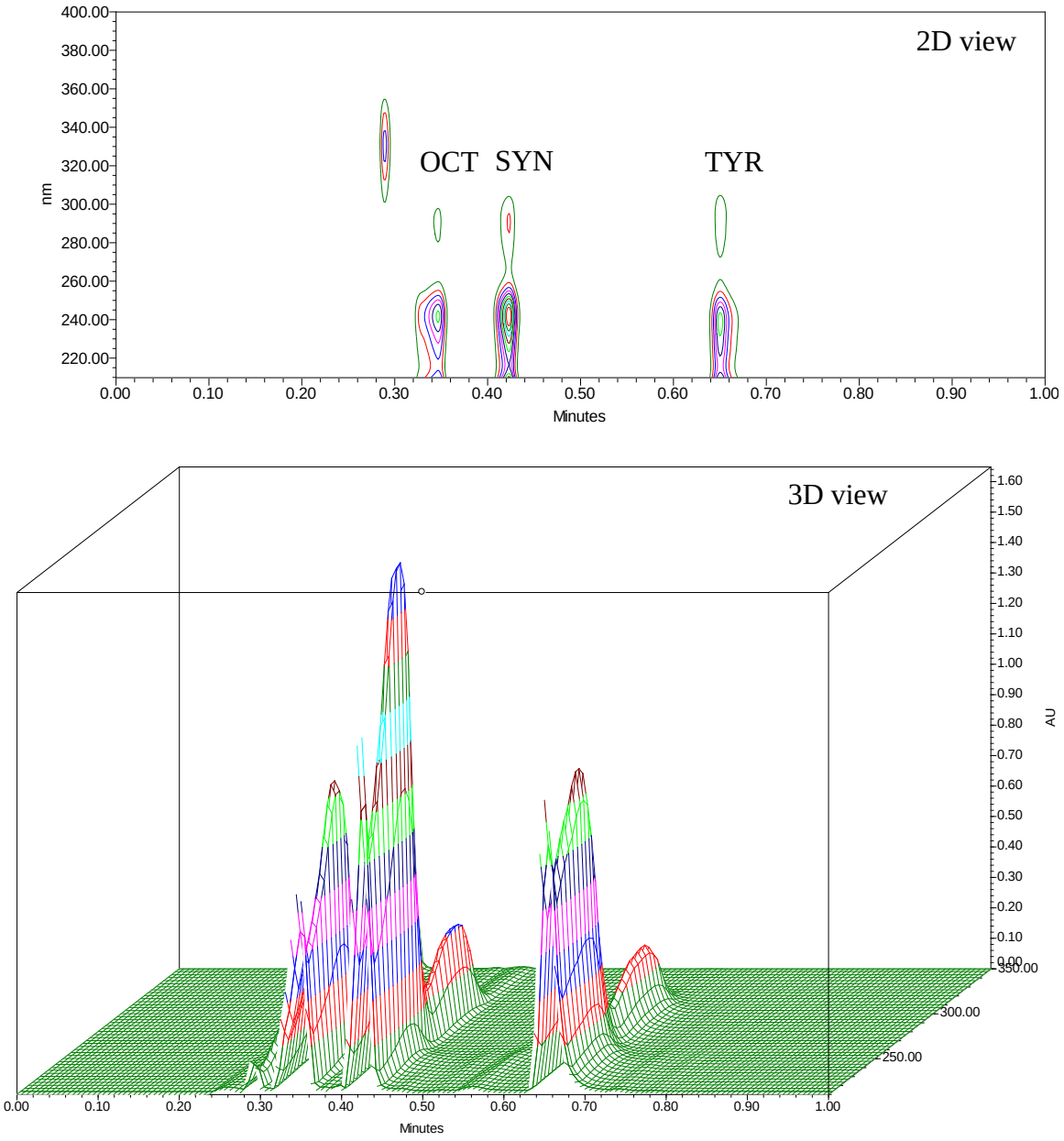


Figure 22 UV detection on a PDA at pH 11 and 35°C where λ_{max} = 242nm for all amines

3.3.6 Optimisation of QDa Mass Detection

Mass spectrometry (MS) has become an essential tool in analytical and pharmaceutical research (D'Hondt, Gevaert, Wynendaele, & De Spiegeleer, 2016). However, factors that limit the widespread use of MS are the high purchasing and operating costs. A high degree of expertise is needed for the optimisation and interpretation of MS data. The QDa is a miniature quadrupole-based MS that is compatible with chromatographic systems. The detector is pre-optimised without the sample specific adjustments typical of traditional mass spectrometers (detailed in Chapter 1). A problem with working with a PWS matrix is the potential co-elutions from other compounds. Unlike other UV detectors, the use of a mass detector minimises the risk of unexpected co-elutions by determining compounds via its specific mass.

Figure 23 shows the order of elution were octopamine (RT= 1.5 mins), synephrine (RT= 2.5 mins) and tyramine (RT= 5.4 mins). The IS eluted between synephrine and tyramine at 4.1 minutes. Peaks were completely resolved with limited background interference. A starting condition blank was used to monitor signs of carryover from high concentration standards/samples.

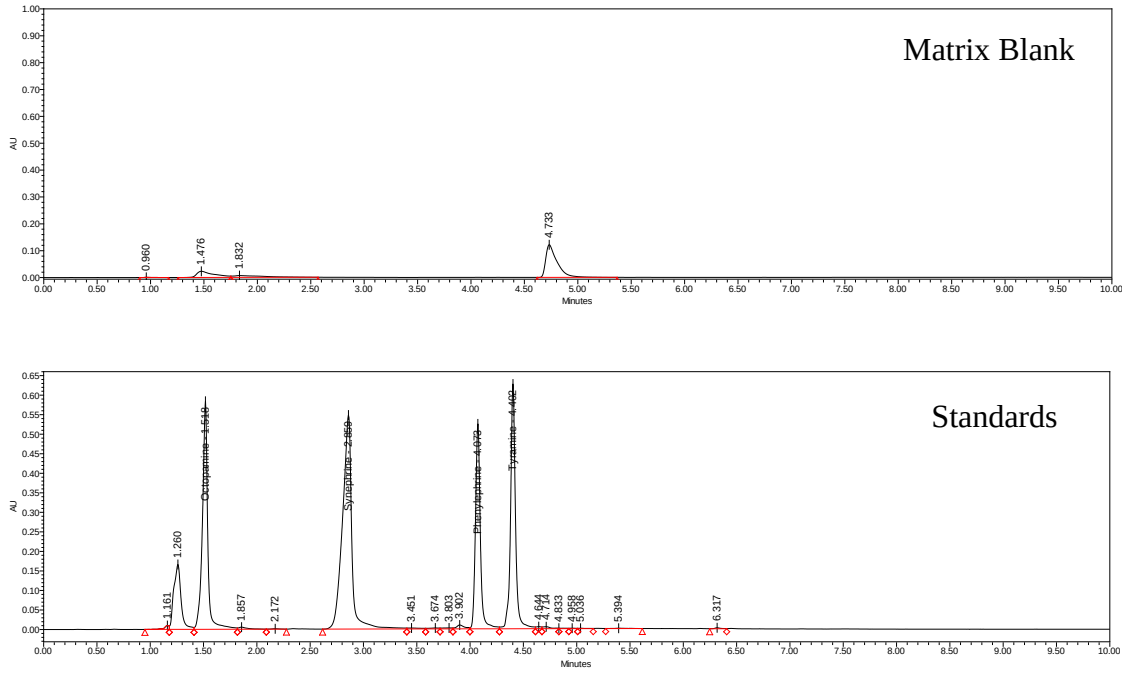


Figure 23 Chromatograph of matrix blank and standards in a matrix blank with the elution order of Octopamine (t = 1.5 mins), Synephrine (t = 2.5 mins), Phenylephrine (t= 4.0 mins) and Tyramine (t = 4.4 mins).

One of the manipulable parameters of the QDa was the cone voltage. The cone voltages of 1 V-30 V were trialled and are shown in Figure 24. The cone voltage of the mass detector was optimised to 15 V where it had the best mass detection with the least amount of in-source fragmentation.

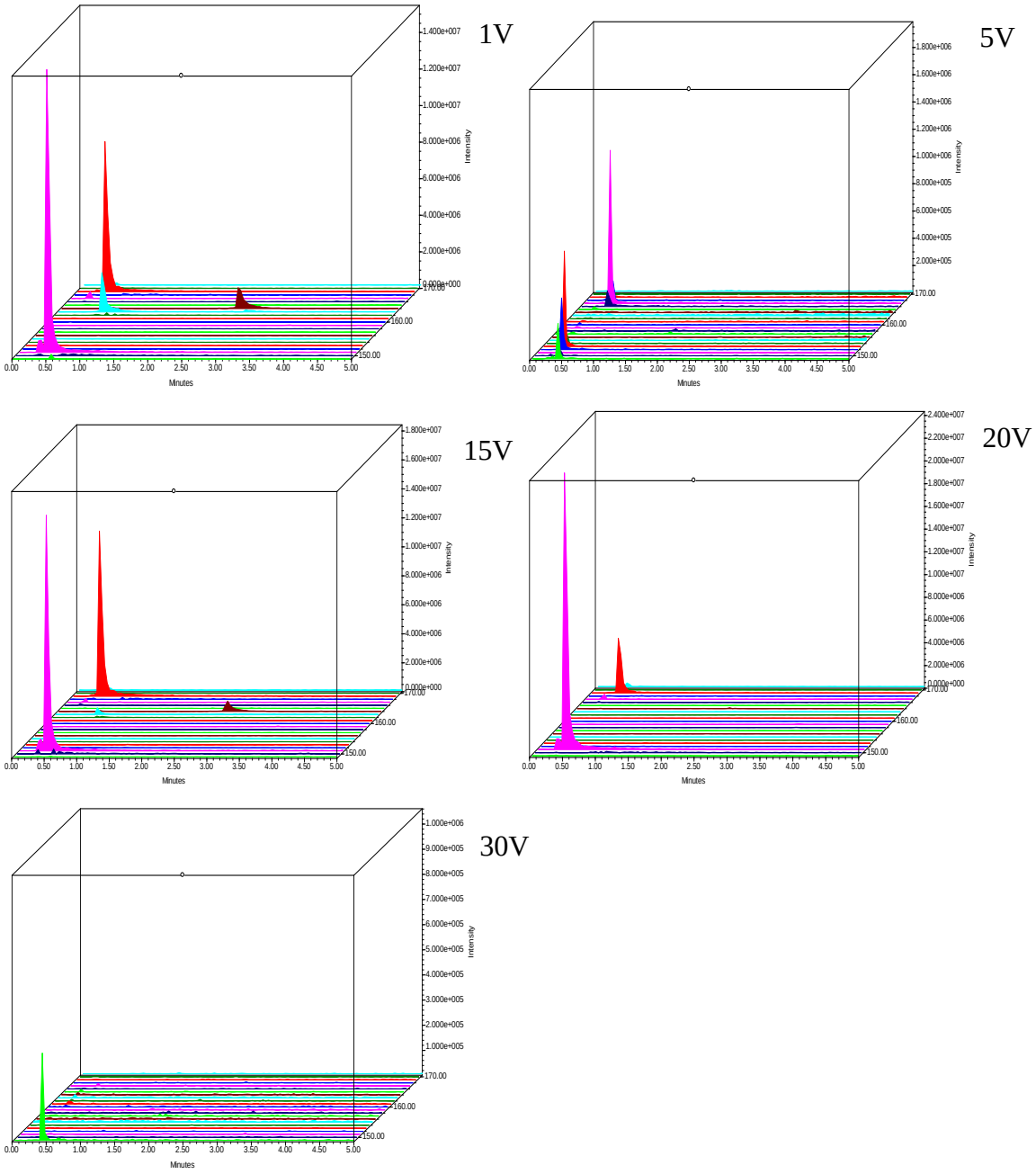


Figure 24 3D chromatographs of synephrine at different cone voltages; 1 V (a); 5 V (b); 15 V (c); 20 V (d); 30 V (e). m/z= 150 (front)and m/z= 168 (back) were observed

During the optimisation procedure, the positive ion mode, producing $[M+H]^+$, was preferred over the negative ion mode ($[M-H]^-$) for these basic compounds due to the greater signal intensity of amines when detected in positive ion mode. Selectivity of the method was confirmed by comparing the elution of external standards to a mix of standards into a dietary supplement blank.

The extra dimension of *m/z* on the Z-axis is a rapid output that aids in identifying and confirming the presences of the biogenic amines based on their specific masses. The mass spectra of the amines are represented in Figure 25. The mass of each compound was observed; there was a noticeable consistent fragmentation of 18 *m/z* for all compounds except for tyramine which had a mass fragment of 17 *m/z*. Octopamine had a mass of 136 and 154 *m/z*; synephrine and phenylephrine had a mass fragment of 150 and 168 *m/z*; and tyramine was 138 and 121 *m/z*. The fragmentation of synephrine, phenylephrine and octopamine was likely a result of a $[M+H - H2O]^+$ ion cleavage on the β-carbon resulting in the loss of a *m/z* = 18. However, the fragmentation of tyramine was likely due to a loss of a NH_3 on the alpha carbon, resulting in $[M+H – NH_3]^+$ (*m/z* 121). This was attributed to the ability of the *p*-hydroxyl group to stabilise the transition state on the pathway to the formation of the ion (Zhao, Shoeib, Siu, & Hopkinson, 2006).

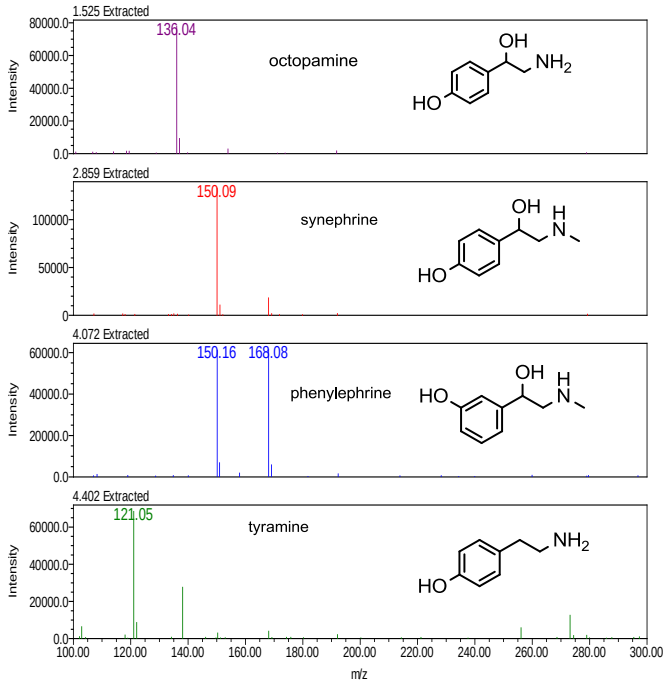


Figure 25 Mass spectra of the detected standards on a QDa with observed fragmentation occurring

A representative 3-dimensional (3D) chromatographic plot depicting the mass to charge ratio (*m/z*) vs intensity for each analyte is presented in Figure 26. A MS scan was used to determine the presence of any contaminant or other compounds within the analytical range of 100 Da to 300 Da. A well-resolved separation was observed between the two synephrine geometric isomers, *m*-synephrine (phenylephrine) and *p*-synephrine, which has not been commonly observed in other studies (Roman et al., 2007; Santana et al., 2008). These isobaric amines are difficult to resolve chromatographically due to their similar structures physical/chemical properties as well as identical masses (*m/z* 168.1) ((Table 5), Chapter 1).

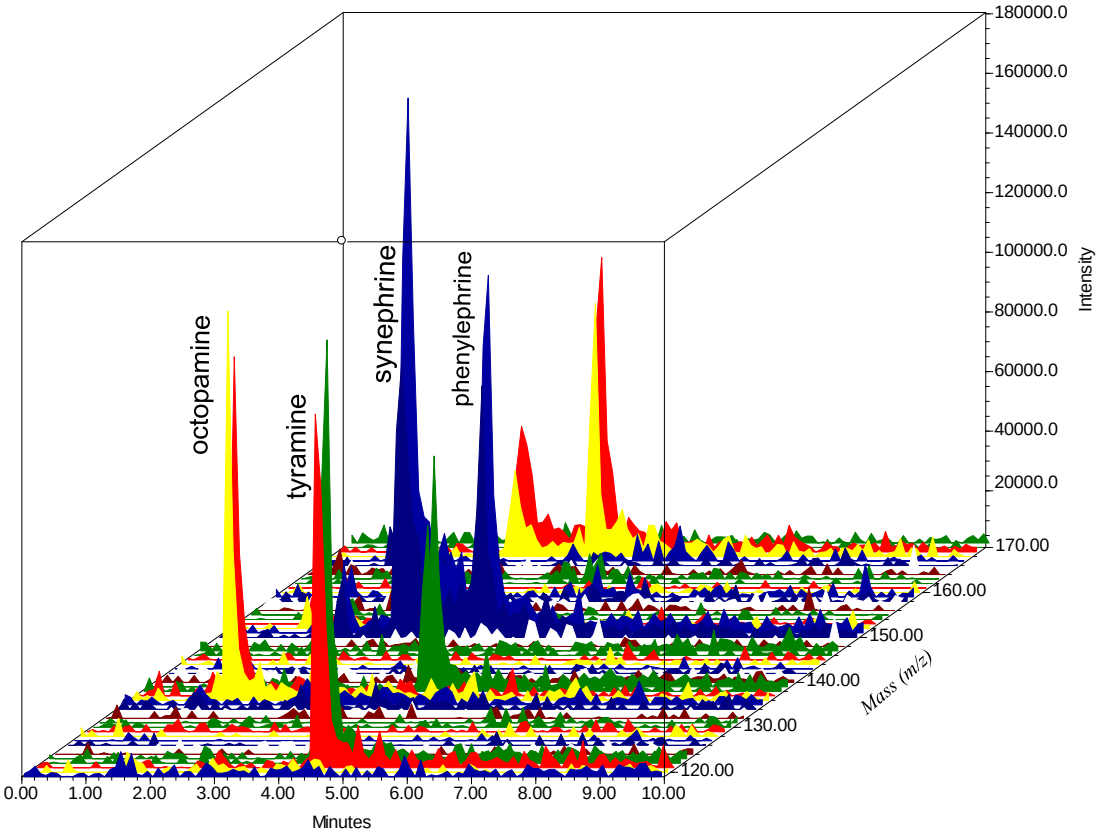


Figure 26 3D plot of the standards present in the spiked matrix blank with the elution order of octopamine (t = 1.5 mins), synephrine (t = 2.5 mins), phenylephrine (t= 4.0mins) and tyramine (t = 4.4 mins)

Figure 27 depicts the combined representation of PWS A which had been spiked with 100 μg/mL of each standard plus internal standard. Peak broadening, was observed in the case of synephrine. This suggests a degree of dispersion known as 'overloading' was present for synephrine. This seems understandable since synephrine is the major adrenergic stimulant in CA.

A peak at a RT of 5.1 minutes, thought to be caffeine was detected in the PWS sample and subsequently supported by a mass of *m/z* 195.1. Other peaks were detected at 4.6 minutes, 4.7 minutes and 5.07 minutes; however, since their masses were greater than 300 Da, they were not detected in the total ion chromatogram (TIC). These peaks could potentially correspond to other compounds such as β-Alanine and other amino acids, also commonly found in PWS. Similar mass fragmentation was observed in the detected amines; this adds a confirmatory element to the chromatographic data found.

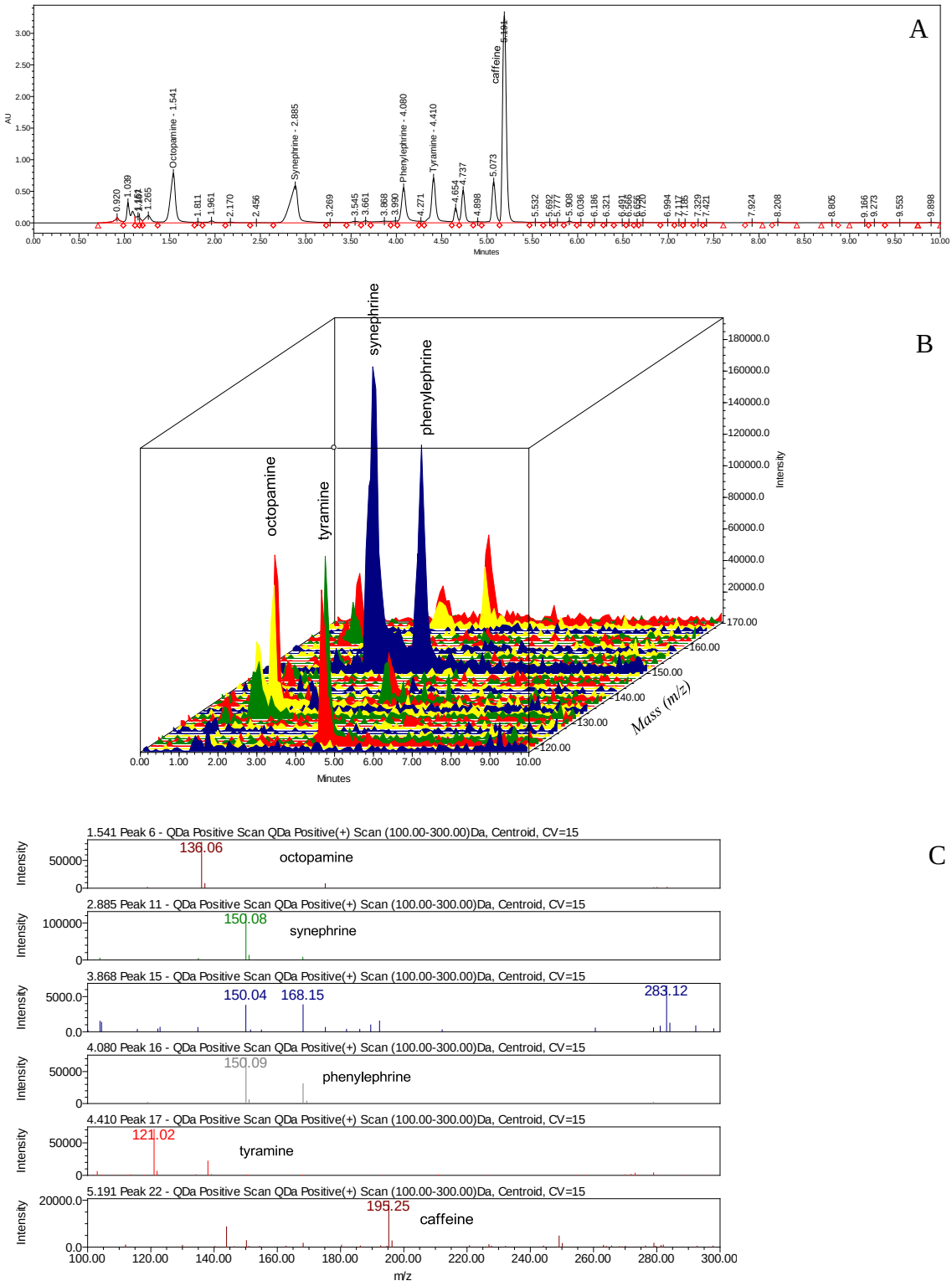


Figure 27 (A) Chromatograph of standards in a CA-containing PWS sample with the elution order of Octopamine (t = 1.5 mins), Synephrine (t = 2.5 mins), Phenylephrine (t= 4.0mins) and Tyramine (t = 4.4 mins). (B) 3D plot of the standards present in the matrix blank. (C) Mass fragments of sample at 15 V.

3.3.7 Optimised HPLC Conditions for CA Separation

Based on the optimised chromatographic and detection parameters, the final chromatographic conditions were determined. The optimised conditions are summarised in the Table 10 below:

Table 10 Summary of optimised HPLC conditions

Optimisation parameter	Selected conditions
Column	Xbridge BEH C18 (2.5 µm, 3.0 x 100 mm)
Organic modifier	Acetonitrile
Flow rate	1.5 mL/min
pH	11 adjusted with NH_4OH
Temperature	40°C
UV (λ)	242 nm
Mass detection range (TIC)	100-300 Da

The column used was an Xbridge C18 (2.5 µm, 3.0 x 100 mm, i.d., Waters, Ireland) protected by a guard column (Xbridge C18, 2.5µm, 3.0 x 20mm i.d., Waters, Ireland). The components were separated using a gradient elution protocol where mobile phase A consisted of water, mobile phase B of acetonitrile, and mobile phase C of H_2O with 1% NH_4OH. All solvents were pH adjusted to 11. The flow rate was maintained at 1.5 mL/min while the column temperature was set to 40°C and sample temperature was set at 25°C. The run time was 10 minutes under gradient conditions (Table 11) based on the retention time of tyramine (the most non-polar of the constituents under investigation). The injection volume was 10 µL. There was a 5-minute delay between injections and UV detection was at 242 nm. Data acquisition with the QDa mass detector was performed in positive mode under the following conditions: total ion current (TIC) between *m/z* 100-300; capillary voltage, 3.5kV; cone voltage, 15V. Single Ion Recording (SIR) was set for positive mode masses of synephrine (168.2 *m/z*), IS (168.2 *m/z*), octopamine (154.1 *m/z*) and tyramine (138.2 *m/z*).

Table 11 HPLC separation gradient used for UV-MS analysis of caffeine and derivatives

Time(min)	Mobile phase A (Water) (%)	Mobile phase B (ACN) (%)	Mobile phase C (Water + 1% NH_4OH (%)
Initial Conditions	83.0	2.0	15.0
1.00	83.0	2.0	15.0
7.00	20.0	65.0	15.0
8.00	20.0	65.0	15.0
8.50	83.0	2.0	15.0

3.4 Analytical Validation of CA by HPLC/UV

The method validation parameters, described in Chapter 2 (Section 2.3) were in accordance with the ICH guidelines (ICH Expert Working Group, 1994).

3.4.1 Linearity, LOD, LOQ

Linearity was determined as a linear regression using the least-square method on a set of dilutions for each standard solution with phenylephrine as the internal standard. The results are shown in Table 12. The linear ranges of the amines were 5 µg/mL to 100 µg/mL. Poor linearity was observed outside this range, and the representative linearity curves of concentrations at 2 µg/mL and 200 µg/mL are found in Appendix 10- 12. An intra-day 6-point standard curve for each analyte, the slope and y-intercept, correlation coefficient (r^2) and RSD % of the standard curve were determined based on the parameters described in Chapter 2. Figure 28 depicts the standard curves for of synephrine, octopamine and tyramine. The correlation coefficients were synephrine (r^2 = 0.998 ± 0.002), octopamine (r^2 = 0.999±0.001) and tyramine (r^2 = 0.997±0.003). Limits of detection (LOD) and Limits of Quantitation (LOQ) were determined, as described in Chapter 2, based on the signal-to-noise ratio (S/N) of the standards at their lowest concentration of 5.0µg/mL (Table 12). The LOD determined for synephrine octopamine and tyramine were 1.64 µg/mL, 1.50 µg/mL and 0.45 µg/mL, respectively. The LOQ ranged from 5.45 µg/mL, 5.00 µg/mL and 1.51 µg/mL for synephrine, octopamine and tyramine.

Table 12 Method calibration data for linearity of synephrine, octopamine and tyramine

Standards	Calibration range (µg/mL)	Linearity Equation	Correlation coefficient (r^2)	LOD (µg/mL)	LOQ (µg/mL)
Synephrine	5.0 -100	*y = 2.6228x + 0.971*	0.998 ± 0.002	1.637	5.457
Octopamine	5.0-100	*y = 1.636x - 0.6659*	0.999±0.001	1.501	5.004
Tyramine	5.0- 100	*y = 1.4786x - 4.9618*	0.997±0.003	0.454	1.514

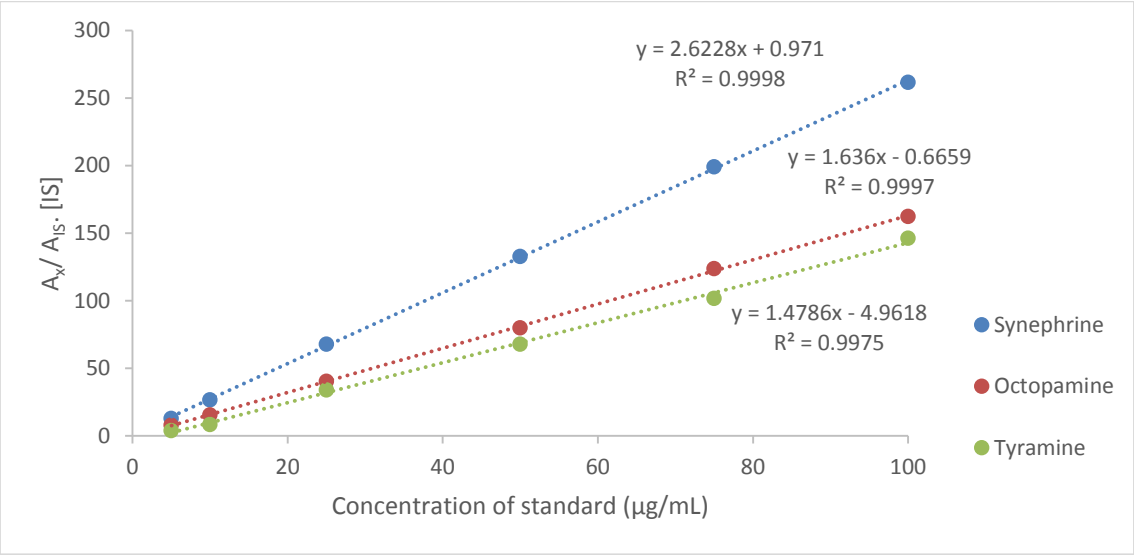


Figure 28 Standard Curves for synephrine, octopamine and tyramine. Ax = area of response of analyte; AIS= area of IS response; [IS]= concentration of IS

3.4.2 Accuracy & Precision

Data presented in Table 13 was used to determine the accuracy of the method. The accuracy for the spiked samples varied from 77% to 107%, with standard deviations ranging from 0.1% to 9.4%. These values were acceptable according to the ICH, however further improvements were made. A matrix blank consisting of a PWS without *CA* was spiked at three concentration levels to check for the presence of peaks with similar retention times to the test analytes in PWS. Apart from synephrine, lower accuracy was observed at lower concentrations such as 5 µg/mL which was at the LOD, this could potentially impact the LOQ of this pilot method. This could be due to operator error during sample spiking as the available low volume pipette could vary in accuracy. Decreased accuracy was also observed at the higher end of the concentration range. This could be a result of column over-loading at 100 µg/mL.

Table 13 also depicts the results of the intra-day and inter-day precision and extraction recovery experiments. The intra-day precision was calculated to be between the range of 2.1-4.1% for synephrine, 2.9-3.0% for octopamine, and 1.4 to 5.3% for tyramine. The inter-day precision data for synephrine was found to be between 1.5- 13.5%, 2.4-7.5% for octopamine and 1.5-5.2% for tyramine. RSD% values less than 15% are acceptable under the FDA guidelines for pharmaceutical determination. However, the 13.5% variation for synephrine at LOD could be improved by using a higher amount of spiked standard or increasing the LOD amount.

Table 13 Intra- and Inter-day precision, accuracy and analytical recovery of standards

Standards	Spiked amounts (µg/mL)	Intra-day precision (RSD %)	Inter-day precision (RSD %)	Recovery (%) (mean ± SD)	Accuracy (%) (mean ± SD)
Synephrine	5	4.2	13.4	102.8 ± 3.3	105 ± 9.0
	50	3.4	1.5	100.6 ± 0.2	101 ± 1.0
	100	2.1	1.5	93 ± 0.3	90 ± 0.1
Octopamine	5	3	7.4	87 ± 10.8	77 ± 9.4
	50	3	7.5	112 ± 7.9	107 ± 1.4
	100	2.9	2.4	132 ± 0.2	80 ± 0.6
Tyramine	5	5.3	5.2	82 ± 0.5	91 ± 0.7
	50	2.4	2.1	102 ± 1.0	106 ± 2.8
	100	1.4	1.5	106 ± 0.5	91 ± 0.6

3.4.3 Recovery

A matrix blank was used to determine the amount of analyte recovered after sample preparation (PWS without CA). 10 samples (n= 10) were prepared, spiked with standard solutions and extracted with the sample preparation method. Each concentration of both sets were analysed in 3 replicates. The percent recovery was calculated by comparing the peak areas of the corresponding standard solutions to the calibration curve (Figure 28). The extraction recovery showed values between 93 to 102% for synephrine, 87 to 132% for octopamine, and 82 to 106% for tyramine amines. The yield recoveries above 100% could potentially be caused by pipetting inconsistencies. Increasing the sample size could help reduce the effects of these errors. At lower concentrations, the amounts of analyte recovered were lower than the higher spiked concentrations except for synephrine. This could possibly be due to loss during sample preparation (Poole, 2003). This could be resolved by using a higher concentration of non-polar solvent during the sample preparation.

3.5 Quantification of Adrenergic Amines in CA-containing PWS.

Table 14 details the quantities of adrenergic amines determined in the PWS samples per gram as well as per respective serving size. The *p*-synephrine content within the PWSs analysed varied from 0.15 mg/g to 0.89 mg/g; octopamine on average 0.16 mg/g; and tyramine averaged at 0.4 mg/g. The concentration of *p*-synephrine was in agreement with other reported methods of between 0.001 mg/g to 8.65 mg/g (Avula, Upparapalli, Navarrete, et al., 2005; Ganzera et al., 2005; Marchei et al., 2006; Pellati & Benvenuti, 2007; Pellati et al., 2002; Roman et al., 2007; Slezak et al., 2007).

A standard reference material of CA (SRM 3258) contained 8.65 mg/g of synephrine, 0.14 mg/g of octopamine and 0.026 mg/g of tyramine (Putzbach et al., 2007). The ratios of biogenic amine found within were used as a reference to contrast with the analysed supplements. The difference in concentration of *p*-synephrine determined was likely due to the lack of uniformity and lot-lot variability of PWS manufacturing (Slezak et al., 2007)

Supplement B (360Rage) and D (ErgoBlast) had the highest amount of octopamine of 44 mg/serve. In Australia, there are currently no legal maximum limits for octopamine concentration in dietary supplements octopamine is found naturally in CA extracts in amounts between 0.12 mg/g to 0.8 mg/g (Sander et al., 2008). It was evident that it was not in agreeance to the ratios found in a SRM. The high amount of octopamine determined in this study suggests it may have been added rather than from that within the plant extract. Octopamine is a banned substance for use in athletic competitions due to its stimulatory properties whereas synephrine is a monitored substance (World Anti-doping Agency, 2015).

Tyramine is not listed on any of the labels of the supplements tested. The highest amount of tyramine quantified in the tested PWS was 0.69 mg/g (PumpHD). The FDA suggests an acceptance level between 100-800 mg/g, and a toxicity level over the amount of 1080 mg/kg tyramine intake (Nout, 1994). Tyramine has been used as an experimental tool to study the mechanisms of noradrenaline release from sympathetic neurones and as an index of peripheral sympathetic nerve function (Broadley, 2010). Note the quantified levels of tyramine are higher than the ones present in the previously mentioned SRM 3258. It is possible that tyramine determined in this supplement was also added to enhance the overall stimulatory effect of the PWS.

Table 14 Synephrine, Octopamine and Tyramine content in PWS (mg/g) (mean ± RSD %)

Samples	Amount of amines found in PWS (mg/g) (mean ± RSD %)			Amount of amines found in PWS (mg/serve) (mean ± RSD %)		
	Synephrine	Octopamine	Tyramine	Synephrine	Octopamine	Tyramine
A (*MusclePrime*)	0.25 ± 9.3	0.10 ± 10.2	0.10 ± 2.5	4.69 ± 9.3	1.97 ± 10.2	1.95 ± 2.5
B (*Gold-Standard Preworkout*)	0.31 ± 5.6	4.46 ± 12.7	0.52 ± 0.4	3.11 ± 5.6	44.62 ± 12.7	5.17 ± 0.4
C (*360Rage)*	0.15 ± 2.9	0.28 ± 4.6	0.28 ± 1.1	1.06 ± 2.9	2.03 ± 4.6	2.05 ± 1.1
D (*ErgoBlast)*	0.70 ± 1.5	8.90 ± 10.1	0.28 ± 2.0	3.49 ± 1.5	44.52 ± 10.1	1.39 ± 2.0
E (*PumpHD)*	0.89 ± 1.8	0.65 ± 3.8	0.69 ± 3.9	8.30 ± 1.8	6.54 ± 3.8	6.90 ± 3.9

3.6 Conclusion

A simple, rapid procedure to determine levels of synephrine, octopamine and tyramine in CA-containing PWS has been presented. The procedure involved a HPLC-UV/MS method with a total run time of 10 minutes on a C18 Xbridge column. Neither extensive derivatization nor sample preparation procedures were required. The amounts of synephrine found were 0.25 – 0.89 mg/g comparable to other studies ranging from 0.001 mg/g to 8.65 mg/g (Avula, Upparapalli, Navarrete, et al., 2005; Ganzera et al., 2005; Marchei et al., 2006; Pellati & Benvenuti, 2007; Pellati et al., 2002; Roman et al., 2007; Slezak et al., 2007). However, octopamine and tyramine were found in higher amounts than standard reference materials at 8.9 mg/g and 0.69 mg/g.

It was difficult to compare the exact amount of octopamine and tyramine as most of the CA was labelled under a proprietary blend. Octopamine is a doping substance that is illicit in sport when detected in urine. According to Thevis et al. (2012), octopamine was eluted at concentrations at an order of magnitude less from the consumed amount. The LOD/LOQ determined for this method is such that its usability could also be extended to PWS screening for concentrations of octopamine and synephrine in biological matrices such as urine for doping analyses as it was in agreeance to Thevis et al. (2012).

Chapter 4 A Rapid HPLC-MS Protocol for Caffeine and Dimethylxanthines in Pre-workout Supplements

4.1 Background

Significant stimulatory effects from the consumption of PWS is provided by the xanthine component, specifically caffeine (1,3,7-trimethylxanthine) and the 3 dimethylxanthines (DMX). These compounds have become an accepted and prevalent dietary constituent for many people and, with increasing evidence of adverse effects arising from overconsumption, regulatory bodies have sought to impose limits on their incorporation into foods and supplements. Caffeine is the only methylxanthine subject to specific product labelling requirements – the details of which vary depending on country of manufacturing origin. In Australia products containing caffeine are required to state the amount and also carry a warning. A recent review on caffeine toxicity suggested that undeclared herbal components in most supplements could expose consumers to an increased risk of caffeine toxicity (Musgrave et al., 2016). The multi-stimulant component nature of PWS is a potential factor contributing to the delay in attributing specific adverse effects to one compound or another. Such increased PWS consumption beyond athletes and fitness enthusiasts into recreational use by the general population (Gibson, 2014), and concomitant the increased adverse health reports may result in more stringent caffeine content/labelling requirements leading to an increased demand for analysis of products and/or biological samples. It is therefore important to develop an accurate, rapid, cost-effective, sensitive and transferrable method for the detection and quantification of caffeine and the DMX isomers that may be suitable for both supplement and biological matrices.

A number of analytical methods have been used for the analysis and quantification of caffeine and its DMX derivatives. A search of the literature revealed that RP-HPLC-UV was the most common analytical method used for caffeine and DMX analysis. Beauchamp et al. sourced 9 caffeine-containing PWS products using LC–quadrupole time-of-flight MS (QTOF/MS) using an Agilent Poroshell C-18 column (2.1 x 100 mm, 2.7 µm), on a 12 minute gradient of water and acetonitrile adjusted with 0.05% formic acid, was used to identify and quantify substances in the PWS samples. The average caffeine recovery was 88.25 ± 13.41% compared to the labelled caffeine content (Beauchamp et al., 2016). The additional use of a TIC confirmed the absence of any other stimulants based on their current database of 45 amphetamines and 8 phenethylamines. Chromatography conducted by the Beauchamp method was

performed on a UPLC system which, while sensitive, has high setup and operating costs. A more cost effective method of caffeine and DMX analysis is HPLC-UV which is likely responsible for the prevalence of published methods using this technique. Recent studies suggested good chromatographic results, approaching that seen in UPLC, can be achieved on a HPLC system by using stationary phases with small particle size such as 2 µm (DeStefano, Boyes, Schuster, Miles, & Kirkland, 2014; Swartz, 2005).

A number of researchers, however, have noted difficulty in separating the three DMX isomers by RP-HPLC (Randon et al., 2010). The three DMX are configurational isomers (Figure 2, Chapter 1) and therefore have identical masses. This presents a challenge for analytical separation without involving costly and time-consuming derivatization protocols. Most methods which involve separation of caffeine in food do not report the separation of paraxanthine (PX) and theophylline (TP) (Bispo et al., 2002; Hasegawa et al., 2009; Kanazawa et al., 2000; Martinez-Lopez et al., 2014; Thomas et al., 2004). This may be because PX is not derived from plant products. Bispo et al., developed a method to determine caffeine, TB and TP in beverages and urine samples using HPLC-UV. The method involved a Bondesil C18 column with a methanol-water-acetic acid gradient at 0.7 mL/min and UV detection at 273 nm. The LOD was 0.1 pg/mL for all three methylxanthines. This method was versatile enough to be used for levels up to 350 µg/mL of caffeine, 32 µg/mL of TB and 47 µg/mL of TP. This method did not involve additional sample derivatization but was not specific for PX, the main metabolite of caffeine. The incorporation of mass detection would enhance structure confirmation for the DMX derivatives, especially when present in a urine matrix. Our strategy, employing a RP-HPLC system with combined UV and in-line QDa mass detector aimed to confirm the identity and masses of all four compounds without derivatisation. Additionally, we aimed to achieve sufficiently high sensitivity as to allow the method to be transferable between various matrices, either biological matrices or supplements

The aim of this present study was to develop a rapid and adaptable HPLC-UV/MS method for the determination and quantification of caffeine and its DMX derivatives, paraxanthine (PX), theobromine (TB) and theophylline (TP) and apply it to the analysis of these compounds in a variety of Australian-manufactured and overseas-sourced PWS.

4.2 Experimental

4.2.1 Chemicals and Reagents

The chemicals and reagents used in this chapter are detailed in General Methods (Chapter 2, section 2.2). The 5 PWS selected were KardioFire (A); 360Rage (360°Cut Performance Supplements) (B); PumpHD (BPISports) (C); MusclePrime (AllMax Nutrition) (D) and DyNO (RSP Nutrition) (E). The PWS samples A, B and D were purchased online (manufactured in the USA) while samples C and E were sourced from a local health store in South-East Queensland, Australia.

4.2.2 Standard Preparation

Stock solutions (1 mg/mL) were prepared for all standards as well as internal standard (IS) and stored in 4°C until analysis. Etofylline *(7-(2, hydroxyethyl) theophylline)* is a synthetic structural analogue of the natural methylxanthines and has been used in previous chromatographic methods (Buyuktuncel, 2010; Johansson, Gronrydberg, & Schmekel, 1993; Kanazawa et al., 2000; Naline, Flouvat, Advenier, & Pays, 1987; Nirogi, Kandikere, Shukla, Mudigonda, & Ajjala, 2007). A linear dilution was prepared for each standard between 1 µg/mL and 200 µg/mL. The results of concentrations outside this linear range will be discussed in section 4.4.1. The PWS samples were prepared using the SPE method detailed in Chapter 2.

4.3 Method Development

Following the method development process depicted in Figure 8 (Chapter 2), various parameters such as different column chemistries, mobile phase composition, temperature and gradient combinations were optimised prior to the quantification of xanthines in PWS.

The details of this process are outlined in the following sections to attain the best conditions for separation.

4.3.1 Effect of Column Chemistries

To compare the effect on analyte retention of the two columns a series of 100µg/mL standard mix injections were run using an initial mobile phase gradient A) 0.1% Formic Acid: 95 – 0% and B) 0.1% Formic Acid: 5 – 100% over a period of 10 minutes with a flow rate of 1mL/min at 30°C The two columns tested were a T3 HSS C18 column (5 µm, 3.0 x 150 mm, i.d.) and a Xselect CSH phenyl-hexyl (3.5 µm, 4.6 x 100 mm, i.d.). The phenyl-hexyl column showed higher resolution than the C-18 column. This could be due to increased π-π interaction that allows superior retention (Gibson & Fowler, 2014).

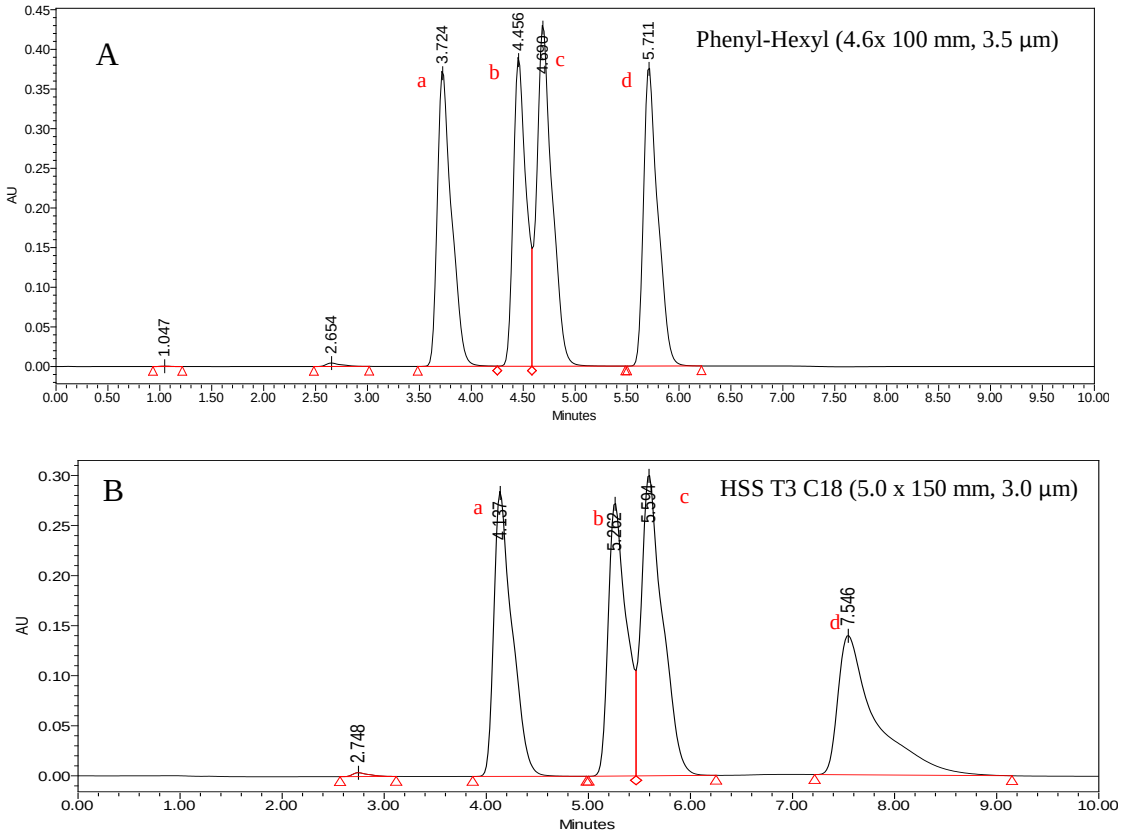


Figure 29 Comparison of columns: (A) Xselect CSH phenylhexyl (3.5µm, 4.6 x 100mm,* i.d.*) and (B) T3 HSS C18 (5 µm, 3.0 x 150 mm,* i.d.*). The standard compounds are represented by: (a)* TB*; (b)* PX*; (c)* TP*; (d) caffeine

The elution order of the xanthines from the two columns was TB, PX, TP followed by caffeine (Figure 29). The results of the chromatography are summarised in Table 15. The order correlates well with the predicted order of elution given the dielectric for each compound (See, Table 3 of Chapter 1).

Table 15 Summary of effects of column chemistry on Caffeine and DMX

Compound	RT on Phenyl-hexyl column (mins)	RT on HSS T3 column (mins)
Theobromine	3.72	4.14
Paraxanthine	4.45	5.26
Theophylline	4.69	5.59
Caffeine	5.71	7.55

The peaks observed on the C18 chromatogram were broader than those seen on the phenyl-hexyl. As previously discussed, a number of factors could contribute to peak broadening. The particle size of the C18 column (5 µm) was larger than the particle size of the phenyl-hexyl (3.5 µm) column. Eddy diffusion could explain the observed peak broadening since the larger particle size of the C18 column could result in a slower rate of analyte flow.

An increased RT was observed for the analytes on the phenyl-hexyl column. The phenyl functional group of the phenyl-hexyl column could introduce potential π-π stacking interactions which would cause analytes to be retained longer on the phenyl-hexyl column (Yang et al., 2005). However, there are additional factors that could explain the longer retention time in the C18 column. The pore diameters for both columns were 130 Å, but the internal diameter of the phenyl hexyl column (4.6 mm) was larger than the C18 column (3.0 mm). The smaller internal diameter of the packing material would have resulted in a slightly higher flow rate in the C18 column to decrease peak broadening. However, the C18 column was longer (150 mm) than the phenyl-hexyl column (100 mm). The 50 mm difference in column length could increase the rate of interaction between stationary phase and xanthines in the C18 column.

Both columns were able to partially separate the isomers, TP and PX. However, further developments will be discussed in later sections of this chapter. On the basis of the observed shorter retention times and decreased amount of peak broadening, the Xselect phenyl-hexyl column was selected as the preferred column for this analysis.

4.3.2 Effect of pH

Caffeine and the DMXs have two to three of its four nitrogen bonds methylated, and can only serve as a weak hydrogen bond acceptor. The conjugate acid of caffeine has a pKa of -0.16, which means at acidic pH, a majority of the caffeine species will be in the deprotonated form. In contrast to the analysis of the amines, caffeine and DMX are neutral at pH 2. The two acidic modifiers trifluroacetic acid (TFA) and formic acid (FA) were tested to confirm which would produce superior chromatography.

Better peak resolution was observed with TFA (Figure 30). The retention of the xanthines with the two pH modifiers was relatively similar. However, TFA also causes ion suppressing effects during MS which results in signal reduction (Annesley, 2003). For this reason, formic acid was selected as an alternative modifier to TFA as it was a volatile modifier capable of producing good peak shape at pH 3.

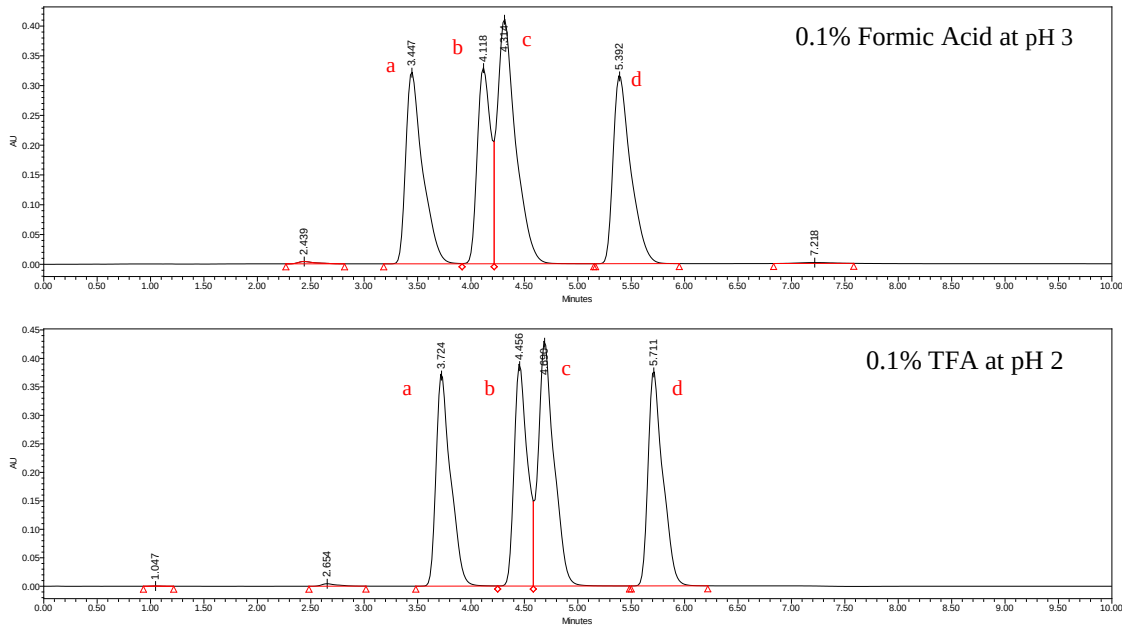


Figure 30 Chromatographs of caffeine and dimethylxanthine adjusted to pH 3 (0.1% formic acid) and pH 2 (0.1% TFA). The standard compounds are represented by: (a)* TB*; (b)* PX*; (c)* TP*; (d) caffeine

4.3.3 Effect of Sampling Rate

The sampling rate of the UV-Vis detector produced interesting results on peak resolution. Studies based on the accuracy of the numerical integration of peak area report that at least 10 data points per second were required when a Gaussian peak was digitized (Wahab et al., 2016).

The results from an initial sampling rate of 1 point/second are shown in Figure 31. This was the default sampling rate used in earlier investigations for xanthine analysis producing relatively broad, non-Gaussian peaks for all the analytes. However, when the sampling rate was increased to 40 points/second, there was a noticeable increase in peak resolution and symmetry. A detailed description of the theory of data acquisition is beyond the scope of this study however for a description of the importance of sampling rate has been reported by Wahab et. al (2016). With the increased improvement in peak shape of the analytes, a sampling rate of 40 points/second was used for further optimisation steps.

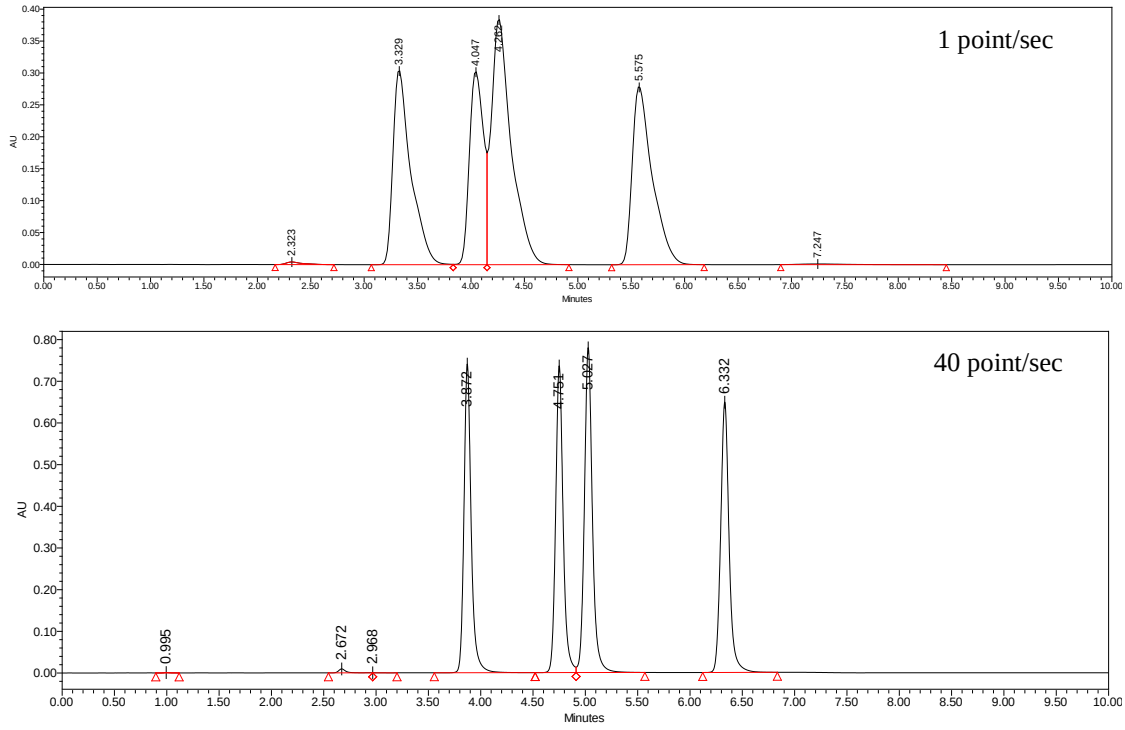


Figure 31 Chromatographs of caffeine and* DMX *at 1 point/sec and 40 point/sec

4.3.4 Effect of Organic Modifier

The extent to which the type of organic solvent and concentration thereof had on retention time was investigated. Acetonitrile was compared with methanol to see which produced the best chromatography. Etofylline was added to the standard mix of caffeine and DMX to observe the changes in resolution with organic modifier use.

Figure 32 shows the differences in chromatography produced by changing between methanol and acetonitrile. A narrower peak width was observed for TP and PX when using acetonitrile compared to methanol. More hydrogen bonds occur between methanol and TP/PX which allowed greater separation between the two compounds. A 15% (v/v) amount of methanol at starting conditions and gradient shift to 60% (v/v) was shown to completely resolve the compounds without interference from other components in the supplements.

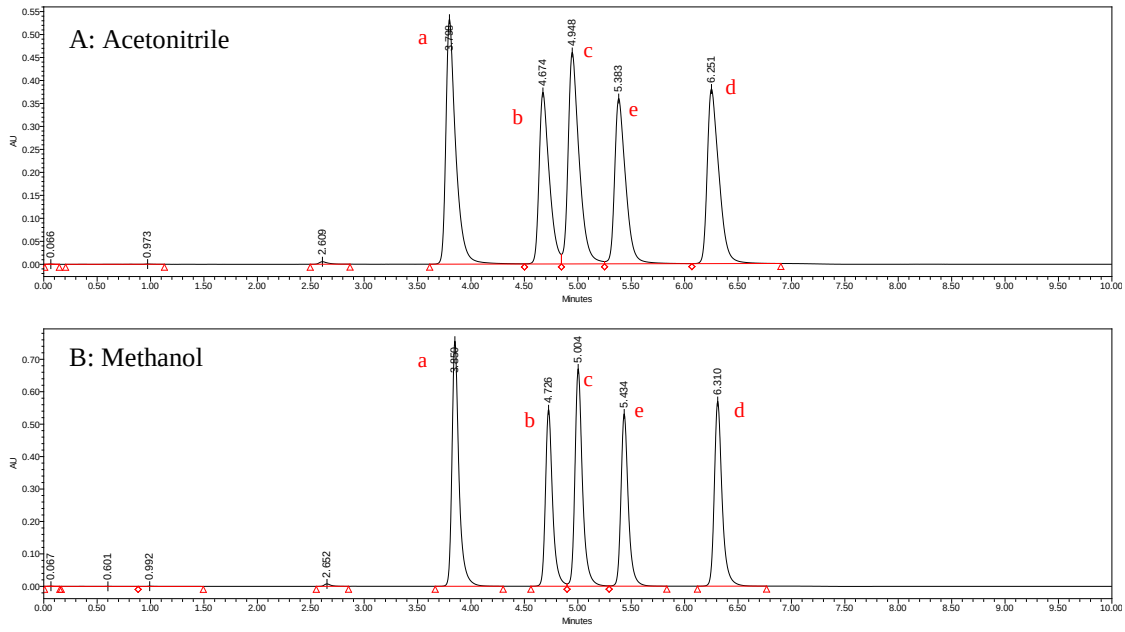


Figure 32 Chromatographs achieved from the two organic modifiers: (A) Acetonitrile as an organic modifier and (B) methanol as the organic modifier. The standard compounds are represented by: (a)* TB*; (b)* PX*; (c)* TP*; (d) caffeine;(e) etofylline

4.3.5 Effect of Temperature

Optimising the column temperature is an important step in HPLC method development. The previously selected gradient was trialled with different column temperatures, ranging from 20°C to 35°C. Figure 33 shows a representative diagram of the effects of temperature on the peak formed by caffeine. It was observed that there was a decrease in retention time and improvement in peak shape as the temperature increased. The higher temperature reduces the viscosity of the organic phase, which would allow for a greater flow rate within the column (Harris, 2016). However, at 35°C, the separation between TP and PX decreased. This could be caused by the decreased rate of mass transfer of the two isomers on the stationary phase with the increased laminar flow. However, further improvements on temperature optimisation could be achieved by plotting the separation factor (k) values and comparing it based on the resolution equation (R) (Equation 1, Chapter 2).

As a result of the good peak symmetry, capability to separate TP and PX and reduced retention times, 30°C was the preferred temperature for the gradient method.

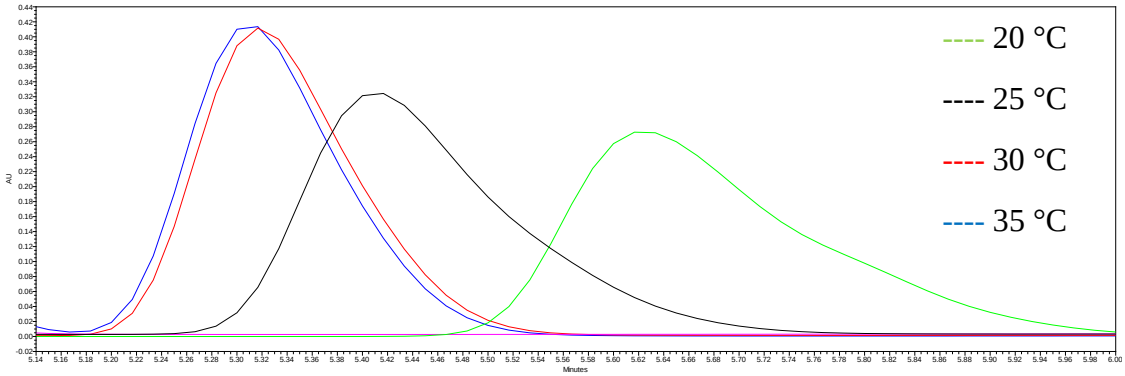


Figure 33 Chromatogram of the effect of temperature on the separation of caffeine on phenyl-hexyl column

4.3.6 Optimisation of the UV-Vis Detector

Based on a search of the literature, the most prevalent wavelength for the analysis of caffeine in dietary chromatography ranged between 270 nm and 280 nm as mentioned in Chapter 1. The maximum wavelength (λ_{max}) for caffeine was determined for caffeine and DMX on a Beckman UV spectrophotometer and found to be 272 nm. (Data not shown)

4.3.7 Representative Chromatograms

Figure 34 shows the order of elution was TB, PX, TP, etofylline and caffeine with the retention times of 3.8, 4.7, 5.0, 5.4 and 6.3 minutes respectively. Peaks were completely resolved with limited background interference. A water blank was used to monitor signs of carryover from high concentration standards/samples.

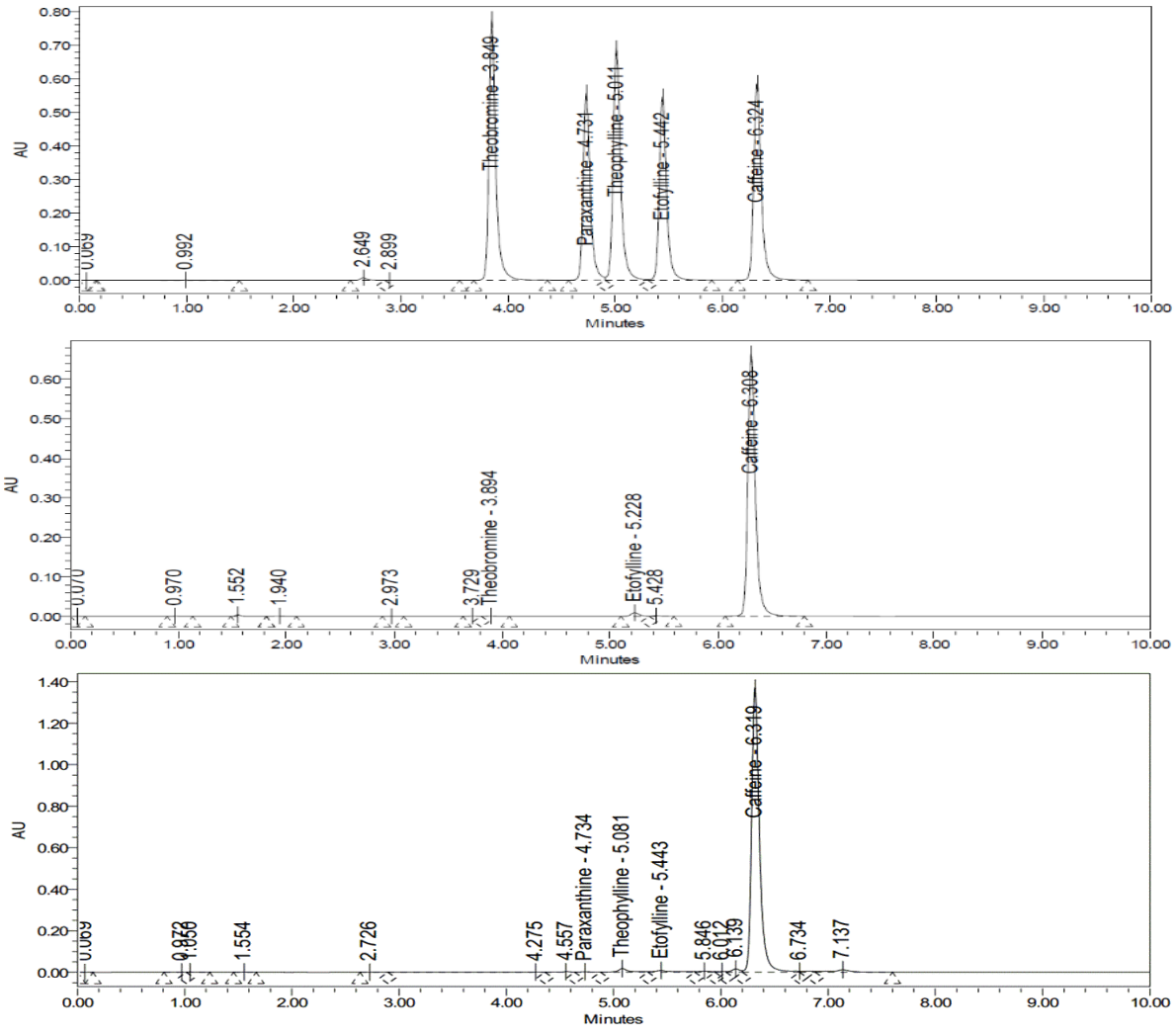


Figure 34 Representative chromatograms or caffeine and* DMX *standards at high concentrations of (100 µg/mL) (top); chromatogram of caffeine present in pre-workout supplement A (middle); and chromatogram of caffeine,* TP *and* PX *present The approximate retention times:* TB= *3.7 min,* PX= *4.6 min,* TP= *4.9 min, etofylline (I.S.) = 5.3 min and caffeine= 6.2 min. On phenyl-hexyl column at λ=272 nm

Selectivity of the method was confirmed by UV-detection and QDa-MS by injecting a starting condition blank and a dietary supplement blank. Representative 3-dimensional (3D) chromatographic plot depicting the additional dimension of mass to charge ratio (*m/z*) is presented in Figure 35, where the standards are colour coded into the following; caffeine (red); IS (blue); TB, TP, and PX (green). The *m/z* aids in identifying and confirming the presence of the methylxanthines by specific masses.

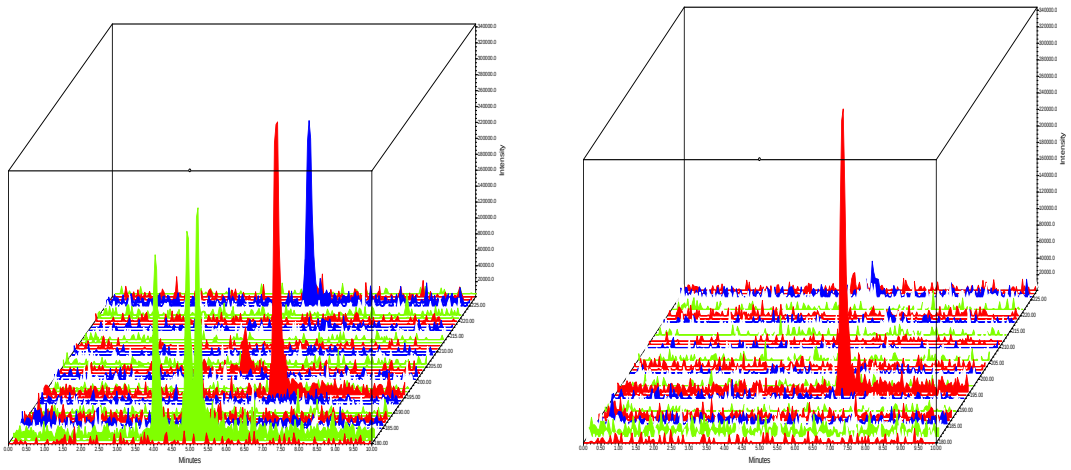


Figure 35 3D chromatogram/ mass plot. X-axis =time (min), Y-axis =Intensity of m/z signal, Z-axis = mass to charge ratio (m/z). Caffeine (red);* TB, PX, *and* TP *(green); etofylline (blue) is present in standards (left). Whereas only caffeine and etofylline is present in the spiked pre-workout sample A (right)

The specific masses detected at the apex of individual compounds (Figure 36) were detected simultaneous to the UV spectra (Figure 37). TIC was used to describe the presence of any minor components that were overlooked within the range of 100Da to 600 Da. The SIR was set to the masses of *m/z* 181.1, for the detection of TB, TP and PX; *m/z* 195.1 for the detection of caffeine; and *m/z* 225.1 for the detection of etofylline. The method showed well-resolved separation between PX and TP, not commonly observed in other studies (Bispo et al., 2002; Hasegawa et al., 2009; Kanazawa et al., 2000; Martinez-Lopez et al., 2014; Thomas et al., 2004). These isobaric DMXs are difficult to resolve due to their chemical similarities and identical mass (*m/z* 181.1). Since PX is not derived from plants and therefore not expected in PWS, its detection was not specifically determined. Whilst this is most likely true in the case of PWS, developing a rapid method which does resolve these compounds provides a distinct advantage in specificity as well as being adaptable to biological matrices.

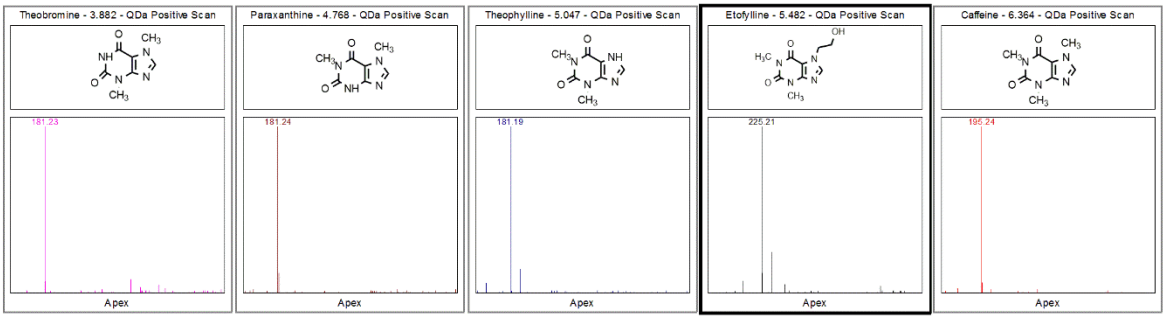


Figure 36 Mass detected at individual peaks of compounds,* TB, PX, TP, *etofylline, and caffeine

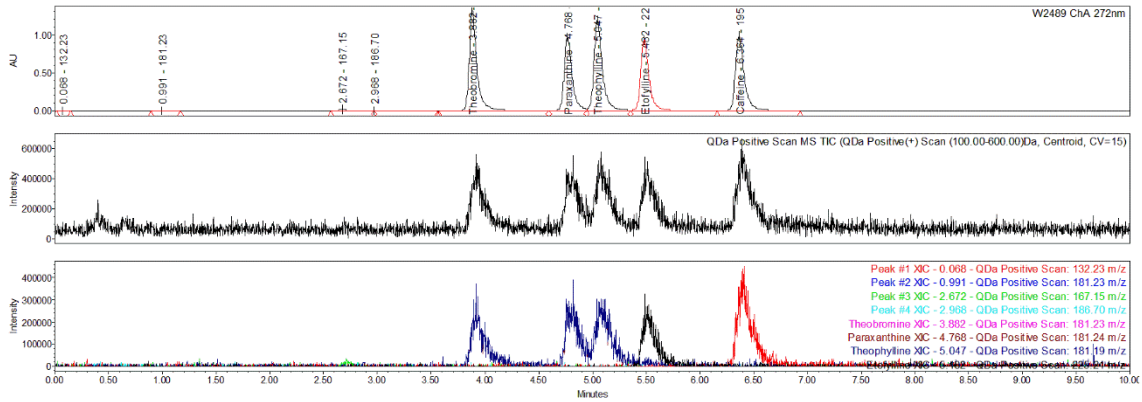


Figure 37 Chromatogram of standards (top) coupled with the Total Ion Chromatogram (TIC) scanning from a range of 100 Da to 600 Da (middle) and Single Ion recording (SIR) of respective compounds

4.3.8 Optimised HPLC/UV-MS Procedure

Based on the optimised chromatographic and detection parameters, the final chromatographic conditions were determined. The optimised conditions are summarised in the Table 16 below:

Table 16 Summary of optimised HPLC conditions

Optimisation parameter	Selected conditions
Column	Xselect CSH phenyl-hexyl (3.5 µm, 4.6 x 100 mm)
Organic modifier	Methanol
Flow rate	1 mL/min
pH	3.0 adjusted with formic acid
Temperature	30°C
UV (λ)	272 nm
Mass detection range (TIC)	100-600 Da

The chromatographic column used was an Xselect CSH phenyl-hexyl (3.5 µm, 4.6 x 100 mm, i.d., Waters, Ireland) protected by a guard column (Xselect CSH phenyl-hexyl 3.5µm, 4.6 x 20 mm). The components were separated using a gradient elution protocol where mobile phase A consisted of a mixture of water with 0.1% formic acid whilst mobile phase B comprised methanol with 0.1% formic acid. Both solvents were pH adjusted to 4.5.

The flow rate was maintained at 1 mL/min while the column temperature was set to 30°C and sample temperature was set at 25°C. The run time was 10 minutes under gradient conditions (Table 17) based on the retention time of caffeine (the most non-polar of the constituents under investigation). There was a 3-minute delay between injections and UV detection was at 272 nm.

Data acquisition with the QDa mass detector was performed in positive mode under the following conditions: total ion current (TIC) between *m/z* 100-600; capillary voltage, 3.5 kV; cone voltage, 15 V. Single Ion Recording (SIR) was set for positive mode masses of caffeine (195.1), IS (225.2) and respective derivatives (181.5).

Table 17 HPLC separation gradient used for UV-MS analysis of caffeine and derivatives

Gradient elution

Time(min)	**Mobile phase A (Water + 0.1% Formic Acid) (%)**	**Mobile phase B (Methanol + 0.1% Formic Acid) (%)**
0	85	15
5	70	30
5.5	85	15

4.4 Method Validation

The method validation parameters were highlighted in the Methods chapter (Section 2.3). Where the method was validated according to the ICH guidelines (ICH Expert Working Group, 1994)

4.4.1 Linearity

Linearity was determined using the least-square method (linear regression) for each of the standards. The assay showed linearity between the range 1 µg/mL to 200 µg/mL as reported in Table 18. The loss of analyte beyond 0.1 µg/mL was amplified and resulted in poor linearity, whereas concentration of 500 µg/mL resulted in peak overload and poor correlation coefficient (data not shown). Figure 38 shows a chromatogram of the xanthines at 8 concentration levels. Standards were injected at the beginning of each sample injection sequence and after every 10 sample injections, and at the end of each sequence. This provided an intra-day 8-point standard curve generated with each analyte, the slope and y-intercept, correlation coefficient (r^2) and %RSD of the standard curve (Figure 38). Linearity was observed in this range for each compound (correlation coefficients (r^2) $\geq$ 0.9998 for all except for TB which had an $r^2 \geq$ 0.9914). The deviation TB's correlation coefficient could be an issue of one concentration point (50 µg/mL) (

Figure 39). This could be a result of a random error cause by improper pipetting.

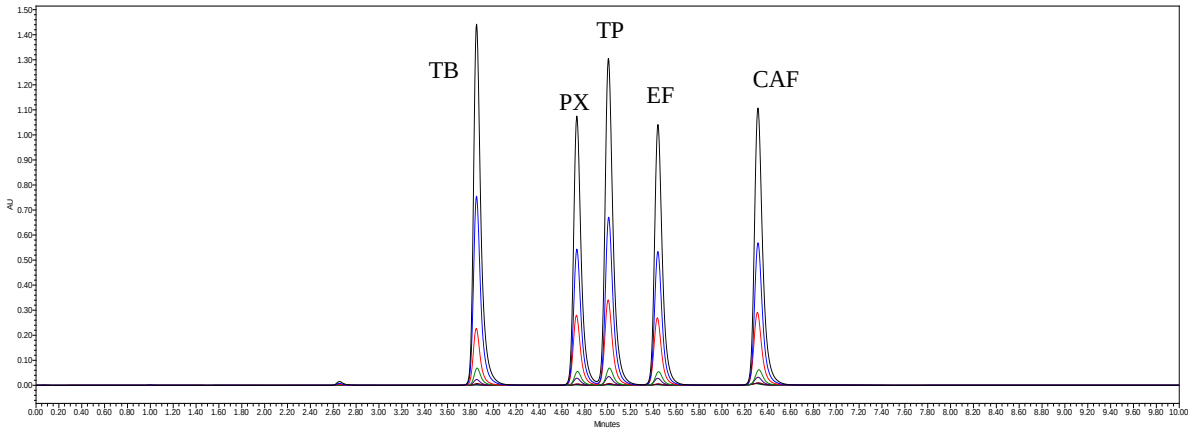


Figure 38 Chromatograph of caffeine and 3* DMX *standards at 8 different concentrations

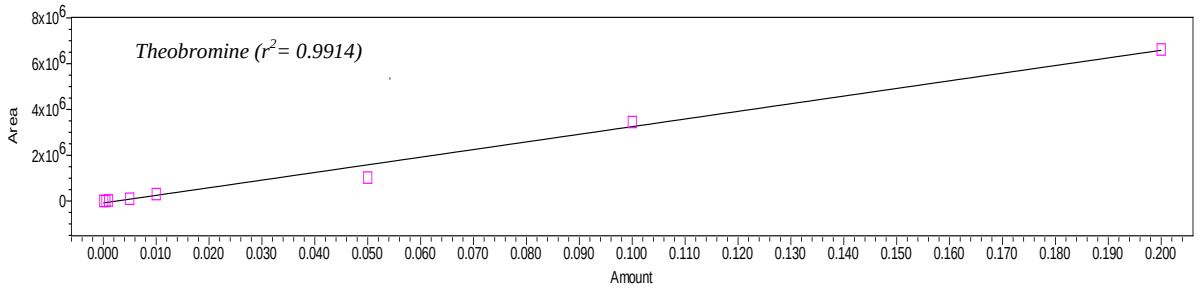


Figure 39 Intra-day 8 point standard curve for TB. Points represent average of 3 replicates

4.4.2 Detection and Quantification Limits

LOD and LOQ were determined based on their respective signal-to-noise ratio (S/N) of the standards at their lowest concentration of 0.1 µg/mL, where the desired S/N for the LOD is 3:1, whereas the desired S/N for the LOQ is 10:1 (Table 18). The LOD of the 3 DMX ranged from 0.02 to 0.4 µg/mL whereas the LOD determined for caffeine was 2.1 µg/mL and LOQ of 6.5 µg/ mL. The higher LOD of caffeine would suggest that the concentrations of less than 5 µg/mL were not suitable for linear calibration. However, this could be improved by avoiding low volume pipette injection, as there could be potential loss of caffeine at 1µg/mL. The reported amounts of caffeine and its DMX derivatives found in urine, ranged from 2 µg/mL to 15 µg/mL, which give potential for this method to be used for caffeine analysis in biological fluids (Buyuktuncel, 2010). The LOD and LOQ also make this method suitable for caffeine doping analysis in sport where 12 µg/mL of caffeine is considered a doping agent when this amount is found in urine (Del Coso, Muñoz, & Muñoz-Guerra, 2011).

Table 18 LOD/ LOQ for caffeine and derivatives

Standards	Calibration range (µg/mL)	Correlation coefficient (r^2)	LOD (µg/mL)	LOQ (µg/mL)
Caffeine	0.1-200	0.999±0.002	2.19	6.46
Etofylline	0.1-200	0.999±0.001	0.10	0.31
Paraxanthine	0.1-200	0.999±0.002	0.02	0.07
Theobromine	0.1-200	0.991±0.004	0.47	1.43
Theophylline	0.1-200	0.999±0.003	0.09	0.28

4.4.3 Precision

The repeatability was determined by preparing 8 replicates of standards at 100µg/mL. The results are reported in Table 19 with the %RSD. Repeatability, according to the association of analytical chemists (AOAC) should not exceed 1.25% RSD, our repeatability values were considered satisfactory for the retention times as indicated by %RSD range of 0.06 – 0.25%, peak height ranging from 1.11 -1.14% and also the peak area indicated by the %RSD range of 0.40-0.59%. This indicates that for a single-laboratory assay acceptable precision was achieved. However, additional validation by incorporating 'between laboratory' testing for parameters such as intermediate precision would be further strengthen the overall validation of this method.

Table 19 Intra-day precision assay of standard and analytical recovery of IS in samples

Analyte	Repeatability (n = 8, mean)					
	RT (min)	%R.S.D	Peak area ($x10^6$) (mean + S. D.)	%R.S.D	Peak Height ($x10^5$) (mean + S. D)	%R.S.D
Caffeine	6.31	0.06	2.89±0.01	0.4	5.76 ±0.70	1.21
Paraxanthine	4.73	0.14	2.54±0.01	0.43	5.51 ±0.06	1.14
Theobromine	3.81	0.25	3.44 ± 0.02	0.54	7.67 ± 0.06	1.21
Theophylline	5.01	0.14	3.30 ± 0.02	0.59	6.79 ± 0.08	1.15
Etofylline	5.44	0.13	2.55 ± 0.01	0.55	5.39 ± 0.06	1.11

4.4.4 Accuracy

Within-batch accuracy evaluations were determined by spiking PWS with etofylline (IS) Accuracy was assessed using 3 concentrations (5, 25 and 50 µg/mL) of I.S on 3 separate days. The standards were prepared in triplicate at each level of concentration. The quantitative recoveries are shown in Table 20 where the recoveries ranged from 81.8 – 89.2%, with %RSD 0.5 – 2.57%. The recoveries obtained after solid phase extraction at different concentrations showed that there were only slight variations within supplements and the results obtained for intra-assay accuracy based on etofylline were satisfactory (ICH Expert Working Group, 1994).

Table 20 Analytical recovery of etofylline (IS) spiking in PWS

Amount of Spiked IS (µg/mL)	Determined amount of IS (µg/mL)	Recovery of IS (%) (mean ± S. D., n=3)	%R.S.D
5	4.09	81.78 ± 5.30	2.57
25	22.3	89.20 ± 1.89	0.50
50	44.1	88.19 ± 5.17	1.10

4.5 Determination of Caffeine and DMX in PWS samples

The RP-HPLC-UV/MS method described was used for the quantitative determination of caffeine and DMX in a set of PWS samples. The results are shown in Table 21 as mean and standard deviation (S.D.) of three replicate samples in the same batch. The concentration of caffeine quantified ranged from 9.89 mg/g size to 25.30 mg/g size. TB was present in products A, D and E. TP and PX were present (at low levels) only in supplement C, which did not contain TB.

Table 21 Caffeine and methylxanthine content (mean± S.D., n= 3) in dietary supplements

Products	Caffeine (mg/g)	Theobromine (mg/g)	Theophylline (mg/g)	Paraxanthine (mg/g)
A (*KardioFire*)	20.10 ± 1.81	1.12 ± 0.15	N/A	N/A
B (*360Rage*)	15.70 ± 1.53	N/A	N/A	N/A
C (*PumpHD*)	25.30 ± 1.21	N/A	0.31± 0.02	0.03 ± 0.01
D (*MusclePrime*)	9.89± 0.39	2.57 ± 5.77	N/A	N/A
E (*DyNO*)	24.00 ± 0.16	3.03 ± 0.10	N/A	N/A

A comparison was made between the levels of caffeine and DMX in the sample set of PWS and that found on their labels (if present) (Table 22). The amount of caffeine determined was calculated to reflect per serving quantity to compare directly with the labelled dose which varied from 7 g to 19 g. Caffeine was not listed on the label of product A despite containing 201 mg of caffeine. Products B and C listed caffeine to be present as part of a proprietary blend where 157 mg of caffeine was found in product B and 192 mg of caffeine was found in product C. The labels of the products D and E listed caffeine in the form of 'caffeine anhydrous' as 100 mg and 300 mg respectively, the amount of caffeine determined in product D was 188 mg/dose while 180 mg/dose was found in product E.

Table 21 clearly shows evidence of the presence of PX in one of the randomly selected PWS, albeit in low amounts. Since it is a primary metabolite of caffeine and found in much higher levels than caffeine in plasma or urine samples, separation from TP was imperative to avoid over estimation of TP. While a complete understanding of the physiological effects of PX are not well researched reports of increased skeletal muscle contractility have been described (Hawke et al., 2000; Orru et al., 2013). An additional source of TP and PX could be attributed by the actions of filamentous fungi. Hakil et.al observed the demethylation of caffeine to TP via the *Aspergillus* and *Penicillium* genus strains of fungi (Hakil, Denis, Viniegra-González, & Augur, 1998). *PumpHD* contains Theobroma cacao extracts likely to be the source of caffeine. Potential degradation of caffeine to these DMX compounds could occur during the storing or processing phase of product manufacturing. The purity and potential co-elution of the compounds were monitored by tracking the masses found in the leading, apex and tailing of each peak. The advantage of incorporating a quadrupole mass detector can be observed in the form of peak purity at the apex in Figure 35. Mass spectral data was used to identify the components with unique masses in the mixture such as caffeine (*m/z* 195.2) and etofylline (*m/z* 225.2). However, confirming the presence of the isobaric DMX (*m/z* 181.2) required combining completely resolved peaks from UV data and mass detection.

Table 22 Amount of caffeine present compared to respective label contents

Products	Amount of Caffeine Quantified (mg/mL)	Caffeine listed on the label (mg) (per serving size)	Actual amount of caffeine (mg)(per serving size)
A (*KardioFire*)	20.10 ± 1.81	Not listed	201.44 ± 18.1
B (*360Rage*)	15.70 ± 1.53	Proprietary Blend	156.96 ± 15.3
C (*PumpHD*)	25.30 ± 1.21	Proprietary Blend	189.60 ± 9.06
D (*MusclePrime*)	9.89± 0.39	100	187.89 ± 7.14
E (*DyNO*)	24.00 ± 0.16	300	179.86 ± 1.20

4.6 Application and Usability of the Method

The FDA takes a liberal approach to the control of caffeine content and labelling in dietary supplements whereby caffeine is only required to be listed as an ingredient. In some other countries such as Australia, however, caffeine-containing dietary supplements, such as PWS, fall outside the jurisdiction of the Food Standards Code of Australia and often contain amounts that would otherwise breach the Code (For example, an energy drink is allowed to have a maximum of 80 mg/serving) ("Australia New Zealand Food Standards Code – Standard 2.9.4 – Formulated supplementary sports foods," 2016). Instead, as complementary medicines, these products are regulated by the TGA and must state the quantity per dose as well as carry a safety warning for vulnerable populations such as those under 18 years or who are pregnant. As highlighted in Table 22, the supplement with the highest caffeine content (201 mg) (Supplement A) did not list caffeine on its label. Furthermore, for those products that quoted specific amounts, much higher levels of caffeine were detected (except for supplement E).

PX was specifically found in supplement C albeit in low levels, which was surprising since, as previously mentioned, it is not a plant based compound. Understandably, no specific listings for any of the DMX were present on the PWS labels - manufacturers having no such requirement to do so by either the FDA or the TGA. Caffeine is often mentioned as part of a 'proprietary blend' where only the weight of the total blend is required to be listed (U.S. Food and Drug Administration (FDA), 1994). The discrepancy between the stated quantities and those quantified here not only makes it difficult for people to discern their total daily stimulant intake but also raises concerns for the public health and safety of consumers due to the potential for adverse effects. This analysis highlights a discrepancy between labelled and actual caffeine content and provides support for an argument for tighter regulation.

4.7 Conclusion

We have developed a simple, rapid procedure to determine levels of caffeine and the three DMX derivatives, TB, PX and TP in PWS. The procedure involved a HPLC-UV/MS method that has a total run time of 10 minutes. Recently, a number of studies used HPLC (Andrews et al., 2007; Hadad, Salam, Soliman, & Mesbah, 2012; Haller, Duan, Benowitz, & Jacob, 2004; Roman, Hildreth, & Bannister, 2013; Thomas et al., 2004) and UPLC (Beauchamp et al., 2016; Hasegawa et al., 2009) to quantify the amount of caffeine specifically in dietary supplements, caffeine powders and weight loss supplements and found the caffeine content in the products analysed were variable to that quoted on their labels. Our results are in agreeance with the trends reported in those studies. Note that the HPLC methods mentioned above were subject to long run times compared to our short run time of 10 minutes. For high throughput analyses, this translates to a considerable cost saving without compromising sensitivity. The sensitivity reported in this method confers potential applicability to supplements as well as biological matrices. However, further improvement of the LOD and LOQ of caffeine is required to improve the sensitivity of the method. To our knowledge, this is the first implementation of a mass-detector on a HPLC method specific to determining caffeine in PWS.

Chapter 5 Discussions, Future Directions and Conclusion

5.1 General Discussion

The overall goal of this thesis was to determine the amounts of selected stimulants in PWS, both the biogenic amines derived from CA extracts as well as caffeine and its DMX derivatives. To achieve these aims, two HPLC-UV/MS protocols were developed. Details of the development and optimisation were discussed in Chapter 3 and Chapter 4 for the detection of biogenic amines and the detection of caffeine and DMX derivatives in PWS respectively. Usability of and limitations to these methods are discussed later in this chapter.

These analytical protocols were developed to answer the following research questions:

1) Do CA-containing PWS comply with the 30 mg per dose limit of synephrine set by the TGA?
2) Is there a discrepancy in synephrine concentrations between overseas sourced and locally manufactured PWS?
3) Are the levels of caffeine measured in a range of CA-containing PWS products, consistent with that listed on their labels?

Research question one was answered by determining the amounts of synephrine found in CA-containing PWS and comparing them to the maximum limit of 30 mg/serve limit set by the TGA. The concentrations of synephrine in all five PWS found were between 1.06 to 8.30 mg/serve. Hence, all PWS supplements tested did not exceed this regulated amount.

The levels of synephrine measured in Australian-made and internationally sourced PWS were compared. The highest concentration of synephrine was found in a locally made PWS (*PumpHD*) (8.30 mg/serve), whereas the lowest concentration of synephrine was found in an internationally sourced PWS (360Rage) at 1.06 mg/serve. A larger sample size was needed to form a trend between the discrepancies of Australian and internationally made PWS.

Finally, the amount of caffeine and its DMX derivatives were determined and contrasted between local and internationally manufactured PWS. The average caffeine content within all PWS was quite similar despite country of origin. Interestingly, a locally made PWS contained traces of TP and PX. These were likely formed during the manufacturing process.

The remainder of this chapter is devoted to a general discussion of the methods developed, their use and limitations and an interpretation of the results of the research in terms of relevance and future directions

5.1.1 Adrenergic Amines Found in CA-containing PWS

Synephrine, as the main biogenic amine in CA, is classed as a Schedule 4 substance (prescription only medicine) in Australia with a RDD of 30 mg. PWS are considered complementary medicines and their importation and use is regulated by the TGA. A rise in popularity of this type of product for recreational use has been accompanied by an increase in adverse health reports and prompted warnings by regulatory bodies. Therefore, there was a need to investigate whether the amounts of synephrine in CA-containing PWS meet the 30 mg RDD and if there was a discrepancy between locally and internationally sourced PWS.

Once developed, the validated HPLC-UV/MS protocol was used to determine the levels of adrenergic amines, synephrine, tyramine and octopamine in a selection of PWS. These included MusclePrime (AllMax Nutrition) (A); Gold Standard Preworkout (OptimumNutrition) (B); 360Rage (360°Cut Performance Supplements) (C); ErgoBlast (ErgoGenix) (D) and PumpHD (BPISports) (E). A matrix blank (Beast Mode (Beast Sport's Nutrition) was included which lacked CA and could help eliminate matrix-borne errors in the extraction protocol. The PWS samples A, C and D were purchased online (manufactured in the USA) while samples B and E were manufactured in Australia.

The chromatography involved a gradient elution over a fast, 10-minute run time (A: water and B: acetonitrile, adjusted to pH 11 with 0.1% ammonium hydroxide) on an Xbridge BEH C18 column. Detection was facilitated with use of a UV/Vis-detector (242 nm) coupled with mass confirmation via an in-line single quadrupole (QDa) mass detector in positive electrospray ionisation (ESI) scan mode. This method proved to be successful in achieving an important feature or reduced analysis time, making it suitable for high-throughput analyses. Additionally, improved chromatography was achieved by using a less common approach to the analysis of adrenergic amines - the inclusion of a basic modifier at high pH. The increase mobile phase pH of 11 meant the use of IPRs was avoided which translates to cost saving for initial IPR purchase as well as eliminating the long equilibration times that accompany IPR use. Furthermore, since ammonium hydroxide is volatile, it is suitable for LC-MS, unlike IPRs. We also showed how the novel implementation of QDa mass detection was able enhances structure confirmation of the adrenergic amines.

Calibrations for each compound were linear between the range 5 µg/mL to 100 µg/mL with determination coefficients (r^2) greater than 0.997. The intra-day precision was calculated to be between the range of 2.1-4.1% for synephrine, 2.9-3.0% for octopamine, and 1.4 to 5.3% for tyramine. According to the FDA guidelines for pharmaceutical determination, RSD% values of less than 15% were considered satisfactory (FDA, 2013). The accuracy was determined by adding a known amount of standard to a blank matrix according to the ICH standards guidelines. In this analysis, the accuracy of all the amines was between 77-105%. A majority of the accuracy values were acceptable In the case of octopamine and tyramine there was a trend for lower accuracy at the LOD (5 µg) as well as the higher range of 100 µg/mL (80%). This could possibly be due to loss during sample preparation or simply experimental error in technique. This former could be improved by using a higher concentration of non-polar solvent (ACN) during the sample preparation to aid in the retention of the compounds during the SPE process.

Table 23 summarises the amount (reported as mg/g) of synephrine, octopamine and tyramine found in our tested PWS and compared against the concentrations obtained in a standard reference material (SRM 3258) by Putzbach et al. (Putzbach et al., 2007).

Table 23 Synephrine, octopamine and tyramine content in PWS and SRM 3258(mg/g)

Compounds of interest	Concentration of amines found in samples (mg/g) (mean ±RSD %)					Yield from SPE (%)	Amount of amines found in SRM 3258 (mg/g)
	A	*B*	C	D	*E*		
Synephrine	0.25 ± 9.3	0.31 ± 5.6	0.15 ± 2.9	0.70 ± 1.5	0.89 ± 1.8	98.0± 1.2	8.65
Octopamine	0.10 ± 10.2	4.46 ± 12.7	0.28 ± 4.6	8.90 ± 10.1	0.65 ± 3.8	110.3 ± 6.3	0.14
Tyramine	0.10 ± 2.5	0.52 ± 0.4	0.28 ± 1.1	0.28 ± 2.0	0.69 ± 3.9	96.7 ± 0.6	0.026

**PWS highlighted in red were Australian-made products*

The concentrations of biogenic amines in certified standard reference materials were presented in mg/g of a standardized source of a CA fruit. The ratios of the biogenic amine within the standard were approximately 10: 01: 0.02 (mg/g) for synephrine: octopamine: tyramine (Putzbach et al., 2007). Thevis et.al measure a similar ratio of biogenic amines in a single CA extract where the ratios were 5: 0.09: 0.03 (mg/g) (Thevis et al., 2012). We reported our findings in mg/g of supplement where the exact quantities of CA extract added were unknown. When contrasted to our findings, it was evident that the ratios of biogenic amines found were significantly higher for octopamine and tyramine, but lower for synephrine.

To compare concentrations of actives between PWS samples, we calculated according to their serving size (Table 24)

Table 24 Amount of amines found calculated to the serving size of the PWS (mg/serve)

	Amount of amines found in PWS (mg/serve) (mean ± RSD %)		
Samples	Synephrine	Octopamine	Tyramine
A (*MusclePrime*)	4.69 ± 9.3	1.97 ± 10.2	1.95 ± 2.5
B (*Gold Standard Preworkout*)	3.11 ± 5.6	44.62 ± 12.7	5.17 ± 0.4
C (*360Rage)*	1.06 ± 2.9	2.03 ± 4.6	2.05 ± 1.1
D (*ErgoBlast)*	3.49 ± 1.5	44.52 ± 10.1	1.39 ± 2.0
E (*PumpHD)*	8.30 ± 1.8	6.54 ± 3.8	6.90 ± 3.9

**PWS highlighted in red were Australian-made products*

Levels of *p*-synephrine were in line with other reported methods of between 0.001 mg/g to 8.65 mg/g (Avula, Upparapalli, Navarrete, et al., 2005; Ganzera et al., 2005; Marchei et al., 2006; Pellati & Benvenuti, 2007; Pellati et al., 2002; Roman et al., 2007; Slezak et al., 2007). *PumpHD*, an Australian-made PWS, had the highest levels of synephrine (8.3mg/ serve), whereas the lowest level of synephrine (1.06mg/ serve) was found in *360Rage*, an internationally manufactured PWS. A much larger sample size would be required for testing before any trend or conclusion could be made in relation to comparing Australian to overseas-made PWS. There would also be scope to test locally sourced and internationally sourced variants of the same brand to observe the discrepancies between them. After recalculating the amounts found in our analysis to its respective serving size, it was observed that all synephrine found did not exceed the TGA's RDD of 30 mg/serve.

Octopamine was detected in high concentrations (44 mg/serve) in one locally-made PWS (*Gold standard Preworkout*) and one made overseas-made PWS (*ErgoBlast*). Octopamine was found in CA extracts in amounts between 0.12 mg/g to 0.8 mg/g (Sander et al., 2008). The high level of octopamine determined was not in line with the apparent ratio of the biogenic amines found in the SRM and thus in these PWS suggests it may have been added rather than simply being present as a component of CA. Currently, there no legal limits or RDDs for octopamine use in dietary supplements in Australia although, due to its stimulatory properties, it is a banned substance in athletic competitions (World Anti-doping Agency, 2015). WADA has not stated a specific acceptable level of octopamine above which penalties apply. With further improvements to sensitivity for detection biological matrices, this method could find use in the quantification of octopamine in the anti-doping arena.

Tyramine, while not listed on any PWS labels was detected in all samples tested. The highest level of tyramine quantified was 0.69 mg/g in a locally made PWS. However, when calculated to its serving size, the concentration of tyramine is 6.9 mg/serve, higher than some synephrine found in the other PWS. Tyramine has been attributed to microbial decarboxylases on amino acids such as tyrosine during handling and packaging or during the preparation of these PWS (Farah, 2012). *PumpHD* specifically uses *Theobroma cacao* seed extract, which is prepared by roasting *Theobroma cacao* seeds. This process could release and increase the amount of amino acid decarboxylation, attributing to the higher amount of tyramine. Like octopamine, there are no current restrictions on the maximum limits for tyramine in supplements.

5.1.2 Caffeine and DMX Found in PWS

In Australia, manufacturer are required to list caffeine on product labels however there are no set maximum limits for PWS. For food additives however, the FSANZ has set the RDD for caffeine to 210 mg/day (approximately 3 cups of coffee/day). For the accurate quantification of total stimulants in PWS, one must include caffeine and its DMX derivatives in the analysis. In addition, since some products do provide actual levels of caffeine on their labels, comparisons were made the measured levels.

To address the third research question a rapid HPLC-UV/MS protocol was developed and validated for the quantification of caffeine and DMX in locally and overseas made PWS.

The chromatography involved a gradient elution over a fast, 10-minute run time (A: water and B: acetonitrile, adjusted to pH 4.5 with 0.1% formic acid) on a CSH phenyl-hexyl column, detected with a UV/Vis-detector (272 nm) coupled with mass confirmation on a QDa mass detector in positive ESI mode. Repeatability, according to the AOAC should not exceed 1.25% RSD ((AOAC), 2012), our repeatability values were considered satisfactory for the retention times as indicated by %RSD range of 0.06 – 1.14% These results are in line with the standards set out by ICH for acceptable single-laboratory assay precision. However, additional validation by incorporating 'between laboratories' testing would strengthen the overall validation of this method.

The quantitative recoveries ranged from 81.8 – 89.2%, with %RSD 0.5 – 2.57% which is satisfactory according to the ICH guidelines. However, yield improvements are indicated given noticeable losses during the sample preparation. This could be improved by using a higher concentration of non-polar solvent (ACN) during the sample preparation.

Table 25 shows the content of caffeine and DMX (mg/g) in the sample PWS. How these four compounds are introduced into the PWS depends on whether they're part of a plant extract or a separate additive. For example, if from plant origin, then PX should not be expected to be present. Interestingly PX was found in one locally-made PWS *(PumpHD)* albeit at low levels. While a complete understanding of the physiological effects of PX are not well researched reports of increased skeletal muscle contractility have been described (Hawke et al., 2000; Orru et al., 2013). An additional source of TP and PX could be attributed by the actions of filamentous fungi. Hakil et.al observed the demethylation of caffeine to TP via the *Aspergillus* and *Penicillium* genus strains of fungi (Hakil et al., 1998). *PumpHD* contains Theobroma cacao extracts likely to be the source of caffeine. Potential degradation of caffeine to these DMX compounds could occur during the storing or processing phase of product manufacturing.

Table 25 Caffeine and methylxanthine content in PWS (mean +/- SD) (mg/g PWS)

Products	Caffeine (mg/g)	Theobromine (mg/g)	Theophylline (mg/g)	Paraxanthine (mg/g)
A	20.10 ± 1.81	1.12 ± 0.15	N/A	N/A
B	15.70 ± 1.53	N/A	N/A	N/A
C	25.30 ± 1.21	N/A	0.31± 0.02	0.03 ± 0.01
D	9.89 ± 0.39	2.57 ± 5.77	N/A	N/A
E	24.00 ± 0.16	3.03 ± 0.10	N/A	N/A

**PWS highlighted in red were Australian-made products*

Comparison of measured caffeine to labelled quantities if present was made by adjusting to serving size (Table 26).

Table 26 Amount of caffeine present compared to its PWS label

Products	Amount of Caffeine Quantified (mg/mL)	Caffeine listed on the label (mg) (per serving size)	Actual amount of caffeine* (mg)(per serving size)
A (*KardioFire*)	20.10 ± 1.81	Not listed	201.44 ± 18.1
B (*360Rage*)	15.70 ± 1.53	Proprietary Blend	156.96 ± 15.3
C (*PumpHD*)	25.30 ± 1.21	Proprietary Blend	189.60 ± 9.06
D (*MusclePrime*)	9.89± 0.39	100	187.89 ± 7.14
E (*DyNO*)	24.00 ± 0.16	300	179.86 ± 1.20

**Average 181.15 ±9.03 RSD %*

***PWS highlighted in red were Australian-made products*

Interestingly, despite the country of manufacture of the PWS, the average caffeine content was quite similar with a relatively low RSD%. Noticeably however the amount per serving size is just under the RDD of a maximum of 210 mg per day mentioned above. The implication for consumers is that it is likely, with so many other sources of caffeine in the modern diet that over consumption may regularly occur. In addition, two or more servings of the PWS per day can easily raise caffeine consumption into dangerously high levels.

In some instances, there are discrepancies between measured product content from that stated on product labels. This finding is consistent with other studies which found similar differences (Beauchamp et al., 2016; Gurley et al., 2004).

The protocol successfully separated TP and PX. As explained earlier, TP and PX are isobaric isomers that differ in the position of a methyl group (as with TB). The difficulty this presents to the analyst is reflected in the paucity of studies which show their clear resolution. Researchers often explain this omission by the absence of PX in food and plants. The use of the QDa mass detector aided in the confirmation of these isomers during quantitation in PWS supplements however this was only possible once peak resolution has been achieved. Since PX was detected in one of the supplement (C), it is clear that attention should be given to this analyte regardless of its origin. Otherwise levels of TP could be overestimated. It is possible that PX has been specifically added in order to increase the overall stimulatory effect of caffeine in PWS. Further testing in a higher sample number is required to confirm whether this was a 'one-off' finding in PWS formulations or a more common practice.

Apart from its reliability and fast run time, this method has the potential for applicability to both commercial products and biological fluids since it completely resolves the isobaric compounds, TP and PX. However, further improvements and validations are required by conducting validations on a larger sample size. Since the typical caffeine content in urine is in the range 2 µg/mL to 15 µg/mL, there is scope for this method to be transferrable to the analysis of caffeine and its metabolites in biological fluids This would be of benefit to sporting regulatory bodies, such as Australian sport anti-doping agency (ASADA) or WADA, where screening is conducted for particularly for caffeine levels above 12 µg/mL. However, by only measuring caffeine in the urine and not its metabolites, underestimation of caffeine content originally consumed is likely.

5.2 Limitations

A number of limitations should be mentioned. First and foremost, the number of PWS selected was too small for any trends or conclusions to be drawn with respect to comparing locally made to overseas manufactured products. A much larger sample size would be required to draw such conclusions. Notwithstanding the above, the results obtained from this pilot data could be used to justify further work extending the scope for a much larger sample set. In addition, the batch of supplements were sourced in 2015 but had not surpassed their expiry date. There was still potential for component degradation could result in an under determination of the analytes as the nature of handling and storage of PWS prior to purchase was unknown.

An improvement for the quantification of caffeine and DMX could have been in the way in which the IS (etofylline) was used. In this protocol only, the IS was used for analyte recovery determination during sample preparation rather than the normal function in calibration to account for variability due to analyte losses in storage or treatment for example.

The QDa mass detector was able to scan the masses of compounds ranging from 100Da to 1300Da. The benefits of linking the LC system to the QDa detector were the enhanced ability to confirm structure identity. While single-quadruple magnet systems such as this are unable to reach anywhere near the degree of accurate mass detection or range of structure detection possible with more complex, tandem MS systems, they can be a powerful tool when used as a mass detection tool. Fragmentations can be tracked with a confidence level of ±0.1 Da. This is not satisfactory for many analyses. However their comparative cheaper cost makes them an attractive option depending on the requirements for use.

Additional experimental errors may have occurred during the sample preparation phase. It was evident that the analytical recoveries after the SPE were especially low at lower concentrations. This indicates a problem with small volume delivery. This could be improvements to technique, adjusting to larger volumes or by further validating the SPE method protocol by optimising the organic elution phase. The use of a centrifugation and extraction method could have been beneficial to the determination of stimulants due to the complex matrix of the PWS.

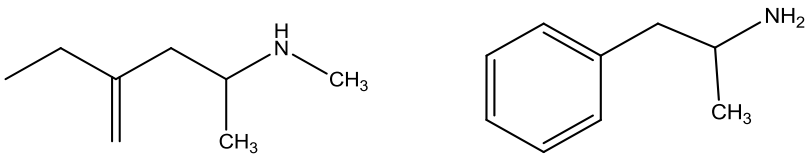
5.3 Future Directions for the Analysis of Stimulants in PWS

To improve the sensitivity of the amphetamine-like compound protocol, Hydrophilic Interaction Chromatography (HILIC) could be used. HILIC has been used in the analysis of small polar compounds such as pharmaceutical, toxins, plant extracts and other compounds important to food and pharmaceutical industries. (Jandera, 2011; Karatapanis, Fiamegos, & Stalikas, 2011; McCalley & Neue, 2008).

A small number of studies have used HILIC as a means of separation for ephedrine and synephrine. A first study by Heathon, investigated the suitability of bare silica HILIC as an alternative to conventional RP-HPLC for the fast chromatographic separation of ephedrine isomers. The study reported the effects of chromatographic parameters including the proportion of acetonitrile, buffer, pH and the column temperature in HILIC separation (Heaton et al., 2012). Faster retention times and symmetrical peaks were achieved on the HILIC method over HPLC conditions coupled with the advantage of MS compatibility.

This was followed by a more comprehensive study by Jovanovic et al who investigated parameters such as mobile phase and other process parameters on HILIC separation of ephedrine and synephrine (Jovanović, Rakić, Ivanović, & Jančić–Stojanović, 2015). The method successfully separated the diasteroisomers, ephedrine and pseudoephedrine as well as the positional isomers of synephrine and phenylephrine. However, the linear calibration was not reported and the correlation coefficients were r^2= 0.990 could be improved by optimising the range of standards used. Sakai et al used a zwitterionic monolith column paired to an electrochemical detection system. The authors demonstrated the use of electron capture dissociation (ECD) in HILIC with a LOD of 3.7 pg (Sakai et al., 2016). Future work implementing a HILIC column, for the analysis of the adrenergic amines within CA should be undertaken and compared to the current method to improve the sensitivity in detecting these compounds in PWS or biological matrices.

Further analytical studies could be undertaken to detect other synthetic stimulants, not of plant origin but reported to be present in recently released PWS. These reports describe banned supplements containing novel synthetic stimulants – many of which lack appropriate evidence for safety in humans (Archer et al., 2015; Cohen, 2012; Venhuis et al., 2014). Synthetic stimulants such as DMAA, amphetamine and methamphetamine analogues have often been sold as a compound within herbal extracts. DMAA ($C_7H_{17}N$) is an aliphatic amine with a pharmacophoric structure similar to amphetamine (Figure 40). Its sympathomimetic mechanism of action is similar to amphetamine. A study conducted in 2013, was the first to characterise the oral pharmacokinetic profile of DMAA, where it was found that DMAA did not impact heart rate, blood pressure or body temperature (Schilling, Hammond, Bloomer, Presley, & Yates, 2013). However, this short-term study only involved eight healthy men providing blood samples collected over a 24 hour period. One subject was excluded due to reported abnormal DMAA levels.



1,3-Dimethylamylamine (DMAA) Amphetamine

Figure 40 Structural simiarities between DMAA and amphetamine

A challenge with detecting DMAA and its analogues by the common technique of UV spectrophotometry is their lack of a chromophore. In addition, their presence in PWS is often masked by claims that they are constituents of natural plant extracts and so are not listed individually. For example, DMAA has been labelled as present in geranium oil (Zhang, Woods, Breitbach, & Armstrong, 2012), methamphetamine analogue, N, α-diethylphenylethylamine (DEPEA) labelled as dendrobium orchid (Cohen, Travis, & Venhuis, 2014) and 1,3-dimethylbutylamine (DMBA) was marketed as an extract of Pouchung tea (Cohen, Travis, & Venhuis, 2015).

Finally, receptor binding studies for these adrenergic amines are quite limited, which limits a more complete understanding of their pharmacology. As mentioned in chapter 1, synephrine has been described to be an α-AR agonist (Carpene et al., 1999). However, the specific subtypes of α-adrenoceptor binding have yet to be explored. This presents a clear gap in knowledge and an opportunity for the investigation into the receptor binding properties of these amines using either cell-based and or tissue-based experiments. Computational approaches such as molecular docking and dynamics could search for unique features of the receptor subtypes which may help explain any observed differences in binding and/or function. Molecular dynamic simulations could be used to computationally derive the binding affinities and compared to the known proposed (α1-, α1-, β2-, β3-) adrenergic and trace amine-associated receptor 1 (TAAR-1).

5.4 Concluding Remarks

The aims and research questions have been addressed by the development of two rapid, accurate and validated HPLC protocols for the quantitation of 1) adrenergic amines present in CA-containing PWS and 2) caffeine and DMX present in PWS. To the best of my knowledge, these methods were the first to implement a QDa detector for rapid mass detection in-line with UV detection on a R*P*-HPLC system for quantification of these compounds. Levels of synephrine in both locally and overseas- made PWS were compliant with the TGA's regulation of a limit of 30 mg/serve. A greater sample size of CA-containing PWS is likely required to determine any real differences in synephrine levels between locally and overseas-made PWS.

It was hypothesised that the amounts of caffeine found in overseas made PWS would be higher than locally sourced PWS. This was not the case however, significant variability was observed in the levels of caffeine found in both locally and overseas made PWS when compared to the stated quantity on its label. A further highlight of this study was the development of a method with the ability to resolve the isobaric isomers, TP and PX. PX was also found in one of the overseas supplement albeit in small amounts. It was noted that when the compounds were present as a 'proprietary blend' this poses an obstacle to accurately compare the quantities of the key ingredients with their labels. Future research into the quantification of these amines and other synthetic stimulants on a HILIC could result in greater sensitivity of the methods especially when coupled to a mass detector.

References

Australia New Zealand Food Standards Code – Standard 2.9.4 – Formulated supplementary sports foods (2016).

AOAC (2012). Guidelines For Dietary Supplements And Botanicals. *AOAC International*.

Andrews, K. W., Schweitzer, A., Zhao, C., Holden, J. M., Roseland, J. M., Brandt, M., Douglass, L. (2007). The caffeine contents of dietary supplements commonly purchased in the US: analysis of 53 products with caffeine-containing ingredients. *Anal Bioanal Chem, 389*(1), 231-239. doi: 10.1007/s00216-007-1437-2.

Annesley, T. M. (2003). Ion suppression in mass spectrometry. *Clin Chem, 49*(7), 1041-1044.

Aranda, M., & Morlock, G. (2007). Simultaneous determination of caffeine, ergotamine, and metamizol in solid pharmaceutical formulation by HPTLC-UV-FLD with mass confirmation by online HPTLC-ESI-MS. *J Chromatogr Sci, 45*(5), 251-255.

Arbo, M. D., Larentis, E. R., Linck, V. M., Aboy, A. L., Pimentel, A. L., Henriques, A. T., Limberger, R. P. (2008). Concentrations of *p*-synephrine in fruits and leaves of Citrus species (Rutaceae) and the acute toxicity testing of *Citrus aurantium* extract and *p*-synephrine. *Food Chem Toxicol, 46*(8), 2770-2775. doi: 10.1016/j.fct.2008.04.037.

Arbo, M. D., Schmitt, G. C., Limberger, M. F., Charao, M. F., Moro, A. M., Ribeiro, G. L., Limberger, R. P. (2009). Subchronic toxicity of *Citrus aurantium* L. (Rutaceae) extract and *p*-synephrine in mice. *Regul Toxicol Pharmacol, 54*(2), 114-117. doi: 10.1016/j.yrtph.2009.03.001

Arch, J. R. (2002). beta(3)-Adrenoceptor agonists: potential, pitfalls and progress. *Eur J Pharmacol, 440*(2-3), 99-107.

Archer, J. R., Dargan, P. I., Lostia, A. M., van der Walt, J., Henderson, K., Drake, N., . . . Kicman, A. T. (2015). Running an unknown risk: a marathon death associated with the use of 1,3-dimethylamylamine (DMAA). *Drug Test Anal, 7*(5), 433-438. doi: 10.1002/dta.1764

Astrup, A. (2000). Thermogenic drugs as a strategy for treatment of obesity. *Endocrine, 13*(2), 207-212. doi: 10.1385/ENDO:13:2:207

Australia New Zealand Food Standards Code – Standard 2.9.4 – Formulated supplementary sports foods (2016).

Avula, B., Upparapalli, S. K., & Khan, I. A. (2005). Enantiomeric separation of adrenergic amines in Citrus species, related genera and dietary supplements by capillary electrophoresis. *Chromatographia, 62*(3-4), 151-157. doi: 10.1365/s10337-005-0598-4

Avula, B., Upparapalli, S. K., Navarrete, A., & Khan, I. A. (2005). Simultaneous quantification of adrenergic amines and flavonoids in *Citrus aurantium*, various Citrus species, and dietary supplements by liquid chromatography. *J AOAC Int, 88*(6), 1593-1606.

Babic, S., Horvat, A. J. M., Pavlovic, D. M., & Kastelan-Macan, M. (2007). Determination of pK(a) values of active pharmaceutical ingredients. *Trac-Trends in Analytical Chemistry, 26*(11), 1043-1061. doi: 10.1016/j.trac.2007.09.004

Bagatela, B. S., Lopes, A. P., Cabral, E. C., Perazzo, F. F., & Ifa, D. R. (2015). High-performance thin-layer chromatography/desorption electrospray ionization mass spectrometry imaging of the crude extract from the peels of *Citrus aurantium* L. (Rutaceae). *Rapid Commun Mass Spectrom, 29*(16), 1530-1534. doi: 10.1002/rcm.7246

Bailey, R. L. (2014). Estimating caffeine intake from energy drinks and dietary supplements in the United States (vol 72, pg 9, 2014). *Nutrition Reviews, 72*(11), 735-735. doi: 10.1111/nure.12158

Barak, L. S., Salahpour, A., Zhang, X., Masri, B., Sotnikova, T. D., Ramsey, A. J., . . . Gainetdinov, R. R. (2008). Pharmacological characterization of membrane-expressed human trace amine-associated receptor 1 (TAAR1) by a bioluminescence resonance energy transfer cAMP biosensor. *Mol Pharmacol, 74*(3), 585-594. doi: 10.1124/mol.108.048884

Beauchamp, G. A., Lin, T., Zeng, W. Z., Hendrickson, R. G., & Gerona, R. (2016). Quantitative analysis of powdered caffeine products purchased from the Internet using liquid chromatography-quadrupole time-of-flight mass spectrometry. *Clin Toxicol (Phila), 54*(4), 339-343. doi: 10.3109/15563650.2016.1138225

Bell, A., Dorsch, K. D., Mccreary, D. R., & Hovey, R. (2004). A look at nutritional supplement use in adolescents. *Journal of Adolescent Health, 34*(6), 508-516. doi: Doi 10.1016/S1054-139x(03)00348-3

Benowitz, N. L. (1990). Clinical-Pharmacology of Caffeine. *Annual Review of Medicine, 41*, 277-288. doi: DOI 10.1146/annurev.me.41.020190.001425

Beyer, J., Peters, F. T., Kraemer, T., & Maurer, H. H. (2007). Detection and validated quantification of nine herbal phenalkylamines and methcathinone in human blood plasma by LC-MS/MS with electrospray ionization. *J Mass Spectrom, 42*(2), 150-160. doi: 10.1002/jms.1132

Bispo, M. S., Veloso, M. C. C., Pinheiro, H. L. C., De Oliveira, R. F. S., Reis, J. O. N., & De Andrade, J. B. (2002). Simultaneous determination of caffeine, theobromine, and theophylline by high-performance liquid chromatography. *J Chromatogr Sci, 40*(1), 45-48.

Bloomberg Business. (2015). Executive Profile : Sidney Stohs Ph.D., from http://www.bloomberg.com/research/stocks/private/person.asp?personId=24704214&privcapId=22729045

Borowsky, B., Adham, N., Jones, K. A., Raddatz, R., Artymyshyn, R., Ogozalek, K. L., Gerald, C. (2001). Trace amines: identification of a family of mammalian G protein-coupled receptors. *Proc Natl Acad Sci U S A, 98*(16), 8966-8971. doi: 10.1073/pnas.151105198

Bouchard, N. C., Howland, M. A., Greller, H. A., Hoffman, R. S., & Nelson, L. S. (2005). Ischemic stroke associated with use of an ephedra-free dietary supplement containing synephrine. *Mayo Clin Proc, 80*(4), 541-545. doi: 10.1016/S0025-6196(11)63207-2

Broadley, K. J. (2010). The vascular effects of trace amines and amphetamines. *Pharmacology and Therapeutics, 125*(3), 363-375. doi: 10.1016/j.pharmthera.2009.11.005

Broadley, K. J., Akhtar Anwar, M., Herbert, A. A., Fehler, M., Jones, E. M., Davies, W. E., Ford, W. R. (2009). Effects of dietary amines on the gut and its vasculature. *Br J Nutr, 101*(11), 1645-1652. doi: 10.1017/S0007114508123431

Brown, C. M., McGrath, J. C., Midgley, J. M., Muir, A. G., O'Brien, J. W., Thonoor, C. M., Wilson, V. G. (1988). Activities of octopamine and synephrine stereoisomers on alpha-adrenoceptors. *Br J Pharmacol, 93*(2), 417-429.

Brown, N. J., Porter, J., Ryder, D., & Branch, R. A. (1991). Caffeine Potentiates the Renin Response to Diazoxide in Man - Evidence for a Regulatory Role of Endogenous Adenosine. *Journal of Pharmacology and Experimental Therapeutics, 256*(1), 56-61.

Brunton, L., Lazo, J., & Parker, K. (2005). *The Pharmacological Basis of Therapeutics* (11th ed.). New York: McGraw-Hill.

Burn, J. H., & Rand, M. J. (1958). The action of sympathomimetic amines in animals treated with reserpine. *J Physiol, 144*(2), 314-336.

Buyuktuncel, E. (2010). Simultaneous Determination of Theobromine, Paraxanthine, Theophylline, and Caffeine in Urine by Reversed-Phase High-Performance Liquid Chromatography with Diode Array UV Detection. *Analytical Letters, 43*(16), 2518-2524. doi: Pii 930390242

Cabezas, C., Simao, A., Bermudez, C., Varela, M., Pena, I., Mata, S., Alonso, J. L. (2013). Conformational analysis of octopamine and synephrine in the gas phase. *J Phys Chem A, 117*(23), 4907-4915. doi: 10.1021/jp4032223

Calabuig-Hernandez, S., Garcia-Alvarez-Coque, M. C., & Ruiz-Angel, M. J. (2016). Performance of amines as silanol suppressors in reversed-phase liquid chromatography. *J Chromatogr A, 1465*, 98-106. doi: 10.1016/j.chroma.2016.08.048

Carpene, C., Galitzky, J., Fontana, E., Atgie, C., Lafontan, M., & Berlan, M. (1999). Selective activation of beta3-adrenoceptors by octopamine: comparative studies in mammalian fat cells. *Naunyn Schmiedebergs Arch Pharmacol, 359*(4), 310-321.

Caruso, J., Charles, J., Unruh, K., Giebel, R., Learmonth, L., & Potter, W. (2012). Ergogenic Effects of β-Alanine and Carnosine: Proposed Future Research to Quantify Their Efficacy. *Nutrients, 4*(7), 585-601. doi: 10.3390/nu4070585

Castells, C. B., & Castells, R. C. (1998). Peak distortion in reversed-phase liquid chromatography as a consequence of viscosity differences between sample solvent and mobile phase. *Journal of Chromatography A, 805*(1–2), 55-61. doi: http://dx.doi.org/10.1016/S0021-9673(98)00042-9

Chen, J. J., Swope, D. M., & Dashtipour, K. (2007). Comprehensive review of rasagiline, a second-generation monoamine oxidase inhibitor, for the treatment of Parkinson's disease. *Clinical Therapeutics, 29*(9), 1825-1849. doi: 10.1016/j.clinthera.2007.09.021

Chen, M. L., Zheng, M. M., & Feng, Y. Q. (2010). Preparation of organic-inorganic hybrid silica monolith with octyl and sulfonic acid groups for capillary electrochromatograhpy and application in determination of theophylline and caffeine in beverage. *Journal of Chromatography A, 1217*(21), 3547-3556. doi: 10.1016/j.chroma.2010.03.032

Chen, X., Liu, L. Y., Deng, H. W., Fang, Y. X., & Ye, Y. W. (1981). [The effects of *Citrus aurantium* and its active ingredient N-methyltyramine on the cardiovascular receptors (author's transl)]. *Yao Xue Xue Bao, 16*(4), 253-259.

Chess-Williams, R. G., Grassby, P. F., Broadley, K. J., & Sheridan, D. J. (1987). Cardiac alpha- and beta-adrenoceptor sensitivity and binding characteristics after chronic reserpine pretreatment. *Naunyn Schmiedebergs Arch Pharmacol, 336*(6), 646-651.

Chizzali, E., Nischang, I., & Ganzera, M. (2011). Separation of adrenergic amines in *Citrus aurantium* L. var. amara by capillary electrochromatography using a

novel monolithic stationary phase. *J Sep Sci, 34*(16-17), 2301-2304. doi: 10.1002/jssc.201100186
Clement, B. a., Goff, C. M., & Forbes, T. D. a. (1998). Toxic amines and alkaloids from Acacia rigidula. *Phytochemistry, 49*(5), 1377-1380.
Cohen, P. A. (2012). DMAA as a dietary supplement ingredient. *Archives of internal medicine, 172*(13), 1038-1039. doi: 10.1001/archinternmed.2012.1677
Cohen, P. a., Travis, J. C., & Venhuis, B. J. (2014). A methamphetamine analog (N,α-diethyl-phenylethylamine) identified in a mainstream dietary supplement. *Drug Testing and Analysis, 6*(7-8), 805-807. doi: 10.1002/dta.1578
Cohen, P. A., Travis, J. C., & Venhuis, B. J. (2015). A synthetic stimulant never tested in humans, 1,3-dimethylbutylamine (DMBA), is identified in multiple dietary supplements. *Drug Test Anal, 7*(1), 83-87. doi: 10.1002/dta.1735
Colker, C. M., Klalman, D. S., Torina, G. C., & Perlis, T. (1999). Effects of *Citrus aurantium* extract, caffeine and St. John's wort on body fat loss, lipid levels, and mood states in overweight healthy adults. *Curr. Ther. Res. Clin. Exp., 60*(3), 145-153.
D'Hondt, M., Gevaert, B., Wynendaele, E., & De Spiegeleer, B. (2016). Implementation of a single quad MS detector in routine QC analysis of peptide drugs. *Journal of Pharmaceutical Analysis, 6*(1), 24-31. doi: http://dx.doi.org/10.1016/j.jpha.2015.09.002
David, J. C., & Coulon, J. F. (1985). Octopamine in invertebrates and vertebrates. A review. *Prog Neurobiol, 24*(2), 141-185.
de Aragao, N. M., Veloso, M. C., Bispo, M. S., Ferreira, S. L., & de Andrade, J. B. (2005). Multivariate optimisation of the experimental conditions for determination of three methylxanthines by reversed-phase high-performance liquid chromatography. *Talanta, 67*(5), 1007-1013. doi: 10.1016/j.talanta.2005.04.066
de Oliveira, A. L., de Paula, M. N., Comar, J. F., Vilela, V. R., Peralta, R. M., & Bracht, A. (2013). Adrenergic metabolic and hemodynamic effects of octopamine in the liver. *International Journal of Molecular Sciences, 14*(11), 21858-21872. doi: 10.3390/ijms141121858
Del Coso, J., Muñoz, G., & Muñoz-Guerra, J. (2011). Prevalence of caffeine use in elite athletes following its removal from the World Anti-Doping Agency list of banned substances. *Applied Physiology, Nutrition, and Metabolism, 36*(4), 555-561. doi: 10.1139/h11-052
DeStefano, J. J., Boyes, B. E., Schuster, S. A., Miles, W. L., & Kirkland, J. J. (2014). Are sub-2 mum particles best for separating small molecules? An alternative. *J Chromatogr A, 1368*, 163-172. doi: 10.1016/j.chroma.2014.09.078
Di Lorenzo, C., Ceschi, A., Kupferschmidt, H., Lude, S., De Souza Nascimento, E., Dos Santos, A., . . . Restani, P. (2015). Adverse effects of plant food supplements and botanical preparations: a systematic review with critical evaluation of causality. *Br J Clin Pharmacol, 79*(4), 578-592. doi: 10.1111/bcp.12519
Di Lorenzo, C., Dos Santos, A., Colombo, F., Moro, E., Dell'Agli, M., & Restani, P. (2014). Development and validation of HPLC method to measure active amines in plant food supplements containing *Citrus aurantium* L. *Food Control, 46*, 136-142. doi: 10.1016/j.foodcont.2014.05.017
Dolan, J. W. (2008). Ion-Pairing : Blessing or Curse? , from http://www.chromatographyonline.com/ion-pairing-blessing-or-curse
Dorsey, J. G., Cooper, W. T., Wheeler, J. F., Barth, H. G., & Foley, J. P. (1994). Liquid chromatography: theory and methodology. *Anal Chem, 66*(12), 500R-546R.

Duffy, A. (2012). New drug banned on Bowen Basin mine. from http://www.miningaustralia.com.au/news/new-drug-banned-on-bowen-basin-mine

Dwyer, J. T., Allison, D. B., & Coates, P. M. (2005). Dietary supplements in weight reduction. *Journal of the American Dietetic Association, 105*(5), S80-S86. doi: 10.1016/j.jada.2005.02.028

Eichner, A., & Tygart, T. (2016). Adulterated dietary supplements threaten the health and sporting career of up-and-coming young athletes. *Drug Test Anal, 8*(3-4), 304-306. doi: 10.1002/dta.1899

Eliason, M. J., Eichner, A., Cancio, A., Bestervelt, L., Adams, B. D., & Deuster, P. A. (2012). Case Reports: Death of Active Duty Soldiers Following Ingestion of Dietary Supplements Containing 1,3-Dimethylamylamine (DMAA). *Mil Med, 177*(12), 1455-1459. doi: 10.7205/Milmed-D-12-00265

Eudy, A. E., Gordon, L. L., Hockaday, B. C., Lee, D. A., Lee, V., Luu, D., Ambrose, P. J. (2013). Efficacy and safety of ingredients found in preworkout supplements. *Am J Health Syst Pharm, 70*(7), 577-588. doi: 10.2146/ajhp120118

FDA. (2013). Guidance for Industry: Bioanalytical Method Validation

Farah, A. (2012). Coffee Constituents *Coffee* (pp. 21-58): Wiley-Blackwell.

Ferrer-Lorente, R., Cabot, C., Fernández-López, J.-A., & Alemany, M. (2005). Combined effects of oleoyl-estrone and a β3-adrenergic agonist (CL316,243) on lipid stores of diet-induced overweight male Wistar rats. *Life Sci, 77*(16), 2051-2058. doi: http://dx.doi.org/10.1016/j.lfs.2005.04.008

Ford, M. J., Deibel, M. A., Tomkins, B. A., & Van Berkel, G. J. (2005). Quantitative thin-layer chromatography/mass spectrometry analysis of caffeine using a surface sampling probe electrospray ionization tandem mass spectrometry system. *Analytical Chemistry, 77*(14), 4385-4389. doi: 10.1021/ac050488s

Fredholm, B. B. (2011). Methylxanthines. Heidelberg: Springer-Verlag.

Fregly, M. J., Kelleher, D. L., & Williams, C. M. (1979). Adrenergic activity of ortho-, meta-, and para-octopamine. *Pharmacology, 18*(4), 180-187.

Fugh-Berman, A., & Myers, A. (2004). *Citrus aurantium*, an ingredient of dietary supplements marketed for weight loss: Current status of clinical and basic research. *Exp Biol Med (Maywood), 229*(8), 698-704.

Ganzera, M., Lanser, C., & Stuppner, H. (2005). Simultaneous determination of Ephedra sinica and *Citrus aurantium* var. amara alkaloids by ion-pair chromatography. *Talanta, 66*(4), 889-894. doi: 10.1016/j.talanta.2004.12.045

Gatti, R., & Lotti, C. (2011). Development and validation of a pre-column reversed phase liquid chromatographic method with fluorescence detection for the determination of primary phenethylamines in dietary supplements and phytoextracts. *J Chromatogr A, 1218*(28), 4468-4473. doi: 10.1016/j.chroma.2011.05.044

Gatti, R., Lotti, C., Morigi, R., & Andreani, A. (2012). Determination of octopamine and tyramine traces in dietary supplements and phytoextracts by high performance liquid chromatography after derivatization with 2,5-dimethyl-1H-pyrrole-3,4-dicarbaldehyde. *J Chromatogr A, 1220*, 92-100. doi: 10.1016/j.chroma.2011.11.060

Gay, M. L., Niemann, R. A., & Musser, S. M. (2006). An isotopically labeled internal standard liquid chromatography-tandem mass spectrometry method for determination of ephedrine alkaloids and synephrine in dietary supplements. *J Agric Food Chem, 54*(2), 285-291. doi: 10.1021/jf051735w

Gibbons, S. (2012). Legal Highs novel and emerging psychoactive drugs: a chemical overview for the toxicologist. 15-24. doi: 10.3109/15563650.2011.645952

Gibson, C. (2014). In Good Health: 2014 Complementary Medicines Industry Survey. ACT, Australia: Complementary Medicines Australia.

Gibson, C. M., & Fowler, P. W. (2014). Aromaticity of caffeine, xanthine and the dimethyl xanthines. *Tetrahedron Letters, 55*(13), 2078-2081. doi: http://dx.doi.org/10.1016/j.tetlet.2014.02.027

Gill, A. (2015). *Poisons Standard July 2015*. Australia. Standard For The Uniform Scheduling Of Medicines And Poisons (2016).

Grandy, D. K. (2007). Trace amine-associated receptor 1 - Family archetype or iconoclast? *Pharmacology & Therapeutics, 116*(3), 355-390. doi: 10.1016/j.pharmthera.2007.06.007

Greenway, F., de Jonge-Levitan, L., Martin, C., Roberts, A., Grundy, I., & Parker, C. (2006). Dietary herbal supplements with phenylephrine for weight loss. *J Med Food, 9*(4), 572-578. doi: DOI 10.1089/jmf.2006.9.572

Gurley, B. J., Gardner, S. F., Hubbard, M. A., Williams, D. K., Gentry, W. B., Carrier, J., Shah, A. (2004). In vivo assessment of botanical supplementation on human cytochrome P450 phenotypes: *Citrus aurantium*, Echinacea purpurea, milk thistle, and saw palmetto. *Clin Pharmacol Ther, 76*(5), 428-440. doi: 10.1016/j.clpt.2004.07.007

Gurley, B. J., Steelman, S. C., & Thomas, S. L. (2015). Multi-ingredient, caffeine-containing dietary supplements: history, safety, and efficacy. *Clin Ther, 37*(2), 275-301. doi: 10.1016/j.clinthera.2014.08.012

Haaz, S., Fontaine, K. R., Cutter, G., Limdi, N., Perumean-Chaney, S., & Allison, D. B. (2006). *Citrus aurantium* and synephrine alkaloids in the treatment of overweight and obesity: an update. *Obes Rev, 7*(1), 79-88. doi: 10.1111/j.1467-789X.2006.00195.x

Hadad, G. M., Salam, R. A. A., Soliman, R. M., & Mesbah, M. K. (2012). Rapid and simultaneous determination of antioxidant markers and caffeine in commercial teas and dietary supplements by HPLC-DAD. *Talanta, 101*, 38-44. doi: 10.1016/j.talanta.2012.08.025

Hakil, M., Denis, S., Viniegra-González, G., & Augur, C. (1998). Degradation and Product Analysis of Caffeine and Related Dimethylxanthines by Filamentous Fungi. *Enzyme and Microbial Technology, 22*(5), 355-359. doi: http://dx.doi.org/10.1016/S0141-0229(97)00205-6

Haller, C. A. (2000). Adverse Cardiovascular And Central Nervous System Events Associated With Dietary Supplements Containing Ephedra Alkaloids. *N Engl J Med, 343*(25), 1833-1838.

Haller, C. A., Benowitz, N. L., & Jacob, P. (2005). Hemodynamic effects of ephedra-free weight-loss supplements in humans. *American Journal of Medicine, 118*(9), 998-1003. doi: 10.1016/j.amjmed.2005.02.034

Haller, C. A., Duan, M. J., Benowitz, N. L., & Jacob, P. (2004). Concentrations of ephedra alkaloids and caffeine in commercial dietary supplements. *J Anal Toxicol, 28*(3), 145-151.

Haller, C. A., Jacob, P., & Benowitz, N. L. (2002). Pharmacology of ephedra alkaloids and caffeine after single-dose dietary supplement use. *Clinical Pharmacology & Therapeutics, 71*(6), 421-432. doi: 10.1067/mcp.2002.124523

Haller, C. A., Jacob, P., & Benowitz, N. L. (2004). Enhanced stimulant and metabolic effects of combined ephedrine and caffeine. *Clinical Pharmacology & Therapeutics, 75*(4), 259-273. doi: 10.1016/j.clpt.2003.11.375

Harris, D. C. (2016). *Quantitative Chemical Analysis* (9th ed.). New York, NY 10010: W. H. Freeman and Company.

Hasegawa, T., Takahashi, K., Saijo, M., Ishii, T., & Nagata, T. (2009). Rapid Determination of Theophylline, Theobromine and Caffeine in Dietary Supplements Containing Guarana by Ultra-Performance Liquid Chromatography. *Journal of the Food Hygienic Society of Japan, 50*(6), 304-310.

Hawke, T. J., Allen, D. G., & Lindinger, M. I. (2000). Paraxanthine, a caffeine metabolite, dose dependently increases [Ca(2+)](i) in skeletal muscle. *J Appl Physiol (1985), 89*(6), 2312-2317.

Heaton, J., Gray, N., Cowan, D. A., Plumb, R. S., Legido-Quigley, C., & Smith, N. W. (2012). Comparison of reversed-phase and hydrophilic interaction liquid chromatography for the separation of ephedrines. *Journal of Chromatography A, 1228*, 329-337. doi: http://dx.doi.org/10.1016/j.chroma.2011.09.026

Heftmann, E. (2004). *Chromatography: Fundamentals and applications of chromatography and related methods* (Vol. 69A). The Netherlands: Elsevier

Hibino, T., Yuzurihara, M., Kase, Y., & Takeda, A. (2009). synephrine, a component of Evodiae Fructus, constricts isolated rat aorta via adrenergic and serotonergic receptors. *J Pharmacol Sci, 111*(1), 73-81.

Hodgson, A. B., Randell, R. K., & Jeukendrup, A. E. (2013). The Metabolic and Performance Effects of Caffeine Compared to Coffee during Endurance Exercise. *Plos One, 8*(4). doi: ARTN e5956110.1371/journal.pone.0059561

Hoffman, J. R., Kang, J., Ratamess, N. A., Hoffman, M. W., Tranchina, C. P., & Faigenbaum, A. D. (2009). Examination of a pre-exercise, high energy supplement on exercise performance. *J Int Soc Sports Nutr, 6*, 2-2. doi: 10.1186/1550-2783-6-2

Holmes, R. O., Jr., & Tavee, J. (2008). Vasospasm and stroke attributable to ephedra-free xenadrine: case report. *Mil Med, 173*(7), 708-710.

Hung, S. K., Hillier, S., & Ernst, E. (2011). Case reports of adverse effects of herbal medicinal products (HMPs): a quality assessment. *Phytomedicine, 18*(5), 335-343. doi: 10.1016/j.phymed.2010.07.007

Hurlbut, J. A., Carr, J. R., Singleton, E. R., Faul, K. C., Madson, M. R., Storey, J. M., & Thomas, T. L. (1998). Solid-phase extraction cleanup and liquid chromatography with ultraviolet detection of ephedrine alkaloids in herbal products. *J AOAC Int, 81*(6), 1121-1127.

Hwa, J., & Perez, D. M. (1996). The unique nature of the serine interactions for alpha 1-adrenergic receptor agonist binding and activation. *J Biol Chem, 271*(11), 6322-6327.

Ibrahim, K. E., Couch, M. W., Williams, C. M., Fregly, M. J., & Midgely, J. M. (1985). *m*-octopamine Normal occurence with *p*-octopamine in mammalian sympathetic Nerves.pdf (pp. 1862-1867).

ICH Expert Working Group. (1994). *Validation of Analytical Procedures: Text and Methodology*

Jandera, P. (2011). Stationary and mobile phases in hydrophilic interaction chromatography: a review. *Anal Chim Acta, 692*(1-2), 1-25. doi: 10.1016/j.aca.2011.02.047

Johansson, I. M., Gronrydberg, M. B., & Schmekel, B. (1993). Determination of Theophylline in Plasma Using Different Capillary Electrophoretic Systems. *Journal of Chromatography A, 652*(2), 487-493. doi: Doi 10.1016/0021-9673(93)83270-3

Jordan, R., Midgley, J. M., Thonoor, C. M., & Williams, C. M. (1987). Beta-Adrenergic Activities of Octopamine and Synephrine Stereoisomers on Guinea-Pig Atria and Trachea. *Journal of Pharmacy and Pharmacology, 39*(9), 752-754.

Jovanović, M., Rakić, T., Ivanović, D., & Jančić–Stojanović, B. (2015). Optimization of the Separation of Ephedrine, Pseudoephedrine, Phenylephrine, and Synephrine by Hydrophilic Interaction Liquid Chromatography Employing Experimental Design Methodology. *Instrumentation Science & Technology, 43*(2), 156-169. doi: 10.1080/10739149.2014.910670

Kanazawa, H., Atsumi, R., Matsushima, Y., & Kizu, J. (2000). Determination of theophylline and its metabolites in biological samples by liquid chromatography-mass spectrometry. *Journal of Chromatography A, 870*(1-2), 87-96. doi: Doi 10.1016/S0021-9673(99)00891-2

Karatapanis, A. E., Fiamegos, Y. C., & Stalikas, C. D. (2011). A revisit to the retention mechanism of hydrophilic interaction liquid chromatography using model organic compounds. *J Chromatogr A, 1218*(20), 2871-2879. doi: 10.1016/j.chroma.2011.02.069

Kim, H. J., Kwak, B. M., Ahn, J. H., & Park, J. S. (2014). Simultaneous determination of synephrine and N-Methyltyramine in orange fruit and juice from korean market by UPLC-FLD. *Korean Journal of Food Science and Technology, 46*(3), 276-282. doi: 10.9721/KJFST.2014.46.3.276

Kleinau, G., Pratzka, J., Nurnberg, D., Gruters, A., Fuhrer-Sakel, D., Krude, H., . . . Biebermann, H. (2011). Differential modulation of Beta-adrenergic receptor signaling by trace amine-associated receptor 1 agonists. *PLoS One, 6*(10), e27073. doi: 10.1371/journal.pone.0027073

Kockler, D. R., McCarthy, M. W., & Lawson, C. L. (2001). Seizure activity and unresponsiveness after hydroxycut ingestion. *Pharmacotherapy, 21*(5), 647-651. doi: DOI 10.1592/phco.21.6.647.34542

Kole, J., & Barnhill, A. (2013). Caffeine Content Labeling: A Missed Opportunity for Promoting Personal and Public Health. *Journal of caffeine research, 3*(3), 108-113. doi: 10.1089/jcr.2013.0017

Kusu, F., Matsumoto, K., Arai, K., & Takamura, K. (1996). Determination of synephrine enantiomers in food and conjugated synephrine in urine by high-performance liquid chromatography with electrochemical detection (vol 235, pg 191, 1996). *Anal Biochem, 236*(2), 378-378.

Kusu, F., Matsumoto, K., & Takamura, K. (1995). Direct Separation and Determination of Synephrine Enantiomers by High-Performance Liquid-Chromatography with Electrochemical Detection. *Chemical & Pharmaceutical Bulletin, 43*(7), 1158-1161.

Lake, C. R., Rosenberg, D., & Quirk, R. (1990). Phenylpropanolamine and Caffeine Use among Diet Center Clients. *Int J Obes (Lond), 14*(7), 575-582.

Leveritt, M. D., Quinlivan, A., Irwin, C., Grant, G. D., Dukie, S., & Desbrow, B. (2012). Red Bull Energy Drink and Anhydrous Caffeine have Similar Benefits for Cycling Time Trial Performance. *Med Sci Sports Exerc, 44*, 722-722.

Liles, J. T., Dabisch, P. A., Hude, K. E., Pradhan, L., Varner, K. J., Porter, J. R., . . . Kadowitz, P. J. (2006). Pressor responses to ephedrine are mediated by a direct mechanism in the rat. *J Pharmacol Exp Ther, 316*(1), 95-105. doi: 10.1124/jpet.105.090035

Liu, Y., Xu, F., Zhang, Z., Yang, C., Song, R., & Tian, Y. (2009). Analysis of synephrine in da-cheng-qi decoction by HPLC employing precolumn

derivatization with 9-fluorenylmethyl chloroformate. *J Chromatogr Sci, 47*(10), 925-930.
Liu, Y. M., Cao, J. T., Zheng, Y. L., & Chen, Y. H. (2008). Sensitive determination of norepinephrine, synephrine, and isoproterenol by capillary electrophoresis with indirect electrochemiluminescence detection. *J Sep Sci, 31*(13), 2463-2469. doi: 10.1002/jssc.200800034
Luo, G. M., Tan, X. H., Xu, L. F., Yang, Y. Q., & Yang, S. L. (2008). [Study on fingerprints of *Citrus aurantium* from different places by capillary electrophoresis]. *Zhongguo Zhong Yao Za Zhi, 33*(20), 2362-2364.
Ma, G., Bavadekar, S. A., Schaneberg, B. T., Khan, I. A., & Feller, D. R. (2010). Effects of Synephrine and β-Phenethylamine on Human α-Adrenoceptor Subtypes. *Planta Med, 76*(10), 981-986. doi: 10.1055/s-0029-1240884
Marchei, E., Pellegrini, M., Pacifici, R., Palmi, I., & Pichini, S. (2005). Development and validation of a high-performance liquid chromatography-mass spectrometry assay for methylxanthines and taurine in dietary supplements. *J Pharm Biomed Anal, 37*(3), 499-507. doi: 10.1016/j.jpba.2004.11.013
Marchei, E., Pichini, S., Pacifici, R., Pellegrini, M., & Zuccaro, P. (2006). A rapid and simple procedure for the determination of synephrine in dietary supplements by gas chromatography-mass spectrometry. *J Pharm Biomed Anal, 41*(4), 1468-1472. doi: 10.1016/j.jpba.2006.03.035
Martin, A. J. P., & Synge, R. L. M. (1941). A new form of chromatogram employing two liquid phases: A theory of chromatography. 2. Application to the micro-determination of the higher monoamino-acids in proteins. *Biochemical Journal, 35*(12), 1358-1368.
Martinez-Lopez, S., Sarria, B., Gomez-Juaristi, M., Goya, L., Mateos, R., & Bravo-Clemente, L. (2014). Theobromine, caffeine, and theophylline metabolites in human plasma and urine after consumption of soluble cocoa products with different methylxanthine contents. *Food Research International, 63*, 446-455. doi: 10.1016/j.foodres.2014.03.009
Mattoli, L., Cangi, F., Maidecchi, A., Ghiara, C., Tubaro, M., & Traldi, P. (2005). A rapid liquid chromatography electrospray ionization mass spectrometry(n) method for evaluation of synephrine in *Citrus aurantium* L. samples. *J Agric Food Chem, 53*(26), 9860-9866. doi: 10.1021/jf051270+
McCalley, D. V., & Neue, U. D. (2008). Estimation of the extent of the water-rich layer associated with the silica surface in hydrophilic interaction chromatography. *J Chromatogr A, 1192*(2), 225-229. doi: 10.1016/j.chroma.2008.03.049
Meinhart, A. D., Bizzotto, C. S., Ballus, C. A., Prado, M. A., Bruns, R. E., Teixeira, J., & Godoy, H. T. (2010). Optimisation of a CE method for caffeine analysis in decaffeinated coffee. *Food Chem, 120*(4), 1155-1161. doi: 10.1016/j.foodchem.2009.11.048
Mikes, O., Vespalex, R. (2011). Mobile Phases for Liquid-Liquid Chromatography. In K. Macek, Deyl, Z., Janak, J. (Ed.), *Liquid Column Chromatography- A Survey of Modern Techniques and Applications*

(Vol. 3, pp. 234-245). New York, NY: Elsevier Scientific
Mitenko, P. A., & Ogilvie, R. I. (1973). Rational Intravenous Doses of Theophylline. *New England Journal of Medicine, 289*(12), 600-603. doi: Doi 10.1056/Nejm197309202891202
Moawad, F. J., Hartzell, J. D., Biega, T. J., & Lettieri, C. J. (2006). Transient blindness due to posterior reversible encephalopathy syndrome following ephedra

overdose. *Southern Medical Journal, 99*(5), 511-514. doi: DOI 10.1097/01.smj.0000215739.90211.3b

Monteiro, J. P., Alves, M. G., Oliveira, P. F., & Silva, B. M. (2016). Structure-Bioactivity Relationships of Methylxanthines: Trying to Make Sense of All the Promises and the Drawbacks. *Molecules, 21*(8). doi: 10.3390/molecules21080974

Musgrave, I. F., Farrington, R. L., Hoban, C., & Byard, R. W. (2016). Caffeine toxicity in forensic practice: possible effects and under-appreciated sources. *Forensic Science Medicine and Pathology, 12*(3), 299-303. doi: 10.1007/s12024-016-9786-9

Myers, D. P., Hetrick, E. M., Liang, Z., Hadden, C. E., Bandy, S., Kemp, C. A., . . . Baertschi, S. W. (2013). On-column nitrosation of amines observed in liquid chromatography impurity separations employing ammonium hydroxide and acetonitrile as mobile phase. *Journal of Chromatography A, 1319*, 57-64. doi: http://dx.doi.org/10.1016/j.chroma.2013.10.021

Naline, E., Flouvat, B., Advenier, C., & Pays, M. (1987). Determination of Theophylline and Its Metabolites in Plasma and Urine by Reversed-Phase Liquid-Chromatography Using an Amine Modifier. *Journal of Chromatography-Biomedical Applications, 419*, 177-189. doi: Doi 10.1016/0378-4347(87)80276-1

National Drugs and Poisons Schedule Committee. (2003). *Proposed Changes/Additions To Parts 1 To 3 And Part 5 Of The Standard For The Uniform Scheduling of Drugs And Poisons*.

Neue, U. D., Wheat, T. E., Mazzeo, J. R., Mazza, C. B., Cavanaugh, J. Y., Xia, F., & Diehl, D. M. (2004). Differences in preparative loadability between the charged and uncharged forms of ionizable compounds. *Journal of Chromatography A, 1030*(1–2), 123-134. doi: http://dx.doi.org/10.1016/j.chroma.2003.11.048

Niemann, R. A., & Gay, M. L. (2003). Determination of ephedrine alkaloids and synephrine in dietary supplements by column-switching cation exchange high-performance liquid chromatography with scanning-wavelength ultraviolet and fluorescence detection. *J Agric Food Chem, 51*(19), 5630-5638. doi: 10.1021/jf0302052

Nirogi, R. V. S., Kandikere, V. N., Shukla, M., Mudigonda, K., & Ajjala, D. R. (2007). A simple and rapid HPLC/UV method for the simultaneous quantification of theophylline and etofylline in human plasma. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences, 848*(2), 271-276. doi: 10.1016/j.jchromb.2006.10.035

Nout, M. J. R. (1994). Fermented foods and food safety. *Food Research International, 27*(3), 291-298. doi: http://dx.doi.org/10.1016/0963-9969(94)90097-3

Oberlender, R., & Nichols, D. E. (1991). Structural variation and (+)-amphetamine-like discriminative stimulus properties. *Pharmacology Biochemistry and Behavior, 38*, 581-586. doi: 10.1016/0091-3057(91)90017-V

Onakpoya, I., Davies, L., & Ernst, E. (2011). Efficacy of herbal supplements containing *Citrus aurantium* and synephrine alkaloids for the management of overweight and obesity: A systematic review. *Focus on Alternative and Complementary Therapies, 16*(4), 254-260. doi: 10.1111/j.2042-7166.2011.01115.x

Orru, M., Guitart, X., Karcz-Kubicha, M., Solinas, M., Justinova, Z., Barodia, S. K., . . . Ferre, S. (2013). Psychostimulant pharmacological profile of paraxanthine, the main metabolite of caffeine in humans. *Neuropharmacology, 67*, 476-484. doi: 10.1016/j.neuropharm.2012.11.029

Palamar, J. (2011). How ephedrine escaped regulation in the United States: a historical review of misuse and associated policy. *Health Policy, 99*(1), 1-9. doi: 10.1016/j.healthpol.2010.07.007

Pang, V., & Ko, C. (2006). *The Maze of Caffeine & You*. Retrieved from http://www.odec.ca/projects/2006/pang6v2/pages/body.htmL

Pawar, R. S., Grundel, E., Fardin-Kia, A. R., & Rader, J. I. (2014). Determination of selected biogenic amines in Acacia rigidula plant materials and dietary supplements using LC-MS/MS methods. *J Pharm Biomed Anal, 88*, 457-466. doi: 10.1016/j.jpba.2013.09.012

Pellati, F., & Benvenuti, S. (2007). Chromatographic and electrophoretic methods for the analysis of phenethylamine [corrected] alkaloids in *Citrus aurantium*. *J Chromatogr A, 1161*(1-2), 71-88. doi: 10.1016/j.chroma.2007.05.097

Pellati, F., Benvenuti, S., & Melegari, M. (2005). Enantioselective LC analysis of synephrine in natural products on a protein-based chiral stationary phase. *J Pharm Biomed Anal, 37*(5), 839-849. doi: 10.1016/j.jpba.2004.09.008

Pellati, F., Benvenuti, S., Melegari, M., & Firenzuoli, F. (2002). Determination of adrenergic agonists from extracts and herbal products of *Citrus aurantium* L. var. amara by LC. *J Pharm Biomed Anal, 29*(6), 1113-1119.

Penzak, S. R., Jann, M. W., Cold, J. A., Hon, Y. Y., Desai, H. D., & Gurley, B. J. (2001). Seville (sour) orange juice: synephrine content and cardiovascular effects in normotensive adults. *J Clin Pharmacol, 41*(10), 1059-1063.

Poole, C. F. (2003). New trends in solid-phase extraction. *TrAC Trends in Analytical Chemistry, 22*(6), 362-373. doi: http://dx.doi.org/10.1016/S0165-9936(03)00605-8

Putzbach, K., Rimmer, C. A., Sharpless, K. E., & Sander, L. C. (2007). Determination of Bitter Orange alkaloids in dietary supplements standard reference materials by liquid chromatography with ultraviolet absorbance and fluorescence detection. *J Chromatogr A, 1156*(1-2), 304-311. doi: 10.1016/j.chroma.2006.12.027

Qi, B. L., Liu, P., Wang, Q. Y., Cai, W. J., Yuan, B. F., & Feng, Y. Q. (2014). Derivatization for liquid chromatography-mass spectrometry. *Trac-Trends in Analytical Chemistry, 59*, 121-132. doi: 10.1016/j.trac.2014.03.013

Randon, J., Huguet, S., Demesmay, C., & Berthod, A. (2010). Zirconia based monoliths used in hydrophilic-interaction chromatography for original selectivity of xanthines. *Journal of Chromatography A, 1217*(9), 1496-1500. doi: http://dx.doi.org/10.1016/j.chroma.2009.12.077

Rhidian, R. (2011). Running a risk? Sport supplement toxicity with ephedrine in an amateur marathon runner, with subsequent rhabdomyolysis. *BMJ Case Rep, 2011*. doi: 10.1136/bcr.11.2011.5093

Riksen, N. P., Rongen, G. A., & Smits, P. (2009). Acute and long-term cardiovascular effects of coffee: Implications for coronary heart disease. *Pharmacology & Therapeutics, 121*(2), 185-191. doi: 10.1016/j.pharmthera.2008.10.006

Roeder, T. (1999). Octopamine in invertebrates. *Prog Neurobiol, 59*(5), 533-561.

Roman, M. C., Betz, J. M., & Hildreth, J. (2007). Determination of synephrine in bitter orange raw materials, extracts, and dietary supplements by liquid chromatography with ultraviolet detection: single-laboratory validation. *J AOAC Int, 90*(1), 68-81.

Roman, M. C., Hildreth, J., & Bannister, S. (2013). Determination of Catechins and Caffeine in Camillia sinensis Raw Materials, Extracts, and Dietary Supplements by HPLC-UV: Single-Laboratory Validation. *J AOAC Int, 96*(5), 933-941. doi: 10.5740/jaoacint.10-488

Rossato, L. G., de Pinho, P. G., Silva, R., Carmo, H., Carvalho, F., Bastos, M. D., . . . Remiao, F. (2010). Development and validation of a GC/IT-MS method for simultaneous quantitation of para and meta-synephrine in biological samples. *J Pharm Biomed Anal, 52*(5), 721-726. doi: 10.1016/j.jpba.2010.02.022

Sakai, Y., Kotani, A., Umemura, T., Mori, Y., Kusu, F., Yamamoto, K., & Hakamata, H. (2016). Electrochemical Determination of Synephrine by Hydrophilic Interaction Liquid Chromatography Using a Zwitterionic Monolith Column. *Electroanalysis, 28*(9), 1947-1950. doi: 10.1002/elan.201600150

Sander, L. C., Putzbach, K., Nelson, B. C., Rimmer, C. A., Bedner, M., Thomas, J. B., . . . Betz, J. M. (2008). Certification of standard reference materials containing bitter orange. *Anal Bioanal Chem, 391*(6), 2023-2034. doi: 10.1007/s00216-008-2074-0

Santana, J., Sharpless, K. E., & Nelson, B. C. (2008). Determination of para-synephrine and meta-synephrine positional isomers in bitter orange-containing dietary supplements by LC/UV and LC/MS/MS. *Food Chem, 109*(3), 675-682. doi: 10.1016/j.foodchem.2007.12.076

Santos, V. G. F., Santos, V. R. F., Felippe, L. J. C., Almeida, J. W., Bertuzzi, R., Kiss, M. A. P. D. M., & Lima-Silva, A. E. (2014). Caffeine Reduces Reaction Time and Improves Performance in Simulated-Contest of Taekwondo. *Nutrients, 6*(2), 637-649. doi: 10.3390/nu6020637

Schaneberg, B. T., & Khan, I. A. (2004). Quantitative and qualitative HPLC analysis of thermogenic weight loss products. *Pharmazie, 59*(11), 819-823.

Schilling, B. K., Hammond, K. G., Bloomer, R. J., Presley, C. S., & Yates, C. R. (2013). Physiological and pharmacokinetic effects of oral 1,3-dimethylamylamine administration in men. *BMC Pharmacol Toxicol, 14*, 52. doi: 10.1186/2050-6511-14-52

Scott, R. P. W. (1996). *Chromatographic Detectors: Design, Function and Operation*. New York, NY: CRC Press.

Shawky, E. (2014). Determination of synephrine and octopamine in bitter orange peel by HPTLC with densitometry. *J Chromatogr Sci, 52*(8), 899-904. doi: 10.1093/chromsci/bmt113

Shrivas, K., & Wu, H. F. (2007). Rapid determination of caffeine in one drop of beverages and foods using drop-to-drop solvent microextraction with gas chromatography/mass spectrometry. *J Chromatogr A, 1170*(1-2), 9-14. doi: 10.1016/j.chroma.2007.09.020

Simmons, A. (2013). Fitness buffs turning to 'speed-like' supplements. from http://www.abc.net.au/news/2012-01-17/pre-workout-supplements/3774122

Slezak, T., Francis, P. S., Anastos, N., & Barnett, N. W. (2007). Determination of synephrine in weight-loss products using high performance liquid chromatography with acidic potassium permanganate chemiluminescence detection. *Anal Chim Acta, 593*(1), 98-102. doi: 10.1016/j.aca.2007.04.038

Smith, P. F., Smith, A., Miners, J., McNeil, J., Proudfoot, A. (2000). The Safety Aspects Of Dietary Caffeine (1 ed.). Canberra, Australia: Australia New Zealand Food Authority.

Smith, T. B., Staub, B. a., Natarajan, G. M., Lasorda, D. M., & Poornima, I. G. (2014). Acute Myocardial Infarction Associated with Dietary Supplements. *41*(1), 70-73.

Stadheim, H. K., Kvamme, B., Olsen, R., Drevon, C. A., Ivy, J. L., & Jensen, J. (2013). Caffeine Increases Performance in Cross-Country Double-Poling Time Trial

Exercise. *Med Sci Sports Exerc, 45*(11), 2175-2183. doi: 10.1249/MSS.0b013e3182967948
Stahl, C. E., Borlongan, C. V., Szerlip, H., & Szerlip, M. (2006). No pain, no gain - exercise-induced rhabdomyolysis associated with the performance enhancer herbal supplement ephedra. *Medical Science Monitor, 12*(9), Cs81-Cs84.
Stohs, S. J., Preuss, H. G., Keith, S. C., Keith, P. L., Miller, H., & Kaats, G. R. (2011). Effects of *p*-Synephrine alone and in Combination with Selected Bioflavonoids on Resting Metabolism, Blood Pressure, Heart Rate and Self-Reported Mood Changes. *Int J Med Sci, 8*(4), 295-301.
Stohs, S. J., Preuss, H. G., & Shara, M. (2011). The safety of *Citrus aurantium* (bitter orange) and its primary protoalkaloid *p*-synephrine. *Phytother Res, 25*(10), 1421-1428. doi: 10.1002/ptr.3490
Stohs, S. J., Preuss, H. G., & Shara, M. (2012). A review of the human clinical studies involving *Citrus aurantium* (bitter orange) extract and its primary protoalkaloid *p*-synephrine. *Int J Med Sci, 9*(7), 527-538. doi: 10.7150/ijms.4446
Swartz, M. E. (2005). Ultra performance liquid chromatography (UPLC): An introduction. *Lc Gc North America*, 8-14.
Talik, P., Krzek, J., & Ekiert, R. J. (2012). Analytical Techniques Used for Determination of Methylxanthines and their Analogues—Recent Advances. *Separation & Purification Reviews, 41*(1), 1-61. doi: 10.1080/15422119.2011.569047
Tang, F., Tao, L., Luo, X., Ding, L., Guo, M., Nie, L., & Yao, S. (2006). Determination of octopamine, synephrine and tyramine in Citrus herbs by ionic liquid improved 'green' chromatography. *J Chromatogr A, 1125*(2), 182-188. doi: 10.1016/j.chroma.2006.05.049
TGA. (2016a). Alerts. from https://www.tga.gov.au/alerts
TGA. (2016b). Reporting adverse events. from https://www.tga.gov.au/reporting-adverse-events
The Federal Insitute for Risk Assessment. (2013). *Health Assessment of sports and weight loss products containing synephrine and caffeine*. (004/2013).
Therapeutic Goods Administration. (2013). Listed Complementary Medicines. Retrieved 21/5/15, 2015, from https://www.tga.gov.au/listed-complementary-medicines
Thevis, M., Koch, A., Sigmund, G., Thomas, A., & Schanzer, W. (2012). Analysis of octopamine in human doping control samples. *Biomed Chromatogr, 26*(5), 610-615. doi: 10.1002/bmc.1705
Thomas, J. B., Yen, J. H., Schantz, M. M., Porter, B. J., & Sharpless, K. E. (2004). Determination of caffeine, theobromine, and theophylline in standard reference material 2384, baking chocolate, using reversed-phase liquid chromatography. *J Agric Food Chem, 52*(11), 3259-3263. doi: 10.1021/jf030817m
U.S. Food and Drug Administration (FDA). (1994). *Dietary Supplement Health and Education Act of 1994*.
Upadhyay, M. (2014). *Food Standards Agency*. Retrieved from www.food.gov.uk/sites/default/files/multimedia/pdfs/letters/acacia-rigidula-interested-parties-letter.pdf
Uysal, U. D., Aturki, Z., Raggi, M. A., & Fanali, S. (2009). Separation of catechins and methylxanthines in tea samples by capillary electrochromatography. *J Sep Sci, 32*(7), 1002-1010. doi: 10.1002/jssc.200800634
Vaclavik, L., Krynitsky, A. J., & Rader, J. I. (2014). Mass spectrometric analysis of pharmaceutical adulterants in products labeled as botanical dietary supplements

or herbal remedies: a review. *Anal Bioanal Chem, 406*, 6767-6790. doi: 10.1007/s00216-014-8159-z

Venhuis, B., Keizers, P., van Riel, A., & de Kaste, D. (2014). A cocktail of synthetic stimulants found in a dietary supplement associated with serious adverse events. *Drug Test Anal, 6*(6), 578-581. doi: 10.1002/dta.1664

Verpeut, J. L., Walters, A. L., & Bello, N. T. (2013). *Citrus aurantium* and Rhodiola rosea in combination reduce visceral white adipose tissue and increase hypothalamic norepinephrine in a rat model of diet-induced obesity. *Nutr Res, 33*(6), 503-512. doi: 10.1016/j.nutres.2013.04.001

Viana, C., Zemolin, G. M., Lima, F. O., de Carvalho, L. M., Bottoli, C. B., & Limberger, R. P. (2013). High-performance liquid chromatographic analysis of biogenic amines in pharmaceutical products containing *Citrus aurantium*. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess, 30*(4), 634-642. doi: 10.1080/19440049.2013.775606

Viana, C., Zemolin, G. M., Müller, L. S., Molin, T. R. D., Seiffert, H., & de Carvalho, L. M. (2015). Liquid chromatographic determination of caffeine and adrenergic stimulants in food supplements sold in brazilian e-commerce for weight loss and physical fitness. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 33*(1), 1-9. doi: 10.1080/19440049.2015.1112040

Wahab, M. F., Dasgupta, P. K., Kadjo, A. F., & Armstrong, D. W. (2016). Sampling frequency, response times and embedded signal filtration in fast, high efficiency liquid chromatography: A tutorial. *Anal Chim Acta, 907*, 31-44. doi: http://dx.doi.org/10.1016/j.aca.2015.11.043

Wainscott, D. B., Little, S. P., Yin, T., Tu, Y., Rocco, V. P., He, J. X., & Nelson, D. L. (2007). Pharmacologic characterization of the cloned human trace amine-associated receptor1 (TAAR1) and evidence for species differences with the rat TAAR1. *J Pharmacol Exp Ther, 320*(1), 475-485. doi: 10.1124/jpet.106.112532

Wang, F., Liu, X.-K., Lai, S., Fang, J., & Semin, D. (2011). Chromatographic studies of unusual on-column degradations of aniline compounds on Xbridge Shield RP18 column in high pH aqueous mobile phase. *Journal of Chromatography A, 1218*(22), 3502-3510. doi: http://dx.doi.org/10.1016/j.chroma.2011.04.004

Wang, X. L., & Zhao, L. H. (2015). Simultaneous determination of ten components in Xianglian Huazhi Pill by HPLC. *Chinese Traditional and Herbal Drugs, 46*(21), 3190-3193. doi: 10.7501/j.issn.0253-2670.2015.21.011

Waters. (2016). BEH (Ethylene Bridged Hybrid) Technology.from http://www.waters.com/waters/en_US/BEH-(Ethylene-Bridged-Hybrid)-Technology/nav.htm?cid=134618172&locale=101

Watson, D. G., Midgley, J. M., Chen, R. N., Huang, W., Bain, G. M., Mcdonald, N. M., Mcghee, C. N. J. (1990). Analysis of Biogenic-Amines and Their Metabolites in Biological Tissues and Fluids by Gas Chromatography-Negative Ion Chemical Ionization Mass-Spectrometry (Gc-Nicims). *J Pharm Biomed Anal, 8*(8-12), 899-904. doi: Doi 10.1016/0731-7085(90)80139-G

World Anti-doping Agency. (2015). The 2015 Prohibited List. (January), 1-10.

Xie, Z., Vallender, E. J., Yu, N., Kirstein, S. L., Yang, H., Bahn, M. E., . . . Miller, G. M. (2008). Cloning, expression, and functional analysis of rhesus monkey trace amine-associated receptor 6: evidence for lack of monoaminergic association. *J Neurosci Res, 86*(15), 3435-3446. doi: 10.1002/jnr.21783

Yang, M., Fazio, S., Munch, D., & Drumm, P. (2005). Impact of methanol and acetonitrile on separations based on pi-pi interactions with a reversed-phase

phenyl column. *J Chromatogr A, 1097*(1-2), 124-129. doi: 10.1016/j.chroma.2005.08.028

Yang, T., Zeng, F. Q., Ge, Q., Xiong, Q., Guo, F., & Zhang, X. G. (2004). [The negative temperature effect of UV absorbance on C60 in different solvents]. *Guang Pu Xue Yu Guang Pu Fen Xi, 24*(2), 180-182.

Zacharis, C. K., Kika, F. S., Tzanavaras, P. D., & Fytianos, K. (2013). Development and validation of a rapid ultra high pressure liquid chromatographic method for the determination of methylxanthines in herbal infusions. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences, 927*, 218-222. doi: 10.1016/j.jchromb.2013.01.011

Zhang, Y., Woods, R. M., Breitbach, Z. S., & Armstrong, D. W. (2012). 1,3-Dimethylamylamine (DMAA) in supplements and geranium products: natural or synthetic? *Drug testing and analysis, 4*(12), 986-990. doi: 10.1002/dta.1368

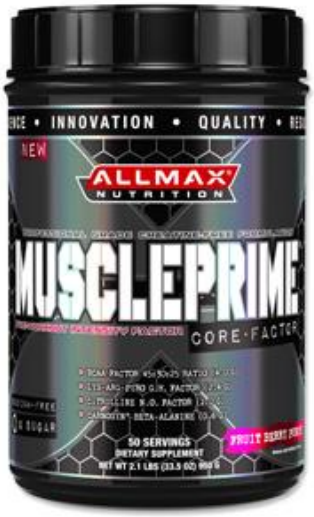
Zhao, J. F., Shoeib, T., Siu, K. W. M., & Hopkinson, A. C. (2006). The fragmentation of protonated tyrosine and iodotyrosines: The effect of substituents on the losses of NH3 and of H2O and CO. *International Journal of Mass Spectrometry, 255*, 265-278. doi: 10.1016/j.ijms.2006.03.012

Ziegleder, G., Stojacic, E., & Stumpf, B. (1992). [Occurrence of beta-phenylethylamine and its derivatives in cocoa and cocoa products]. *Z Lebensm Unters Forsch, 195*(3), 235-238.

Zulli, A., Smith, R. M., Kubatka, P., Novak, J., Uehara, Y., Loftus, H., . . . Kruzliak, P. (2016). Caffeine and cardiovascular diseases: critical review of current research. *Eur J Nutr, 55*(4), 1331-1343. doi: 10.1007/s00394-016-1179-z

Appendices

Appendix 1: Muscle Prime (Allmax Nutrition) label



PURPOSE: FORMULATED TO INCREASE DRIVE, ENERGY, FOCUS & STAMINA.

Recommended Use: Mix 1 scoop with 4-6 oz of water and drink before workouts.

Warnings: For adult use only. Use only as directed. May cause mild and temporary skin level flushing and/or itching in some people. Do not consume with any other stimulant containing products. Each scoop contains the equivalent of about 1 cup of coffee. Do not take if you have any pre-existing medical conditions including but not limited to: high or low blood pressure, hypertension, cardiac arrhythmia, history of heart related ailments, stroke, heart, liver or thyroid disease, anxiety, depression, seizure disorder, psychiatric disease, diabetes, pernicious anemia, prostate enlargement or if you are taking an MAO inhibitor or any other medication. Do not use if you are pregnant, nursing. Discontinue use if you experience any adverse reactions (nervousness, chest pain, tremors, sleeplessness, loss of appetite or nausea, heart palpitations or other similar symptoms). To avoid sleeplessness, do not take within 4 hours of sleeping.

KEEP OUT OF REACH OF CHILDREN AND PETS. DO NOT USE THIS PRODUCT IF THE TAMPER EVIDENT SHRINK BAND IS MISSING OR DAMAGED. STORE IN A COOL DRY PLACE.

Supplement Facts

Serving Size (1 scoop): 19 g
Servings Per Container: 30

Amount Per Serving		% DV‡
Calories	13	
Calories from Fat	0	
Total Fat	0	0%
Cholesterol	0 mg	0%
Sodium	0 mg	0%
Potassium	220 mg	6%
Total Carbohydrate	4 g	3%
Dietary Fiber	0 g	0%
Sugars	0 g	†
Protein	0 g	0%
Vitamin A	0 mg	0%
Vitamin C	60 mg	100%
Vitamin E	7.5 IU	25%
Calcium	200 mg	20%
Iron	0 mg	0%
Niacin	20 mg	100%
Vitamin B6	0.8 mg	40%
Vitamin B12	6 mcg	100%
Magnesium	200 mg	50%
Selenium	60 mcg	86%

MUSCLEPRIME® CORE•FACTOR

45:30:25 BCAA:BETA•A MYO•SYN FACTOR 4,600 mg †
BCAA Blend [L-Leucine (1,800 mg); L-Valine (1,200 mg); L-Isoleucine (1,000 mg)], Beta-Alanine (as CarnoSyn®) (600 mg)

SYN•OCT™ STIM•FACTOR 3,118 mg †
L-Tyrosine (1,500 mg); Taurine (1,257 mg); SYN•OCT™ Factor (190 mg) [Caffeine Anhydrous (160 mg), Synephrine (20 mg), Octopamine (10 mg)]; N-Acetyl L-Tyrosine (158 mg), Phosphatidylserine (12.5 mg); Vitamin B12 (as methylcobalamin) (6 mcg)

G.H. FACTOR 2,400 mg †
LYS-ARG-PYRO G.H. Blend [Lysine HCl (1,200 mg), ARG•PYRO Blend (Arginine Pyroglutamate, Arginine AKG (2:1), Arginine HCl, L-Arginine Base) (1,200 mg)]

N.O. FACTOR 1,368 mg †
Citrulline Malate (1,000 mg), Arginine AKG (2:1) (300 mg), Niacin (20 mg), American Ginseng (7% Ginsenosides) Extract (12 mg), Co-Enzyme Q10 (12.5 mg), Cordyceps (10 mg), Hawthorne Berry (8 mg), Ginkgo Biloba (24% Ginkgosides) Extract (5 mg), Vitamin B6 (as pyridoxine HCl) (0.8 mg)

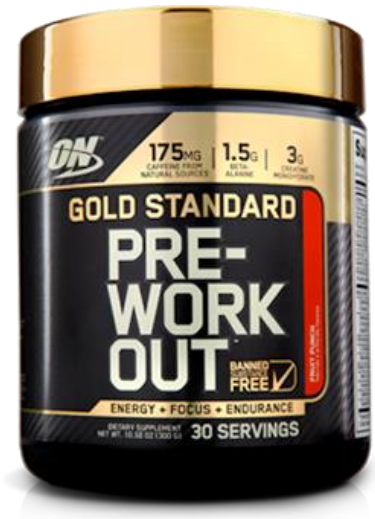
POS•ION FACTOR (K^+/Ca^{2+}/Mg^{2+}) 620 mg †
Potassium (as dipotassium phosphate) (220 mg), Calcium (as calcium citrate, dicalcium & calcium carbonate) (200 mg); Magnesium (as magnesium citrate & oxide) (200 mg)

ANTI•OX FACTOR 138 mg †
Vitamin C (as calcium ascorbate) (60 mg), N-Acetyl L-Cysteine (50 mg), Alpha Lipoic Acid (20 mg), Vitamin E (as dl-tocopheryl acetate) (7.5 IU) (8 mg), Selenium (as selenomethionine) (60 mcg)

‡ Percent Daily Values (DV) are based on a 2,000 calorie diet.
† Daily value not established.
Not a significant source of Vitamin A and Iron.

OTHER INGREDIENTS: Maltodextrin, Natural and Artificial Flavors, Citric Acid, Sucralose.
ALLERGY ALERT: Manufactured in a facility that also processes the following allergens; milk, soy, wheat, eggs and peanuts.

Appendix 2 Gold standard Preworkout (Optimum Nutrition) label



GOLD STANDARD PRE-WORKOUT

Serving Size	1 Scoop (10 g)		2 Scoops (20 g)	
Servings Per Container	30		15	
Amount Per Serving		**% Daily Value**		**% Daily Value**
Calories	5		10	
Total Carbohydrate	1 g	1%*	2 g	1%*
Vitamin D (as Cholecalciferol)	500 IU	125%	1,000 IU	250%
Thiamin (as Thiamin HCl)	2 mg	133%	4 mg	267%
Niacin (as Nicotinic Acid)	20 mg	100%	40 mg	200%
Vitamin B6 (as Pyridoxine HCl)	2 mg	100%	4 mg	200%
Folic Acid	200 mcg	50%	400 mcg	100%
Vitamin B12 (as Cyanocobalamin)	10 mcg	167%	20 mcg	333%
Pantothenic Acid (as D-Calcium Pantothenate)	10 mg	100%	20 mg	200%
Muscle Matrix^				
Creatine Monohydrate (as Creapure®)	3 g	†	6 g	†
AstraGIN® Proprietary Blend (*Astragalus membranaceus* Extract (root) & *Panax notoginseng* Extract (root))	25 mg	†	50 mg	†
Performance Composite^				
L-Citrulline Malate	1.5 g	†	3 g	†
Beta-Alanine (as CarnoSyn®)	1.5 g	†	3 g	†
Energy & Focus Complex^				
Acetyl-L-Carnitine HCl	375 mg	†	750 mg	†
N-Acetyl-L-Tyrosine	250 mg	†	500 mg	†
Caffeine	175 mg	†	350 mg	†
Citrus Bioflavonoids	100 mg	†	200 mg	†

* Percent Daily Values are based on a 2,000 calorie diet.
† Daily Value not established.

OTHER INGREDIENTS: Natural & Artificial Flavors, Citric Acid, Malic Acid, Silicon Dioxide, Calcium Silicate, Caffeine (from Tea and/or Coffee Bean), Gum Blend (Cellulose Gum, Xanthan Gum, Carrageenan), Sucralose, Tartaric Acid, Acesulfame Potassium, FD&C Yellow #5, FD&C Blue #2.

DIRECTIONS: Mix each scoop with 6-8 oz of water and consume 20-30 minutes before training. Start with 1 scoop or less to assess your tolerance.

DO NOT EXCEED 2 SCOOPS PER DAY.

Appendix 3: 360Rage (360°Cut Performance Nutrition) label

Supplement Facts

Serving Size: 7.3g (1 Scoop)
Servings Per Container: 30

	Amount Per Serving	%DV
360 PUMP Matrix:	**3650mg**	†
Beta Alanine, Citrulline Malate, Agmatine, Grape (Vitis vinifera) Seed Extract, Maritime Pine Tree (Pinus pinaster) Bark Extract, L Norvaline		
360 INTENSITY Matrix:	**1985mg**	†
Tyrosine, Caffeine Anhydrous (1,3,7 Trimethylxanthine), Synephrine HCL, Hordenine, Huperzine A (from Whole Plant Toothed Clubmoss (Huperzia serrata) Herb Extract)		

† Percent Daily Values not established.

Other Ingredients: Natural and artificial flavors, debittering magnesium, sucralose, citric acid, and silicon dioxide.

Appendix 4: ErgoBlast (Ergo Genix) label

Supplement Facts

Serving Size: 1 Scoop (5 g) Servings Per Container 30

	Amount per Serving	%DV
Vitamin B6 (as Pyridoxine Hydrochloride)	20 mg	1000%
Vitamin B12 (as Cyanocobalamin)	60 mcg	1000%
Zinc (as Zinc Arginate Chelate)	10 mg	66%
Sodium (as Sodium Citrate)	24 mg	1%
Creatine HCl (Con-Cret®)	1,000 mg	†
Agmatine Sulfate (AgmaMax®)	500 mg	†
ErgoStim Blend: N-Acetyl L-Tyrosine, Caffeine Anhydrous, L-Tyrosine, Mucuna Pruriens Extract (L-Dopa), Theobromine, N-Methyltyramine Hydrochloride, Higenamine Hydrochloride, Guarana Seed Extract, Ubidecarenone (Co-Q10), Huperzine 1%	1,600 mg	†
Total Amount of Caffeine Per Serving: 300 mg		

* Percent Daily Values are based on a 2,000 calorie diet. † Daily Value not established

Appendix 5: PumpHD (BPI Sports) label

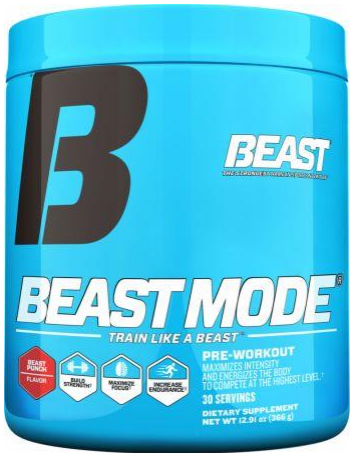
Supplement Facts

Serving Size 1 Scoop (10 grams)
Servings Per Container 25

Amount Per Serving		% Daily Value
Calories	25	
Total Carbohydrate	5 g	2%†
Niacin (as nicotinic acid)	20 mg	100%
Vitamin B-6 (as pyridoxine HCl)	10 mg	500%
Folic Acid	800 mcg	200%
Vitamin B-12 (as cyanocobalamin)	500 mcg	8,334%
Iron (as ferrous fumarate)	9 mg	50%
Sodium (as sodium phosphate)	100 mg	4%
Potassium (as potassium phosphate)	220 mg	6%
CycloCreatine PCr	1 g	**
Glycerol 2X-RS™ (Double Strength bonded glycerol monostearate 2X-reuptake system™)	1 g	**
Andrographis (leaf) [Andrographolides and related Terpenoids]	1 g	**
Citrulline Silicate	1 g	**
Adaptogenic and Erythropoietin (EPO) Matrix (proprietary)	**1 g**	**
Green tea leaf catechin 98% extract, Cordyceps extract [std. to Cordycepin], Rhodiola root extract, Echinacea purpurea (aerial parts) extract, Quercetin dihydrate, ferrous fumarate.		
"THE PUMP EXPERIENCE" Energy with Liposomal Technology (proprietary)	**500 mg**	**
Theobroma cacao extract (seed), *Citrus aurantium* extract (fruit), *Plumbago zeylanica* extract (root), Phosphotidylcholine.		

† Percent Daily Values are base don a 2,000 calorie diet.
** Daily Value not established.

Appendix 6: BeastMode (Beast Sports Nutrition) label



Supplement Facts

Serving Size: 1 Scoop (14.4 g)
Servings Per Container: 45

	Amount Per Serving	% DV*
Calories	30	
Total Carbohydrates	7 g	2%
Vitamin B1 (as Thiamine Hydrochloride)	60 mg	4000%
Vitamin B2 (as Riboflavin)	7 mg	412%
Niacin (as Niacinamide)	6 mg	30%
Vitamin B6 (as Pyridoxine Hydrochloride)	4.2 mg	210%
Vitamin B12 (as Methylcobalamin)	280 mcg	4667%
Calcium (as Silicate and Phosphate)	66 mg	7%
Magnesium (as Oxide)	71 mg	18%
Sodium (as Phosphate)	157 mg	7%
Potassium (as Phosphate)	45 mg	1%
Mass & Endurance Blend *with* Muscle Volumizing Agents	4263 mg	**
Glycerol MonoStearate, L-Arginine Alpha-Ketoglutarate 2:1, CarnoSyn® Beta Alanine, Agmatine Sulfate, Cinnulin PF® (Patented bark extract).		
Neurostimulating Co-Factors	767 mg	**
Choline Bitartrate, L-Tyrosine, Bacopa Monniera (leaf), Ginkgo Biloba (leaf).		
Energy & Intensity Blend *with* High Potency B-Complex	277 mg	**
Coffea Robusta (bean), Nelumbo Nucifera (seed) Extract, Vitamin B1 (Thiamine HCl),Vitamin B2 (as Riboflavin), Vitamin B3 (Niacinamide), Vitamin B6 (Pyridoxine HCl),Vitamin B12 (Methylcobalamin)		

*Percent Daily Value Based on as 2,000 Calorie Diet
**Daily Value Not Established

Other Ingredients: Glucose Polymers, Citric Acid, Natural & Artificial Flavors, Sodium Citrate, Sucralose, Purple Carrot Extract (for color), Acesulfame Potassium.

Appendix 7: KardioFire (Panthera Pharma) label

KARDIO FIRE 350 GMS

SUPPLEMENT FACTS

Serving Size: 10 GMS (1 scoop)		
Serving / container: 35	Amount / Serving	% Daily Value*
Calories	0	+
Fat	0	+
Total Carbohydrate	0	+
Sugar	0	+
Protein	0	+

KARDIO MATRIX

Carnosyn Beta Alanine	4000 mgs	+
BCAA	3000 mgs	+
(2:1:1 L-Leucine, L-Valine, Iso-Leucine)		
L-Glutamine	1000 mgs	+

FIRE MATRIX

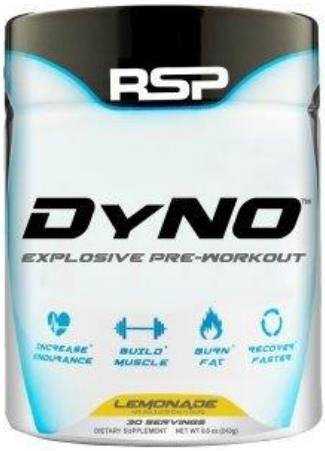
625 mg +

Raspberry Ketone
Caffiene Anhydrous
Citrus Aurantium
L-phenylalanine
L-tyrosine
Acetyl L-carnitine

*Percent Daily Value are based on a 2000 calories diet.
+Daily Value not established

Other Ingredients :
Citric Acid, Natural And Artificial Color, Sucralose, Artificial Flavour

Appendix 8: DyNO (RSP Nutrition) label



Serving Size: 1 Scoop (8.1g)
Servings Per Container: 30

Amount Per Serving		% Daily Value
Vitamin C (as Calcium Ascorbate)	60 mg	100%
Vitamin B3 (as Niacin)	75 mg	375%
Vitamin B6 (as Pyridoxine HCl)	.5 mg	25%
Folic Acid	200 mcg	50%
Vitamin B12 (as Cyanocobalamin)	6 mcg	100%
Vitamin B5 (as D-Calcium Pantothenate)	10 mg	100%
Calcium (as Dicalcium Phosphate)	64 mg	6%
Citrulline Malate	2,000 mg	†
Beta Alanine (CarnoSyn™)	2,000 mg	†
Agmatine Sulfate (AgmaMAX™)	500 mg	†
Focus Blend	1,670 mg	†
Taurine, L-Glycine, L-Tyrosine, N-Acetyl L-Tyrosine		
Acceleration Matrix	516 mg	†
Caffeine Anhydrous, Dicaffeine Malate (Infinergy™), Phenylethylamine Hydrochloride, N-Methyltyramine Hydrochloride, Higenamine Hydrochloride, Black Pepper Fruit Extract (BioPerine®), Rutaecarpine, Rhodiola Root		

* Percent Daily Values are based on 2,000 calories diet
† Daily value not established

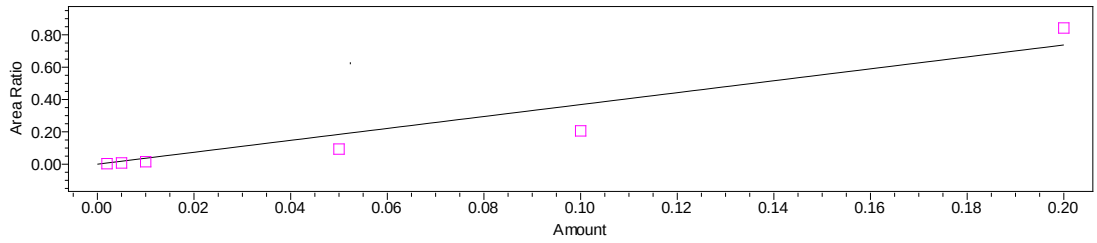
Other Ingredients:
Natural Flavors, Sucralose, Silicon Dioxide, Citric Acid, Acesulfame Potassium, and Red #40.

CAUTION: Product contains 400mg of pure Caffeine

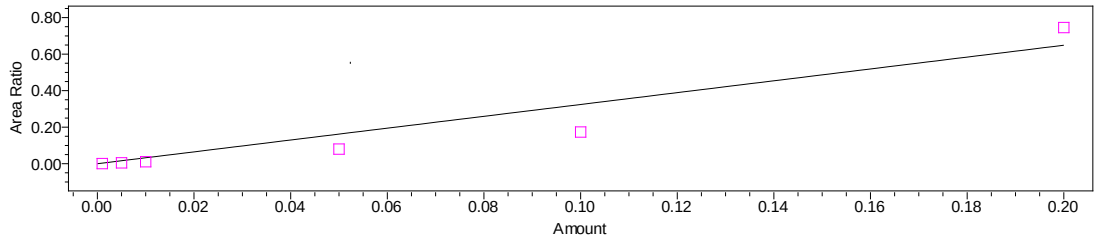
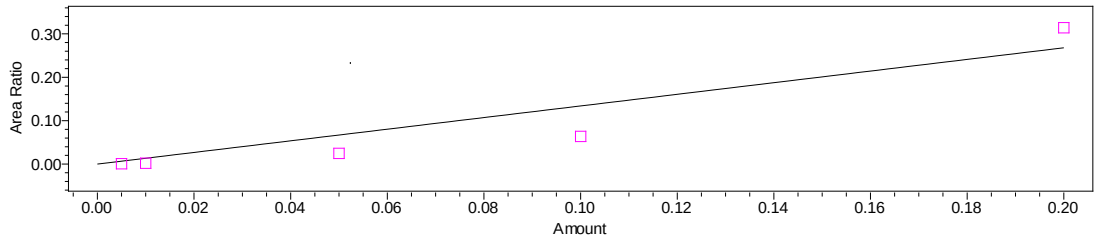
Appendix 9: Scheduling 1-10 that is currently implemented by the Therapeutic Goods Administration of Australia

Schedule 1	This Schedule is intentionally blank.
Schedule 2	**Pharmacy Medicine** – Substances, the safe use of which may require advice from a pharmacist and which should be available from a pharmacy or, where a pharmacy service is not available, from a licensed person.
Schedule 3	**Pharmacist Only Medicine** – Substances, the safe use of which requires professional advice but which should be available to the public from a pharmacist without a prescription.
Schedule 4	**Prescription Only Medicine, or Prescription Animal Remedy** – Substances, the use or supply of which should be by or on the order of persons permitted by State or Territory legislation to prescribe and should be available from a pharmacist on prescription
Schedule 5	**Caution** – Substances with a low potential for causing harm, the extent of which can be reduced through the use of appropriate packaging with simple warnings and safety directions on the label.
Schedule 6	**Poison** – Substances with a moderate potential for causing harm, the extent of which can be reduced through the use of distinctive packaging with strong warnings and safety directions on the label.
Schedule 7	**Dangerous Poison** – Substances with a high potential for causing harm at low exposure and which require special precautions during manufacture, handling or use. These poisons should be available only to specialised or authorised users who have the skills necessary to handle them safely. Special regulations restricting their availability, possession, storage or use may apply
Schedule 8	**Controlled Drug** – Substances which should be available for use but require restriction of manufacture, supply, distribution, possession and use to reduce abuse, misuse and physical or psychological dependence.
Schedule 9	**Prohibited Substance** – Substances which may be abused or misused, the manufacture, possession, sale or use of which should be prohibited by law except when required for medical or scientific research, or for analytical, teaching or training purposes with approval of Commonwealth and/or State or Territory Health Authorities
Schedule 10	**Substances of such danger to health as to warrant prohibition of sale, supply and use** - Substances which are prohibited for the purpose or purposes listed for each poison.

Appendix 10 : Calibration curve of synephrine from concentrations 2 µg/mL to 200 µg/mL. r^2 = 0.9129



Appendix 11 : Calibration curve of octopamine from concentrations 2 µg/mL to 200 µg/mL. r^2 = 0.9061



Appendix 12 : Calibration curve of tyramine from concentrations 2 µg/mL to 200 µg/mL. r^2 = 0.87217