DOCTORAL THESIS

An Investigation into the Authenticity of Citrus Aurantium-Listing Pre-Workout Supplements and Functional Evaluations of their Trace Amines on the Vasculature.

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An Investigation into the Authenticity of *Citrus Aurantium*-Listing Pre-Workout Supplements and Functional Evaluations of their Trace Amines on the Vasculature

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ABSTRACT

Pre-workout supplements (PWS) are stimulatory multi-ingredient dietary supplements used to improve athletic performance. The stimulatory effects of these supplements typically arise from ingredients such as caffeine and phenethylamine alkaloids, commonly derived from a variety of plant extracts such as *Citrus aurantium L.* (Bitter orange). It contains natural phenethylamines such as synephrine, octopamine, and tyramine, which are structurally related to ephedrine. Additionally, these amines exist in trace amounts in the mammalian system and are called trace amines. The safety of PWS has come into question following reports of cardiovascular adverse events associated with PWS consumption, such as arrhythmias, myocardial infarctions, and aortic dissections. While there is some evidence that the inclusion of *C. aurantium* extracts is beneficial for weight loss and exercise, the distinct mechanisms of action of each trace amines on different components of the mammalian vascular system remain poorly understood.

PWS are categorised and regulated as *complementary medicines* in Australia. Manufacturers are not bound to provide evidence for their efficacy and have much less stringent labelling requirements than pharmaceutical drugs. Additionally, manufacturers of PWS are not required to reveal the quantities of stimulatory ingredients on the label when part of a *proprietary blend*, which makes it difficult to determine, which ingredient contributes to an ergogenic effect. However, trace amines in *C. aurantium* generally exhibit a unique ratio of amines and a predominance of a particular enantiomer, which can be useful in differentiating from other non-natural sources. Hence, two main aims were developed to answer a set of questions raised from these observations.

The first aim was to investigate the comparative vascular effects of trace amines (synephrine, octopamine and tyramine) found in *C. aurantium*-listing PWS on three main arteries (mesenteric, coronary, and renal) that contribute to blood pressure. *In vivo* models have been previously explored but have failed to yield a clear explanation of the mechanisms of action of the trace amines on the vascular smooth muscle tissue. Therefore, the scope of the study was limited to the determination of vascular effects on isolated porcine tissues and elucidated

their respective mechanisms of action with functional assays involving various antagonists, including the recently developed TAAR1 antagonist EPPTB. These studies concluded that the three trace amines elicited their contractile effects from complex mechanisms of action that was different across the three selected arteries investigated. Interestingly, the contractile responses to synephrine were affected by TAAR1 blockade, suggesting that synephrine may mediate its effects via this pathway in addition to the direct α_1-adrenoceptor activity.

The next aim of the study was to determine the levels of trace amines found in *C. aurantium*-listing PWS and whether they correlate with their labels and are from an authentic natural source. Two quantitative analytical methods for assessing the authenticity of *C. aurantium*-containing PWS were developed and validated using HPLC-UV-MS. The study first compared the ratios of the trace amines in PWS to that found in *C. aurantium.* Secondly, the compared enantiomeric ratio of synephrine in the PWS sample set to that found in *C. aurantium* standardised reference materials. These studies showed that only three out of twelve evaluated *C. aurantium* PWS contained authentic sources of plant material. Additionally, the values of the amines were not consistent with their labelled amounts.

In conclusion, this thesis showed that the combined results of total synephrine quantified in *C. aurantium* PWS could elicit vascular responses, whereas there were negligible amounts of octopamine and tyramine. Therefore, some of the observed adverse cardiovascular events may be attributed to synephrine in *C. aurantium* PWS, but further vascular effects from other stimulants such as caffeine or synthetic sympathomimetics need further exploration. The variability of stimulant levels in PWS carries a risk to consumers, particularly those with cardiovascular comorbidities such as obesity.

KEYWORDS

Citrus aurantium, Pre-workout supplements, HPLC, Functional Assays, Coronary, Mesenteric, Renal, vasculature, TAAR1,

DECLARATION BY AUTHOR

This thesis is submitted to Bond University in fulfilment of the requirements of the degree of Doctor of Philosophy by Research.

I declare that the research presented within this thesis is a product of my own original ideas and work and contains no material which has previously been submitted for a degree at this university or any other institution, except where due acknowledgement has been made.

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Signature:

Date: 30 August 2021

DECLARATION BY CO-AUTHORS

The following people and institutions contributed to the publication of work undertaken as a part of this thesis.

i) Andy Hsien Wei Koh (AK),

ii) Russell Chess-Williams (RCW)

iii) Anna Elizabeth Lohning (AL)

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5. Koh AHW, Chess-Williams R, Lohning AE. Renal artery responses to trace amines: Multiple and differential mechanisms of action. Life Sciences. (2021) 15; 277:119532. PMID: 33891943. https://doi.org/10.1016/j.lfs.2021.119532

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- Koh, A.H.W., (August 2018). The cardiovascular effects of trace amines found in pre-workout supplements. Queensland State 3-Minute Thesis competition, Gold Coast, Australia

- Koh, A.H.W., Chess-Williams, R., Lohning, A.E. (December 2017). *Functional Evaluations of Synephrine and Octopamine - Stimulants in Pre-Workout Supplements.* Poster presentation at Australian Society of Clinical and Experimental Pharmacology and Toxicology annual meeting, Brisbane, Australia. Toxicology division finalist.
- Koh, A.H.W., Chess-Williams, R., Lohning, A.E. (November 2017). *Functional Evaluations of Synephrine and Octopamine - Stimulants in Pre-Workout Supplements.* Oral presentation at the Gold Coast Health and Medical Research Conference, Gold Coast, Australia.

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LIST OF ABBREVIATIONS

µg	Microgram
µM	Micromolar
ACN	Acetonitrile
ARTG	Australian Register for Therapeutic Goods
BBB	Blood Brain Barrier
BfR	The Federal Institute for Risk Assessment (Germany)
CA	*C. aurantium*
CHO	Chinese Hamster Ovary cell
CNS	Central Nervous System
COMT	Catechol methyl transferase
DMAA	1,3-Dimethylamine
DMSO	Dimethyl sulfoxide
EPPTB	N-(3-Ethoxyphenyl)-4-(1-pyrrolidinyl)-3-(trifluoromethyl)benzamide
ESI	Electron Spray Ionisation
FDA	U.S. Food and Drug Administration
FSANZ	Food Standards Australia and New Zealand
GCPR	G-coupled protein receptor
HPLC	High Pressure Liquid Chromatography
ICH	The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
IPR	Ion-pair Reagent
LNNA	L-Nω-Nitroarginine
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitor
mg	Milligram
mL	Millilitre
mM	Micromolar
MS/MS	Triple quadrupole tandem MS
NA	Noradrenaline
NO	Nitric oxide
ng	Nanogram
NH4OH	Ammonium Hydroxide
NIST	National Institute for Standards and Technology
OCT	Octopamine
*p*EC50	Potency
PEA	Phenethylamine
PWS	Pre-workout Supplement(s)
QDa	Quadrupole Diode array
RDD	Recommended Daily Dose
RP HPLC	Reverse phased High Pressure Liquid Chromatography
RSD	Relative Standard Deviation
SPE	Solid Phase extraction
SRM	Standard Reference Material
SYN	Synephrine
TAAR-1	Trace Amine Associated Receptors
TGA	Therapeutic Goods Administration
TYR	Tyramine
UPLC	Ultra-High Pressure Liquid Chromatography
UV	Ultraviolet
WADA	World Anti-Doping Agency
α-AR	α -adrenoceptor
β-AR	β-adrenoceptor

CHAPTER 1: INTRODUCTION

1.1 PRE-WORKOUT SUPPLEMENTS

Consumers use dietary supplements for various reasons, including improving health, increasing exercise performance or energy levels, and enhancing weight loss. Their use is becoming increasingly prevalent among athletes and recreational consumers alike. According to a National Health Survey conducted in Australia between 2014-2015, nearly half of the Australian population had taken at least one dietary supplement per week, three times higher than levels in 1995 (O'Brien et al., 2017). This trend of dietary supplement consumption reflects the economic growth of the dietary supplement industry, which is currently worth $133.1 billion and is projected to grow by a further 9.6% by the year 2024 (Zayets, 2019).

Pre-workout supplements (PWS) are a subset of dietary supplements that have gained the interest not only of athletes but the wider community, health professionals, and researchers alike (Denham, 2021; Gurley et al., 2015; Jagim et al., 2019). These water-soluble powders are consumed prior to exercise and typically consist of many diverse, active ingredients such as caffeine, creatine, beta-alanine, taurine, and stimulatory ingredients from various botanical sources (Eudy et al., 2013). Consumers mainly use PWS to improve exercise performance and promote weight loss (Gurley et al., 2015; Jagim et al., 2019). However, there has been mixed evidence on the efficacy and ergogenic benefits of ingesting PWS in adults. In some studies, PWS ingestion improved anaerobic exercise performance in trained athletes (Hoffman et al., 2009; Jung et al., 2017a; Jung et al., 2017b; Tinsley et al., 2017) but other studies showed no difference in exercise performance to placebo-controlled trained athletes (Bloomer et al., 2011; Erickson et al., 2020; Nelson et al., 2019; Outlaw et al., 2014). These studies are often limited by their small cohorts (< 30 participants), a narrow population of trained athletes, and inconsistent brands of PWS with different proportions of active ingredients. Caffeine is usually a common ingredient among these studies, but the quantity of caffeine and other botanical ingredients was not comparable. The varying multi-ingredient composition of the PWS used in the studies mentioned above may lead researchers to draw different conclusions on pre-workout supplement’s efficacy.

Labels of PWS are often incomplete, and levels of individual actives are not consistently listed or, if they are, not always accurate (Cohen & Ernst, 2010; Pawar & Grundel, 2017; Pawar et al., 2020). These ingredients are often part of a *proprietary blend* where only relative proportions of stimulatory compounds in a total amount may be disclosed. This means that consumers are often unaware of what may be contained therein and unclear of the amount of stimulant being consumed. Australian labelling requirements for non-prescription medicines were updated in 2019 and now require some over the counter and complementary medicine labels to contain advisory statements that detail specific risks related to the use of the medicines (Therapeutic Goods Administration, 2019). The Required Advisory Statements for Medicine Labels (RASML) register can be accessed online and is available for the public at https://www.legislation.gov.au/Details/F2021L01888. However, overseas PWS manufacturers are not obliged to adhere to these labelling requirements, which may present a problem to the average consumer.

A misconception exists within the general population that herbal ingredients found in PWS are safe for consumption because they may be from a natural source (Ekar & Kreft, 2019). A recent survey in young adults (n = 869) in the United States of America found that some pre-workout supplement consumers actively take more than the recommended dose (32%, n = 278) and other recreational activities apart from sport (3.4%, n= 30) (Jagim et al., 2019). The off-label use of these supplements has led to some public health concerns in Australia. For example, long-shift workers of the Bowen Basin mine in Western Australia were reported to consume PWS to prolong their alertness, enabling them to work long or consecutive shifts (Duffy, 2012). Subsequently, the coal mine management banned the use of the pre-workout supplement, *Jack3d*, because of the perceived risk involved in operating heavy machinery while fatigued. Whereas athletes and gym-goers may generally be considered in good health and perhaps less likely to succumb to adverse cardiovascular events due to pre-workout supplement use, older individuals and/or those overweight with pre-existing health conditions may be at a higher risk for adverse effects. This highlights the need to not only determine the mechanisms of action of the active ingredients but also to establish the safety/risk profile of the individual compounds in PWS.

Ephedra was a popular ingredient in PWS for around ten years (1994-2004) due to tangible weight-loss and ergogenic effects, but its use had a significant level of controversy (Palamar, 2011). *Ephedra sinica* (Ephedra) is a herb traditionally used in Chinese medicine for centuries and a source of ephedrine alkaloids that mimic the activity of amphetamine on the central nervous system of the human body (Haller et al., 2004) (Figure 1).

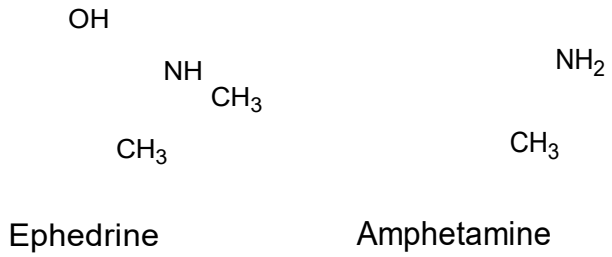


Figure 1: Structure of ephedrine in comparison to amphetamine

The ingestion of Ephedra-listing supplements has been associated with a range of reported cardiovascular adverse events such as cardiac arrhythmias (Dwyer et al., 2005; Haller, 2000), myocardial infarction (Haller, 2000; Smith et al., 2014), and strokes (Bouchard et al., 2005; Haller et al., 2002; Holmes & Tavee, 2008). The rise in adverse health reports against Ephedra-listing supplements led the United States' Food and Drug Administration (FDA) to ban Ephedra as a permissible ingredient in dietary supplements (Pawar & Grundel, 2017). However, the FDA's ban on ephedrine alkaloids led to multiple ephedra-free formulations that contained alternative stimulants and sympathomimetics.

A notable example was the availability of PWS containing a group of synthetic, aliphatic stimulants (1,3-dimethylamylamine (DMAA); 1,3-dimethylbutylamine (DMBA); and 1,5-dimethylhexylamine (DMHA); Figure 2) though they were claimed to be derived from natural sources such as Geranium oil or Pouchong Tea (Cohen et al., 2021; Cohen et al., 2015; Di Lorenzo et al., 2013). However, public health concerns about the use and abuse of pre-workout supplement consumption containing DMAA were raised by the FDA, who took immediate action to review and ban DMAA following the death of two U.S. military personnel from cardiac arrest related to DMAA-listing PWS (Eliason et al.,

2012). Due to the reactive nature of supplement regulators, new experimental stimulants will likely continue to appear in place of banned ingredients.

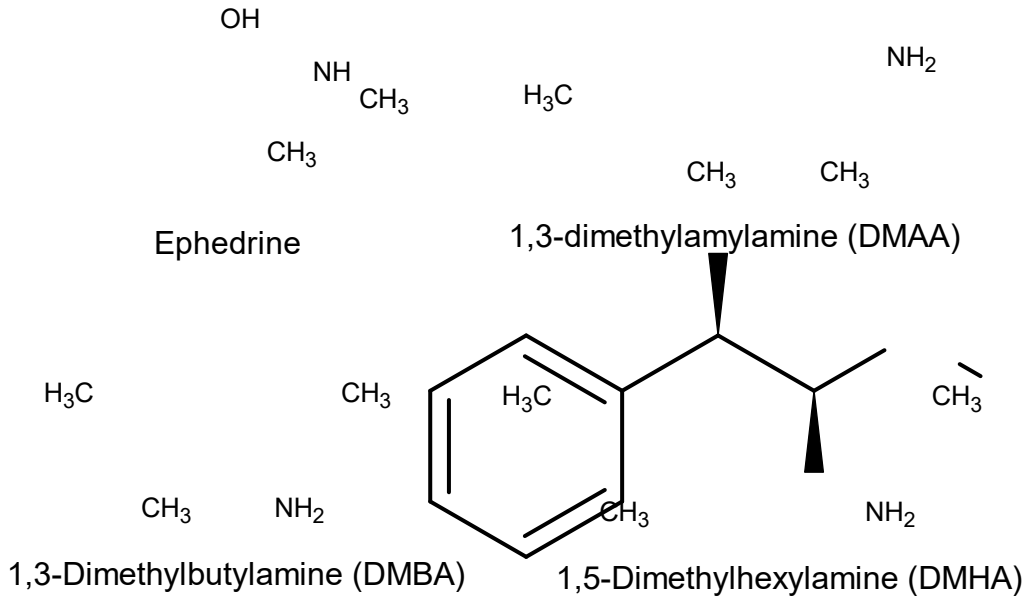


Figure 2: Structural comparisons between ephedrine and banned synthetic stimulants found in pre-workout supplements

1.2 *CITRUS AURANTIUM* AS A STIMULANT IN PRE-WORKOUT SUPPLEMENTS

Citrus aurantium L. (Bitter orange, Seville orange, and Neroli) belongs to the Rutaceae family, and is a hybrid between *Citrus maxima* (Pomelo) and *Citrus reticula* (Mandarin) (National Centre for Complementary and Integrative Health, 2013). *C. aurantium* originates in tropical Asia, but cultivars of the plant are available in numerous countries such as Brazil, the United States of America and Guinea. The fruits and leaves of the plant has been used in traditional Chinese medicines such as *Zhi Shi,* to treat gastrointestinal pain and constipation (Lin et al., 2011). *C. aurantium* has been included in preparations of PWS as an ephedra-free ergogenic and weight-loss agent (Rossato et al., 2011).

The main volatile components in the flowers and peel of *C. aurantium* are the flavonoids, hesperidin, and naringin, whereas the non-volatile alkaloid components include *p*-synephrine, *p*-octopamine, and tyramine, which are structurally similar to ephedrine and noradrenaline (Figure 3). In dietary supplements, the peels of immature *C. aurantium* are extracted and are usually standardised for their synephrine content (6% or 10 % of synephrine) (Bakhiya et al., 2017).

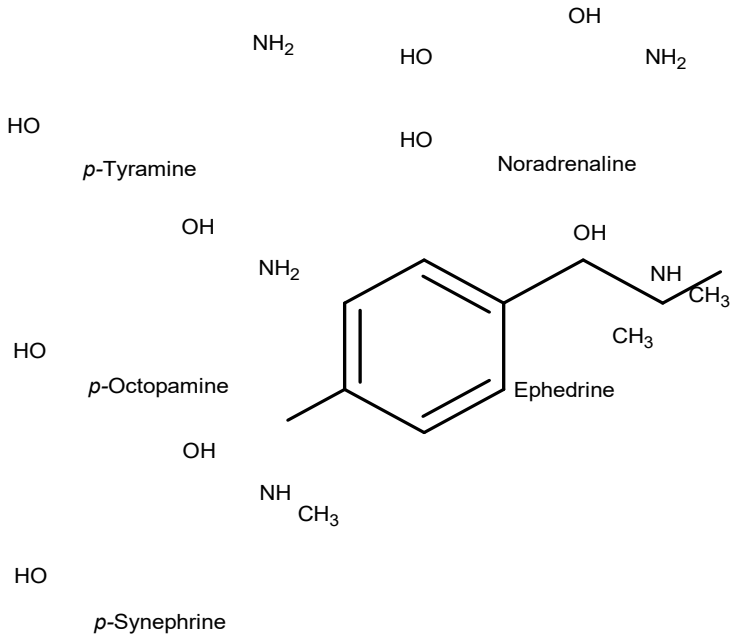


Figure 3: Structural comparison between synephrine, octopamine, and tyramine to ephedrine and noradrenaline

Apart from its purported ergogenic effects, *C. aurantium* has also been marketed as a weight-loss agent based on *in vitro* and rodent studies showing that synephrine and octopamine had β_3-adrenoreceptors binding properties (Takagi et al., 2018). β_3-adrenoreceptor activation in mice has shown anti-obesity effects attributed to an enhanced uncoupling protein-1 (UCP-1) pathway leading to lipolysis and thermogenesis (Coman et al., 2009).

In mammals, there are three types of adipose tissues: white, brown, and beige. These tissues have different functions, morphologies, and protein expressions. White adipose tissues (WAT) store energy in the form of lipids, which can be oxidised for cellular metabolism. Brown adipose tissue (BAT) has thermogenic properties, which helps regulate body temperature and is activated either by cold temperatures or pharmacological stimulation (Chen et al., 2016). Beige adipose tissue is usually co-expressed with WAT but has thermogenic properties similar to BAT (Chen et al., 2016). The thermogenic properties of brown and beige adipose tissues have been attributed to the stimulation of β_3-adrenoreceptors, but their distribution differs between species and age (Chen et al., 2016). In addition, species-related differences include ligand binding properties, bioavailability, cross-reactivity with other β-adrenoreceptors that may result in different responses to β_3-adrenoreceptor activation. Certainly, adverse effects can result from co-activation of β_1- and β_2-adrenoreceptors, such as tachycardia and tremor (Connacher et al., 1990).

There is limited information on molecular and biochemical role of human brown adipose tissue due to limited access to human brown adipose tissue biopsies for primary cell cultures (Riis-Vestergaard et al., 2020). In humans, β_3-adrenoreceptors are found in a range of tissues, including adipose tissue, the heart, urinary and gall bladder, brain, skeletal muscle (Leitner et al., 2017). Certain β_3-adrenoreceptor agonists have been trailed clinically for weight loss with limited results (Riis-Vestergaard et al., 2020); however, the β_3-adrenoreceptor agonist, *Mirabegron*, registered for use in overactive bladder, showed increased β_3-adrenoreceptor activation (vasodilation) and resting metabolic rate (Chapple et al., 2014). Currently there is some evidence that *p*-synephrine supplementation showed modest fat oxidation when taken in conjunction with moderate-intensity exercise (Gutierrez-Hellin et al., 2016a; 2016b; 2018; 2020). However, the role of *C. aurantium* in β_3-adrenoreceptors activation leading to weight-loss is not clear.

Specific studies that explore β_3-adrenoceptor activation in rodent and mammalian cells and isolated tissues are elaborated in section 1.6 of the thesis.

The safety of *C. aurantium* extracts and synephrine remains an ongoing debate. Several case reports have been associated with the intake of *C. aurantium* extract-containing supplements. The cardiovascular-related side effects produced from *C. aurantium*-containing products include myocardial infarction (Nykamp et al., 2004; Thomas et al., 2009; Unnikrishnan et al., 2018), angina (Gange et al., 2006), vasospasm, and stroke (Bouchard et al., 2005; Holmes & Tavee, 2008), ventricular fibrillation (Stephensen & Sarlay, 2009), arterial hypertension (Moaddeb et al., 2011), apical ballooning syndrome (Chung et al., 2013b) and aortic dissection (Doctorian & Do, 2017).

Overall, the patients described in the medical case reports were healthy and ingested *C. aurantium*-containing supplements for weight loss and energy. However, the products consumed contained several other pharmacologically active extracts, including other stimulants such as caffeine (Bouchard et al., 2005; Chung et al., 2013; Nykamp et al., 2004). Although synephrine or *C. aurantium* extracts were present, there was insufficient evidence to show that these constituents were the sole cause of the reported side effects. The citations used in the case reports relied on the limited pharmacological studies available surrounding *C. aurantium* or its constituent trace amines.

Adulterating dietary supplements with synthetic phenethylamines or pharmaceuticals is not an uncommon practice amongst manufacturers, and an in-depth investigation into the cases of and motivation for adulteration has been highlighted in a review by Rocha et al. (2016). An adulterated supplement is often labelled to contain natural sources of active ingredients but contains the addition of illegal substances - the intentional swap or misidentification of plant material. A study conducted by the FDA found that in a random sample of 59 *C. aurantium* PWS, five supplements were adulterated with the synthetic amine, methyl-synephrine (up to 240 mg/ serving), and iso-propyl-octopamine, both of which are not safe for consumption in dietary supplements (Pawar et al., 2020). With increasing consumption of dietary supplements in general, the safety in production and marketing, labelling compliance, and levels of

active ingredients have been of concern among regulators and health professionals (Cohen et al., 2018; Crighton et al., 2019; White, 2020).

REGULATION OF CITRUS AURANTIUM AND SYNEPHRINE SUPPLEMENTS IN AUSTRALIA AND OVERSEAS

In Australia, pre-workout supplements are regulated by the Therapeutic Goods Administration (TGA), part of the Australian Government's Department of Health. Prior to November 2020, PWS were considered complementary medicines under the Therapeutic Goods Act of 1989, as the herbal material (i.e., plant extracts) were considered "*a therapeutic good consisting principally of one or more designated active ingredients, which have an established identity or traditional use"* (Therapeutic Goods Administration, 2013). However, according to the latest amendment of the Therapeutic Goods Act of 1989, all sports supplements, including PWS with a therapeutic claim or containing high-risk ingredients, will be regulated as medicines (Therapeutic Goods Administration, 2020).

The TGA uses a risk-based approach to regulate complementary medicines whereby products containing herbal materials that present a higher risk should 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.' Medicines and drugs are categorised in the Standard for the Uniform Scheduling of Medicines and Poisons (SUSMP) (Appendix Table 2). Synephrine is a Schedule 4, prescription-only drug, with a maximum allowable dose of 30 mg/serving in any preparation (Rebera, 2020). The other amines in *C. aurantium,* such as octopamine and tyramine, have not been added on the SUSMP or have a recommended daily dose.

The TGA also conducts post-market regulatory monitoring of products to ensure safety, quality, and efficacy for locally manufactured but not overseas manufactured PWS (Therapeutic Goods Administration, 2013). The TGA has a pharmacovigilance program, which involves assessing adverse events reported by consumers, health professionals, or scientific experts to the TGA. When a risk is identified by the TGA, a warning statement or recall of the product from the market can occur. The Database of Adverse Event Notifications (DAEN, https://www.tga.gov.au/database-adverse-event-notifications-daen) from January 2010 to March 2021 had twenty-six adverse events reports were made for supplements that contain *C. aurantium* (Appendix Table 1). From the small number of voluntary reports, it appears that there was a range of individuals between 20 to 78 years old who experienced a broad range of cardiovascular adverse effects. Some of the ingredients were not listed on the ARTG since the manufactures were from overseas; this is out of the jurisdiction of the TGA. However, the voluntary reporting method may be inefficient, as general consumers do not necessarily know where to look for such information.

Similar to the TGA, Health Canada also employs a risk-based approach in evaluating the safety and efficacy of products. Evidence for the results derived from a single six-week study concluded that at a maximum daily dose of 320mg caffeine and 40mg synephrine, participants did not show harmful effects (Colker et al., 1999). In contrast to Canada's safety assessment of PWS, in 2013, Germany's Federal Institute for Risk Assessment (BfR) established a maximum safe dosage of synephrine of 25.7mg (The Federal Institute for Risk Assessment. Since some PWS contains caffeine and *C. aurantium* extracts, which 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.

The Netherlands' National Institute for Public Health and the Environment recently conducted a safety assessment on synephrine-containing products but could not establish a fixed maximum safe dosage (Biesterbos et al., 2019). The Dutch authorities have a similar view to the German BfR, where sports supplements, such as PWS, carried an elevated risk to their consumer. Moreover, since the target market was likely individuals undergoing physical exertion, the added strain on the cardiovascular system was higher. As mentioned, adverse effects from the use of weight-loss

supplements containing *C. aurantium* could be amplified and pose a greater risk for an overweight, ageing population with likely pre-existing cardiovascular problems.

1.3 VASCULAR SYSTEM

The cardiovascular system is a dynamic and well-organised system in which signal transduction plays a critical physiological and pathophysiological role (Touyz et al., 2018). The cellular elements of the heart and vasculature have an array of specific receptors with complex intracellular responses that react to external stimuli. Since cardiovascular effects are one of the main concerns surrounding pre-workout supplement use, it is essential to understand their action on the molecular targets of some of the main components in *C. aurantium*.

Arterial tone modulates the perfusion of vital organs and is regulated by various neurohumoral ligands and their receptors on the vascular smooth muscle or endothelial cells (Welsh & Longden, 2017). The arterial system is comprised of a hierarchy of vessels ranging from large diameter elastic arteries to smaller muscular arteries and arterioles. The arteries close to the heart have the most significant percentage of elastic fibres throughout its tunic layers and are known as elastic arteries (or conduit arteries, Figure 4). Examples of conduit arteries are the aorta, pulmonary trunk, and common iliac arteries, usually larger than 10 mm in diameter in humans (White, 2021). The abundance of elastic collagen fibres allows the vessel to expand and recoil in response to increased cardiac output. As the vessel extends further away from the heart, the percentage of elastic fibres in the tunica intima decreases, and the percentage of vascular smooth muscle increases. These medium-sized arteries are described as muscular arteries (or distributing vessels), typically ranging from 0.41 mm to 10 mm in diameter and delivering blood to small resistance vessels. The thick tunica media allows for precise control of blood vessel diameter, and the vasoconstriction of these arteries plays a significant role in blood pressure regulation (Leloup et al., 2015). Vascular smooth muscle cells have varying distributions of adrenergic receptors (adrenoceptors) that contribute to the contraction and relaxation of arteries

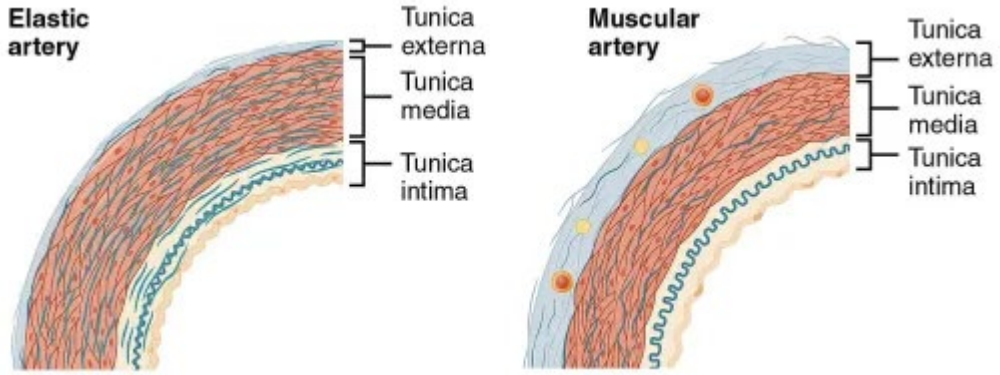


Figure 4: ***Blood vessel structure differences between an elastic artery (left) and a muscular artery (right). The walls of the blood vessels consist of three layers: the tunica intima, the tunica media, and the tunica adventitia. Image re-produced from Betts et al., 2013, Anatomy and Physiology, OpenStax CNX under Creative Commons Attribution license.***

1.3.1 Role of the adrenergic system in vascular smooth muscle contraction

The adrenergic nervous system is one of the main neurohormonal systems that regulate cardiovascular function and vascular tone (Santulli & Iaccarino, 2016). Adrenoceptors belong to the guanine nucleotide-binding G protein-coupled receptor (GPCR) superfamily. Generally, GPCRs consist of one extracellular N-terminal domain, ligand binding site, seven transmembrane (α-helical) domains, three intra- and three extracellular loops, and one intracellular C-terminal domain (Manglik & Kruse, 2017).

There are two classes of adrenoceptors: α-adrenoceptors and β-adrenoceptors, which have distinct effects due to their tissue distribution and intracellular signalling components (Figure 5). Heteromeric G proteins, coupled to the C-terminal (intracellular) receptor, contain alpha, beta, and gamma subunits and can be subdivided into four families according to their alpha subunits. G_s (stimulatory) and G_i (inhibitory) regulate adenylyl-cyclase activity leading to increased or decreased cyclic AMP (cAMP) levels and smooth muscle relaxation or contraction, respectively. Alternatively, G_q proteins activate phospholipase C leading to increased intracellular calcium release and smooth muscle contraction, while $G_{12/13}$ can activate small GTPase families (Kamato et al., 2015).

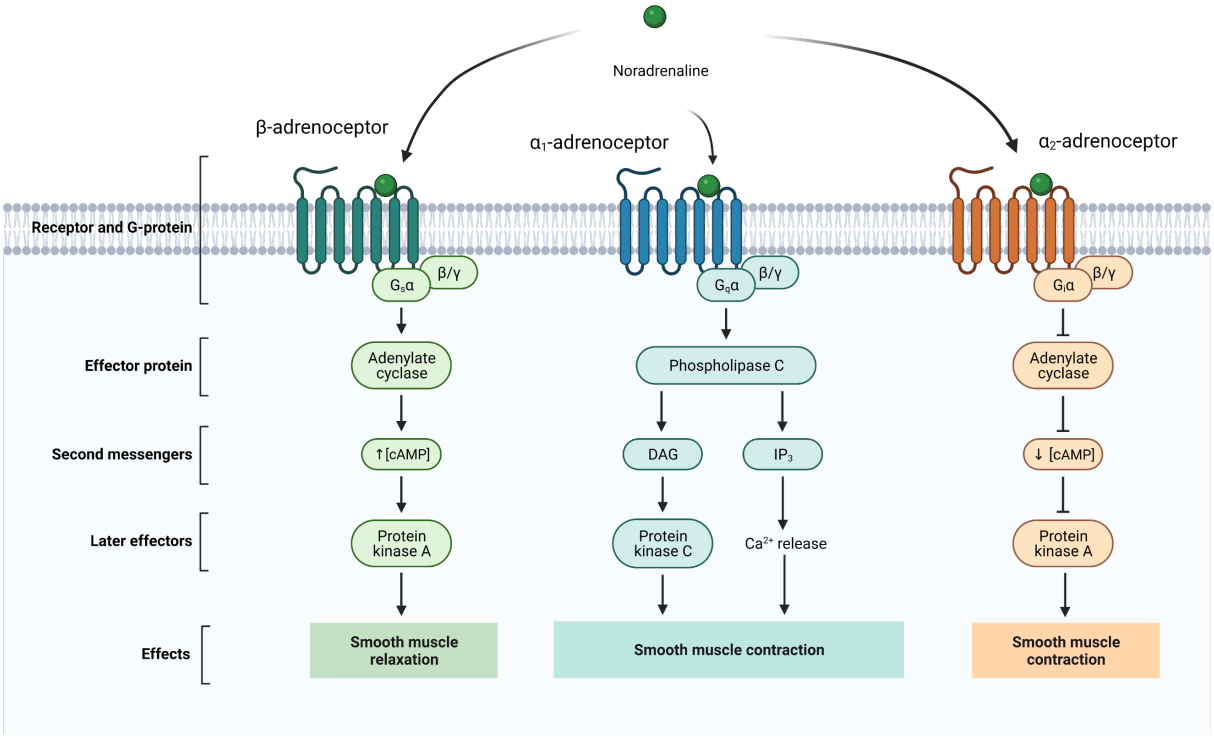


Figure 5: The mechanism of action of adrenergic receptors. Second messengers, inositol-1,4,5-triphosphate (IP3); diacylglycerol (DAG); cyclic adenosine monophosphate (cAMP). Stimulatory pathways (→); inhibitory pathways (⊣); Increased concentration (↑); decreased concentration (↓) Created by Author using BioRender

The α-adrenoceptor subtypes are classified by their anatomical locations (Langer, 1980; Timmermans and van Zwieten, 1981) and the relative affinities of agonists and antagonists (Alexander et al., 2011). The α-adrenoceptor are subdivided into two subfamilies in which α_1-adrenoceptors (G_q coupled receptors) are mostly found post-synaptically on vascular smooth muscle cells and mediate vasoconstriction in certain blood vessels (Rudner et al., 1999). Pre-synaptic α_2-adrenoceptors (G_i-coupled receptors) activation decreases endogenous noradrenaline release during nerve stimulation (Shepperson et al., 1982), whereas post-synaptic α_2-adrenoceptor activation inhibits adenylyl cyclase, which facilitates smooth muscle contraction.

The trace amines, synephrine, octopamine, and tyramine, found in *C. aurantium* have been shown to mediate vasoconstrictor responses by either an indirect or direct mechanism of actions predominantly on α_1-adrenoceptors (Section 1.6). Therefore, it is vital to establish the various distribution of α_1-adrenoceptors and their subtypes present in the vasculature. Although the vascular system expresses all three α_1-adrenoceptor subtypes, specific subtypes are predominant in different arteries. The three subtypes of α_1-adrenoceptors (α_{1A}, α_{1B}, α_{1D}) have been identified in functional and molecular biological studies (Table 1).

Table 1: Table of α_1-adrenoceptor distribution or expression in vasculature in different species

α_1-Adrenoceptor subtype	**Vascular tissue distribution or expression**	**Species**
α_1A	Resistance arteries (Salomonsson et al., 2001)	Rat
	Mesenteric arteries (Nourian et al., 2008)	Rat
	Gastroepiploic artery (Han et al., 2003)	Human
	Subcutaneous arteries (Jarajapu et al., 2001)	Human
α_1B	Aorta, caudal, femoral, illiac, renal and mesenteric arteries (Piascik et al., 1997)	Rat
	Somatic arteries (Price et al., 1994)	Human
	Carotid artery (Zhang et al., 2004)	Mouse
α_1D	Aorta (Leech & Faber, 1996)	Rat
	Femoral artery (Hrometz et al., 1999)	Rat
	Carotid artery (de Andrade et al., 2006)	Rat
	Epicardial coronary arteries (Jensen et al., 2009)	Human
	Aorta (Rudner et al., 1999)	Human
	Renal artery (Cohn et al., 2008)	Mouse
	Coronary artery (Chalothorn et al., 2003)	Mouse
	Femoral artery (Zacharia et al., 2005)	Mouse

The α_{1A}-adrenoceptors are often found in mesenteric arteries (Salomonsson et al., 2001) and renal arteries (Hrometz et al., 1999). In smaller resistance arteries, the α_{1A}-adrenoceptors activation results in rapid vascular adjustments resulting from autonomic activation (Stassen et al., 1998). On the other hand, the α_{1D}-adrenoceptor subtype is more commonly found in larger arteries such as the aorta, carotid, and coronary arteries (Jensen et al., 2009; Leech & Faber, 1996; Rudner et al., 1999). The activation of α_{1D}-adrenoceptor in these arteries has a longer-acting vasoconstrictive effect that continues even after agonist removal, preventing sudden changes in vessel

diameter during variations in circulating levels of catecholamines (Flacco et al., 2013; Gisbert et al., 2000). However, the α_{1B}-adrenoceptor subtype appears to be distributed in multiple arteries (Piascik et al., 1997), but knockout mice studies have shown that α_{1B}-adrenoceptors play a minimal role in vascular homeostasis (Cavalli et al., 1997).

A few studies suggest that the trace amines could mediate their effects via β-adrenoceptors (Section 1.6). There are three subtypes of the β-adrenoceptor family (β_1-, β_2-, & β_3-subtypes). All β-adrenoceptor subtypes positively coupled to adenylate cyclase via activation of G_s protein, which increases cellular levels of cAMP. The increase in cAMP is usually associated with vascular smooth muscle relaxation (Tanaka et al., 2005). However, β-adrenoceptors are not limited to just the vascular smooth muscle of blood vessels, as β_1- and β_2-adrenoceptors have been characterised in all three layers of blood vessels as evidenced by the presence of β-adrenoceptor expression in the coronary endothelial cells (Stephenson & Summers, 1987), adventitia, perivascular nerves, and the coronary tunica media of the dog (Molenaar et al., 1988).

β_3-adrenoceptors are found in a variety of human adipose tissues (white, brown, and beige) (Lelias et al., 1993; Weyer et al., 1999), human heart (Krief et al., 2005) and endothelium of coronary arteries (Dessy et al., 2004). The activation of the β_3-adrenoceptor has been associated with vascular smooth muscle dilation on human coronary arteries (Dessy et al., 2004), rat aortae (Matsushita et al., 2003) and a reduced inotropic effect of the human heart (Gauthier et al, 2000). β-adrenoceptor activation in the cardiac muscle differs from vascular smooth muscle where cAMP activates a cAMP-dependant protein kinase that phosphorylates L-type calcium channels, increasing intracellular Ca^{2+}. The increased calcium concentration enhances the release of calcium from the sarcoplasmic reticulum. The intracellular calcium binds to troponin-C, which enhances cardiac contractile force (inotropy) and increases heart rate (chronotropy) (Figure 6).

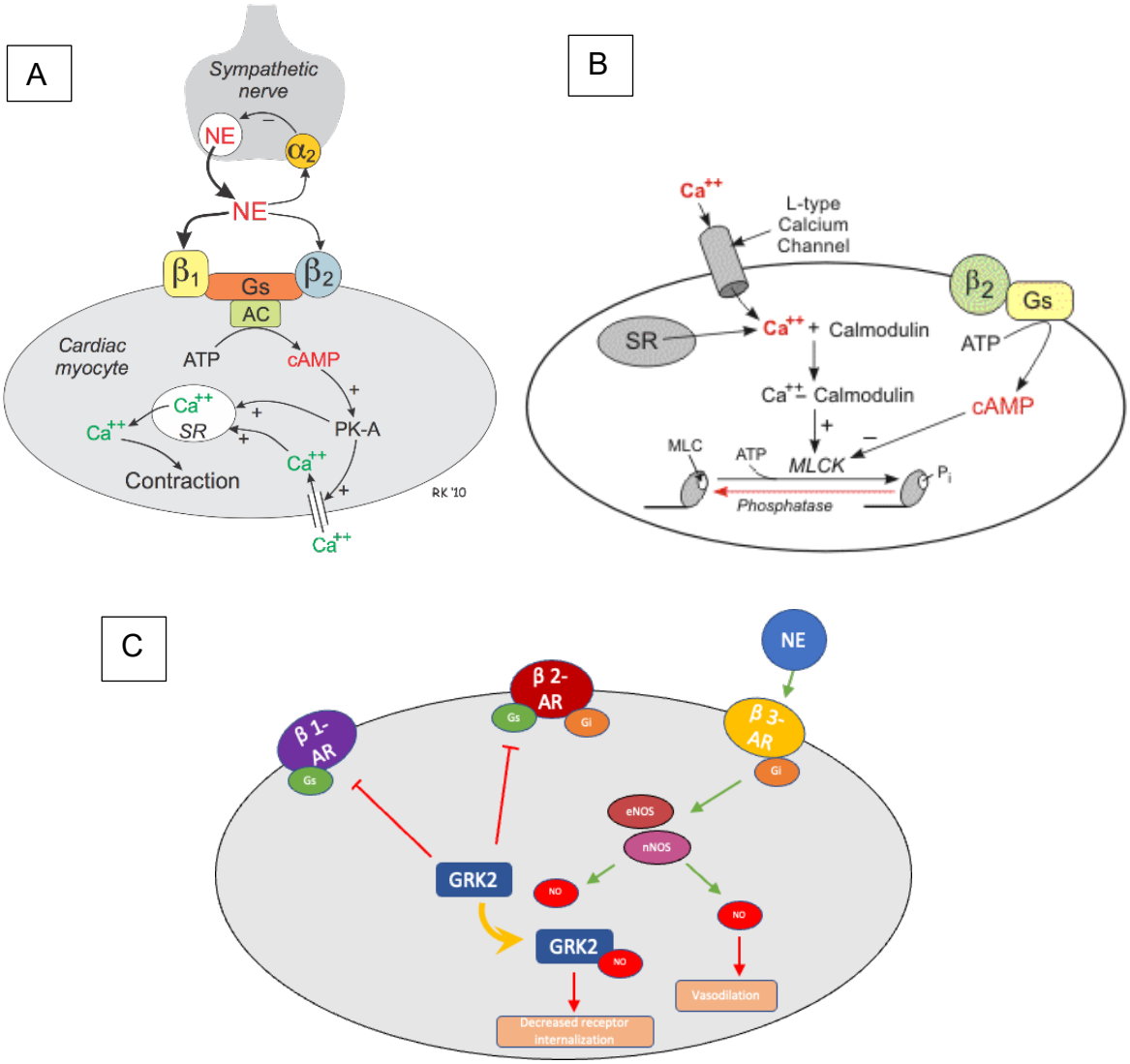


Figure 6: The effect of β-adrenoceptor stimulation on cardiac muscle (A); vascular smooth muscle (B); and the effect of β3-adrenoceptor activation on nitric oxide (c). Abbreviations: NE, noradrenaline; NO, nitric oxide; Gs, G-stimulatory protein; AC, adenylyl cyclase; PK-A, cAMP-dependent protein kinase; SR, sarcoplasmic reticulum; MLC, myosin light chain; GRK2, G protein-coupled receptor kinase. Image A and B were re-produced with permissions from Klabunde, 2012, β-adrenoceptor agonists, CVPhysiology.

1.3.2 TRACE AMINE-ASSOCIATED RECEPTOR ON VASCULAR SIGNALLING

The classical biogenic amines (adrenaline, noradrenaline, serotonin, histamine, and dopamine) are distinct from *p*-tyramine, β-phenylethylamine, tryptamine, and octopamine. The latter group of amines are that present at nanomolar levels in mammals and are called trace amines. The structural and chemical comparisons of these amines are elaborated in Section 1.4.

Trace amine receptors (TA) are rhodopsin-like G-protein-coupled receptors first discovered in 2001 by two separate groups searching for novel 5-HT_1-like and catecholamine receptors by genomic screening (Borowsky et al., 2001; Bunzow et al., 2001). Later, other GPCRs with similar sequences to TA1 and TA2 subtypes were discovered but did not bind to the typical endogenous trace amines (tyramine or β-phenethylamines) (Lindemann et al., 2005). These atypical receptors were later re-classified as trace amine-associated receptors (TAARs) where other endogenous ligands may be involved. For example, trace amine-associated receptor 1 (TAAR1) was classified according to its affinity for the endogenous trace amines, tyramine, β-phenethylamine, and octopamine in addition to dopamine.

TAARs are a family of vertebrate, rhodopsin-like GPCRs that has nine sub-families (TAAR1-9) (Lindermann et al., 2005; Borowsky et al., 2001; and Grandy, 2007). Within these nine sub-families, there is a wide range of TAAR distribution that varies between species. Humans express functional variants of 6 of the TAAR family members (TAAR1, 2, 5, 6, 8 and 9), which are predominantly found in the limbic and olfactory system (Berry et al., 2017). From these sub-families, four TAAR subtypes have been expressed in the heart, namely TAAR 1, 2, 4, and 8. However, the functional roles of the subtypes on the vasculature have not yet been classified.

TAAR1 expression has been reported in mice (Borowsky et al., 2001; Grandy, 2007), rats (Bunzow et al., 2001), and rhesus monkey brains (Xie et al., 2008). Initial studies with RT-PCR showed TAAR1 is expressed in mouse central nervous system regions such as the amygdala, cerebellum, hippocampus, dorsal root ganglia, and hypothalamus (Borowsky et al., 2001). Further investigations using *in* situ hybridization identified TAAR1 mRNA levels in several monoaminergic regions such as the substantia nigra, locus coeruleus, and dorsal raphe nucleus (Borowsky et al., 2001).

Currently, several studies have focused on the expression and functional role of TAAR1 on the mesolimbic system (Gainetdinov et al., 2018) but not in the vascular system.

The possible role of trace amines and amphetamines mediating contractile effects on vascular tissues via TAAR was pioneered by the Broadley laboratory (Broadley, 2010; Broadley et al., 2013; Fehler et al., 2010; Anwar et al., 2012; Herbert et al., 2008). The pharmacological effects of trace amines are attributed to indirectly acting sympathomimetic activity, where endogenous noradrenaline is released from sympathetic neurons. In the vasculature, vasoconstrictor effects are produced from the activation of post-junctional α_1-adrenoceptors (Broadley, 1996). However, this mechanism was suggested to be an oversimplification of the typical responses to trace amines as it did not fully explain the remnant responses to tyramine in rabbit (Hudgins and Fleming, 1966) and rat aorta (Maling et al., 1997) in the absence of endogenous noradrenaline. Furthermore, contractile responses to trace amines in isolated rat aorta (Fehler et al., 2010) and pig coronary arteries (Herbert et al., 2008) were not affected by the α_1-adrenoceptor blockade or cocaine-sensitive neuronal uptake. Hence, the possibility of the vasoconstriction being mediated by trace amine-associated receptors was hypothesised.

An important study by Fehler et al. (2010) showed evidence that TAAR1 and TAAR4 proteins were expressed in the aorta of rats. Moreover, the mRNA of TAAR1 was confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR). Additionally, the study reported that contractile responses to β-phenethylamine were not affected α_1- or β-adrenoceptor blockade and was resistant to the noradrenaline uptake inhibitor, cocaine. The authors hypothesised that TAAR1 mediated the contractile effects of β-phenethylamine and other trace amines, but without a selective antagonist, further investigations into the involvement of TAAR1 on the rat aorta were not possible. To date, there have been no further investigations into the possible role of TAAR in mediating vascular effects.

1.4 CHEMICAL STRUCTURE OF TRACE AMINES AND STRUCTURE-ACTIVITY RELATIONSHIP WITH ADRENOCEPTORS

Since the effects of PWS are purported to be mediated through adrenoreceptor activation, this section is devoted to considering structural differences between the classical catecholamines and trace amines, synephrine, octopamine, and tyramine, found in *C. aurantium*-containing PWS, which underpin their different affinities and activity. Trace amines are substituted phenethylamines where seemingly minor variations in structure result in different chemical and physical properties (Table 2, Figure 7)

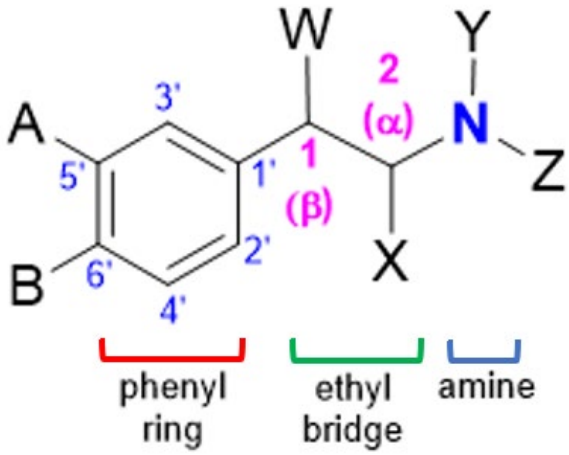


Figure 7: β-phenethylamine structural backbone and the various positions for substation

The similarity in structure between the trace amines to amphetamine and ephedrine 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). The trace amines and catecholamines share a structural motif with a β-phenethylamine backbone that consists of a phenyl-ring, ethyl-bridge, and an amine group. The chemical additions or substitutions of β-phenethylamine influence the mechanism of action, receptor selectivity, and their absorption, metabolism, and duration of action.

For direct-acting sympathomimetic amines, maximal activity is seen in β-phenethylamine derivatives that contain (a) a catechol (where A & B are hydroxyl groups) and (b) a (β)-OH substitution on the ethylamine portion of the molecule (Ruffolo et al., 1983). Such structural features are seen in the catecholamines such as noradrenaline and adrenaline (Table 2). The trace amines, tyramine, octopamine, and synephrine lacks a catechol group but exists with the hydroxyl group located on the para-position (position β in Figure 7) on the phenyl ring (Mattoli et al., 2005; Pellati et al., 2005; Rossato et al., 2010).

Maximal α- and β-adrenoceptor activity depends on the presence of the 5' and 6' hydroxyl groups on the aromatic ring and 1β-OH on the ethyl-bridge, such as catecholamines. Compounds with one or both OH groups are not metabolized by catechol methyl transferase (COMT) and have a longer duration of action. On the other hand, the loss of both aromatic OH groups would generally lead to a compound to act by causing noradrenaline release from sympathetic nerve terminals and more significant CNS activity such as amphetamine.

Substitutions on the amine group determine α- or β-adrenoceptor selectivity. Both primary and secondary amines are more selective for adrenoceptor binding than tertiary amines or quaternary ammonium salts. However, the length and size of the amine (Y or Z) substitutions increase selectivity for β-adrenoceptor activation. For example, adrenaline is more selective for β-adrenoceptor compared to noradrenaline. Of the three trace amines of interest, only synephrine contains a methyl group in position Y (or Z), which indicates its potential for β-adrenoceptor activity. This explains synephrine's reported activity on β_3-adrenoceptors over tyramine or octopamine (Takagi et al., 2018).

Optical isomerism of substituted hydroxyl groups on the ethyl-bridge plays an important role in the interaction of adrenergic agonists. Based on the Easson-Stedman hypothesis, The R-(-)-configuration at position (1β) on the ethyl bridge is typically more potent than the 1S-counterpart of phenylethylamine-derived agonists (Ruffolo et al., 1983). Naturally derived synephrine and octopamine have been reported to exist predominately with the 1R-configuration of the β-OH group (Pellati et al., 2005; Tanaka et al., 2019). Indeed, the R-enantiomers of *p*-synephrine and *p*-octopamine were shown to be 1-2 orders of magnitude less potent than the S-enantiomers on α_1-adrenoceptors found on the rat aortae (Brown et al., 1988).

Given the structure-activity relationship so described above, it could be predicted that synephrine and octopamine would act as direct α-adrenoceptor agonists on the vasculature. In contrast, tyramine would be less likely given the loss of the β-carbon hydroxyl group. Nonetheless, experimental data on the functional assays conducted for these amines will be elaborated on in section 1.6 of this chapter.

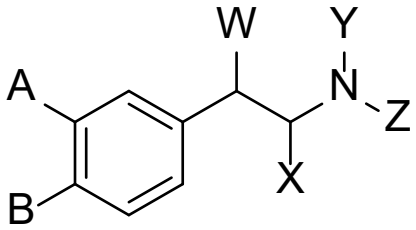


Table 2 Chemical properties of different compounds with a phenethylamine backbone. Chemical data obtained from (Wishart et al., 2018)

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	$C_9H_{13}NO_2$	167.1	H	OH	OH	H	CH_3	H	-0.45	9.76	52.5	3	3	3	1
Phenylephrine	$C_9H_{13}NO_2$	167.1	OH	H	OH	H	CH_3	H	-0.31	8.97	52.5	3	3	3	1
Tyramine	$C_8H_{11}NO$	137.2	H	OH	H	H	H	H	0.86	10.41	46.20	2	3	3	0
Octopamine	$C_8H_{11}NO_2$	153.2	H	OH	OH	H	H	H	-0.59	9.64	66.48	2	3	3	1
Adrenaline	$C_9H_{13}NO_3$	183.2	OH	OH	OH	H	H	H	-0.54	8.55	72.72	3	4	4	1
Nor-adrenaline	$C_9H_{13}NO_3$	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

1.5 PHARMACOKINETICS OF SYNEPHRINE, OCTOPAMINE, AND TYRAMINE

The biosynthetic pathway for trace amines, synephrine, octopamine and tyramine begins with the aromatic amino acid, L-tyrosine and has been of interest because they participate in the synthesis of the catecholamines, dopamine, noradrenaline, and adrenaline (Grandy, 2007) (Figure 8).

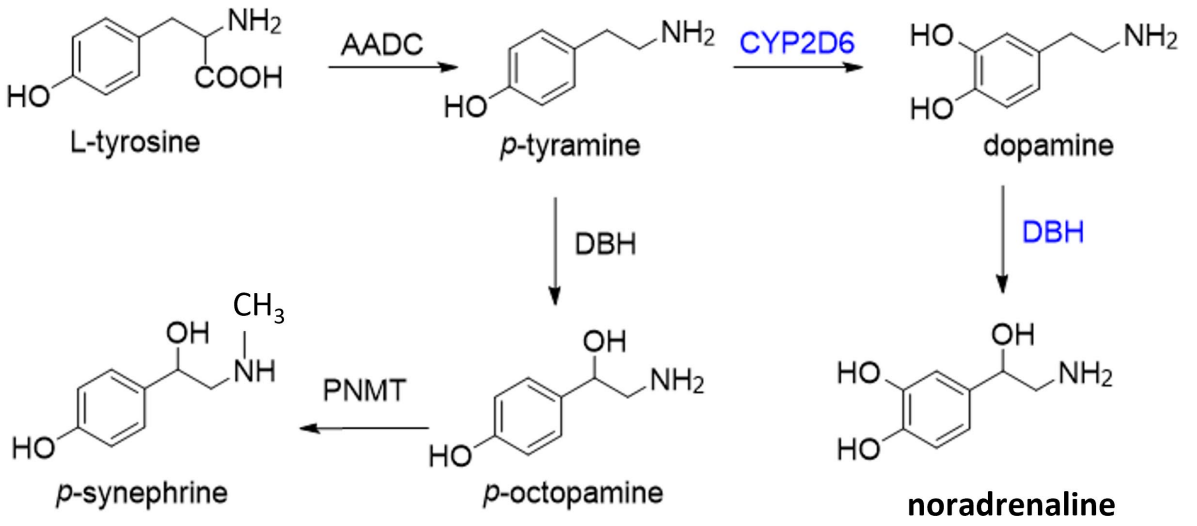


Figure 8: Biosynthetic pathway of trace amines and catecholamines

Trace amines and catecholamines share a similar biosynthetic pathway and are metabolised by monoamine oxidases (MAO), which deaminates primary and secondary amines (Broadley, 2010). Trace amines are found throughout the central nervous system, but the endogenous levels of trace amines are several hundred-fold below catecholamines (Berry, 2004). Even though the rate of synthesis of trace amines is similar to dopamine and noradrenaline(Durden & Philips, 1980; Paterson et al., 1990), the high turnover rate of trace amines by MAO results in low nanomolar tissue concentrations of trace amines (Berry, 2004). Plasma levels of the trace amines have been found to vary from 2.5 to 7.5 ng/mL for tyramine, from 0.9 to 14 ng/mL for synephrine, and from 0.6 to 6.9 ng/mL for octopamine (D'Andrea et al, 2003).

MAO catalyses the oxidative deamination of a wide variety of monoamines (exogenous and endogenous) with significant roles in metabolising neurotransmitters and detoxification. It exhibits two subtypes, A and B, with differing substrate specificity for the various neurotransmitters, although both MAO-A and MAO-B can metabolize dopamine and tyramine.

MAO inhibitors (MAOI), used to treat depression and nervous system disorders, prevent inactivation of the transmitters displaced from the vesicles within the terminal. MAO inhibition enhances the action of dietary tyramine, for example, as the gastrointestinal tract and liver MAO generally degrade it before entering the systemic circulation. Inhibition may be specific to one isoform and reversible or irreversible. The dietary tyramine-provoked hypertensive crisis is a well-known interaction associated with irreversible MAO-A inhibition (Chen et al., 2007). Similarly, The use of MAO-inhibitors would impair the metabolism of synephrine and potentially cause hypertension due to its prolonged vasoconstriction (Grandy, 2007).

The oxidation of synephrine and octopamine by MAO results in the production of the aldehyde, *p*-hydroxymandelic acid, and hydrogen peroxide, which could result in oxidative stress by the production of reactive oxygen species (Grandy, 2007) (Figure 9**Error! Reference source not found.**). This fact has drawn attention to the role of MAO products as neurotoxins and implicated in the development of neurodegenerative disease. Further degradation of *p*-hydroxymandelic acid by aldehyde dehydrogenase results in glycols and carboxylic acids.

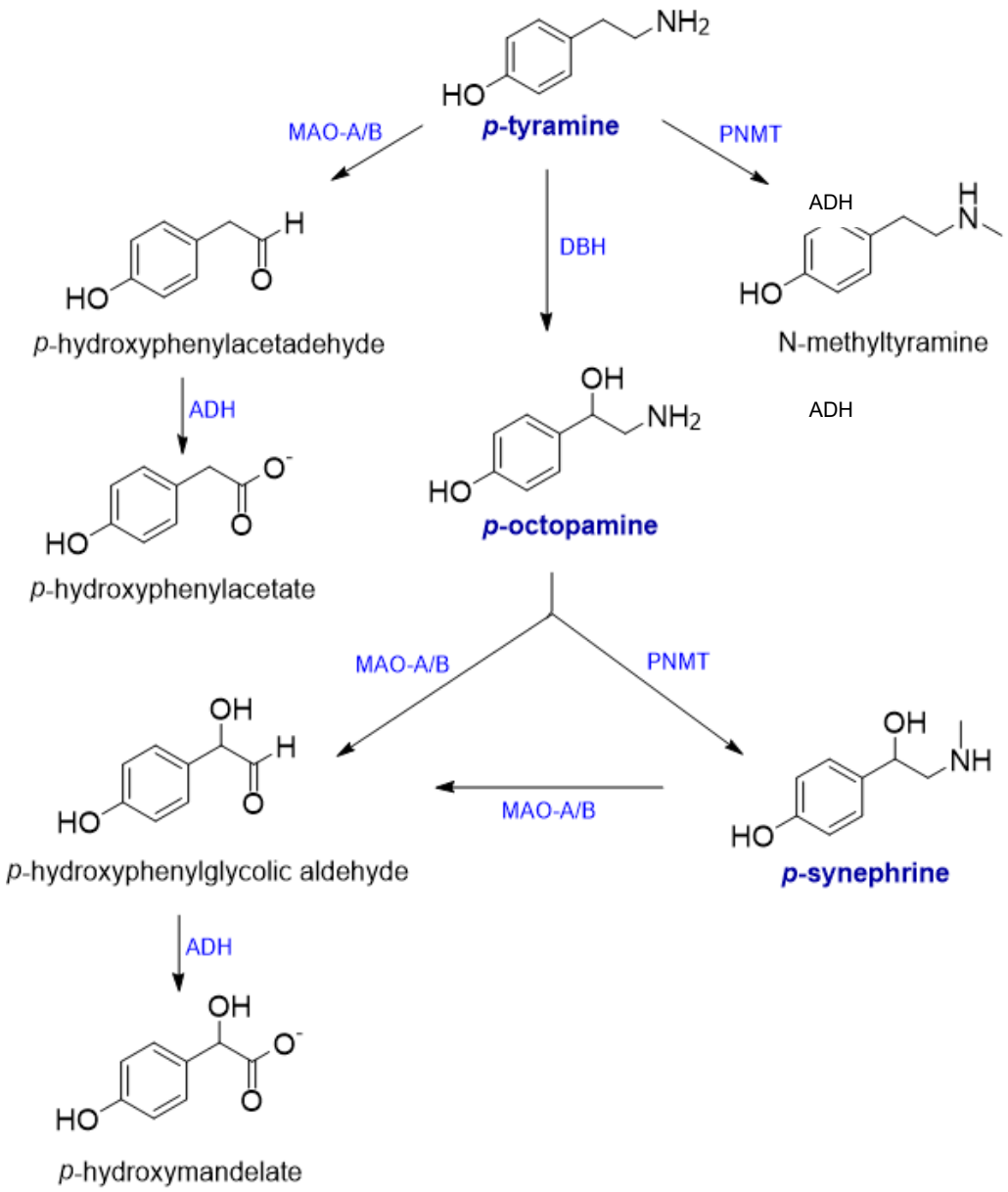


Figure 9: The degradation pathway of trace amines in humans. MAO represents the monoamine oxidases; ADH represents the aldehyde dehydrogenase; PNMT represents the phenyl-ethanolamine-N-methyl transferase

A study in 1983 that investigated the composition of urine after oral ingestion of synephrine found 47% sulphated synephrine, 30% hydroxymandelic acid, 12% conjugated synephrine 6% hydroxyphenyl glycol 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 et al., 1996).

In-vitro studies have shown that *p*-synephrine isomers are taken up by Caco-2 cells, suggesting that they can be absorbed by the gastrointestinal tract (Rossato et al., 2010). However, poor bioavailability of *p*-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). Thevis et al. (2012) showed that orally administered 150 mg synephrine showed a low peak urine concentration of 142 µg/mL after 4 hours in healthy adults. No urine concentration of octopamine was detected from the ingestion of synephrine. Therefore, the amines synephrine and octopamine were subject to rapid first-pass metabolism.

C. aurantium extracts contain flavonoids, specifically 6',7'-di-hydroxy-bergamotin, a cytochrome P4503A4 (CYP3A4) selective antagonist (Fugh-Berman & Myers, 2004). As CYP3A4 metabolises a majority of existing drugs, such as MAOI (anti-depressants), it is not advisable to take *C. aurantium*-containing PWS if taking prescription MAOIs (Fugh-Berman & Myers, 2004).

1.6 OBSERVED EFFECTS OF SYNEPHRINE, OCTOPAMINE AND TYRAMINE AND THEIR POSSIBLE MECHANISMS OF ACTION

1.6.1 SYNEPHRINE

Synephrine is a trace amine found naturally in the body or can enter the body after consuming most citrus or citrus-containing products. Endogenously, synephrine is a product of tyrosine metabolism and is present in trace amounts (3 ng/mL) in healthy adults (D'Andrea et al., 2017). In most citrus plants, synephrine is present in various amounts ranging from 8.8 to 77.5 mg/g (Nelson et al., 2007). Synephrine is a chiral compound that exists predominantly as the R-(-)-isomer in most plants (Pellati & Benvenuti, 2007). Although there is no record of a predominant enantiomer in humans, one enantiomer would likely predominate during endogenous biosynthesis in a similar way to that seen in plants due to stereoselectivity (Finefield et al., 2012). Orally ingested synephrine has a 22% bioavailability with a two-hour biological half-life in humans (Hengstmann & Aulepp, 1978). Synephrine is the active component of oxedrine, used in Europe as a drug to treat hypotension. The Australian government listed oxedrine as a Schedule 4 drug in 2002 with a recommended daily dose of 30 mg (National Drugs and Poisons Schedule Committee, 2003).

EXPERIMENTAL OBSERVATIONS OF SYNEPHRINE-RELATED EFFECTS

I) Animal studies

In vivo animal studies on rats and mice have been used to evaluate the systemic effects of synephrine as a weight-loss agent or on the cardiovascular system. Most of the studies used *C. aurantium* as the source of synephrine, while some investigated the effects of pure synephrine in rodents (Table 3). This sub-section summarises the literature on the cardiovascular effects of synephrine, some central nervous system effects and signs of toxicity induced by synephrine either alone or in the form of *C. aurantium* extract. The effects on the cardiovascular system are the focus of this thesis.

a) Cardiovascular effects

In rats, pure synephrine affects blood pressure but not heart rate in a dose dependent manner. Intravenously administered synephrine (0.095, 0.19, 0.38 and 1 mg/kg/min) has been shown to dose-dependently increase mean arterial pressure but did not affect heart rate in spontaneously hypertensive rats (Huang et al., 1995). A similar observation was observed in healthy rats that were given 10 mg/kg and 50mg/kg of synephrine via oral gavage (Hansen et al., 2012; Hansen et al., 2013). However, there were significant increases in heart rate when synephrine was administered in the form of C. aurantium extract or in the presence of caffeine. Hence, this suggest that the chronotropic effects were from synergistic interactions between the trace amines found in C. aurantium and caffeine rather than pure synephrine.

Synephrine, administered in the form of *C. aurantium* extracts, had a more significant increase in blood pressure and heart rate than pure-synephrine. Repeated oral doses of *C. aurantium* extracts (dosed to 10 and 50 mg/kg synephrine) had a more significant dose-dependent increase in heart rate and blood pressure than pure synephrine in healthy rats (*n* = 16/group) (Hansen et al., 2012; 2013). However, no change in heart rate or blood pressure were observed when rats (*n* = 6/group) were given oral doses of *C. aurantium* extract (dosed to 0.8 and 1.2 mg/kg synephrine) (Calapai et al.,1999). Rats that were given *C. aurantium* extracts had electrocardiogram (ECG) abnormalities, which were not observed in the studies with pure synephrine. These observations suggest that the greater pressor response was from synephrine activity but ECG alterations were likely due to a synergistic effect of other phenethylamines found in *C. aurantium* extracts such as octopamine and tyramine.

The pressor effects of either synephrine or *C. aurantium* extract were further enhanced when caffeine (25 mg/kg) was co-administered in normal or exercised rats (Hansen et al., 2012; 2013). This was expected as caffeine is an adenosine receptor (A_{1}/A_{2A}) antagonist. Adenosine typically inhibits the release of catecholamines from the synapse, and its inhibition by caffeine increases catecholamine concentrations that act on post-junctional receptors that mediate vasoconstriction and thus causing an increase in blood pressure (Nehlig et al., 1992). The typical physiological response to exercise is increased blood flow and decreased peripheral resistance to enhance cardiac output, increasing systolic blood pressure (Silva and Zanesco, 2010).

It is important to note when interpreting *in vivo* animal experiments, and it is not appropriate to scale the dose (mg/kg) given to animals directly to humans (Nair & Jacob, 2016) as is the case for synephrine in several reviews concerning the safety of *C. aurantium* (Stohs, 2017; Stohs et al., 2020). This is because the pharmacokinetic properties, body surface area and functional systems are species-dependent. Information on translating doses between species for appropriate doses for clinical trials has been outlined by Nair and Jacob (2016). The authors of the study demonstrated sample calculations by allometric scaling for estimating the Human Equivalent Dose (HED) are as follows:

$$\text{HED (mg/kg)} = \text{Animal dose (mg/kg)} \times (\text{Animal } K_m / \text{ Human } K_m)$$

*K_m = correction factor for each species derived from dividing the average body weight (kg) of species to its body surface area (m^2). The Human K_m = 37 kg/m^2

For example, the cardiovascular effects in rats (K_m = 6 kg/m^2) were observed when 10 mg/kg of pure synephrine was administered. Therefore, the HED that is appropriately scaled is 1.62 mg/kg synephrine. A seventy-kilogram person would need to ingest a minimum of 113.5 mg dose of pure synephrine to experience similar cardiovascular effects. However, cardiovascular effects have been observed at lower doses of 49 mg of synephrine in humans (section 1.61.ii.a).

Table 3 Summary of in vivo animal studies with synephrine

Species/ Study type	Amount of synephrine or *C. aurantium* extract (mg/kg body weight)	Cardiovascular effects		Systemic effects	Proposed mechanism of action	Author
		Heart rate	Blood Pressure			
Cardiovascular						
Spontaneously hypertensive rats (Sprague-Dawley)	0.095, 0.19 and 0.38 (pure synephrine)	No change	increased	Increased mean arterial pressure and systemic vascular resistance in hypertensive rats	α- adrenergic	Huang et al. (1995)
Rats (Sprague-Dawley)	2.5, 5.0, 10 and 20 (*C. aurantium* extract)	No change	No change	Enlarged QRS complex on ECG	β_2- Adrenergic	Calapai et al. (1999)
Spontaneously hypertensive rats (Sprague-Dawley)	1 mg (pure synephrine)	No change	increased	Increased mean arterial pressure and systemic vascular resistance in hypertensive rats	α- adrenergic	Huang et al. (2001)
Rats (Sprague-Dawley)	10 & 25 (pure synephrine); 10 & 25 (*C. aurantium* extract); 25 (caffeine)	Increased	Increased	Strong effects for the first 7 out of 28 days which weakened over time	α- adrenergic	Hansen et al. (2012)
Rats (Sprague-Dawley)	10 & 50 (pure synephrine); 10 & 50 (*C. aurantium* extract); 25 (caffeine)	Increased	Increased	Increase in blood pressure for up to 8 hours; blood pressure increased further with the caffeine treated rats	α- adrenergic	Hansen et al. (2013)
Rats (Sprague-Dawley)	1, 3.2, 5.6, 10 (*C. aurantium*); 2, 6.4, 20 (Rhodiola rosea)	Increased	Increased	Decrease in weight gain when rats ingested a combination of *C. aurantium* and Rhodiola Rosea	Indirect release of noradrenaline on adrenergic system	Verpeut et al. (2013)
Toxicity						
Rats (Sprague-Dawley)	0.3, 1.0, 3.0 (S-(+)-synephrine or R-(-)-synephrine)	Not recorded	Not recorded	Dose-dependent anti-depressant activity	In direct release of noradrenaline on adrenergic system	Kim et al.(2001)

Mice (Albino CF1)	18, 30, 60, 150, 210, 300 (*C. aurantium* extract); 150, 300, 450 (pure synephrine)	Not recorded	Not recorded	The mice showed signs of gasping, piloerection and salivation with decreased spontaneous locomotor activity	α- adrenergic	Arbo et al. (2008)
Mice (Albino CF1)	24, 120, 240 (*C. aurantium* extract); 30, 300 (pure synephrine)	Not recorded	Not recorded	The mice showed similar signs of distress coupled with an increase in plasma glutathione levels	α- adrenergic	Arbo et al. (2009)
Pregnant and embryonic rats (Sprague-Dawley)	1.0, 2.5, 5.0, 10, 25 (pure synephrine); 10 & 25 (*C. aurantium extract*)	Not recorded	Not recorded	No developmental toxicities but a decrease in maternal weight	N/A	Hansen et al. (2011)
Mice (Albino CF1)	10: 4: 6: 80 w/w ratio (p-synephrine, ephedrine, salicin and caffeine)	Not recorded	Not recorded	Muscle spasms, tremors and seizures with mice treated with highest dose of 400 mg/kg body weight	α- adrenergic	Schmitt et al. (2012)
Rats (Sprague-Dawley)	125, 250, 500, 1000 (*C. aurantium* extract)	Not recorded	Not recorded	Internal organ damage at highest dose (2000 mg/kg)	α- adrenergic	Deshmukh et al. (2017a)
Rats (Sprague-Dawley)	125, 250, 500, 1000 (*C. aurantium* extract)	Not recorded	Not recorded	Increased heart weight and signs of discomfort at 500 mg/kg and above	α- adrenergic	Deshmukh et al. (2017b)

b) Central nervous system

Synephrine affects the central nervous system by displaying antidepressant-like activities in mice. Orally administered racemic synephrine (1 to 10 mg/kg) produced a dose-dependent antidepressant-like effect by reducing immobility on mice (Song et al., 1996). These effects were similarly observed in another study by Kim et al. (2001), who showed that the acute and repeated administration of S-(+)-synephrine (3 mg/kg/day for seven days) showed a significant decrease in duration of immobility. The authors later showed different effects between the naturally occurring R-(-)-synephrine and synthetic S-(+)-synephrine on depressive behaviour. These results suggest that synephrine could elicit an indirect sympathomimetic response by releasing endogenous noradrenaline, which may cause vasoconstriction in blood vessels. However, there is a paucity of data regarding the potential sympathomimetic response of synephrine.

c) Signs of toxicity

An acute or a sub chronic daily dose of pure synephrine (300 mg/kg) and *C. aurantium* (containing up to 300 mg/kg synephrine) caused acute signs of discomfort, salivation, piloerection, gasping, and exophthalmia in mice (Arbo et al. 2008; 2009) and rats (Deshmukh et al. 2017a; 2017b). The acute side effects were reversed in four hours post-administration in both rodent species, but necropsies showed decreased adrenal gland and thymus mass and internal bleeding in the intestines and the lungs. These side effects were suggested to be attributed to systemic α_1-adrenoceptor activation based on the reduction in adrenal gland mass (Duteil et al., 1990; Kikuchi-Utsumi et al., 2013).

The co-administration of a mixture of synephrine, caffeine, ephedrine and salicin (10:80:4:6, w/w, 400 mg/kg) resulted in similar side effects to those observed by Arbo et al. (2008) but with further muscle spasms, tremors and seizures (Schmitt et al., 2012). Interestingly, there was a significant decrease in body temperature in the rats treated with the mixture of stimulants rather than when synephrine was administered alone. It could be possible that the hyperstimulation of α_2-adrenoceptors from the combination of caffeine and ephedrine caused hypothermia (Bill et al., 1989).

The signs of toxicity of synephrine were observed at high doses (>300 mg/kg) in mice and rats (>500 mg/kg). The human equivalent dose of synephrine toxicity would be 24.3 mg/kg (around 1.7g for a seventy-kilogram person). The Australian government has set the maximum daily dose of synephrine in commercial products at 30 mg/day. This suggests that synephrine, consumed following these guidelines, has a low degree of toxicity in humans.

II) HUMAN STUDIES

Only a few human studies have been investigated the cardiovascular effects of pure synephrine. Most clinical trials investigated synephrine's weight-loss or ergogenic effects in healthy adults where cardiovascular parameters were a secondary outcome (Table 4). Similar to the studies on animals, most human trials evaluated the effects of synephrine as a part of *C. aurantium* extract rather than synephrine alone.

a) Cardiovascular effects

In young, healthy subjects, synephrine alone (3 mg/kg) increased heart rate and blood pressure during exercise (Gutierrez-Hellin et al., 2016a; 2016b). However, synephrine did not significantly affect heart rate or blood pressure when measured one-hour post-ingestion. It is not known if these effects were dose-dependent since a single dose was administered.

There is no clear relationship between synephrine, *C. aurantium* and cardiovascular effects. Some studies showed that a single low dose of 47 mg and 54 mg increased heart rate and blood pressure in healthy subjects (Haller et al., 2005; Bui et al., 2006). However, other studies with doses of synephrine up to 100 mg did not affect heart rate or blood pressure (Gougeon et al., 2005; Junget al., 2017a; Min et al., 2005; Ratamess et al., 2017; Shara et al., 2016; Stohs, Preuss, Keith, et al., 2011; Stohs, Preuss, & Shara, 2011). The effects of synephrine were hard to unequivocally determine from human studies as it was administered as a part of *C. aurantium* extract (standardised to 6% synephrine) or citrus-containing products.

The differences in cardiovascular effects observed in studies using synephrine alone or as part of *C. aurantium* may be due to the variability in study design, where there are methods of evaluating heart rate and blood pressure The method of evaluating heart rate and blood pressure were varied between all these studies as some

measured the effects on an hourly (Ratamess et al., 2016; Ratamess et al., 2015; Ratamess et al., 2018), bi-hourly (Shara et al., 2016) or monthly (Kaats et al., 2013) basis, with no clear rationale or justification of their study design. Shara et al. (2018) measured the heart rate, blood pressure and serum *p*-synephrine levels with a five-day interval but failed to report the baseline measurements as a comparative control. The most extensive (n= 75) study by Kaats et al. (2013) evaluated side effects of synephrine by subjective, self-reported Quality of Life questionnaires that were not designed to detect negative treatment-related symptoms.

Cardiovascular effects were observed less frequently in human studies than in rodent studies. Synephrine has a 22% bioavailability in humans (Hengstmann & Aulepp, 1978), but this has not been determined in rodents. The rate of synephrine metabolism between the two species may result in differences in cardiovascular response. The cardiovascular effects of synephrine in humans were also challenging to interpret due to the variability of study design, lack of a dose-dependent relationship and number of participants. Further well designed, *in vivo* investigations in both animals and humans are needed to elucidate the cardiovascular effects of synephrine in both species.

Table 4 Summary of human studies conducted with synephrine (administered in the form of *C. aurantium*). *Administered in the form of pure p-synephrine.

Author	Number of participants (n)	Amount of synephrine (mg)	Amount of caffeine (mg)	Observed effects	
				Heart rate	Blood pressure
Gougeon et al. (2005)	18	27	0	No significant change	No significant change
Haller et al. (2005)	10	47	0	Increased	Increased
		5.5	239		
Bui et al. (2006)	15	54	0	Increased	Increased
Haller et al. (2008)	10	21	304	Increased	Increased
Seifert et al. (2011)	23	13	176	No significant change	No significant change
Stohs et al. (2011)	50	50	0	No significant change	No significant change
Kaats et al. (2013)	75	49	0	No significant change	No significant change
Sahara et al. (2016)	18	49	0	No significant change	Not recorded
Gutierrez-Hellin et al (2016a)	13	240*	0	Increased	Increased
Gutierrez-Hellin et al (2016b)	12	240*	0	Increased	Increased
Ratamess et al. (2016)	12	100	100	Increased when caffeine was added	Not recorded
Ratamess et al. (2017)	16	100	100	No significant change	Lower diastolic blood pressure in synephrine treated group

Jung et al. (2017)	25	20	284	No significant change	No significant change
Shara et al. (2018)	16	49	0	No significant change	No significant change
Ratamess et al. (2018)	16	104	233	No significant change	Increased systolic and diastolic blood pressure in treated groups
Gutierrez-Hellin et al. (2020)	14	240*	0	No significant change	Slight decrease in mean arterial blood pressure

MECHANISMS OF ACTION OF SYNEPHRINE FROM IN VITRO EXPERIMENTS

Isolated tissue and cellular experiments (Table 5) give a valuable insight into the physiological outcomes (contraction or relaxation) of a drug that may be relevant to the body. Experimental studies have shown that synephrine can mediate its actions via either direct or indirect adrenoceptor activities. This subsection will elaborate on the proposed mechanism of actions of synephrine as either a direct or indirect sympathomimetic.

I) DIRECT AGONIST ACTIVITY

Synephrine was shown to have direct α_1-adrenoceptor activities on isolated rat aorta (Brown et al., 1988; Varma et al., 1995; Hibino et al., 2009). Synephrine (0.1 μM to 100 μM) has been shown to cause dose-dependent vasoconstrictions that were attenuated by prazosin (100nM to 1 μM), an α_1-adrenoceptor selective antagonist. However, the potency of synephrine was 1000-fold less potent than noradrenaline, whereas the positional isomer (*m*-synephrine or phenylephrine) was only 6-fold less potent on the α_1-adrenoceptor on the rat aorta (Brown et al. 1999). Additionally, Brown and colleagues investigated the stereospecificity of the enantiomers and found that R-(-)-synephrine was a more potent α_1- adrenergic agonist than S-(+)-synephrine by an order of magnitude. These studies indicate that (±)-synephrine is a weak but direct α_1-agonist on rat aorta.

Synephrine was also shown to have α_1- and α_2-adrenoceptor activity on isolated rat liver and portal vein. Peixoto et al. (2012) showed that synephrine (200 μM), as a part of *C. aurantium extract,* caused dose-dependent vasoconstriction of the portal vein of isolated rat liver which was attenuated prazosin (10 μM) and α_2-adrenoceptor antagonist, yohimbine (100 μM). A similar study conducted by de Oliveria et al. (2014) showed that synephrine increased perfusion pressure by increasing intracellular Ca^{2+} concentration and was not affected by the non-selective β-adrenoceptor antagonist, propranolol (10 μM) or β_3-adrenoceptor antagonist, SR59230A. These studies show that synephrine may have activities on both α-adrenoceptors present in rat liver.

In functional studies using human cloned α_{1A}-, α_{2A}- and α_{2C}-adrenoceptor, synephrine was shown to bind weakly to α_1-adrenoceptors (Ma et al., 2010). Synephrine was two orders of magnitude less potent than phenylephrine on α_1-adrenoceptors and four orders of magnitude less potent on α_2-adrenoceptors compared to medetomidine.

However, synephrine reversed forskolin-induced cAMP elevation, suggesting that synephrine may antagonise pre-synaptic α_{2A-} and α_{2C}-adrenoceptors. The antagonism of these α_2-adrenoceptors may increase the vasocontractile effect of noradrenaline.

Synephrine showed weak β_1-adrenoceptor agonist activity on isolated guinea pig atria (Chahl, 1972; Jordan et al.,1987) and ventricular muscle (Ledda et al., 1980). Synephrine was less potent than octopamine and more than 10,000-fold less active on β_1-adrenoceptors than noradrenaline in guinea-pig atria (Chahl, 1972; Jordan et al.,1987). In terms of stereoselectivity, the naturally occurring R-(-)-synephrine was more potent than its S-(+)-synephrine counterpart, similar to their action on the α-adrenoceptors (Jordan et al., 1987). Additionally, synephrine (300 μM) restored excitability and contractility in partially depolarised guinea pig ventricular muscle and was attenuated in the presence of β-adrenoceptor antagonists, practolol and sotalol (Ledda et al., 1980). These studies show that synephrine has a weak direct activity on the β_1-adrenoceptors present in these cardiac tissues. However, it was not clear if there was a dose-dependent response to synephrine or if circulating noradrenaline might influence cardiac tissue.

Synephrine was a weak β_2-adrenoceptor agonist on guinea pig trachea, but a direct agonist on cloned β_2-adrenoceptor expressed cells. In the same study by Jordan et al. (1987), synephrine had weak effects on the bronchodilator response of isolated guinea pig trachea. However, this was later supported by Shi et al. (2009), who showed that synephrine had dose-dependent bronco-protective effects on asthma-induced guinea pigs. Additionally, synephrine dose-dependently increased cAMP concentrations in β_2-adrenoceptor-expressing CHO cells. The effects of synephrine were attenuated in the presence of propranolol (10 μM). The discrepancies in these studies may arise from non-specific effects of synephrine on an isolated tissue compared to the effects observed in an overexpressed cell.

Table 5 Summary of isolated animal tissue studies with synephrine

Isolated Tissue Type/ Cells	Observed effects	Proposed mechanism of action	Author
Rat aorta	The authors suggest the rat aorta vasodilatory response to tyramine > amphetamine > ephedrine > synephrine > octopamine. There was no specific binding of [^{3}H] tyramine to the aortic membrane after inhibition of monoamine oxidase. Tyramine and several other phenethylamines produce relaxation of rat aorta, which does not involve any of the known adrenergic receptors	α- adrenergic	Varma et al. (1995)
Rat cortical slices	S-synephrine also inhibits [^{3}H] noradrenaline uptake in rat cerebral cortical slices, whereas r-synephrine stimulates [^{3}H]noradrenaline release from CNS. The stimulatory effect of r-synephrine on [^{3}H] noradrenaline release inhibited nisoxetine, but not tetrodotoxin, and extracellular calcium depletion had no effect.	In the direct release of noradrenaline on the adrenergic system	Kim et al. (2001)
Rat aorta	Vasoconstrictive effects of Goshyuto were blocked by prazosin, $5HT_{1D}$ antagonist and $5HT_{2A}$ antagonist but not $5HT_{1B}$ or propranolol. The active compound was later identified to be synephrine.	α_{1D}-adrenoceptor and $5HT_{1D}$/ $5HT_{2A}$	Hibino et al. (2009a)
Rat aorta	Vasoconstrictive effects of synephrine were blocked by prazosin, $5HT_{1D}$ antagonist and $5HT_{2A}$ antagonist but not $5HT_{1B}$ or propranolol.	α_{1D}-adrenoceptor and $5HT_{1D}$/ $5HT_{2A}$	Hibino et al. (2009b)
Guinea pig trachea	AS component for β_2- signalling is synephrine. Synephrine showed significant spasmolytic effects on acetylcholine chloride-induced contraction in isolated guinea pig trachea and protected against histamine-induced asthma	β_2- Adrenergic	Shi et al. (2009)
Rat liver	Low concentration of synephrine caused increase gluconeogenesis; high concentration synephrine was inhibitory. *P*-synephrine was partially causing the vasoconstrictive response in the isolated rat liver	Adrenergic system	Peixoto et al. (2012)
Rat liver	Synephrine increased hepatic portal pressure in rat liver increase. The effects were reduced in the presence of prazosin	cAMP and Ca2+ release	de Oliveria et al. (2014)
Mammalian fat cells (Dog, pig, rat, guinea pig, human)	Synephrine was only a partial agonist at β_3-adrenoceptors compared to octopamine.	β_3- adrenergic	Carpene et al. (1999)
Chinese Hamster Ovary (CHO) and Human Embryonic Kidney (HEK) cells expressing α-adrenoceptors	Synephrine was two orders of magnitude less potent than phenylephrine on α_1-adrenoceptors. Synephrine is capable of displacing α2-adrenoceptor antagonist on α_{2A}- and α_{2C}-adrenoceptors	α- adrenergic	Ma et al. (2010)
Rat and human adipocytes	Synephrine caused a weak release of glycerol of rat and human adipocytes. Synephrine was a weak competitor to the α_2-adrenoceptor agonist, bromoxidine	α_2-adrenoceptor	Mercader et al. (2011)

As mentioned, synephrine has been marketed in the supplement industry to be a weight-loss agent – an effect purported to be mediated via β_3-adrenoceptors activity (Bour et al., 2003; Rossato et al., 2011; Stohs, 2017). However, synephrine was a weak direct β_3-adrenoceptor agonist, with greater potencies on rodent adipocytes than in human adipocytes. Synephrine was first shown to stimulate lipolysis more strongly in rodent adipocytes than human adipocytes but with low potencies (pEC50 = 4.38 ± 0.04 (rat) and pEC50 = 4.94 ± 0.14 (human) (Carpene et al., 1999). The effects of synephrine were abolished by propranolol and attenuated by the selective β_3-adrenoceptor antagonist, SR59230A. Another study showed that synephrine was four orders of magnitude less potent than isoprenaline on rat adipocytes and five orders of magnitude less potent than in human adipocytes (Mercader et al., 2011). These studies show that synephrine has direct actions on β_3-adrenoceptors in rodent adipocytes. β_3-adrenoceptors have been reported to play a role in the cardiovascular system, mainly in the myocardium and endothelium, where they modulate cardiac function (Cannavo & Koch, 2017; Dessy et al., 2004; Gauthier et al., 2000). To date, no studies are investigating the effects of synephrine on cardiovascular β_3-adrenoceptors.

Synephrine has been suggested to act as a direct agonist on trace amine-associated receptors on the rat aorta. Varma et al. (1995) found that synephrine was more vasoconstrictive than tyramine in an isolated rat aorta pre-treated with adrenergic receptor (α_1, α_2, β_1 and β_2), 5-hydroxytryptamine, histamine and adenosine antagonists. At the time, Varma et al. proposed tyramine receptors similar to those present in invertebrates. However, Fehler et al. 2010 would later classify these receptors on the rat aorta as trace amine-associated receptors-1 (TAARs). Wainscott et al. (2007) showed that synephrine had a potency of pEC50 = 4.37 on cloned cells expressing human TAAR1, which is relatively weak compared to tyramine (pEC50 =6.4 approximately). To date, no studies are investigating the effects of synephrine in the presence of selective TAAR1 antagonist EPPTB on isolated tissues.

II) INDIRECT (SYMPATHOMIMETIC) ACTIVITY

Kim et al. (2001) showed that S-(+)-synephrine was more potent than R-(-)-synephrine in inhibiting [^{3}H] noradrenaline re-uptake in rat cortical slices. However, R-(-)-synephrine was more effective than S-(+)-synephrine in releasing [^{3}H] noradrenaline from rat cortical slices. Noradrenaline release was markedly inhibited by nisoxetine (a noradrenaline transporter blocker). However, it was not influenced by voltage-gated sodium channel inhibitor, tetrodotoxin or removal of extracellular calcium, which showed that R-(-)-synephrine could act as an indirect sympathomimetic via the release of noradrenaline. The difference in enantiomer activity on noradrenaline uptake and release may result from the possible differential effect of the stereoisomers on vesicular monoamine transporter, which remains to be examined.

In 2009, Hibino et al. in 2009 showed that pure synephrine (0.1 μM to 30 μM) caused dose-dependent vasoconstrictions on isolated rat aortas that were abolished by prazosin (100nM to 1μM) but also partially inhibited by 5-hydroxytryptamine (5-HT) receptors (5-HT_{1D} and 5-HT_{2A}) antagonist, BRL-15,572, but not by the 5-HT_{1B} antagonist, SB-216,641. This was the first study to propose a serotonergic-mediated contractile response to synephrine. The authors proposed that it may be possible that synephrine was eliciting its effects by triggering the release of 5-HT from platelets which then binds to the 5-HT receptors present on the endothelium of the isolated aortas, causing vasoconstriction. 5-HT has been previously shown to cause vasoconstriction of the rat mesenteric artery by inhibiting 4-aminopyridine-sensitive voltage-gated K+ channels (4-AP-sensitive-Kv) via a 5-HT_{2A} receptor pathway. Synephrine and tyramine are structurally similar; thus, it may be possible that synephrine could also elicit vasoconstrictor effects from indirect sympathomimetic pathways.

1.6.2 OCTOPAMINE

p-Octopamine was first identified in the posterior salivary glands of an octopus (*Octopus Vulgaris),* from which its name derives (David & Coulon, 1985). Its action as a neurotransmitter in invertebrates is similar to the physiological activity of adrenaline in mammals (Roeder, 1999). There are also octopamine receptors in invertebrates but do not occur in humans, and studies involving these receptors are not comparable to human studies (Lind et al., 2010).

As outlined in Section 1.5, *p*-octopamine can be produced endogenously (~2.5ng/L in human plasma) or ingested from most citrus containing foods where it is present 0.27 to 4.90 mg/g in standardised dried fruits and *C. aurantium* extracts (D'Andrea et al., 2010; Nelson et al., 2007). Like synephrine, octopamine contains a single chiral centre existing predominantly as the R-(-)-isomer in most plants (Pellati & Benvenuti, 2007). Trace levels of the geometric isomer, *m*-octopamine, is reported to occur naturally in animals, although, to date, it has not been found in plants. As a drug, m-octopamine, known as Norfenefrine, is used to treat urinary incontinence in Europe, Japan and Mexico (Ganellin & Truggle, 1996). Unfortunately, there is limited information on the use of Norfenefrine or its regulation in Australia. The clinical observations and effects of Norfenefrine are shown in Section 1.6.2.ii

EXPERIMENTAL OBSERVATIONS OF OCTOPAMINE-RELATED EFFECTS

I) ANIMAL STUDIES

a) Cardiovascular effects

Only two studies evaluated the cardiovascular effects of octopamine *in vivo*. Delbarre et al. (1982) showed that intravenous administrations of *p*-octopamine (5 µg injection) decreased systolic pressure in hypertensive rats, which were further reduced in the presence of the MAO inhibitor, pargyline. The authors suggested that pre-and postsynaptic α-adrenoceptors did not mediate the effects of p-octopamine Octopamine-induced hypotension was unaffected by α-adrenoceptor inhibitors, piproxan, yohimbine or prazosin. From this study, the researchers proposed that octopamine mediated its effects by receptors that were resistant to catecholamines but did not explore the effects of octopamine in the presence of β-adrenoceptor antagonists.

Fracerelli et al. (2008) showed that octopamine increased heart rate but significantly reduced cardiac output in perfusion experiments on healthy rats. Additionally, the aortic and coronary blood flow was reduced in response to octopamine, suggesting vasoconstrictor effects. The effects of octopamine on the heart rate were attenuated in the presence of the non-selective β-blocker, propranolol. On the other hand, the negative inotropic effects were potentiated in the presence of a tyrosine kinase inhibitor, genistein (37 µM). The response to genistein suggested that the transduction pathway triggered by octopamine were similar to those by 3-iodotyhyronamine (T_1AM) via tyrosine kinases. In the case of octopamine, the negative inotropic effect was confirmed by experiments performed in a papillary muscle preparation, which rules out any interference of changes in heart rate and coronary perfusion. Hence, the authors suggested that the chronotropic effects of octopamine from an adrenergic receptor pathway whereas the negative inotropic effects of octopamine were from a TAAR-mediated pathway.

The negative inotropic effects of octopamine, which is the opposite to what would be expected in cardiac smooth muscle was not discussed by Fracerelli et al. (2008). The α_1-adrenoceptor agonist, phenylephrine has been shown to decrease inotropy in isolated papillary muscles of Guinea pigs (Kim et al., 2005). The

authors suggested that phenylephrine stimulated a PKC dependent pathway that decreases total intracellular concentration of Na+ ions via an α_{1A}-adrenoceptor pathway. These findings were supported by an earlier study by Nagashima et al (1997), that showed that the negative inotropic effects of phenylephrine on rat papillary muscle were attenuated by the selective α_{1A}-adrenoceptor antagonist, WB4101 but was unaffected by the α_{1B}-adrenoceptor antagonist, chloroethylclonidine (CEC). Therefore, the negative inotropic effects of octopamine in Fracerelli et al.'s study could be attributed to direct α_1-adrenoceptor activation similar to that of phenylephrine.

b) Central nervous system effects

Intravenously administered octopamine (50 to 250 µg) dose-dependently increased excitability in rats. The effects of octopamine were enhanced in rats pre-treated with reserpine, which depleted catecholamine stored in the neurons but attenuated when catecholamines neurons were destroyed by 6-hydroxydopamine (200 µg) (Jagiello-Wojtowicz,1979). This study showed that octopamine could elicit CNS effects by direct actions or the release of neuronal noradrenaline and dopamine in the rat brain. In another study, octopamine (10 nM to 10 µM) dose-dependently decreased stress-induced prolactin levels, which were attenuated in the presence of dopamine receptor inhibitors (Becu-Villalobos et al.,1992). Moreover, the salivary flow rates and the amounts of protein secreted increased with non-dose-dependent intravenous administration of octopamine and attenuated by catecholamine blockers and α-adrenoceptor antagonists (Okina et al., 1993). These studies suggest that octopamine mediated its CNS effects by releasing catecholamines and, therefore, might elicit indirect sympathomimetic effects on the vasculature.

A daily intraperitoneal administration of octopamine (81 µM/ kg) resulted in a 19% decrease in the body weight gain in mice (Visentin et al., 2001). The lipolytic effects of octopamine were more pronounced in normal mice than β_3-adrenoceptor deficient mice. The authors showed that octopamine exerted a dual effect on adipocyte glucose transport: counteracting insulin via action via β_3-adrenoceptors, which stimulates basal glucose transport via α_2-adrenoceptors. Additionally, octopamine reduced the eating frequency of these mice via β-

adrenergic stimulation, which resulted in overall weight loss. However, the distribution of β_3-adrenoceptors is species-dependent, and the effects observed in mice may not be directly comparable to humans.

ii) *HUMAN CLINICAL STUDIES*

To date, no human clinical trials have been conducted on *p*-octopamine specifically. However, some studies were conducted on *m*-octopamine (Norfenefrine) due to its use as a sympathomimetic drug in urinary incontinence (Diernaes et al., 1989; Lose & Lindholm, 1984). A small trial conducted on 12 healthy male patients showed that *m-octopamine* increased systolic blood pressure but not heart rate (Boldt et al., 1986). The authors concluded that norfenefrine induced an α-adrenoceptor response and interferes with the autoregulation of noradrenaline release (Schmitt et al., 1983). Clinical trials have shown that a daily dose of *m*-octopamine (90 mg) improved the symptoms of urinary stress incontinence with minimal side effects in 33 women, but the mechanisms of action of *m*-octopamine were not discussed (Diernaes et al.,1989). In a later study by Jorgensen et al. (1991), norfenefrine showed a modest increase in urethral pressure in six patients with stress incontinence. The authors suggested that norfenefrine had weak direct agonist effects, and the observed increase in urethral pressure was likely from an indirect sympathomimetic response. This mechanism of action was distinct from the equivalent *m*-synephrine, phenylephrine, which is a known direct α_1-adrenoceptor agonist and is not used to treat urinary incontinence.

The World Anti-Doping Agency (WADA) currently regards *p*-octopamine as an ergogenic stimulant banned for use in competitive sport (WADA, 2015). However, there is a substantial lack of evidence or understanding of the effects of *p*-octopamine in humans. On the contrary, *p*-synephrine is not considered a stimulant despite evidence of its involvement in the cardiovascular and central nervous system, highlighted in Sections 1.6.1. Hence, further investigations into the specific mechanisms of action of *p*-octopamine are required.

MECHANISMS OF ACTION OF OCTOPAMINE BASED ON IN VITRO EXPERIMENTS

I) DIRECT AGONIST ACTIVITY

Octopamine is a weak direct agonist on α_1-adrenoceptors on rat vas deference, aorta, and cloned cells expressing human α_1-adrenoceptors. Octopamine dose-dependently (1 µM to 15 µM) contracted isolated vas deferens tissue (Celuch & Juorio, 1987). The contractile effects of octopamine were enhanced in the presence of cocaine and yohimbine (1 µM) but were attenuated in the presence of corynanthine (1 µM) and reserpine-pretreatment. In rat aorta, octopamine had weak vasoconstrictor effects that were 1000-fold less potent than noradrenaline (Brown et al., 1988). Additionally, R-(–)-octopamine was more potent than S-(+)-octopamine and racemic octopamine. Richardson et al. (2003) showed that *p*-octopamine was selective for α_{1A}-adrenoceptors whereas *m*-octopamine was selective for α_{1B}-adrenoceptor on cloned cells expressing human α_1-adrenoceptor subtypes (α_{1A}-, α_{1B}- and α_{1D}-adrenoceptor). However, the relative potency to noradrenaline or phenylephrine was not compared. Moreover, the α_1-adrenoceptor subtypes may be overexpressed in the cloned cells, which does not best represent the actual physiological system.

Octopamine showed weak direct α_2-adrenoceptor activity on guinea pig tissues and α_2-adrenoceptor expressing cells. Octopamine inhibited substance P-induced acetylcholine release on guinea pig synaptosome, which was not affected by dopamine receptor antagonist or guanethidine pre-treatment but was attenuated in the presence of α_2-blockers, idazoxan and yohimbine (Chang & Cheng, 1993). On cells that expressed α_{2A}-adrenoceptors, p-octopamine caused a dose-dependent increase in intracellular cAMP but was two times less potent than *m*-octopamine and four times less potent than noradrenaline (Airriess et al., 1997). In a study on mammalian white adipocytes, octopamine increased lipolytic activity and reduced insulin action (Fontana et al., 2000). Although not as potent as noradrenaline, octopamine (1- 100 µM) had less potent on α_2-adrenoceptors present on these fat cells. Unlike the study by Airriess et al. (1997), the adipocytes did not overexpress α_{2A}-adrenoceptors, and the use of α_2-antagonists did not potentiate a lipolytic response.

Octopamine was shown to be a potent β_3-adrenoceptor agonist on mammalian adipocytes. Octopamine dose-dependently reduced glucose uptake by increasing cAMP in rat white adipocytes comparable to specific β_3-adrenoceptor agonist, BRL37344 (Yen et al.,1998). Octopamine was shown to be a potent β_3-adrenoceptor agonist to rat, hamster and dog lipocytes but not human or guinea pig cells based on competition curves to [125I]-iodocyanopindolol (Carpene et al., 1999). These studies showed that octopamine had agonist activities on β_3-adrenoceptors present in mammalian adipocytes via cAMP accumulation, and the role of β_3- should be extended to observations in the vasculature.

Octopamine is a direct agonist on the TAAR1 receptor on rat aorta and left ventricular papillary muscle. As elaborated in section 1.6.2.i.a, a decrease in rat heart inotropy to dose-dependent additions of octopamine (0.1 nM to 10 μM). Furthermore, the vasoconstrictor effects of octopamine were attenuated in the presence of the tyrosine kinase inhibitor, genistein. The authors suggest that octopamine effects were mediated by a non-adrenergic pathway (Frascarelli et al., 2008).

Much later, it was elucidated that the rat aorta expresses mRNA for the TAAR1 subtype (Fehler et al., 2010). The contractile responses to cumulative additions of β-PEA were not affected by the selective α_1-adrenoceptor antagonist, prazosin, the non-selective β-adrenoceptor antagonist, propranolol, or the β_2-selective antagonist ICI-118,551. Furthermore, the pre-contracted aortic ring with a thromboxane TxA2 agonist, U46619, did not have a vasodilatory effect to β-PEA mediated responses. Other possible mechanisms of action were ruled out when a combination of different antagonists, including cocaine and pargyline, did not alter the responses to β-PEA. The authors hypothesised that TAAR1 mediated the vasoconstrictor effects of β-PEA. The effects to β-PEA were compared to non-cumulative additions of tryptamine, p-tyramine, p-octopamine or D-amphetamine in the presence of the mixture of antagonists. The contractile responses to octopamine were similarly potent to β-PEA and was an order of magnitude more potent than tyramine. This meant that octopamine was a more potent agonist for TAAR1 present in the rat aorta.

Broadley et al., (2013) expanded upon Fehler et al's work by expanding the list of agonists to include MDMA, cathinone, and methylphenidate. The experiments were conducted with a cocktail of inhibitors that consisted of prazosin (α_1-antagonist, 1μM), ICI118,551 (β_2-selective, 1 μM), cocaine (catecholamine re-uptake inhibitor, 10 μM) and pargyline (MAO inhibitor, 10 μM). The vasoconstrictor effects of the rat aorta were significantly antagonised by the mixture of inhibitors but not abolished. In agreement with Fehler et al. (2008), the researchers showed that octopamine was either a direct or indirect sympathomimetic at α_1-adrenoceptors with residual contractile responses likely from TAAR1 activation.

II) INDIRECT AGONIST ACTIVITY

Octopamine, dopamine and noradrenaline induced a positive chronotropic and inotropic response on the sinus node artery of canines (Chiba, 1976). The effects of octopamine were attenuated but not abolished by non-selective β-blocker and 5_{HT}-antagonist, alprenolol and serotonin reuptake inhibitor, desmethylimipramine but were not affected by the sodium channel blocker, tetrodotoxin. Rahmani et al. (1987) showed that octopamine had vasoconstrictor effects even after prazosin (1 μM) treatment, suggesting that octopamine mediated its effects from a different mechanism of action to α_1-adrenoceptor. The rank of potencies after pre-treatment with prazosin was p-octopamine> m-octopamine > phenylephrine > o-octopamine (at relative potencies of 2.5: 1.2: 1: 0.75 respectively). The partial agonism of octopamine was supported by Lafi and Leake (1988), who showed that octopamine mediated a mixed direct and indirect agonist activity on the rat vas deferens. Reserpine, guanethidine and alpha-adrenoceptor antagonists were used to distinguish between presynaptic and postsynaptic actions of exogenous dopamine, octopamine, tyramine and noradrenaline on the rat vas deferens. *p*-Octopamine had mixed pre-synaptic actions (releasing endogenous noradrenaline) and post-synaptic actions that caused smooth muscle contraction. It appears that octopamine and synephrine have a similar indirect sympathomimetic action as tyramine. However, further studies are required to establish octopamine's effect on mediating the release of catecholamines in isolated smooth muscle tissue.

1.6.3 TYRAMINE

As mentioned in section 1.5, endogenous tyramine is formed from the amino acid tyrosine by the action of the aromatic amino acid decarboxylase enzyme and a precursor for synephrine and octopamine. It is susceptible to oxidation by monoamine oxidase (MAO) and therefore exists in trace amounts (<50 ng/mL) in the human body. Tyramine is also found in common dietary sources such as chocolate, aged cheese, aged meat, certain fruits and vegetables (Ziegleder et al.,1992). Typically, patients who are prescribed MAO inhibitors are recommended to avoid tyramine rich foods to prevent a hypertensive crisis, sometimes called the "*cheese effect*" (Broadley, 2010). These effects are often characterised by a progressive increase in blood pressure over 30 – 60 minutes after tyramine ingestion, which may manifest into heart palpitations, reduced heart rate, tightness in the chest and pallor (Korn et al., 1986; Lader 1970).

EXPERIMENTAL OBSERVATIONS OF TYRAMINE-RELATED CARDIOVASCULAR EFFECTS

In humans, the pressor response to tyramine is used clinically as a tool to evaluate the degree of tyramine to increase systolic blood pressure by 30 mmHg- this is referred to as the Tyr30 (Blackwell 1963; Blackwell and Mabbitt 1965; Blackwell and Marley 1964). The Tyr30 has been used as a test to measure peripheral adrenergic function. However, the pressor response of tyramine was suggested to be from a cardiac origin rather than through vasoconstriction (Schafers et al., 1997) as tyramine induced increase in blood pressure was inhibited by the β_1-adrenoceptor antagonist, bisoprolol, and not affected by the α_1-adrenoceptor antagonist, doxazosin. Furthermore, the increase in tyramine mediated blood pressure did not cause an increase in heart rate, suggesting that the increase in blood pressure resulted from positive inotropy. In the same study, Schafers et al. (1997) showed that there were increases in heart rate in response to tyramine in the presence of muscarinic antagonist, atropine. This would suggest that chronotropic responses to intravenous tyramine were masked by reflex bradycardia, a parasympathetic vagal reflex that reduces contraction of cardiac muscle.

Similarly, a study by Meck et al. (2003) showed that intravenous tyramine caused a rise in systolic blood pressure and a decrease in total peripheral resistance after intravenously administered tyramine, suggesting that there was a baroreceptor mediated reflex vasodilation in response to increased cardiac output. This observation was in agreement with a separate study by Jacob et al. (2003), who showed that tyramine infusion to healthy subjects caused an increase in systolic but not diastolic blood pressure despite an increase in forearm blood flow, indicating peripheral vasodilation. On the other hand, vasoconstriction is observed when tyramine is administered directly via the brachial artery (Tchakovsky et al., 2002). This paradoxical vasodilation caused by intravenous tyramine may be explained by the local release of vasodilator autacoids such as dopamine (Jacob et al., 2003). Another possible cause for tyramine-induced vasodilation is the co-release of ATP, which regulates blood flow by attenuating sympathetic vasoconstrictor activity (Rosenmeier et al., 2004).

In animals, similar observations have been made for intravenous tyramine action. Khwanchuea et al. (2008) showed that the pressor effects to tyramine in anesthetized rats were antagonised by the α-adrenoceptor antagonist, phentolamine but were not affected by propranolol or atropine. In the same study, tyramine caused an increase in heart rate, but the chronotropic effects were attenuated in the presence of atropine and propranolol. These results suggest that tyramine elicited an indirect sympathomimetic release of endogenous noradrenaline. On isolated guinea-pig vas deferens, tyramine was shown to co-release ATP and noradrenaline (Drissen et al., 1996). However, the ATP release was suggested to be from a non-neuronal α-adrenoceptor mediated response causing peripheral vasodilation. Another possible vasodilator autacoid released by tyramine is nitric oxide. When directly administered, intravenous tyramine caused an increase in blood pressure in the hindlegs of rabbits, which were potentiated in the presence of the nitric oxide synthase inhibitor, N-nitro-L-arginine (LNNA). This suggests that the vasoconstrictor responses to tyramine were indeed affected by the co-release of nitric oxide. Therefore, the pressor effects of tyramine in animals and humans were mediated by either a direct

vasoconstrictor effect when administered directly or from a cardiac origin when an excess of tyramine is present in the systemic circulatory system.

A review by (Gillman 2018) showed that, on average, patients would be able to ingest 10 mg of tyramine on an empty stomach and about 30 mg as a part of a meal to get a 30 mmHg systolic blood pressure increase. Therefore, tyramine avoidance is still recommended for patients who require MAOI. However, although the quantities of tyramine in food have been well characterised, there is not enough information on the cardiovascular effects of tyramine derived from dietary supplements. To elaborate on this, an understanding of tyramine's established mechanism of action as an indirect sympathomimetic and as a direct agonist on TAAR needs to be explained.

MECHANISMS OF ACTION OF TYRAMINE'S PRESSOR RESPONSE

I) INDIRECTLY ACTING SYMPATHOMIMETIC

The established mechanism of action for the responses to tyramine is that it acts as an indirectly acting sympathomimetic via endogenous noradrenaline released from neuronal synapses (Broadley, 2010). The first observations on the effects of tyramine on adrenoceptors by indirect mechanisms were reported by Tainter & Chang (1927), who showed that the contractile responses to tyramine were inhibited by cocaine. Much later, the support for the indirect mechanism of action of tyramine came from the reduced contractile responses to animal pre-treatment to reserpine, which reduces the amount of noradrenaline from vesicles stores (Burn & Rand, 1958). Correlating to the increased concerns of ephedrine, Liles et al. (2006) showed that the pressor effects of ephedrine were mediated by direct α-adrenoceptor stimulation, whereas tyramine was indirectly acting on these receptors.

Upon ingestion, tyramine is subjected to a first-pass clearance by MAO-A in the intestinal lumen and MAO-B in the liver. The mechanisms of tyramine-induced noradrenaline release differ from endogenous noradrenaline release from sympathetic innervation (Goldstein, 2008). During sympathetic innervation, vesicular noradrenaline is released directly into the synapse via exocytosis from nerve stimulation. On the other hand, lipophilic amines such as tyramine promote the leakage of vesicularly stored noradrenaline into the synapse by increasing

the intra-vesicular pH (Langeloh & Trendelenburg, 1987). The displaced noradrenaline in the cytoplasm is transported to the synapse via reverse transport through the cell membrane noradrenaline transporter (NET) (Trendelenburg, 1990). Additionally, excess tyramine in sympathetic vessels can be converted to octopamine via hydroxylation by dopamine-β-hydroxylase, which further contributes to vasoconstriction (Fischer et al., 1965).

Despite the evidence surrounding the sympathomimetic effects of tyramine, some atypical observations suggest other mechanisms involved (Broadley, 2010). For example, the depletion of noradrenaline stores by reserpine was shown only partially to inhibit the pressor responses to tyramine in spinal cats. Similarly, the pre-treatment with reserpine showed residual responses to tyramine in rabbits (Hudgins & Flemming, 1966), and rat aorta (Maling et al., 1971) and atria (Rice et al., 1987), which were initially attributed to direct α_1-adrenoceptor effects. In addition to indirect sympathomimetic behaviours of tyramine, the Broadley laboratory was the first to pioneer investigations into the alternative mechanism of tyramine action (Broadley, 2010). A hallmark of their investigations was that indirect sympathomimetics were particularly sensitive to tachyphylaxis- a feature that shows the gradual decline of responses to the same drug. The progressive depletion of neuronal noradrenaline would decrease vasoconstrictor response due to the lack of catecholamine availability. It was also suggested that certain trace amines have direct actions on non-adrenergic receptors, such as the case of tyramine mediating vasodilatory responses from dopamine release (Jacob et al., 2003). Furthermore, contractile responses to trace amines in isolated rat aorta (Fehler et al., 2010) and pig coronary arteries (Herbert et al., 2008) were not affected by the α_1-adrenoceptor blockade or cocaine-sensitive neuronal uptake. Hence, the possibility of the vasoconstriction being mediated by trace amine-associated receptors was hypothesised.

II) DIRECT TRACE AMINE ASSOCIATED RECEPTOR AGONIST

As previously mentioned in section 1.3.2, TAAR1 expression has been reported in mice (Borowsky et al., 2001; Grandy, 2007), rats (Bunzow et al., 2001), and rhesus monkey brains (Xie et al., 2008). Initial studies with RT-PCR showed TAAR1 is expressed in several mouse brain regions such as the amygdala, cerebellum, hippocampus, dorsal root ganglia, and hypothalamus (Borowsky et al., 2001). TAAR1 activation triggers the accumulation of intracellular cAMP, which stimulates inwardly rectifying K+ channels (Borrowsky et al., 2001; Bunzow et al., 2001). Tyramine has been described as a potent agonist of TAAR1, with β-PEA being more potent than tyramine at human and mouse variants of TAAR1, but the opposite is observed in the rat TAAR1 (Wainscott et al., 2007). The neuroanatomical distribution of TAAR1 relative to other monoamine systems suggest that TAAR1 could regulate monoaminergic transmission via monoamine transporters and presynaptic autoreceptors co-expressed with TAAR1 (Pei et al., 2016).

In the heart tissues of rats, the expression of 5 TAAR subtypes has been detected by RT-PCR (Chiellini et al., 2007). Furthermore, Fehler et al. (2010) showed evidence that TAAR1 and TAAR4 proteins were expressed in the aorta of rats. Tyramine and β-PEA were shown to cause aortic contractile responses in isolated rat aorta (Fehler et al., 2010), and pig coronary arteries (Herbert et al., 2008) were not affected by the α_1-adrenoceptor blockade or cocaine-sensitive neuronal uptake. Furthermore, Frascarelli et al. (2008) showed that tyramine (100 μM) significantly increased heart rate but did not reduce cardiac output in rats, which were consistent in opposition to the earlier established observations of infused tyramine on cardiac tissue. Hence, it was suggested that the dephosphorylation of tyrosine residues might mediate the responses to TAAR stimulation. This was further supported using genistein, a tyrosine kinase inhibitor, which significantly reduced the inotropic and chronotropic effects of tyramine.

In recent developments, Broadley et al. (2013) showed that tyramine could produce a vasocontractile response in rat aortic tissues even in the presence of α-adrenoceptor inhibitor, prazosin (1 μM); β_2-adrenoceptor inhibitor, ICI-118,551(1 μM); NET-inhibitor, cocaine (10 μM); and MAOI, pargyline (10 μM). However, the maximal contractions to tyramine were lesser than that to β-PEA and were suggested to be a partial agonist. In comparison, the responses to β-PEA, amphetamine and octopamine were regarded as full agonists and were inhibited by tyramine, suggesting that these amines acted upon a common receptor. This common receptor was suggested to be TAAR1, giving the evidence that the amines were resistant to adrenoceptor, 5-HT receptor or prostaglandin receptor blockade and the known expression of TAAR1 mRNA in rat aorta by Fehler et al. (2008).

Additionally, trace amines' concentration range to induce vasoconstriction was comparable to those in the porcine coronary artery at 10- 100μM (Herbert et al., 2009). These concentrations were suggested to be compatible with the reported physiological levels required to activate TAAR1 expressed in cell lines. Therefore, given that normal circulating levels of tyramine are in the low nanomolar range, an increase in circulatory tyramine levels due to tyramine consumption in *C. aurantium* PWS could induce further increases in blood pressure. Given that consumers of PWS may not be fully aware of this, those who have altered physiological states such as in obesity or who are currently prescribed MAOI might be at an elevated risk of adverse cardiovascular events.

1.7 QUANTITATIVE ANALYSIS OF TRACE AMINES IN DIETARY SUPPLEMENTS

Following on from understanding the mechanisms of action of the trace amines that contribute to cardiovascular effects and levels required to elicit a response, it is of interest to establish how this may relate to levels in *Citrus-aurantium*-containing PWS. Several studies have been conducted to quantify the trace amines present in *C. aurantium* and *C. aurantium*-containing supplements. The determination of these Citrus alkaloids has been discussed in a previous review by Pellati et al. (2007). This subsection will focus on describing the various methods of detecting trace amines present in *C. aurantium*-containing supplements rather than Citrus juices, fresh fruit or in human plasma samples.

The most common quantitative analysis method has employed chromatography - a physical method of separation that exploits a compound's polarity. According to their affinity for that phase, analysts of interest partition between two phases, stationary or mobile (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 or affinity).

Gas chromatography paired to a mass spectrometer (GC-MS) has been used to detect and quantify synephrine and octopamine (Cabezas et al., 2013; Rossato et al., 2010). Derivatisation is often necessary for polar non-volatile trace amines but adds additional time and cost to the analytical method. Other chromatographic methods such as thin-layer chromatography (TLC) (Bagatela et al., 2015; Shawky, 2014) and ion exchange liquid chromatography (Tang et al., 2006; Thevis et al., 2012) have also been developed for the analysis of synephrine in various citrus preparations. However, these methods require extensive sample preparation, are not easily replicable in commercial laboratories and are not quantitative.

Liquid chromatography (LC), on the other hand, is more suitable for polar compounds (such as the trace amines), and faster analyses are possible with the direct injection of samples through shorter columns. High-Pressure Liquid Chromatography (HPLC) is the most common analytical method for trace amine detection in *C. aurantium* fruit and dietary supplements (Table 6). HPLC methods are approached with various strategies to improve sensitivity and selectivity, such as different types of stationary phase, mobile phase compositions, and various selective detectors systems. HPLC enables analysis in various separation modes, including normal phase, hydrophilic interaction chromatography (HILIC) and ion-exchange chromatography. However, reverse phase HPLC (RP-HPLC) procedures using non-polar octadecyl silica (C_{18}) stationary phases and a polar mobile phase are the most popular for trace amine analysis in *C. aurantium* plant material and supplements. In RP-HPLC, the non-polar analytes will be retained longer on the stationary phase. Based on the $\log_P$ for the trace amines (Table 7), the expected elution order would therefore be octopamine/synephrine/tyramine.

Table 6 Comparison of HPLC/ RP-HLC analytical methods for the determination of trace amines in dietary supplements

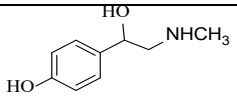
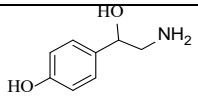
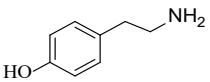
Authors	Compound analysed	Sample preparation	Stationary phase	Mobile phase	Detection	Accuracy (Yield %)	Limits of Quantitation & Detection
(Hurlbut et al., 1998)	Synephrine	Magnetic stirring with dilute mobile phase Solid phase extraction with ISOLUTE non-polar SPE	YMC phenyl column (250 mmx 3.0 mm, 5 µm)	Isocratic A: 0.1M sodium acetate/acetic acid (pH 4.8) B: Acetonitrile with 2% triethylamine	UV, 255 nm	85-102%	N/A
(Niemann & Gay, 2003)	Synephrine	Magnetic stirring with acidified acetone/water/acetonitrile (4:1:1, v/v/v)	Zorbax 300-SCX column (250 mm x 4.6 mm, 5 µm)	Isocratic: 0.4M Sodium phosphate buffer (pH 3.0/water/acetonitrile (50:35:15, v/v/v)	UV: 205,210 and 225 nm	93.2-97.5%	LOQ: 0.016 µg
(Schaneberg & Khan, 2004)	Synephrine	Sonication with acetonitrile/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
(Ganzera et al., 2005)	Synephrine, octopamine, tyramine	Sonication with 0.37% HCl	HyperClone C18 BDS 1 column (100 mm x 4.6, 3µM)	A: aqueous (3 mM SDS) with 0.1% phosphoric acid (pH 4.0) B: Acetonitrile /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)
(Avula, Upparapalli, Navarrete, & Khan, 2005)	Synephrine, octopamine, tyramine, *N*-methyltyramine, hordenine	Sonication with methanol/DMSO (1:1)	Synergi Hydro-RP column (250 mm x 4.6 mm, 4 µm)	A: 0.1 Sodium acetate buffer (pH 5.5) B: Acetonitrile	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)
(Tang et al., 2006)	Synephrine, octopamine, tyramine	Sonication with 0.1M HCl	Spherigel C18 column (250 mm x 4.6 mm, 4 µm)	Isocratic: 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),
(Roman et al., 2007)	Synephrine, octopamine, tyramine, *N*-methyltyramine, hordenine	Sonication with 0.1% H_3PO_4	Luna C18 column (150 mm x 3.0 mm, 5 µm)	A: Aqueous hexanesulfonate in borate buffer 10 mM (pH 8.2) B: Acetonitrile-20mM borate buffer (pH 8.2) (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 for syn, oct and tyr in *C. aurantium* fruit
(Putzbach et al., 2007)	Synephrine, octopamine, tyramine, *N*-methyltyramine, hordenine	PFE and sonication extraction	Discovery HS F5 (200 mm x 4.6 mm, 5 µm)	Isocratic: aqueous 10 mM SDS (pH 2.5) / acetonitrile (72:28, v/v)	UV, 220 nm	N/A	LOD: 4.2 ng (syn), 3.5 ng (oct), 5.9 ng (tyr),5.5 ng (nmt)

(Santana et al., 2008)	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 methanol (70:30, v/v)	UV, 220 nm	N/A	LOD: 11 ng (*p*-synephrine), 10 ng (*m*-synephrine). LOQ: 33 (*p*-synephrine), 30 (*m*-synephrine)
(Gatti & Lotti, 2011)	Synephrine, octopamine, tyramine, *N*-methyltyramine, hordenine	Sonication with water. Pre-column derivatization	Luna C18 column (250 mmx 4.6 mm, 5 µm)	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
Ribiere et al., 2012	Synephrine	Prepared in methanol before mechanical stirring, sonication and centrifugation at 3500 rpm	Acuity BEH C18 column, 100 x 2.1 mm, 1.7 µm	A: 50mM sodium dihydrogen phosphate buffer with 10% phosphoric acid (pH = 3.8) B: Acetonitrile	UV, 223 – 273 nm	N/A	LOQ: 0.1 µg/mL
(Di Lorenzo et al., 2014)	Synephrine, octopamine, tyramine, *N*-methyltyramine, hordenine	Prepared in aqueous HCl 0.1 M: methanol (75:25 v/v) Magnetic stirring	LiChrospher RP-18 column (250 mm x 4 mm, 5 µm)	A: Aqueous SDS + 85% phosphoric acid (pH 4.2) B: Aqueous SDS with acetonitrile (62:38, v/v)	UV, 224 nm.	Average RSD values <15%	LOD: 1.8-7.5 ng/mL LOQ: 6.0-25 ng/mL
Viana et al., 2017	Caffeine, synephrine, octopamine, tyramine, hordenine, ephedrine	Prepared in methanol before dilution with the mobile phase	Thermo Scientific RPLC C18 (250 x 4.6 mm, 5 µm)	Isocratic: water containing 0.1% phosphoric acid (pH = 2.5) : acetonitrile (30:70, v/v)	UV, 220 nm	Recovery 97 – 105%	LOD: 0.2 µg/mL (syn), 0.11 µg/mL (oct), 0.14 µg/mL (tyr)
(Paiga et al., 2017)	Synephrine, ephedrine,	QuEChERS extraction	Kinetex C18 column (150 x 2.6 mm, 1.7 µm)	A: Acetonitrile B: aqueous 5 mM ammonium acetate (pH 4)	UV, 281 nm	Average recovery 70.2 %	LOD: 0.53 µg/L LOQ: 1.75 µg/L
(Avula et al., 2019)	Synephrine, octopamine, tyramine, other phenethylamine compounds	Prepared in methanol prior to centrifugation at 959 x g.	YMC Triart (100 x 2.0 mm, 3 µm)	A: water + 0.1% formic acid B: Acetonitrile + 0.1 % formic acid	QTOF-MS, ESI + mode	N/A	LOD & LOQ: 1 – 1000 ng/mL
(Pawar et al., 2020)	Synephrine, octopamine, tyramine, methyl synephrine, methyltyramine, hordenine	12.5 mL of 1% HCl in 80% aq methanol before sonication for 30 mins.	Acentis Express F5 (100 x 2.1 mm, 2.0 µm)	A: water + 0.1% formic acid B: Acetonitrile + 0.1 % formic acid	Triple Quadrupole MS, ESI + mode	Syn: 111 % Oct: 118 % Tyr: 99%	LOD: 0.3 – 0.7 µg/g LOQ: 1.0 – 2.3 µg/g
(Al-Khadhra 2020)	Synephrine, caffeine, clenbuterol, nandrolone, testosterone, methylhexaneamine	Sonication for 20 mins at 40 °C with water and methanol (1:1, v/v)	Waters C8 (250 x 4.6, 5 µm)	A: 10mM ammonium acetate in water (pH = 4.75) B: 10mM ammonium acetate in methanol	UV, 225 nm	Syn: 100.5%	LOD: 5.9 µg/mL LOQ 18 µg/mL

HIGH PRESSURE LIQUID CHROMATOGRAPHY METHODS

In RP-HPLC, in which the stationary phase is non-polar, retention of the polar amines depends on the protonation state and the pH of the mobile phase. An important factor in separating the trace amines, synephrine, octopamine and tyramine, is their charged state, which has high acid dissociation constants (pKa) ranging from 9.6 to 10.4 (Table 7). Therefore, in an acidic mobile phase (below their pKa), the trace amines will be positively charged, whereas, at a mobile pH higher than their pKa, the trace amine will be neutral. However, the interaction between the positively charged amine with residual silanol groups on the silica surface of the stationary phase can cause poor peak shape and resolution.

Table 7 Comparison of chemical properties of synephrine, octopamine and tyramine. Chemical data was updated by (Wishart et al., 2018).

			
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	9.8	9.6	10.4
Log P	-0.45	-0.59	0.86

One of the most common techniques to reduce silanol interactions is to use a mobile phase with an acidic pH. Santana et al. (2008) developed two LC methods for the baseline separation and quantitative determination of *p*-synephrine and *m*-synephrine. The incorporation of a MS assisted in confirming the identity of the resolved isomers. The chromatography involved a Supelco Discovery HS-F5 (pentaflurophenyl) column (150mm x 4.6mm, 5µm); mobile phase A= 10mmol/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 a LOD of 11 ng. The mass spectrometry set up had gradient conditions with the same mobile phase but with a flow rate of 0.5 mL/min with the detection and quantification was carried out with the use of electrospray-ionization

multiple-reaction monitoring (ESI-MRM). 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 synephrine structural isomers in dietary supplements.

Viana et al. (2017) developed an HPLC-UV-DAD method to detect a range of stimulants (caffeine, synephrine, octopamine, hordenine, tyramine, salicin) ephedrine) in 94 dietary supplements sold in Brazil. The stimulants were extracted using 25 mL of methanol and subsequently diluted 200-fold in the mobile phase. The separation was achieved on a Thermo Scientific C18 column (250 x 4.6 mm, 5 µm) under isocratic conditions of the mobile phase water containing 0.1% phosphoric acid (pH 2.5): acetonitrile (30:70, v/v) with. The UV-Vis detection was achieved at 220 nm. The LOD for synephrine is 0.2 µg/mL octopamine is 0.11 µg/mL, tyramine is 0.14 µg/mL. Amongst the amines, the only synephrine was reported in 4 supplements and ranged from 59 – 127 mg/day. However, this method had a considerable baseline drift with a poor resolution that was not appropriately addressed in their report.

Other frequently used methods of suppressing silanol ion formation employ an ion pair reagent (IPR) such as sodium dodecyl sulphate (SDS), which binds to the silanol groups resulting in improved peak shape and retention behaviour. Studies showed that HPLC separations with IPRs produced resolved chromatography peaks (Ganzera, Lanser, & Stuppner, 2005; Putzbach, Rimmer, Sharpless, & Sander, 2007; Roman, Betz, & Hildreth, 2007; Tang et al., 2006).

Ganzera et al. (2006) have used an ion-pair HPLC method to analyse Ephedra sinica and *C. aurantium* alkaloids in plant material and commercial products. The samples were extracted with 0.37% hydrochloric acid by sonication at ambient temperature. HPLC analysis was performed on a HyperClone C18 BDS 1 column (100 x 4.6 mm, 3 µm), and the gradient mobile phase composed of 3 mM aqueous SDS (pH = 4.0), 0.1% phosphoric acid containing 3 mM SDS and acetonitrile/methanol (2:1. v/v).

Roman et al. (2007) analysed *C. aurantium* alkaloids in raw fruit, extracts and dietary supplement standard reference materials using an ion-pair HPLC method. The alkaloid separation was conducted on a Luna C18 column (150 x 3.0 mm, 5 µm), with

a mobile phase composed of an isocratic mobile phase of acetonitrile/ 20 mM borate buffer (pH 8.2) (20:80%, v/v) with 10 mM sodium 1-hexane sulfonate, at a flow rate of 0.85 mL/min. The total synephrine found in the *C. aurantium* fruit ranged from 8.29 – 26.11 mg/g, *C. aurantium* extracts from 66.58 – 292.3 mg/g and 4.69 -11.98 mg/g in *C. aurantium* supplements. However, the authors noted that the inter-day repeatability was poor for octopamine, tyramine and hordenine. This was the first and only method that had used a high pH buffer, which improved the resolution between tyramine and N-methyltyramine. However, there was a significant level of baseline interference for octopamine and tyramine (Figure 10), and the use of an IPR prohibited further investigations from confirming the identity of octopamine.

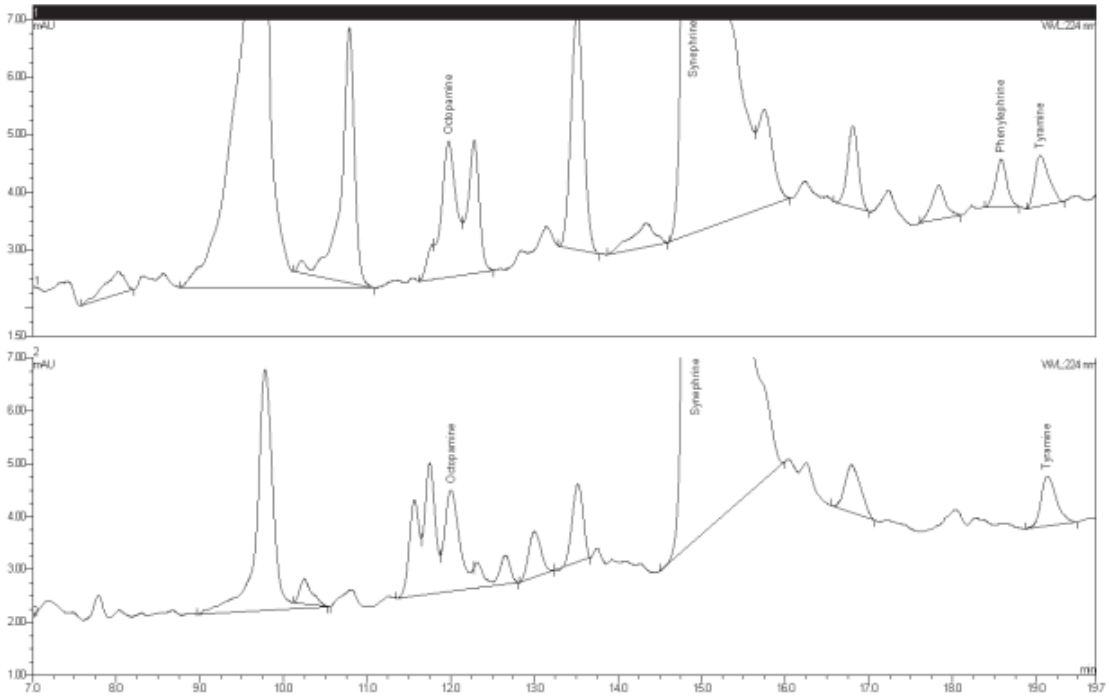


Figure 10: Representative chromatographs showing the interference for octopamine. Figure reproduced from Roman et al., 2007, OpenPMC, PMCID: PMC3207213

Ribere et al. 2012 developed an LC-UV method for the detection of 32 compounds, including synephrine found in 14 weight-loss supplements. The samples were extracted using methanol before 15 minutes of mechanical stirring, sonication, and centrifugation at 3500 rpm. Separation was achieved with an Acuity BEH C18 column, 100 x 2.1 mm, 1.7 µm under gradient conditions with an ion-pair reagent. The mobile phase was A: 50mM sodium dihydrogen phosphate buffer with 10% phosphoric acid (pH = 3.8), and mobile phase B was acetonitrile at a flow rate of 0.35 mL/min. The detection was achieved with a DAD UV-Vis detector set at 223 – 273 nm. The LOQ of synephrine was at 0.1 µg/mL. However, there was some interference with co-eluting metformin. Synephrine was found in 5/14 supplements and ranged from 19 - 29 mg/serving.

A limitation with IPRs use was that it was not compatible with MS detectors, lowered the selectivity and decreased reproducibility. An alternative to IPRs was to derivatize the trace amines before analysis. Gatti et al. (2012) showed that pre-column derivatization with O-phthadialdehyde (OPA) yielded good recoveries ranging from 99.5-101.3% with RSD ranging from 0.8 to 1.2% with well resolved and identifiable peaks. However, derivatization protocols are time-consuming, subject to additional impurities and cause ion-suppression for mass detection (Qi et al., 2014).

In recent developments for HPLC stationary phases, HILIC chromatographic techniques are the most suitable for polar analytes. HILIC columns have a stationary phase similar to normal phase LC (NPLC), but, unlike NPLC, the mobile phase is water-miscible. Compared to RP-HPLC, HILIC separation allows for longer retention of polar analytes. So far, there has only been one developed method that used a HILIC stationary phase to detect synephrine and other basic polar drugs (ephedrine, norephedrine, noradrenaline and norphenylephrine). The separation was conducted on a Luna HILIC stationary phase (250 x 100 mm, 3 µm, 110 Å) under isocratic conditions of the mobile phase, acetonitrile: water (92:8, v/v) with a 10 mM ammonium formate buffer (pH = 3). However, the resolution between analytes was relatively poor, with a significant level of baseline interference (Figure 11). This chromatographic method was only limited to the analysis of basic drug standards and was not applied to any pharmaceutical matrices.

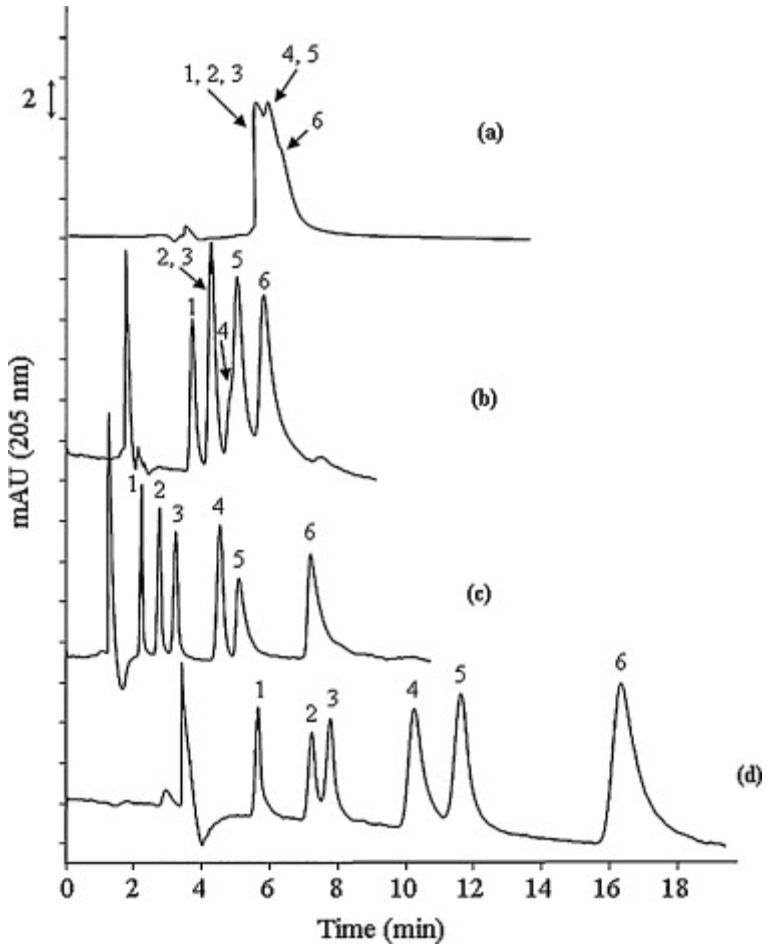


Figure 11: Comparison of the chromatographic separation of the six sympathomimetic drugs on different stationary phases: a) cyanopropyl silica, b) diol silica, c) aminopropyl silica, d) HILIC Luna column. Compounds: 1) ephedrine, 2) norephedrine, 3) synephrine, 4) adrenaline, 5) norphenylephrine, 6) noradrenaline. Figure reproduced with permissions from Aturki et al., 2011.

UPLC AND HYPHENATED METHODS

Chromatographic separation coupled with mass spectrometry (MS) provides a clearer insight into the chemical characterisation of alkaloids in plant and dietary supplement matrices. In recent years, synephrine and other phenethylamines have been quantified using Ultra-High-Pressure LC (or UPLC) systems (Avula et al., 2019; Kim et al., 2014; Pawar et al., 2020; Venhuis et al., 2014), which are capable of faster run times and higher resolution than HPLC. Avula et al., 2019 developed an LC-QToF-MS method for the simultaneous analysis of 111 stimulants, anorectics and other active components in 27 weight-loss and pre-workout supplements. The compounds were extracted with 2 mL of methanol and 30 minutes of sonication, and 30 minutes of centrifugation at 959 x *g*. This procedure was repeated three more times, and the supernatants were combined, and methanol was added to produce a final volume of

10 mL. LC separation was achieved on a YMC Triart (100 x 2.0 mm, 3µm) stationary phase under gradient conditions of mobile phase A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid) at a flow rate of 0.21 mL/min. The LOD for synephrine is 0.01 µg/mL octopamine is 0.5 µg/mL, tyramine is 0.1 µg/mL. Synephrine was found in 12 supplements (0- 182 mg/serving), Octopamine was found in one supplement (0.6 mg/serving), and tyramine was not found in any supplements.

Muller et al., 2019 developed an LC-MS/MS method to simultaneously determine 32 drugs, including synephrine, in 80 dietary supplements. The analytes of interest were extracted with sonication for 15 minutes with 25 mL of methanol. The samples were then diluted 100 times prior to analysis. Separation was accomplished using a Zorbax SB-C18 column (50 x 2.1 mm, 1.8 µm) and a gradient elution of 0.05% formic acid in water and acetonitrile at neutral pH. The limit of detection for synephrine was 1.71 ng/mL, and the LOQ was 2.31 ng/mL with a 108.3% accuracy. Synephrine was found in 9/80 dietary supplements ranging from 0.2- 25.4 mg/daily dose. However, synephrine was eluted close to the solvent front and was co-eluted with amiloride

Pawar et al., 2020 developed an LC-MS/MS method to quantify amines found in *C. aurantium* (synephrine, octopamine, tyramine, N-methyltyramine and hordenine) and synthetic phenethylamines (phenylephrine, methylsynephrine, etilefrine, and isopropyloctopamine) in 59 dietary supplements sold in the US as well as standard reference materials. The compounds of interest were extracted using 1% HCl in 80% aqueous methanol and sonicated for 30 minutes. The samples were then centrifuged for 5 minutes at 2934 relative centrifugal force (RCF), and the supernatant was diluted in a range from 10x to 10000x with 50% acetonitrile. The analytes were separated on an Ascentis Express F5 column (100 x 2.1 mm, 2.0 µm) maintained at 40oC under gradient conditions with the mobile phase A: Water with 0.1% formic acid and B: acetonitrile containing 0.1% formic acid. The LOD and LOQ for synephrine were 0.3 and 1.0 µg/g, octopamine was 07 µg/g and 2.3 µg/g and tyramine were 0.3 and 1.0 µg/g. The average recovery for these amines ranged from 99 – 122%. The study found that synephrine was present in all supplements and ranged from 0.002 – 92 mg/g. N-methyltyramine was the second most abundant compound and was found up to 11mg/g. When factoring serving size, synephrine was found up to 160 mg/serving, N-methyltyramine was 26 mg/serving, octopamine was found at 130 mg/serving, and

hordenine was found up to 60 mg/serving. Tyramine was found in some products up to 1.1 mg/serving.

However, UPLC systems are understandably more costly and are not widely available in most commercial laboratories. Therefore, for many testing facilities with high throughput requirements, HPLC is often preferred over UPLC, especially when paired with multi-wavelength ultraviolet (UV) (e.g. Photodiode Array, PDA) and mass spectrometry (MS) detectors.

SAMPLE PREPARATION PROCEDURES

Due to the complex nature of dietary supplement matrices, sample preparation steps are essential as they impact analyte identification, confirmation, and quantification. Sample preparation includes the isolation and pre-concentration of compounds of interest from various matrices and making the analytes more suitable for separation and detection (Chen et al., 2008). The extent of sample pre-treatment depends not only on the complexity of the sample but the chromatographic conditions of the stationary and mobile phases. For extracting the polar trace amines from dry powdered dietary supplements, some of the most common steps involved were first to dissolve the supplements in an extraction solvent. Next was to assist in the dissolution by sonication, followed by diluting the extracted compounds. In some analyses, an additional partitioning method such as solid-phase extraction (SPE) was used to isolate these compounds before separation with liquid chromatography.

For the extraction of amines from various *C. aurantium* materials and dietary supplements, the advantages of using acidified water and methanol were previously reported (Sander et al., 2008). For dietary supplement analysis, more studies used methanol as an extraction solvent (Avula et al., 2019; Avula et al., 2005; Pawar et al., 2020; Karsten Putzbach et al., 2007; Rebiere et al., 2012; Viana et al., 2016) rather than acidified water (Ganzera et al., 2005; Gatti & Lotti, 2011; Paiga et al., 2017; Roman et al., 2007; Santana et al., 2008) or aqueous acetonitrile (Niemann & Gay, 2003; Schaneberg & Khan, 2004). However, the efficiency of extraction depends on the type of *C. aurantium* used in the dietary supplements. An analysis of extraction efficiency by the NIST showed that ethanolic extracts of *C. aurantium* were more easily dissolved in both methanol and acidic water than *C. aurantium* fruit, which had consistently lower yields (Sander et al., 2008). It was hypothesised that the cell

structure of the plant contributes to the difficulty in extracting the alkaloids. Therefore, acidified extraction solvents were recommended for sample preparation to help degrade these cells to release their contents in the solution. As PWS often do not disclose the type or source of *C. aurantium* used (raw fruit or extract), an acidic extraction solvent should be used.

Sonication is an inexpensive extraction method that creates microenvironments with high temperatures and pressure, which shortens the time for dietary supplements to dissolve in their respective solvents (Priego-Capote & Luque de Castro, 2004). Sonication has been used in most of the summarised methods in Table 6 with varying times and temperatures. However, except for Putzbach et al. (2007), most analytical methods do not explain why those specific conditions were selected. Putzbach et al. (2007) evaluated the efficiency of two different extraction techniques (Soxhlet extraction and sonication extraction) at varying times and extraction solvents on *C. aurantium* standard reference materials. The phenethylamines were separated on an Ace 5 RP C18 column (250 x 4.6 mm, 5 µm) under isocratic conditions with a flow rate of 1mL/min of 72% mobile phase A (aqueous SDS solution; 10 mM, pH = 2.5) and 28% mobile phase B (acetonitrile). The study showed that sonication extraction with hydrochloric acid provided the best extraction results for all three SRMs with the highest yield of synephrine, octopamine, tyramine and *N*-methyltyramine. On the other hand, the Soxhlet extractions worked well for synephrine but resulted in lower octopamine or tyramine levels than the sonication extractions. Therefore, dietary supplements dissolved in hydrochloric acid with 60 minutes of sonication at ambient temperature appeared to be the best extraction condition for these amines.

Solid-phase extraction is a partitioning method between a silica-based solid phase and a liquid phase (sample). Analytes of interest must have a greater affinity for the solid phase than for the sample matrix. Retention may involve nonpolar, polar or ionic interactions. A wide range of SPE sorbents ensures various product forms, including column cartridges, discs and well pates. SPE is one of the most popular sample preparation approaches because it has a high degree of selectivity, requires less solvent, has limited emulsion formation, and is easier to operate over conventional liquid-liquid extraction (LLE). Hurlbut et al. (1998) developed a HPLC method with UV detection to analyse ephedrine-type alkaloids (including noradrenaline,

pseudoephedrine, and synephrine) in six herbal products. The analytes from the herbal products were dissolved in an acidified buffer and isolated on a strong cation exchange propyl sulfonic acid solid-phase extraction (SPEC) column and eluted a high ionic strength sodium acetate buffer. HPLC separation was performed on a YMC phenyl column (250 x 3.0 mm, 5 µm) under isocratic conditions with 0.1 M sodium acetate and acetic acid (pH 4.8) containing triethylamine and 2% acetonitrile, at a flow rate of 0.8 mL/min. Synephrine was detected with a UV-Vis detector set to 255 nm. One supplement out of six contained 13 mg/g of synephrine with an average recovery of 85%. In a separate study, strong cation-exchange SPE (LiChrolut SCX column) was successfully applied to detect synephrine enantiomers in a range of *C. aurantium* fruit and dietary supplements by Pellati et al. (2005). The samples used were dissolved in water before magnetic stirring before SPE. The recovery values of both synephrine enantiomers ranged from 68.3 – 75.3% and were relatively poor. As outlined in the paragraphs above, these recovery values could be improved using an acidic or methanolic extraction solvent.

Novel extraction methods such as Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) extraction had disappointing yields of synephrine with an average of 70%. Paigia et al. (2017) developed a UHPLC-MS/MS method to analyse 26 pharmaceutical compounds, including synephrine, in sixteen weight-loss and dietary supplements. The QuEChERS extraction method involved adding homogenised samples in acidified water (0.1% formic acid) prior to adding acetonitrile, anhydrous MgSO4 and NaCl. A 1 mL aliquot of this mixture was evaporated to dryness before reconstitution with 1 mL of acetonitrile: water mixture (1:9, v/v). The analytes were then separated on a Kinetex C18 column (150 x 2.6 mm, 1.7 µm) under gradient conditions with mobile phase A (0.1% aqueous formic acid with 5 mM ammonium acetate, pH =4) and B (acetonitrile) at a flow rate of 0.3 mL/min. Despite having low detection limits of 0.53 ng/mL, synephrine was quantified in one supplement (0.77 mg/mL). The study's authors showed an interfering peak that co-eluted with synephrine but did not elaborate on the influence of the interfering peak on synephrine quantitation.

ENANTIOSELECTIVE CHROMATOGRAPHIC METHOD

Plant biochemical pathways produce a series of chemicals whose stereochemical configurations are determined by specific enzymatic activity (Fischer et al., 2012). Because of this, most naturally occurring phytochemicals would often have a more significant proportion of one enantiomer over the other. In the case of *C. aurantium*, only a few methods have been developed to detect synephrine enantiomers in citrus in traditional Chinese medicines with electrochemical detection (ECD) (Kusu et al., 1996; Kusu et al., 1995) or on natural products with UV detection (Pellati et al., 2005; Pellati et al., 2002; Tanaka et al., 2019).

Synephrine enantioseparation has been conducted either by direct or indirect methods on HPLC-UV. A direct enantioseparation uses a chiral mobile phase additive or chiral stationary phase to separate the enantiomers of interest. Kusu et al. (1995) were the first to quantify synephrine enantiomers by direct HPLC using a chiral exchange column (Sumichiral OA-5000) with a copper (II) acetate and ammonium acetate mobile phase paired with an electrochemical detector(Kusu et al., 1995). *R*-(-)-synephrine was the only enantiomer found in various citrus fruits (0.07% - 0.40%), crude drugs (0.07%-0.40%) and Chinese medicines (0.03% - 0.11%). Pellati et al. (2002 & 2005) developed and validated a direct HPLC-UV method analysis of synephrine enantiomers in *C. aurantium* and *Evodia* species using a protein-based chiral stationary phase [11, 23]. *R*-(-)-synephrine was the only enantiomer isolated from fresh citrus fruits, while *S*-(+)-synephrine was detected and quantified in dry extracts of *C. aurantium*.

Indirect methods use derivatising agents, such as ion-pair reagents, which react with enantiomers during sample preparation to produce diastereoisomers, separated by conventional RP-HPLC (Gal & Brown, 1986; Tanaka et al., 2019). Tanaka and colleagues derivatised different sections of citrus fruit samples with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosil isothiocyanate (TAG-ITC) before HPLC with UV detection at 254nm (Figure 12).

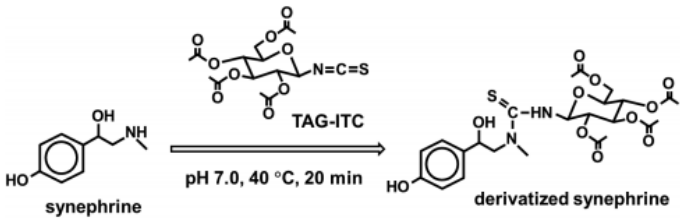


Figure 12: Reaction scheme of synephrine derivatization with TAG-ITC (Tanaka et al., 2019)

Only the *R*-(-)-synephrine enantiomer was found in the exocarp, mesocarp, endocarp and sarcocarp sections of *C. aurantium* at levels below 2.6 mg/g (Tanaka et al., 2019). *S*-(+)-synephrine was found in *Citrus unshiu* and citrus hybrids but were at levels below 1% that of the *R*-(-)-synephrine levels.

Nonetheless, determining the ratio of synephrine enantiomers is an additional means of determining authentic *C. aurantium* use in PWS. A racemic ratio or the presence of high levels of *S*-(+)-synephrine in commercially available PWS is indicative of a non-botanical source. Further developments in determining whether PWS contain authentic sources of *C. aurantium* will be explored in chapter 3.

1.8 RATIONALE, RESEARCH QUESTIONS AND AIMS

C. aurantium-containing supplements have caused cardiovascular-related side effects, raising concerns about its safety (Eudy et al., 2013). In both animals and humans, studies showed that these trace amines could cause an increase in either heart rate or blood pressure, but the mechanism of action of synephrine and octopamine remains unclear. Most *in vitro* studies showed that these amines caused the vasoconstrictor effects on rodent aorta. However, the effects of these trace amines are still unclear on other types of blood vessels that regulate blood pressure in larger animal models such as the pig.

Tyramine is a well-established indirect sympathomimetic, where it mediates its responses on adrenoceptors via noradrenaline (Day, 1967). In most *in vitro* experiments, racemic (±) synephrine and octopamine had weak direct α- and β-adrenoceptor activities, but the experimental design did not consider a tyramine-like effect on arterial tissues. Whether the effects of synephrine and octopamine have direct or indirect sympathomimetic mechanisms, remain unclear.

The trace amines (synephrine, octopamine and tyramine) are naturally present in *C. aurantium,* where synephrine is more abundant than the other trace amines. Moreover, synephrine has a recommended daily dosage of 30 mg/day in Australia. However, given that PWS often list their ingredients in a *proprietary blend*, it is not clear whether the amounts of synephrine in *C. aurantium*-listing pre-workout can elicit a vascular response or if they were within the daily dose limit.

Finally, *C. aurantium* extracts have up to 10 times more R-(-)-enantiomer than S-(+)-enantiomer (Pellati et al., 2005). However, it is unknown if these trace amines in *C. aurantium*-containing PWS have a similar predominance of the R-(-)-enantiomer. The presence of racemic synephrine with a relatively higher proportion of the S-(+)-enantiomer will suggest that it is not from authentic *C. aurantium* extracts

Dietary supplements are not as well-regulated as a food or a pharmaceutical drug, which has led to a number of adulteration cases. A common form of this mis-practice is to illegal spike dietary supplements with pharmaceutical drugs to produce stimulatory effects but claim that the effects to be from a plant material. Another form of adulteration is the substitution of the plant material with an alternative species which

may vary in active compound composition. *Citrus aurantium* extracts have up to 10 times more R-(-)-enantiomer, which is more pharmacologically active than S-(+)-enantiomer on α-adrenergic receptors (Pellati et al., 2005). However, it is not known if these trace amines in *Citrus aurantium*-containing PWS have a similar predominance of the R-(-)-enantiomer to a natural *C. aurantium* plant extract. Therefore, an accurate determination of the composition of amines and their corresponding enantiomers is important to understand the possible effects of *C. aurantium*-listing pre-workout supplements on the cardiovascular system.

RESEARCH QUESTIONS

1. What effects do synephrine and octopamine have on different arteries that regulate blood pressure?
2. What are the mechanisms of action of synephrine and octopamine on arterial tissues, and are they similar to tyramine?
3. What amounts of trace amines are present in pre-workout supplements, and could the ratio of trace amines indicate an authentic source of *C. aurantium*?
4. What is the ratio of synephrine enantiomers in *C. aurantium,* and could the enantiomeric ratio be used to determine authentic plant extract use?
5. Could the amounts of trace amines found in *C. aurantium*-listing pre-workout supplements elicit a contractile response on arteries that regulate blood pressure?

AIMS

1. To investigate the mechanism of action of the trace amines found in *C. aurantium*, synephrine, octopamine and tyramine on different types of arteries with different physiological roles in blood pressure regulation.
2. To develop and validate an HPLC-UV-MS method to quantify the levels of trace amines in *C. aurantium* standard reference material and compare their relative ratio to that found in a sample of *C. aurantium-listing* pre-workout supplements.
3. The second aim of this study was to develop a direct, convenient, and accurate screening method suitable for separating and quantifying synephrine enantiomers in a convenience sample of *C. aurantium*-listing pre-workout supplements.

CHAPTER 2: VASCULAR PHARMACOLOGY OF TRACE AMINES

2.1 INTRODUCTION

As discussed in Chapter 1, *C. aurantium* extracts in most PWS has been linked to several adverse cardiovascular events such as vasospasms (Holmes & Tavee, 2008), arterial hypertension (Moaddeb et al., 2011), and aortic dissection (Doctorian & Do, 2017). The trace amines (synephrine, octopamine and tyramine) are naturally present in most *C. aurantium* extracts, but there was not enough evidence to show that these compounds were the exact cause of the reported adverse effects.

Tyramine is the most researched among the three main trace amines and mediates vasoconstriction in isolated blood vessels. Tyramine dose-dependently induced vasoconstrictions on isolated rat aorta (Broadley et al., 2013; Chan & Chow, 1976; Fehler et al., 2010; Krishnamurty & Grollman, 1972), guinea pig aorta (Maling et al., 1971), and coronary arteries of pigs (Herbert et al., 2008). Additionally, tyramine elicits vasodilation in pre-contracted mesenteric vascular bed of rats (Broadley et al., 2009). The established mechanism of action of tyramine is via an indirectly acting sympathomimetic action that releases neuronal noradrenaline to interact with post-junctional adrenoceptors on the vascular smooth muscle cells of arteries (Goldstein, 2008). Moreover, residual plasma tyramine can be converted into octopamine via hydroxylation by dopamine-β-hydroxylase, which causes further vasoconstriction (Fischer et al., 1965). However, the mostly vasoconstrictive effects of tyramine increase the overall blood pressure but not necessarily heart rate.

The effects of *p*-octopamine are not well understood but have effects similar to noradrenaline on the sympathetic nervous system in invertebrates (Roeder, 1999). Octopamine is a weak direct agonist on α_1-adrenoceptors on rat vas deferens, aorta, and cloned cells expressing human α_1-adrenoceptors. In rat aorta, octopamine had weak vasoconstrictor effects 1000-fold less potent than noradrenaline (Brown et al., 1988). Additionally, R-(–)-octopamine was more potent than S-(+)-octopamine and racemic octopamine. Richardson et al. (2003) showed that *p*-octopamine was selective for α_{1A}-adrenoceptors whereas *m*-octopamine was selective for α_{1B}-adrenoceptor on cloned cells expressing human α_1-adrenoceptor subtypes (α_{1A}-, α_{1B}- and α_{1D}-adrenoceptor). However, the signalling cascade in cloned cells might be overexpressed, and this method may not be the best representation of a physiological system.

Synephrine is the main amine in *C. aurantium* extracts and is suspected of contributing to cardiovascular events as it is a positional isomer to phenylephrine (m-synephrine) and structurally similar to ephedrine (Rossato et al., 2011). On isolated rat aortae, synephrine was shown to have direct α_1-adrenoceptor activities that were attenuated by prazosin (100nM to 1 μM). (Brown et al., 1988; Huang et al.,1995; Varma et al., 1995; Hibino et al 2009). However, synephrine was 1000-fold less potent than noradrenaline, whereas phenylephrine was only 6-fold less potent than noradrenaline in the rat aortae (Brown et al. 1999). On cloned cells expressing human α_{1A}-, α_{2A}- and α_{2C}-adrenoceptors, synephrine showed some receptor binding albeit with low affinities relative to phenylephrine (Ma et al., 2010). However, synephrine reversed forskolin-induced cAMP elevation, which suggested that synephrine may act as an antagonist to pre-synaptic α_{2A}- and α_{2C}-adrenoceptors. The antagonism of these α_2-adrenoceptors may increase the vasocontractile effect of noradrenaline

Although the studies on isolated tissues showed direct adrenoceptor activities, it was not clear if synephrine could also exert indirect sympathomimetic actions. So far, there is only one study by Kim et al. (2001) that showed that R-(-)-synephrine increased spontaneous [^{3}H]noradrenaline release in a concentration-dependant manner. Noradrenaline release was inhibited by nisoxetine (a noradrenaline transporter blocker) but was not influenced by tetrodotoxin or the removal of extracellular calcium. Hence, these results suggested that the R-(-)-synephrine found naturally in *C. aurantium* could potentially act via the release of noradrenaline.

One of the main factors regulating blood pressure is vascular resistance, which is affected by contractions and dilation of the vascular smooth muscle (Touyz et al., 2018). The vascular system is comprised of a hierarchy of vessels ranging from large conduit arteries to capillaries, with varying expressions of functional adrenoceptors. Previous *in vitro* research on trace amines has focused on conduit arteries such as the aorta, which has a greater proportion of α_{1D}-adrenoceptors, and plays a lesser role in blood pressure regulation than peripheral vessels (Piascik et al., 1995). So far, the effects of trace amines are not well described on other vascular tissues that can contribute to an increase in blood pressure. Therefore, three different blood vessels were investigated in this study.

Mesenteric arteries

In humans, the inferior mesenteric artery branches from the abdominal aorta and supplies blood to the distal third of the colon (Shaikh et al., 2021). The mesenteric vasculature consists of a network of arteries that have a dense innervation of sympathetic nerves, which regulates blood flow to the gut during physiological stress such as exercise (Ceppa et al., 2003). Blood flow to the gastrointestinal tract can be drastically reduced (>80%) during exercise, causing a large increase in overall blood pressure (Clausen, 1977). So far, there has been a paucity of data on the effects of trace amines on the mesenteric artery.

The inferior mesenteric artery is a vessel that possesses all three α_1-adrenoceptor subtypes in humans (Shibata et al., 1998). In rats, the vasocontractile responses are mostly mediated via α_{1A}- and α_{1D}-adrenoceptors subtypes in rodents (Stam et al., 1999; Yamamoto & Koike, 2001; Yokomizo et al., 2015). Despite being an indirectly acting sympathomimetic, tyramine failed to elicit a vasoconstrictor response in the perfused mesenteric bed of rats (Anwar et al., 2012). However, tyramine and high concentrations of octopamine caused vasodilatory responses that 5-HT receptor antagonists did not attenuate. The authors of the study had hypothesised that the effects mediated by tyramine and octopamine were likely from post-junctional trace amine-associated receptors (TAARs) present on the mesenteric vasculature. A separate study by Narang et al. (2014) showed that the trace amine, β-phenethylamine (PEA), mediated concentration-dependent vasoconstriction of the mesenteric arteries of rats. The study showed that PEA elicited a dual indirectly acting sympathomimetic and α_1-adrenoceptor antagonist action, where residual vasoconstrictor responses could be mediated by TAAR. Therefore, it is possible that the trace amines could cause vasoconstriction in the mesenteric vasculature, leading to an increase in blood pressure.

Coronary arteries

The coronary circulation is important as it provides the myocardium with oxygen and substrates to ensure the normal function and viability of the heart (Goodwill et al., 2017). The coronary artery is regulated by sympathetic innervation that plays a role in direct or indirect regulation of coronary blood flow (Goodwill et al., 2017). The epicardial coronary arteries, such as the left descending coronary artery, have a high distribution of α_1-adrenoceptors (Chilian, 1991; Chilian et al., 1989), whereas β_2-adrenoceptors are predominant in the intramuscular arteries (Amenta et al., 1991; Toda & Okamura, 1990). Plasma levels of trace amines may be elevated from ingesting PWS containing *C. aurantium,* but not much is known about their immediate effects on the coronary artery.

Tyramine has been shown to cause coronary artery constriction in dogs via the release of noradrenaline (Forman et al., 1984; Kobayashi et al., 1984). In prostaglandin pre-contracted coronary arteries, tyramine (1 µM to 20 µM) induced vasorelaxation, further supporting an indirect mechanism of action (Hayashi & Park, 1984). However, a study by Herbert et al. (2008) showed that tyramine induced concentration-dependent contraction on denuded porcine coronary arteries in the presence of a cocktail of inhibitors of the adrenergic receptors, serotonin receptors, dopamine receptors and noradrenaline reuptake. The authors proposed that the contractile effects mediated by tyramine were from TAAR activation on the porcine coronary artery. However, a selective antagonist for the TAARs was not available at that time to elucidate the possible mechanism of action of trace amines on the coronary artery. Furthermore, there have not been similar investigations of synephrine or octopamine on the coronary artery.

Renal arteries

The kidney is an essential yet complex organ that plays a significant role in blood pressure regulation as it is highly perfused and receives ~25% of cardiac output (Carlström et al., 2015). Renal artery contractions reduce blood flow to the kidney, affecting renal function and increasing systolic blood pressure (Sata et al., 2018). The renal arteries are innervated by efferent nerves, which are predominantly adrenergic, and noradrenaline release mediates vasoconstriction. Furthermore, renal artery contractions affect sodium and water reabsorption in the tubular epithelial cells downstream, which leads to decreased renal blood flow (Sata et al., 2018). Hence, investigating the possible role of trace amines on mediating renal vasocontraction is important to identify possible links to increased blood pressure in humans.

There is a heterogenic distribution of α_{1A}- and α_2-adrenoceptors along the human renal artery (Morrow & Creese, 1986; Pettinger et al., 1987) but a lower expression of β-adrenoceptor, which is predominantly expressed downstream of the renal vasculature (Bailly et al., 1990). In the pig, there is a notable expression of additional α_{1A}- and α_{1B}-adrenoceptors, which affect the influx and release of intracellular Ca^{2+} to cause vascular smooth muscle contraction. (Zhou et al., 1998). In humans, $\alpha_{1A/L}$-adrenoceptors are the main α-adrenoceptor subtype to mediate the vasocontractile responses to noradrenaline (Moriyama et al., 2000).

In addition, the kidney has been reported to express low levels of TAAR-1, TAAR3, TAAR4 and TAAR5 mRNA (Borowsky et al., 2001). However, there is no information on whether trace amines such as tyramine, octopamine or synephrine could mediate a vascular response on the renal artery via TAARs. Currently, no reports are investigating the possible mechanism of action of trace amines on the renal artery or whether synephrine or octopamine could elicit a vasocontractile response.

Significance of porcine vasculature to human vasculature

Pigs share many similarities in physiology and anatomy with humans and are among the most used animal species in translational practice. Like humans, pigs have a similar response to vascular injury and develop intimal hyperplasia to the same extent as humans. Like humans, elderly pigs can develop spontaneous atherosclerosis (Tsang et al., 2016).

Although no animal model can fully replicate the complexity of human pathological conditions, animal models are vital for evaluating disease mechanisms and testing diagnostic technologies and interventions (Genain et al., 2018). Significant insight into cardiovascular molecular and cellular mechanisms has come from small animal models, such as mice, rats, and guinea pigs. However, considerable differences exist in cardiovascular characteristics when rodent models are compared to humans (Milani-Nejad & Janssen, 2014). Therefore, large animal models that approximate human physiology, function, and anatomy are essential to developing discoveries from murine models into clinical therapies (Milani-Nejad & Janssen, 2014).

However, there are limited studies on the distribution of G-coupled protein receptors in pigs and their comparison to humans. It has been assumed that porcine tissue shares similar receptor pharmacology to humans based on how pig vasculature has been used as a suitable replacement for cardiovascular surgeries (Patelis et al., 2017).

2.2 AIM

This study aimed to investigate the mechanism of action of the trace amines found in *C. aurantium*, synephrine, octopamine and tyramine on different types of arteries with different physiological roles in blood pressure regulation.

2.3 MATERIALS AND METHODS

ANIMALS AND TISSUE PREPARATION

Gastrointestinal tracts, hearts and kidneys were obtained from female non-parous White-Landrace bacon pigs (six months old) from the local abattoir (Highchester Meats, Beaudesert, QLD) and transported in ice-cold Krebs-bicarbonate solution to the laboratory. From this collection of tissues, 3 mm segments of either the inferior mesenteric arteries, left descending anterior coronary arteries, or the renal arteries (proximal to the abdominal aorta) were isolated. The isolated circular rings were suspended between stainless-steel hooks and stationary supports in an 8mL organ bath (EZ-baths, Global Towns, CA) containing Krebs-bicarbonate solution (composition in mM: NaCl 118, $NaHCO_3$ 25, glucose 11.7, $MgSO_4$ 2.4, KH_2PO_4 1.2, KCl 1.2, $CaCl_2$ 2.5) maintained at 37°C and continuously oxygenated with 5% CO_2 in oxygen (Figure 13). The rings were mounted under a resting tension of 5 g, and the tension developed by the circular smooth muscle in response to the addition of drugs was measured using isometric force transducers coupled to a PowerLab LabChart® 7 software (ADInstruments, Castle Hill, Australia). The tissues are weighed at the end of the experiment to normalise their developed tensions to tissue weight.

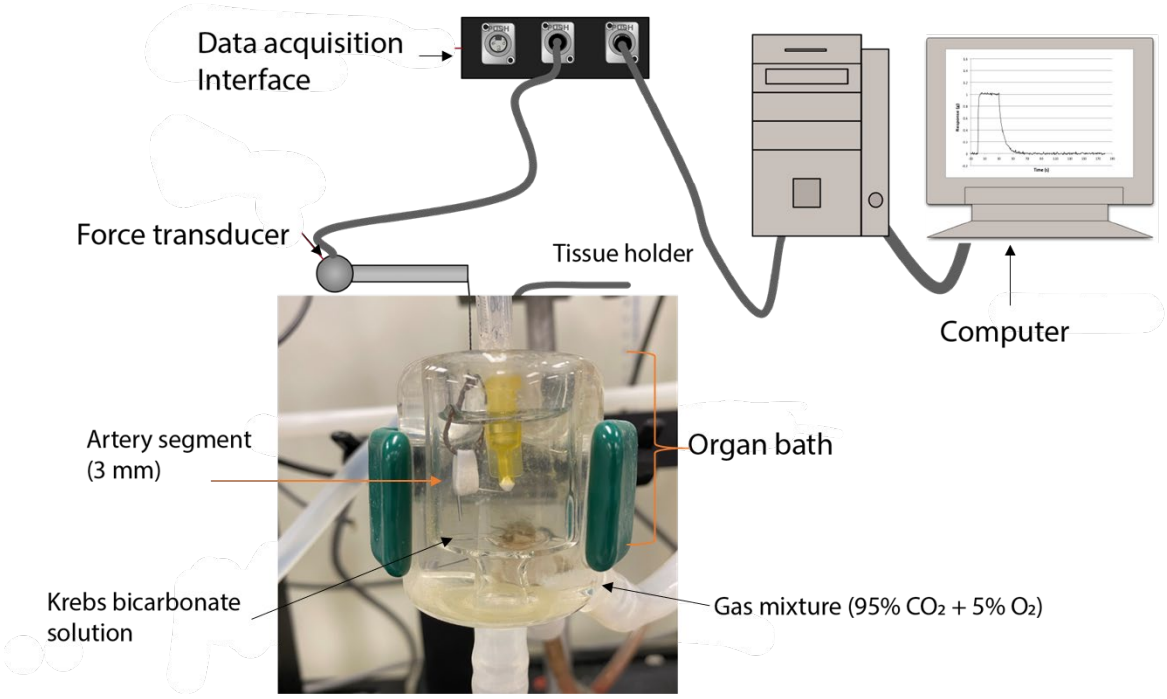


Figure 13: Organ bath set up with a porcine artery ring held to a fixed holder and a suspended hook attached to an isometric force transducer. Data acquisition is displayed on a computer

DRUGS AND CHEMICALS

(±)-*p*-synephrine, (±)-*p*-octopamine hydrochloride, (±)-*p*-tyramine, (+)-phenylephrine hydrochloride, prazosin hydrochloride, propranolol hydrochloride, yohimbine hydrochloride, EPPTB (RO-5212773, N-(3-Ethoxyphenyl)-4-(1-pyrrolidinyl)-3-(trifluoromethyl)benzamide), L-Nω-Nitroarginine (L-NNA), ketanserin tartrate and methiothepin mesylate were obtained from Sigma-Aldrich (Sydney, Australia). All drugs except RO-5212773 were dissolved in distilled water and then diluted with Krebs-bicarbonate solution. RO-5212773 was dissolved in 1% dimethyl sulfoxide (DMSO) before dilution with Krebs-bicarbonate solution. The Krebs-bicarbonate solution was used for all the experiments (Table 8).

Table 8 Concentrations (mM) in Krebs-Henseleit solution and high-concentration potassium chloride Krebs-bicarbonate solution (60mM KCl)

Compound	Normal Krebs-Henseleit solution	High-potassium (K+) Krebs-Henseleit solution
Sodium chloride	118.4 mM	63.0 mM
D-(+)-glucose	11.7 mM	11.7 mM
Sodium bicarbonate	25.0 mM	25.0 mM
Potassium chloride	4.6 mM	60.0 mM
Magnesium sulphate	2.4 mM	2.4 mM
Potassium dihydrogen phosphate	1.2 mM	1.2 mM
Calcium chloride	1.9 mM	1.9 mM

EXPERIMENTAL PROCEDURE

PROTOCOL FOR CONCENTRATION-RESPONSE CURVES

To observe the initial contractile responses to the trace amines, artery segments were equilibrated for 60 minutes with several washes with fresh Krebs-bicarbonate solution before adding either tyramine, synephrine or octopamine in a dose-dependent manner in half logarithmic increments (1 µM – 10 mM). The tissues were washed, and a vehicle control (H_2O or 1% DMSO) was incubated for 30 minutes before repeating the responses to the trace amines. In experimental groups, the tissues were pre-treated with tyramine (3 mM) during the 60-minute incubation period and treated with antagonists in place of the vehicle control that inhibit either α-adrenoceptor, β-adrenoceptor, nitric oxide synthesis, or TAAR1 signalling, separately or combined. At the end of the experiment, a response to a high concentration of potassium chloride (KCl, 60 mM) Krebs-bicarbonate solution was obtained and responses to the amines was expressed as a percentage of this response to KCl Krebs-bicarbonate buffer (%KCl) (Figure 14).

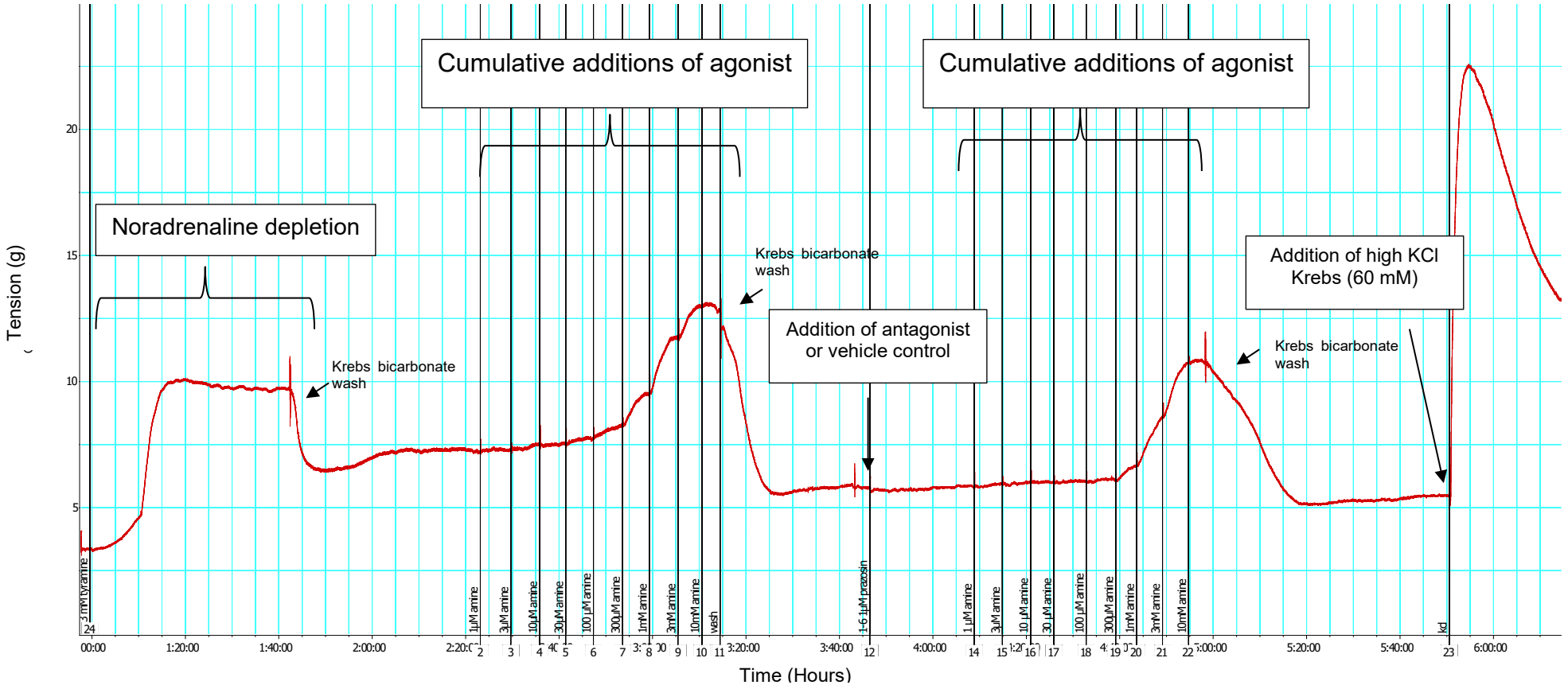


Figure 14: Representative trace of contractile response of noradrenaline-depleted coronary arteries to tyramine in the absence and presence of prazosin (1µM)

INDIRECT SYMPATHOMIMETIC SIGNALLING (NORADRENALINE DEPLETION PROTOCOL)

To investigate these amines' possible indirect sympathomimetic action, the tissues were incubated with a high concentration of tyramine (3 mM) for 60 minutes to deplete pre-synaptic noradrenaline stores. After washout, a cumulative dose-response to the trace amines (1 µM – 10 mM) were obtained. These concentration-response curves (noradrenaline-depleted) were compared to the first cumulative response curves of the respective trace amines (controls). Further experiments investigating the mechanisms of action of the trace amines on their respective arteries were conducted after depleting noradrenaline with 3mM tyramine.

INHIBITION OF DIRECT ADRENERGIC RECEPTOR ACTIVITY

In noradrenaline-depleted tissues, concentration-response curves to the trace amines were obtained. After washout, the tissues were incubated with either prazosin (α_1-adrenoceptor antagonist, 1 µM) or propranolol (β-adrenoceptor antagonist, 1 µM) for 30 minutes before the concentration responses of the trace amines.

INHIBITION OF NITRIC OXIDE SYNTHASE (NOS)

The endothelium plays an important role in maintaining vascular tone. The endothelium releases various vasodilatory factors such as nitric oxide, prostacyclin and endothelium-derived hyperpolarising factor (Sandoo et al., 2010). To investigate the effects of the NO-synthase inhibitor, L-NNA (100 µM) was used in place of the vehicle control.

INHIBITION OF TRACE AMINE ASSOCIATED RECEPTOR 1 (TAAR-1)

After depleting tissues of noradrenaline and blocking nitric oxide synthase, and antagonising α_1- and β-adrenoceptors, significant contractions of the coronary and renal artery to the amines remained; it has been suggested that these responses were evidence for a role for trace amine receptors. For these tissues, the effects of the trace amine-associated receptor 1 antagonist (EPPTB; 100 nM and 100 μM) were examined in noradrenaline-depleted tissues. EPPTB was also examined or in the presence of a cocktail of inhibitors to remove α- and β-adrenoceptor and nitric oxide effects (prazosin 1μM, propranolol 1μM and L-NNA 100μM).

STATISTICAL ANALYSIS

Concentration-response curves were analysed using PRISM 8 (GraphPad Software, San Diego, USA). The data were represented as mean ± standard error of mean (SEM) with *n* indicating the number of animals from which arterial rings were obtained. Comparison of the different concentration-response curves was performed by paired Student's t-test. A two-way ANOVA with a post hoc Dunnett's test was applied to determine differences between multiple data groups. An unpaired Welch's *t*-test was used to compare the maximum contractile responses of tissues to agonist groups. A *p*-value of less than 0.05 was considered statistically significant. Potency was expressed as the *pEC50* value (-log EC50), the molar concentration of the drug producing a response of 50% of the maximum effect.

2.4 RESULTS

STUDY 1: MESENTERIC ARTERY RESPONSES TO TRACE AMINES

EFFECT OF TRACE AMINES RELATIVE TO NORADRENALINE ON MESENTERIC ARTERY

All three trace amines produced concentration-dependent constrictions of the mesenteric arterial rings (Figure 15). Tyramine and synephrine produced similar maximum responses, whilst those to octopamine were significantly greater (unpaired Welch's *t*-test, $p < 0.05$). All three trace amines had similar potencies that were not significantly different (*p*EC50 ranging from 3.25 – 3.91). The endogenous amine, noradrenaline produced greater contractions (85.7 ± 12.2, $n = 6$; $p < 0.01$) and was 100-fold more potent than the three trace amines (*pEC50* = 5.42 ± 0.21, *n*= 6) (Figure 15, Table 9).

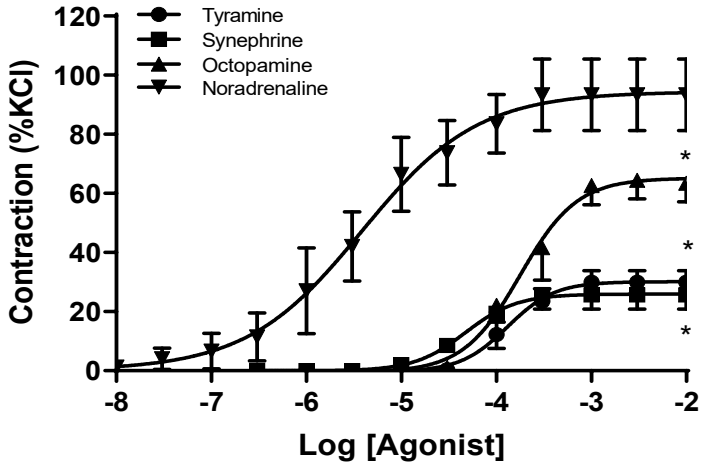


Figure 15 : Initial cumulative-dose responses of trace amines on isolated mesenteric artery. The amines shown are tyramine (●pEC50= 3.39 ± 0.08; n=6), synephrine (■pEC50= 3.82 ± 0.12; n=6) and octopamine (▲pEC50= 3.25 ± 0.12; n=6). The data are presented as percent of contraction to 60 mM potassium chloride (%KCl). (*p<0.05 vs noradrenaline).

THE ROLE OF ENDOGENOUS NORADRENALINE RELEASE IN RESPONSES OF MESENTERIC ARTERY.

In mesenteric arteries, tyramine pre-treatment for 30 minutes nearly abolished subsequent responses to tyramine with a >90% reduction in maximal contraction and a rightward shift of curves (pEC50 value 2.87 ± 0.06, n=6; Student's t-test $p < 0.05$) (Figure 16, Table 9). In tissues depleted of endogenous noradrenaline, the maximal contractions to synephrine were reduced by 58% and maximal responses to octopamine were halved. However, the potencies of either synephrine or octopamine were not affected in noradrenaline-depleted arteries. The contractile responses to exogenous noradrenaline were not significantly affected by tyramine pre-treatment (Table 9).

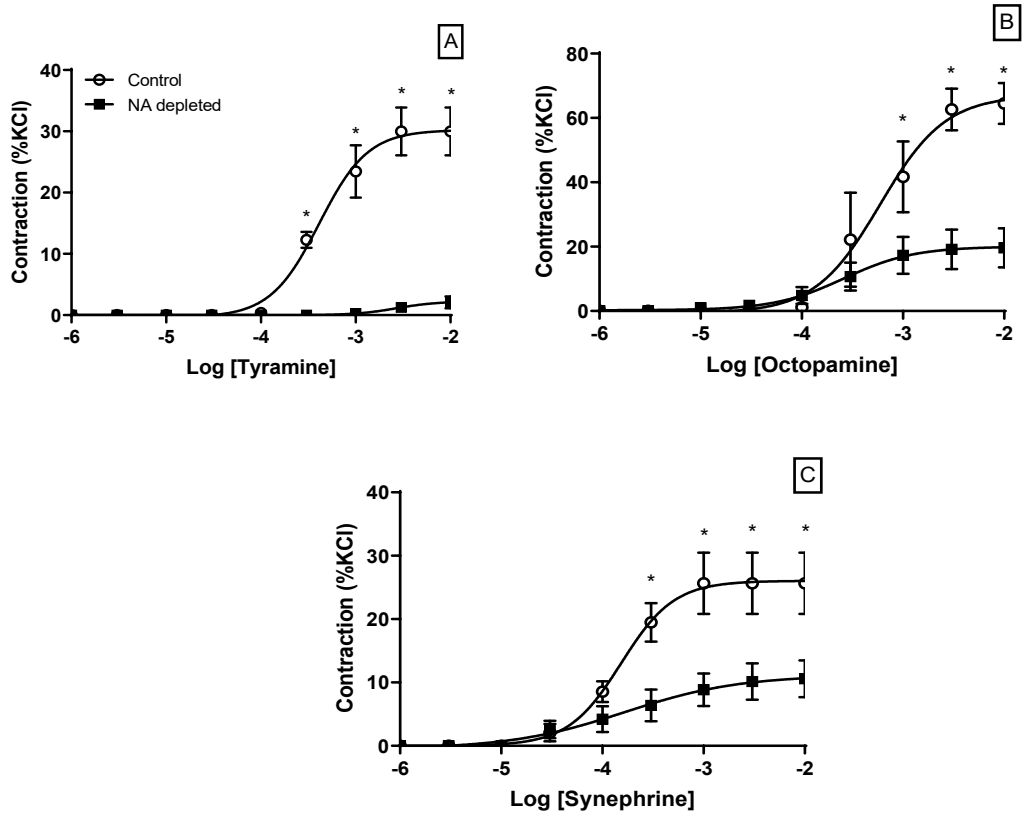


Figure 16: Mean (± sem) concentration-response curves to tyramine (*n= 6)* (A), octopamine (*n= 5)* (B) and synephrine (*n= 6)* (C) on normal (○) or noradrenaline-depleted (■) mesenteric arteries. Two-way ANOVA *p<0.05 vs. noradrenaline-intact controls

To examine whether responses to any of the three amines involved the release of nitric oxide, noradrenaline-depleted tissues were incubated with the nitric oxide synthase inhibitor, L-NNA (100 μM)(Figure 17). None of the responses to the trace amines was affected by the removal of nitric oxide (Table 9). This protocol was not conducted on noradrenaline.

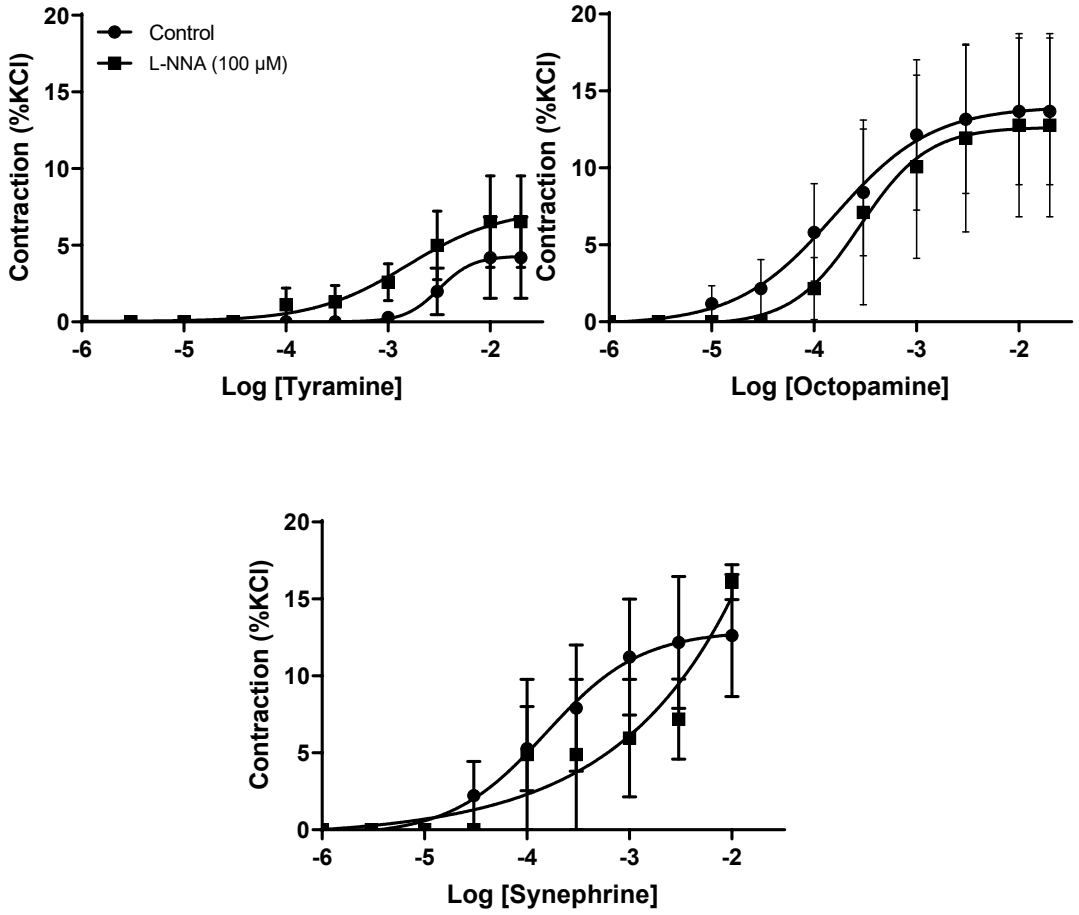


Figure 17 The effect of L-NNA (100uM, ¡) on responses to tyramine (A), synephrine (B) and octopamine (C) in noradrenaline-depleted renal arteries (control, ●). Contractile responses are means ± sem (n = 6 -9) values expressed as a percentage of the contractile response to potassium chloride (60mM). Two-way ANOVA *p<0.05 vs control.

Table 9: Effects of antagonists on the maximum responses and potency values of p-synephrine, octopamine, tyramine, noradrenaline and phenylephrine in porcine mesenteric arteries. Paired Student's t-test *vs.control p< 0.05

Trace amine and interventions	*Controls*		*Treatment*		*Sample number*
	Max contraction (%KCl)	*Potency (pEC50)*	*Max contraction (%KCl)*	*Potency (pEC50)*	*(n)*
Tyramine					
Tyramine pre-treatment (3 mM)	*30.0 ± 3.9*	*3.91 ± 0.14*	*2.9 ± 0.7**	*2.87 ± 0.06**	*6*
Propranolol (1 μM)	*2.9 ± 0.7*	*2.49 ± 0.19*	*2.9 ± 0.6*	*2.88 ± 0.27*	*6*
Prazosin (1 μM)	*2.9 ± 0.7*	*2.49 ± 0.19*	*Abolished**	*Abolished**	*6*
L-NNA (100 μM)	*4.2 ± 2.7*	*2.50 ± 0.23*	*6.5 ± 3.0*	*2.81 ± 0.56*	*4*
Synephrine					
Tyramine pre-treatment (3 mM)	*25.6 ± 4.8*	*3.81 ± 0.12*	*10.9 ± 2.6**	*3.76 ± 0.26*	*6*
Propranolol (1 μM)	*12.5 ± 2.9*	*3.90 ± 0.51*	*16.1 ± 3.8*	*3.45 ± 0.41*	*6*
Prazosin (1 μM)	*9.6 ± 2.6*	*3.81 ± 0.56*	*Abolished**	*Abolished**	*5*
L-NNA (100 μM)	*19.2 ± 10.1*	*4.06 ± 0.68*	*17.4 ± 9.8*	*2.29 ± 1.19*	*4*
Octopamine					
Tyramine pre-treatment (3 mM)	*63.4 ± 6.3*	*3.25 ± 0.12*	*19.9 ± 2.3**	*3.60 ± 0.21*	*5*
Propranolol (1 μM)	*17.1 ± 3.6*	*3.80 ± 0.44*	*15.4 ± 4.4*	*3.46 ± 0.52*	*5*
Prazosin (1 μM)	*14.0 ± 2.8*	*3.80 ± 0.45*	*Abolished**	*Abolished**	*6*
L-NNA (100 μM)	*16.7 ± 9.3*	*3.80 ± 0.44*	*12.8 ± 6.0*	*3.55 ± 0.41*	*6*
Noradrenaline					
Tyramine pre-treatment (3mM)	*85.7 ± 12.2*	*5.42 ± 0.21*	*85.9 ± 6.5*	*5.22 ± 0.13*	*5*
Propranolol (1 μM)	*81.9 ± 5.9*	*5.92 ± 0.14*	*91.4 ± 4.4*	*5.65 ± 0.09*	*5*
Prazosin (1 μM)	*87.7 ± 4.7*	*5.92 ± 0.14*	*81.9 ± 5.2*	*5.10 ± 0.08**	*5*

ROLE OF DIRECT ADRENOCEPTOR STIMULATION IN TRACE AMINE-INDUCED VASOCONSTRICTION IN MESENTERIC ARTERY.

In noradrenaline-depleted artery rings the presence of the α_1-adrenoceptor antagonist prazosin (1 μM) abolished the responses to all three trace amines, tyramine, octopamine and synephrine (Figure 18, Table 9). The maximum contractions in response to noradrenaline in the presence of prazosin were not significantly affected. However, there was a slight but significant rightward shift for the potency value of noradrenaline treated with 1 μM prazosin (*pEC50* = 5.10 ± 0.08, n = 5, $p < 0.05$). Neither maximum contractile responses nor potencies for any of the amines or noradrenaline changed in the presence of propranolol (1 μM) (Figure 18, Table 9).

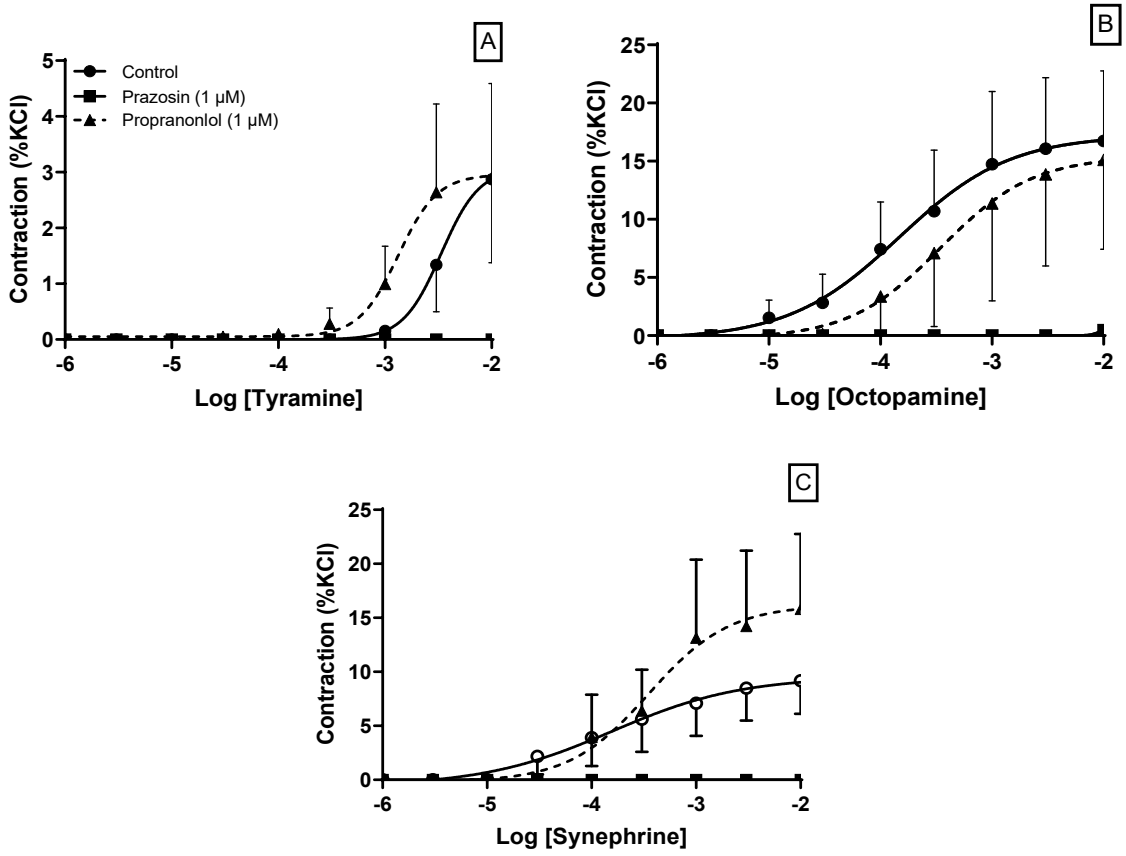


Figure 18: Concentration-response curves to tyramine (A), octopamine (B) and p-synephrine(C) in the absence (○) and presence of prazosin (1 μM, ▪) or propranolol (1 μM, ∆) in NA-depleted mesenteric arterial rings. Data are means ± sem (n=4-6) values expressed as a percentage of the contractile response to potassium chloride (60mM).

STUDY 2: CORONARY ARTERY RESPONSES TO TRACE AMINES

RESPONSES OF THE PORCINE CORONARY ARTERY TO NORADRENALINE AND TRACE AMINES

All three trace amines produced concentration-dependent contractions of porcine coronary arterial rings (Figure 19a). Octopamine and synephrine produced similar maximum responses, whilst those to tyramine were greater, the difference between tyramine and octopamine being statistically significant ($p < 0.05$). The potencies of all three amines were similar and ranged from 3.30 – 3.88 (Table 10). The endogenous amine noradrenaline failed to produce contraction and only relaxations were observed (Figure 19b). These relaxations were converted to contractions in the presence of the β-adrenoceptor antagonist propranolol (1μM). The maximum contractile responses to noradrenaline (33.4 ± 2.7 % of the response to potassium) were significantly greater than those to octopamine (20.3 ± 4.6 %, $p < 0.05$) and synephrine (21.1 ± 7.3 %, $p < 0.05$), but not tyramine (32.3 ± 4.5 %). In the presence of both propranolol (1μM) and the α_1-adrenoceptor antagonist prazosin (1μM) responses to noradrenaline were abolished completely.

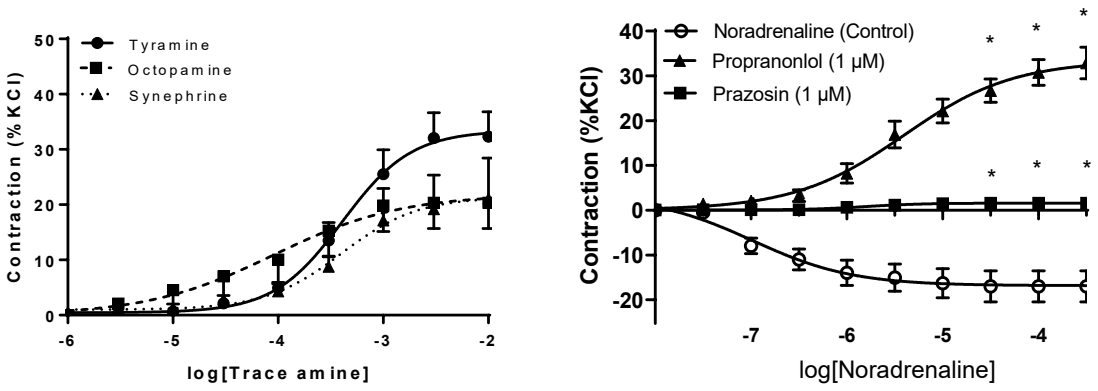


Figure 19: (Left) Concentration-response curves of porcine coronary artery to tyramine, octopamine and p-synephrine (n=6-8). (Right) Concentration-response curves to noradrenaline in the absence of antagonists and in the presence of propranolol (1M) and in the combined presence of propranolol (1 μM) and prazosin (1 μM). Responses are expressed as a percentage of the contractile response to potassium chloride (60 mM). *p<0.05 vs noradrenaline

THE ROLE OF ENDOGENOUS NORADRENALINE RELEASE IN RESPONSES IN CORONARY ARTERY.

Tyramine pre-treatment did not affect the potencies or maximal contractions to either tyramine itself or synephrine (Figure 20, Table 10). Responses to octopamine appeared to be reduced by about 50% and although maximum responses were not significantly altered in depleted tissues, responses to lower concentrations of octopamine were reduced in depleted tissues (Figure 20).

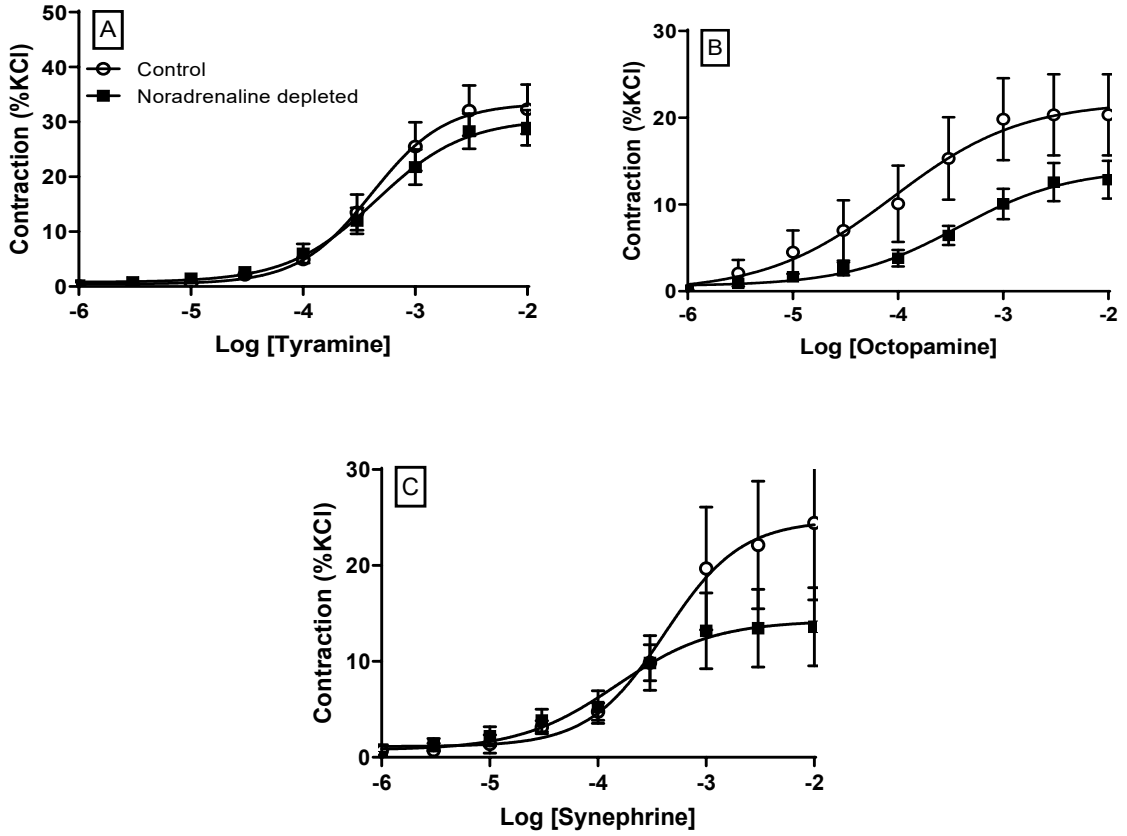


Figure 20 Concentration-response curves to tyramine (A), octopamine (B) and synephrine (C) in control tissues (○) and in arteries previously depleted of neuronal NA (▪). Data are means ± sem values from *n* = 6 to 24 experiments, expressed as a percentage of the contractile response to potassium chloride (60 mM).

ROLE OF ADRENERGIC RECEPTOR BLOCKADE IN NA-DEPLETED CORONARY ARTERY

In noradrenaline-depleted artery rings the presence of the α_1-adrenoceptor antagonist prazosin (1 μM) reduced maximum contractions produced by tyramine, octopamine and synephrine by about half without significantly affecting agonist potency (Figure 21, Table 10). Contractile responses to synephrine were the most significantly affected by prazosin (1μM), but responses were again not completely abolished. Neither maximum contractile responses nor potencies for any of the amines changed significantly in the presence of propranolol (1 μM) (Figure 21).

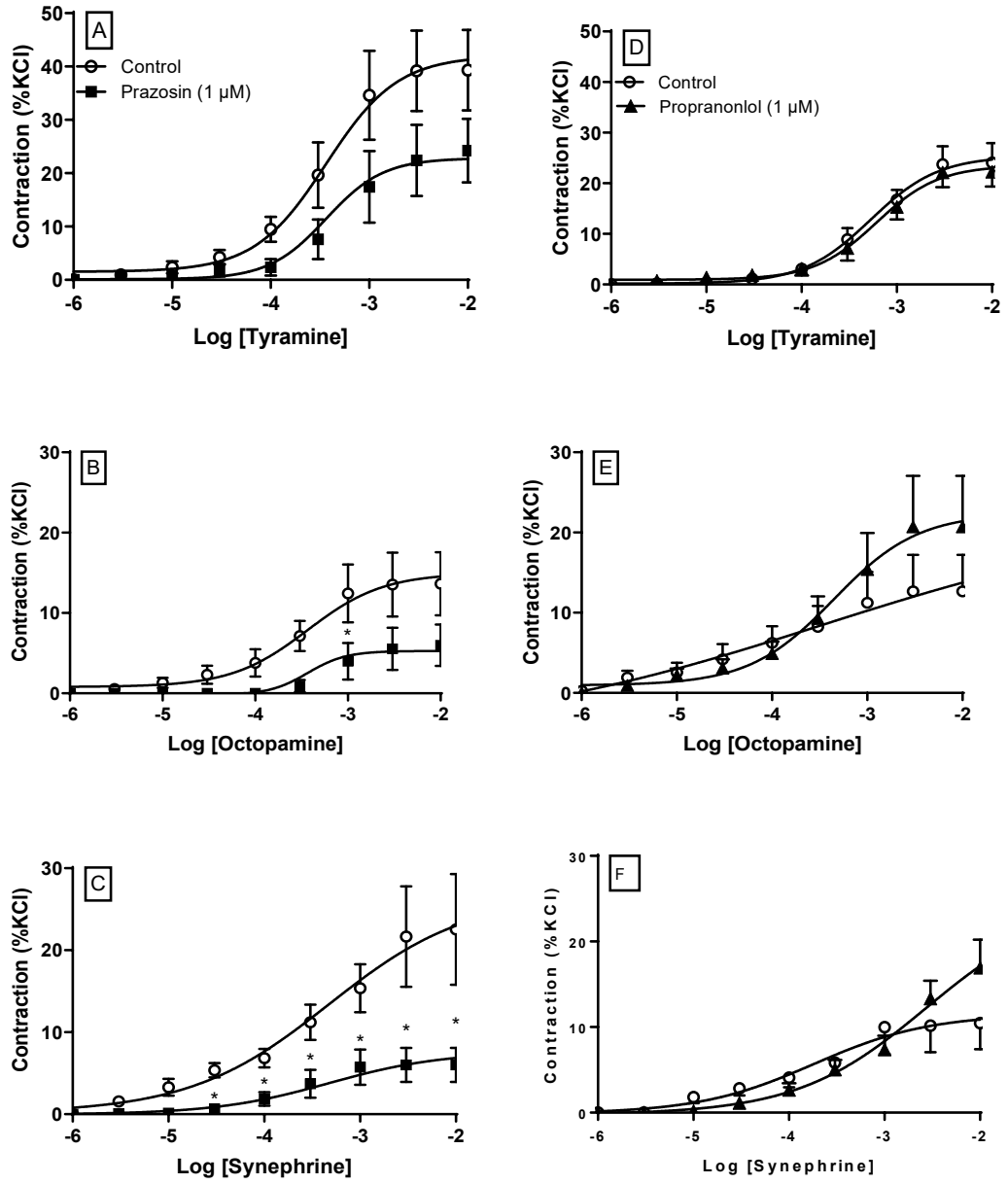


Figure 21: Concentration-response curves to tyramine, octopamine and synephrine in the absence (○) and presence of prazosin (1 μM, ■; A,B,C) or propranolol (1 μM, ▲;D E,F) in NA-depleted coronary arterial rings. Data are means ± sem (n

= 5 -7) values expressed as a percentage of the contractile response to potassium chloride (60mM). Two-way ANOVA *p<0.05 vs control

EFFECT OF NITRIC OXIDE INHIBITION IN NA-DEPLETED CORONARY ARTERY

To examine whether responses to any of the three amines involved the release of nitric oxide, tissues were incubated with the nitric oxide synthase inhibitor, L-NNA (100 μM). None of the responses to these amines was affected by the removal of nitric oxide (Figure 22). The inhibition of NOS did not significantly affect responses to any of the amines indicating they did not stimulate release of NO.

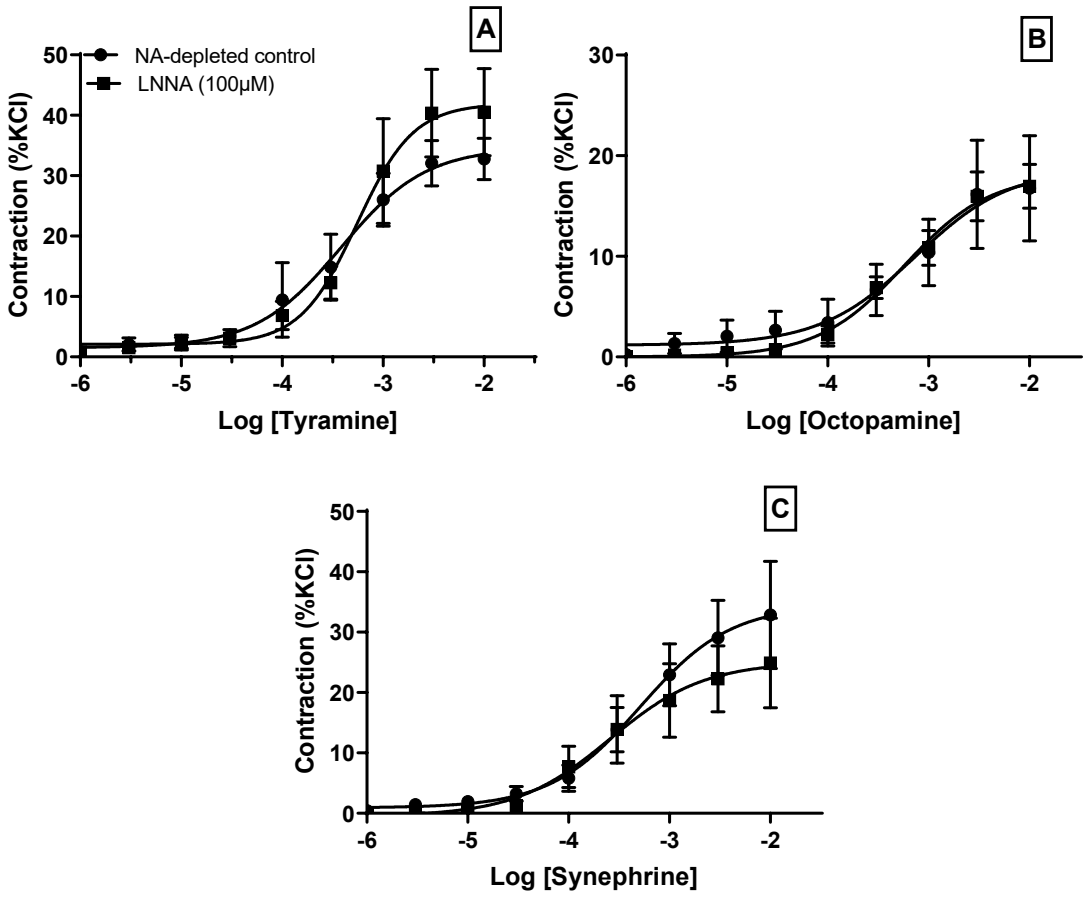


Figure 22: The effect of L-NNA (100uM, ■) on responses to tyramine (A), synephrine (B) and octopamine (C) in noradrenaline-depleted coronary arteries (control, ●). Contractile responses are means ± sem (n = 6 -9) values expressed as a percentage of the contractile response to potassium chloride (60mM).

ROLE OF TRACE AMINE-ASSOCIATED RECEPTOR 1 (TAAR-1) IN TRACE AMINE-INDUCED VASOCONSTRICTION OF CORONARY ARTERY

The trace amine-induced coronary contractions were reduced but were not abolished in the presence of the adrenoceptor antagonists. In noradrenaline-depleted tissues the possible involvement of the TAAR-1 receptor in mediating responses was investigated using the selective TAAR-1 antagonist EPPTB (Figure 23, Table 10). The effects of this antagonist were dependent on the concentration of the antagonist. At the lower concentration (100 nM), EPPTB appeared to enhance maximum responses to tyramine without altering *pEC50* values, but these changes were not statistically significant. The low EPPTB concentration had no effect on responses to synephrine or octopamine.

The higher concentration of antagonist (100 μM EPPTB) halved the maximal responses to octopamine and significantly reduced the contractile responses to tyramine and synephrine. The concentration-response curves to octopamine were also shifted to the right with a significant increase in octopamine *pEC50* values (Figure 23; Table 11).

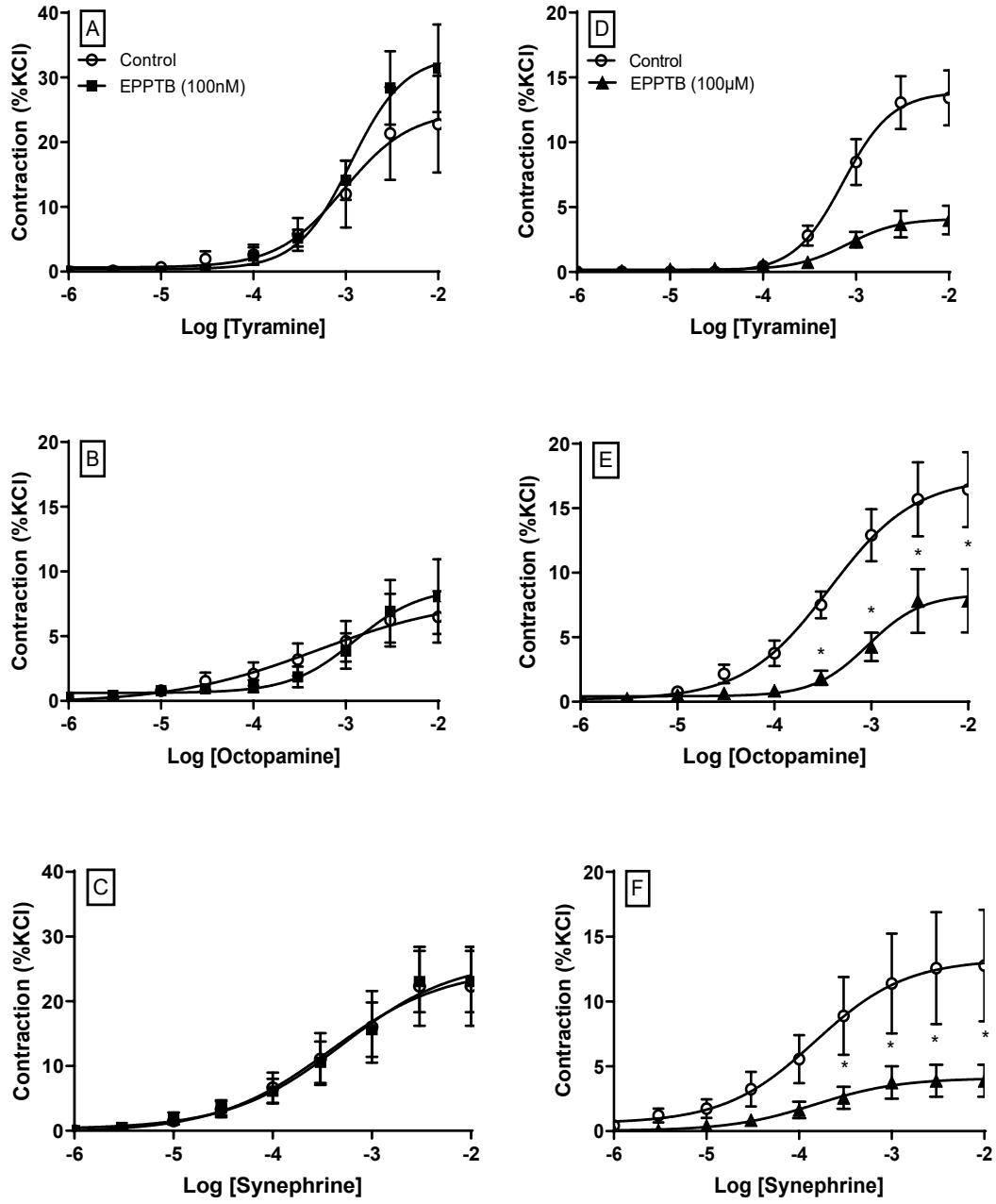


Figure 23: Concentration-response curves to tyramine (A,D), octopamine (B,E) and synephrine (C,F) in the absence (○) and presence of the TAAR-1 antagonist, EPPTB at 100 nM (■;A,B,C) or 100 μM (▲;D,E,F) in porcine coronary arterial rings. Data are means ± sem from *n* = 5 to 8 separate experiments, expressed as a percentage of the contractile response to potassium chloride (60mM) Two-way ANOVA *p<0.05 vs control

Table 10: Effects of antagonists on the maximum responses and potency values of tyramine and synephrine in porcine coronary arteries. Paired parametric Student's t-test *vs. controls $p < 0.05$. **vs. controls $p < 0.01$

Trace amine and interventions	*Controls*		*Treatment*		*Sample number (n)*
	Max contraction (%KCl)	*Potency (pEC50)*	*Max contraction (%KCl)*	*Potency (pEC50)*	
Tyramine					
Tyramine pre-treatment (3 mM)	*32.3 ± 4.5*	*3.30 ± 0.13*	*34.12 ± 6.5*	*3.37 ± 0.12*	*8*
Propranolol (1 µM)	*24.1 ± 3.8*	*3.27 ± 0.12*	*22.3 ± 2.9*	*3.23 ± 0.11*	*5*
Prazosin (1 µM)	*39.3 ± 7.5*	*3.51 ± 0.17*	*24.3 ± 5.9***	*3.26 ± 0.21*	*6*
EPPTB (100 nM)	*22.8 ± 7.5*	*3.03 ± 0.31*	*31.4 ± 6.7*	*2.94 ± 0.14*	*6*
EPPTB (100 µM)	*13.4 ± 2.1*	*3.13 ± 0.09*	*4.0 ± 1.1***	*3.10 ± 0.18*	*7*
L-NNA (100 µM)	*34.6 ± 4.5*	*3.42 ± 0.20*	*41.6 ± 4.1*	*3.27 ± 0.14*	*6*
Synephrine					
Tyramine pre-treatment (3 mM)	*21.1 ± 7.3*	*3.50 ± 0.26*	*13.1 ± 3.4*	*3.55 ± 0.19*	*6*
Propranolol (1 µM)	*10.5 ± 3.0*	*3.74 ± 0.44*	*16.9 ± 0.3*	*2.59 ± 0.29*	*7*
Prazosin (1 µM)	*22.5 ± 6.7*	*3.21 ± 0.80*	*6.0 ± 2.1**	*3.70 ± 0.35*	*6*
EPPTB (100 nM)	*25.1 ± 7.2*	*3.42 ± 0.43*	*27.0 ± 5.1*	*3.54 ± 0.33*	*6*
EPPTB (100 µM)	*12.8 ± 4.3*	*3.82 ± 0.42*	*3.9 ± 1.3**	*3.83 ± 0.36*	*8*
L-NNA (100 µM)	*34.4 ± 6.5*	*3.29 ± 0.28*	*24.8 ± 4.5*	*3.60 ± 0.31*	*6*

Table 11: Effects of antagonists on the maximum responses and potency values of octopamine and noradrenaline in porcine coronary arteries. Paired parametric Student's t-test *vs. controls p < 0.05. **vs. controls p< 0.01

Trace amine and interventions	*Controls*		*Treatment*		*Sample number (n)*
	Max contraction (%KCl)	*Potency (pEC50)*	*Max contraction (%KCl)*	*Potency (pEC50)*	
Octopamine					
Tyramine pre-treatment (3 mM)	*20.3 ± 4.6*	*4.04 ± 0.43*	*13.7 ± 3.9*	*3.47 ± 0.21*	*6*
Propranolol (1 µM)	*12.6 ± 4.5*	*3.85 ± 0.68*	*20.7 ± 6.3*	*3.35 ± 0.34*	*5*
Prazosin (1 µM)	*13.7 ± 3.9*	*3.55 ± 0.27*	*5.9 ± 2.6**	*3.15 ± 0.24**	*6*
EPPTB (100 nM)	*6.5 ± 2.0*	*3.31 ± 0.88*	*8.1 ± 2.9*	*2.94 ± 0.25*	*5*
EPPTB (100 µM)	*16.5 ± 2.9*	*3.43 ± 0.18*	*7.8 ± 2.5**	*3.04 ± 0.20*	*6*
L-NNA (100 µM)	*18.8 ± 6.0*	*3.13 ± 0.49*	*17.6 ± 1.5*	*3.27 ± 0.13*	*5*
Noradrenaline					
Propranolol (1 µM)	*-16.9 ± 1.5*	*6.98 ± 0.48*	*33.4 ± 2.7***	*5.40 ± 0.15**	*6*
Propranolol + Prazosin (1 µM)	*-16.9 ± 1.5*	*6.98 ± 0.48*	*4.0 ± 1.5***	*5.29 ± 0.65**	*6*

STUDY 3: RENAL ARTERY RESPONSES TO TRACE AMINES

All the amines produced concentration-dependent contractions of the porcine renal arterial rings (Figure 24). They produced similar maximum responses with induced responses with a similar potency values ranging from (*pEC50* = 3.7 to 4.0). Contractile responses to tyramine at 300 µM and 1 mM appeared to be lower than synephrine and octopamine, but the values were not statistically different.

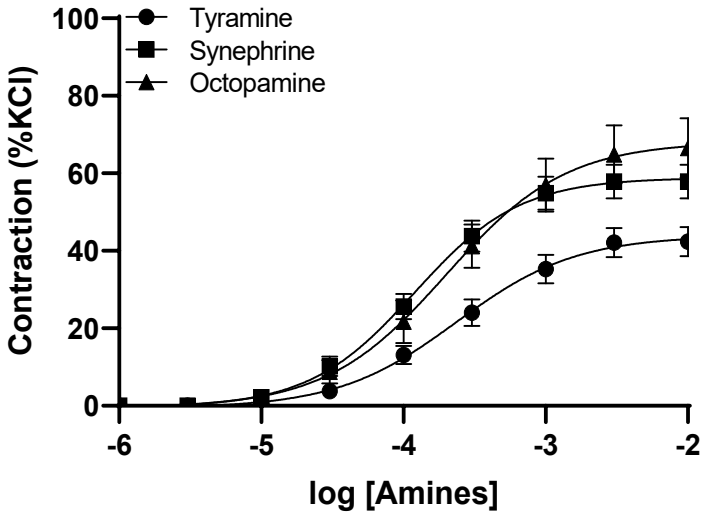


Figure 24 Cumulative concentration-response curves to the trace amines and phenylephrine in isolated renal arteries. Mean responses (±SEM, n=6-12) are expressed as a percentage of the response to 60mM potassium chloride (%KCl).

THE ROLE OF ENDOGENOUS NORADRENALINE RELEASE IN RENAL ARTERY RESPONSES

In tissues depleted of endogenous noradrenaline, the maximal contractile responses to tyramine were reduced by 68% and potency was similarly decreased (Figure 25, Table 12, *n= 12)*. Similarly, the maximum contractions to synephrine were reduced by 35% although for this amine potency was unchanged. In contrast, for octopamine neither maximum contractions nor potency were significantly affected by noradrenaline depletion.

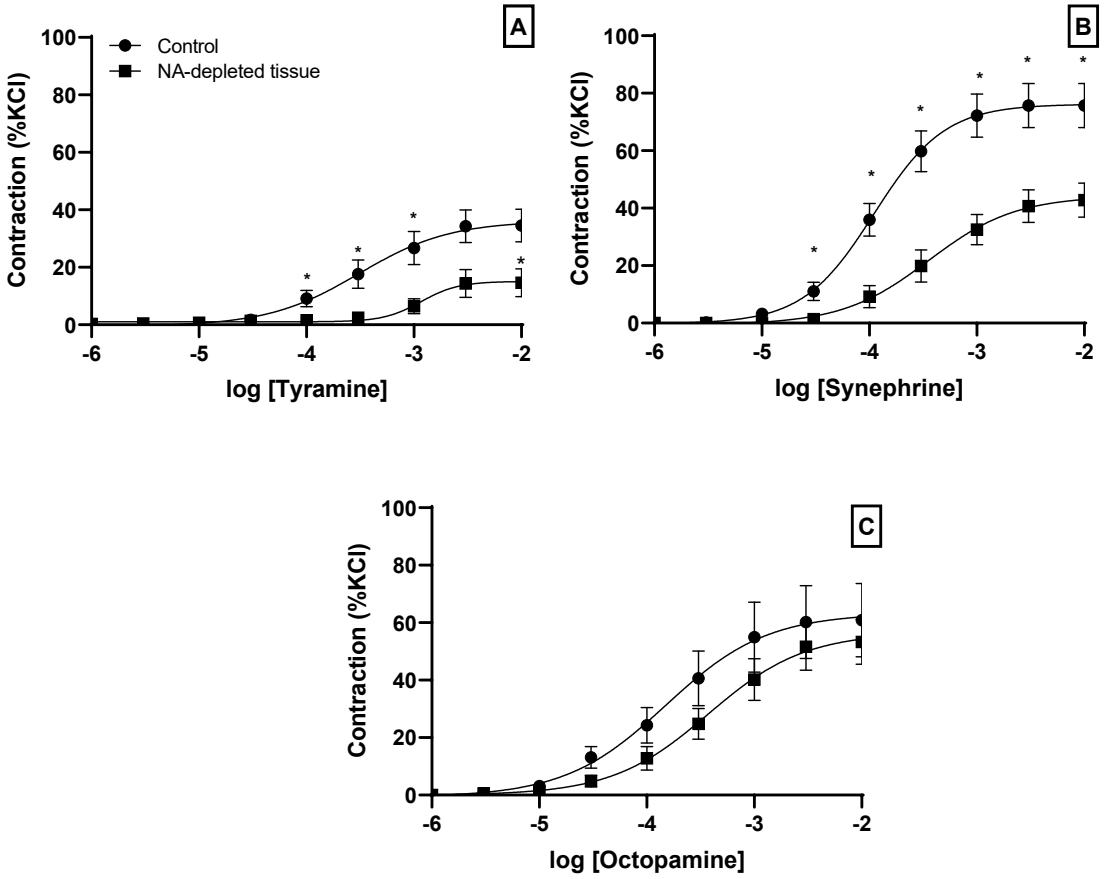


Figure 25 Concentration-response curves to tyramine (A), synephrine (B) and octopamine (C) in renal artery control tissues (●) and in tissues previously depleted of neuronal noradrenaline (■) using prolonged contact (60 mins) with a high concentration of tyramine (3mM). Responses are means ± sem values (n= 4 – 12) expressed as a percentage of the contractile response to potassium chloride (60 mM). Two-way ANOVA *p<0.05 vs control

Table 12 The effects of deleting endogenous noradrenaline stores on renal artery responses to trace amines. Tissues were incubated with tyramine (3mM) for 60 minutes to deplete stores.

Trace amine	*Controls*		*Noradrenaline-depleted Tissues*		*Sample size (n)*
	Max contraction (%KCl)	*Potency (pEC50)*	*Max contraction (%KCl)*	*Potency (pEC50)*	
Tyramine	*36.16 ± 6.73*	*3.51 ± 0.36*	*15.09 ± 4.00**	*2.93 ± 0.42**	*12*
Synephrine	*80.26 ± 11.03*	*3.93 ± 0.33*	*52.59 ± 8.98**	*3.43 ± 0.30*	*6*
Octopamine	*69.36 ± 18.04*	*3.79 ± 0.43*	*60.50 ± 13.35*	*3.28 ± 0.414*	*6*

ROLE OF ADRENERGIC RECEPTOR BLOCKADE IN NA-DEPLETED RENAL ARTERY

In noradrenaline-depleted tissues, the addition of the α_1-adrenoceptor selective antagonist (prazosin, 1μM), caused a rightward shift of concentration-response curves to synephrine and octopamine and reduced maximal contractions to these amines by only 39% and 47% respectively (Figure 26A-C, Table 12) but failed to alter responses to tyramine. Interestingly, the vasoconstrictor responses of renal artery segments, were increased by 38% and 49% for synephrine and octopamine but not tyramine in the presence of the non-selective β-adrenoceptor antagonist, propranolol (1 μM) (Figure 26D-F, Table 12). Moreover, octopamine was more potent after β-adrenoceptor blockade but the potency of synephrine on the renal artery remained the same. The combination of α_1- and β-adrenoceptor blockade caused a significant decrease in tyramine and octopamine maximum response but not potency. The possible role of α_2-adrenoceptors in mediating contractions was also investigated using the selective antagonist yohimbine (100 nM) on depleted tissues. The presence of this antagonist however did not have any effect on the responses to any of the trace amines (Table 13).

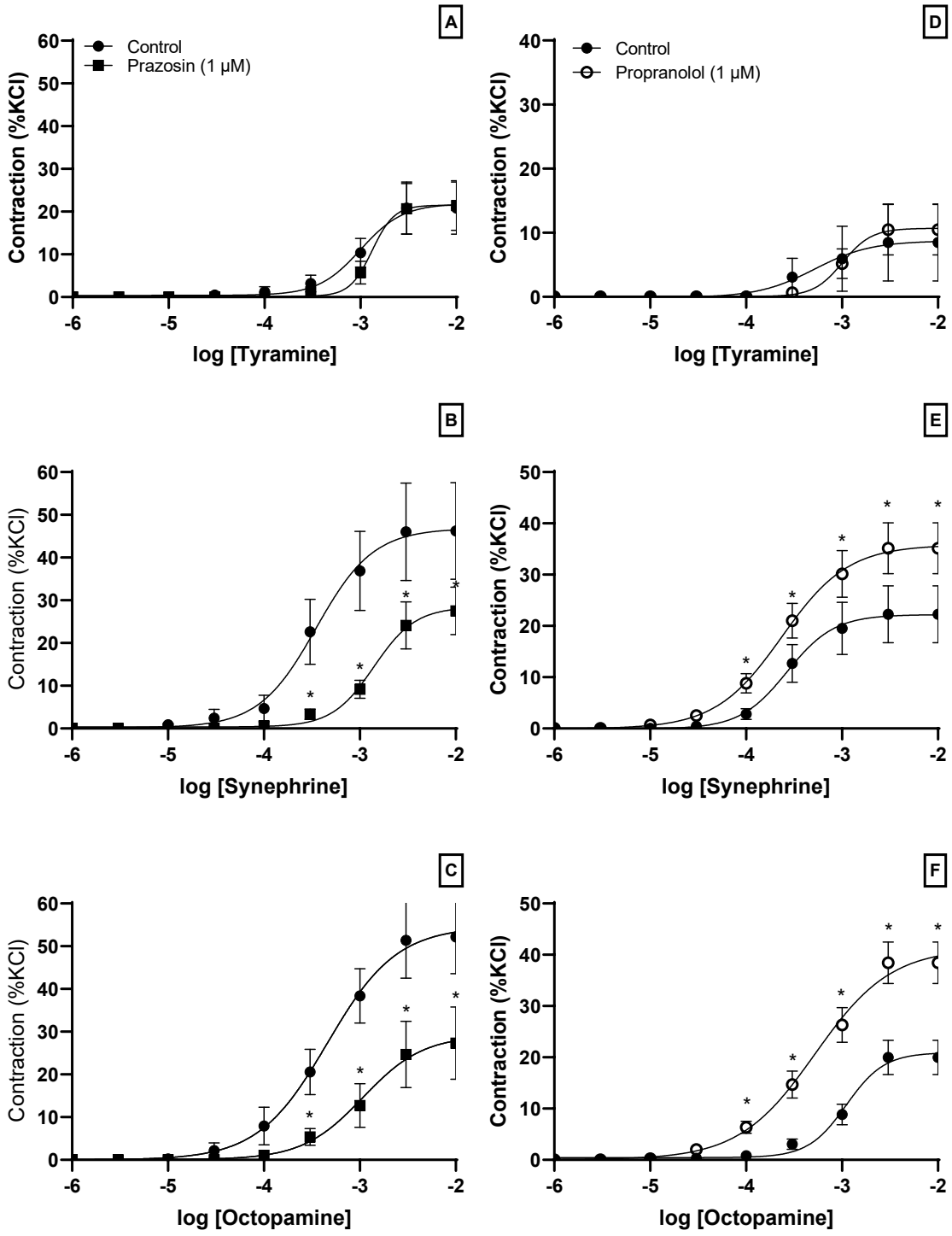


Figure 26 The effect of prazosin (1 μM, A-C, ■) and propranolol (1μM, D-F,○) on concentration-response curves to tyramine (A, D), synephrine (B, E) and octopamine (C, F) in noradrenaline-depleted renal arteries (control, ●). Responses are means ± sem (n = 6 -9) values expressed as a percentage of the contractile response to potassium chloride (60mM). Two-way ANOVA *p<0.05 vs control.

EFFECT OF NITRIC OXIDE INHIBITION IN NA-DEPLETED RENAL ARTERY

To investigate the possible role of nitric oxide in modulating contractile responses to the amines, the responses of noradrenaline-depleted tissues to the amines were examined in the presence of the nitric oxide synthase inhibitor, L-NNA (100 μM). Contractile response of the renal artery to tyramine were not affected by the presence of L-NNA (Figure 27). However, maximum responses to both synephrine and octopamine were doubled in the presence of L-NNA and their potency were significantly increased (Table 13).

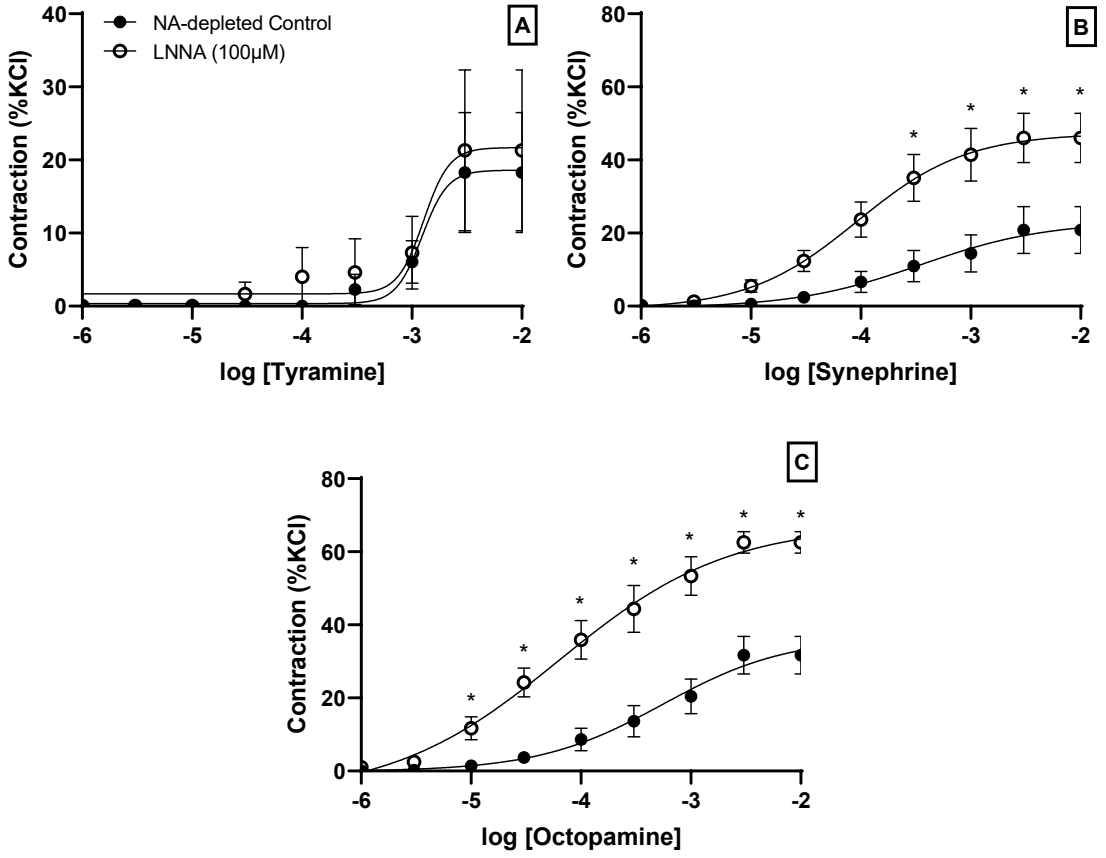


Figure 27. The effect of L-NNA (100uM, ○) on responses to tyramine (A), synephrine (B) and octopamine (C) in noradrenaline-depleted renal arteries (control, ●). Contractile responses are means ± sem (n = 6 -9) values expressed as a percentage of the contractile response to potassium chloride (60mM). Two-way ANOVA *p<0.05 vs control.

Table 13: Effects of antagonists/inhibitors on the maximum responses and potency values of tyramine, synephrine and octopamine in porcine noradrenaline-depleted renal arteries. Maximum responses are expressed as a percentage of the response to KCl (60mM). *P<0.05 versus controls, paired Student's t-test.

Trace amine and interventions	*Controls*		*Treatment*		*n*
	Max contraction (%KCl)	*Potency (pEC50)*	*Max contraction (%KCl)*	*Potency (pEC50)*	
Tyramine					
Propranolol (1 μM)	*8.74 ± 4.74*	*3.29 ± 0.71*	*10.7 ± 2.93*	*2.99 ± 0.37*	*7*
Prazosin (1 μM)	*21.85 ± 5.16*	*2.99 ± 0.45*	*21.51 ± 8.01*	*2.88 ± 0.28*	*9*
L-NNA (100 μM)	*18.60 ± 5.77*	*2.90 ± 0.44*	*21.70 ± 8.25*	*2.90 ± 0.80*	*6*
Prazosin (1 μM) + Propranolol (1 μM)	*40.27 ± 9.26*	*2.97 ± 0.36*	*19.47 ± 2.96**	*2.90 ± 0.34*	*6*
Prazosin (1 μM) + Propranolol (1 μM) + L-NNA (100 μM)	*25.81 ± 13.12*	*3.03 ± 0.46*	*24.67 ± 5.67*	*3.18 ± 0.40*	*6*
Synephrine					
Propranolol (1 μM)	*22.21 ± 4.37*	*3.57 ± 0.32*	*35.81 ± 4.7 **	*3.628 ± 0.24*	*9*
Prazosin (1 μM)	*46.83 ± 10.12*	*3.46 ± 0.36*	*28.43 ± 5.59**	*2.86 ± 0.21**	*8*
L-NNA (100 μM)	*23.17 ± 7.72*	*3.46 ± 0.33*	*47.33 ± 7.39**	*4.03 ± 0.54**	*9*
Prazosin (1 μM) + Propranolol (1 μM)	*53.18 ± 19.08*	*4.01 ± 0.24*	*38.73 ± 7.49*	*3.37 ± 0.37*	*6*
Prazosin (1 μM) + Propranolol (1 μM) + L-NNA (100 μM)	*56.27 ± 15.48*	*3.19 ± 0.57*	*62.16 ± 13.3*	*3.50 ± 0.50*	*8*
Octopamine					
Propranolol (1 μM)	*20.93 ± 3.14*	*2.96 ± 0.18*	*41.31 ± 5.1**	*3.28 ± 0.20**	*9*
Prazosin (1 μM)	*54.61 ± 9.21*	*3.34 ± 0.28*	*29.15 ± 8.21**	*2.97 ± 0.44**	*6*
L-NNA (100 μM)	*36.62 ± 8.83*	*3.26 ± 0.50*	*67.43 ± ± 9.18**	*4.20 ± 0.47**	*9*
Prazosin (1 μM) + Propranolol (1 μM)	*82.17 ± 12.28*	*3.22 ± 0.25*	*38.91 ± 9.23 **	*3.17 ± 0.45*	*6*
Prazosin (1 μM) + Propranolol (1 μM) + L-NNA (100 μM)	*76.81 ± 15.2*	*2.97 ± 0.32*	*57.78 ± 11.15*	*3.268 ± 0.38*	*6*

ROLE OF TRACE AMINE-ASSOCIATED RECEPTOR-1 (TAAR-1) IN RENAL ARTERY VASOCONSTRICTION

Even after depleting tissues of noradrenaline, blocking nitric oxide synthase and antagonising α_1- and β-adrenoceptors, significant contractions of the renal artery to the amines remained, suggesting a role for trace amine receptors. Thus, a role for the TAAR-1 receptor subtype was investigated in depleted tissues in the presence of the inhibitor cocktail (prazosin 1μM, propranolol 1μM, L-NNA 100μM) using the selective TAAR-1 antagonist, EPPTB at either 100nM or 100μM (Figure 28,Table 14). There were no significant differences in maximum contractile responses or potencies of the trace amines at either of the antagonist concentrations (Table 14). However, in the presence of EPPTB (at both concentrations) the antagonist significantly increased ($p<0.05$, $n=6$) the contractile responses to low concentrations of octopamine (10 μM to 100 μM). The TAAR-1 antagonist at the higher concentration had a similar effect on low concentrations of synephrine (Figure 28). This effect with this antagonist was not observed when examining responses to tyramine.

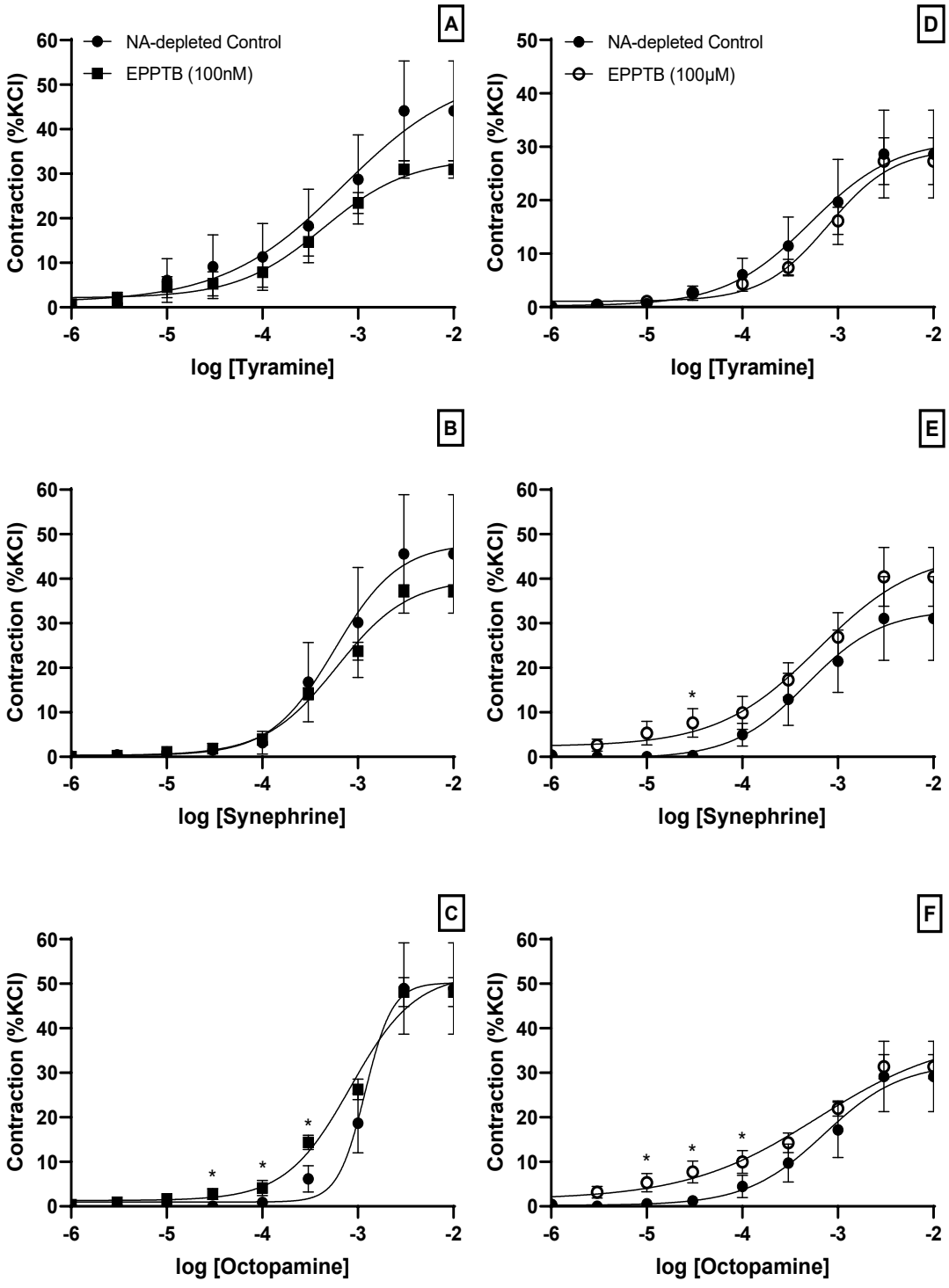


Figure 28: The effect of EPPTB (100nM, A, B, C; ■) and EPPTB (100µM, D, E, F; ○) on concentration-response curves of renal arteries to tyramine (A & B), synephrine (C & D) and octopamine (E & F). Experiments were conducted on noradrenaline-depleted tissues (control, ●) . The antagonists were added in the presence of a cocktail of inhibitors to remove α- and β-adrenoceptor and nitric oxide contributions to responses (prazosin, 1 µM; propranolol, 1 µM; L-NNA, 100 µM). Responses are mean ± sem (n = 6 - 14) values expressed as a percentage of the contractile response to potassium chloride (60mM). Two-way ANOVA *p<0.05 vs control.

ROLE OF SEROTONERGIC RECEPTORS (5HT) IN RENAL ARTERY CONTRACTION

To investigate the possible role of serotonin receptors in mediating contractile responses to the amines, the response of noradrenaline-depleted tissues to the amines were examined in the presence of a mixture of antagonists (prazosin 1μM, propranolol 1μM, L-NNA 100μM) and either with a non-selective 5-HT antagonist, methiothepin (1 μM) or the 5-HT_{2A}-selective antagonist, ketanserin (1 μM). There were no significant changes in both the maximum contractile responses or potencies of the trace amines with either 5-HT antagonists used (Figure 29,Table 14).

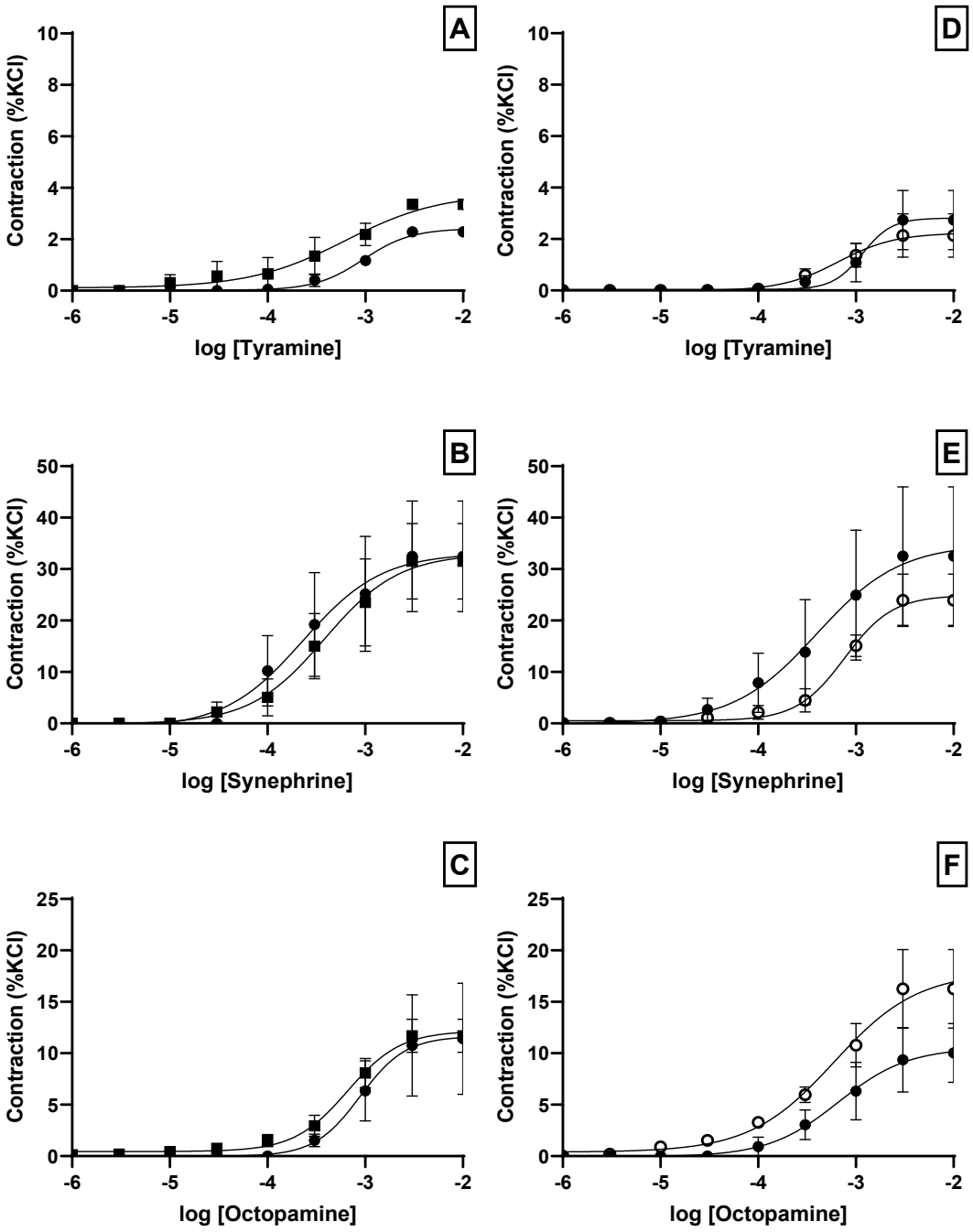


Figure 29: The effect of ketanserin (1 µM, A, B, C; ■) and Methiothepin (1 µM, D, E, F; ○) on concentration-response curves of renal arteries to tyramine (A & B), synephrine (C & D) and octopamine (E & F). Experiments were conducted on noradrenaline-depleted tissues (control, ●). The antagonists were added in the presence of a cocktail of inhibitors to remove α- and β-adrenoceptor and nitric oxide contributions to responses (prazosin, 1 µM; propranolol, 1 µM; L-NNA, 100 µM). Responses are mean ± sem (n = 6 - 14) values expressed as a percentage of the contractile response to potassium chloride (60mM). Student's paired parametric t-test *p<0.05 vs control

Table 14: The effects of the TAAR-1 antagonist EPPTB (10nM and 100μM) and 5-HT antagonists ketanserin and methiothepin (both μM) on the responses to trace amines of the renal artery following isolation of the non-adrenergic, non-nitrergic responses to trace amines. Tissues were depleted of endogenous noradrenaline and then responses to the trace amines obtained in the presence of prazosin (1μM), propranolol (1μM) and L-NNA (100uM). Maximum responses were expressed as a percentage of the response to KCl (60mM).

Trace amine and antagonist	*Controls*		*Treatment*		*n*
	Max contraction (%KCl)	*Potency (pEC50)*	*Max contraction (%KCl)*	*Potency (pEC50)*	
Tyramine					
EPPTB (100nM)	*53.42 ± 19.29*	*3.16 ± 0.29*	*33.51 ± 5.02*	*3.36 ± 0.29*	*5*
EPPTB (100μM)	*31.40 ± 9.65*	*3.29 ± 0.71*	*29.67 ± 4.78*	*3.099 ± 0.25*	*14*
ketanserin (1μM)	*2.42 ± 0.31*	*3.02 ± 0.15*	*3.79 ± 0.95*	*3.21 ± 0.48*	*4*
methiothepin (1μM)	*2.81 ± 0.88*	*2.93 ± 0.47*	*2.24 ± 0.69*	*3.18 ± 0.51*	*4*
Synephrine					
EPPTB (100nM)	*48.02 ± 13.69*	*3.24 ± 0.50*	*39.99 ± 2.88*	*3.22 ± 0.11*	*6*
EPPTB (100μM)	*33.06 ± 9.36*	*3.33 ± 0.55*	*46.24 ± 10.66*	*3.21 ± 0.48*	*6*
ketanserin (1μM)	*33.11 ± 10.72*	*3.64 ± 0.26*	*33.00 ± 8.27*	*3.42 ± 0.48*	*4*
methiothepin (1μM)	*34.84 ± 12.99*	*3.40 ± 0.60*	*24.91 ± 8.45*	*3.11 ± 0.25*	*3*
yohimbine (100nM)	*32.84 ± 6.06*	*3.10 ± 0.29*	*39.07 ± 8.13*	*3.18 ± 0.40*	*8*
Octopamine					
EPPTB (100nM)	*50.20 ± 8.57*	*2.93 ± 0.19*	*52.26 ± 4.7*	*3.08 ± 0.13*	*6*
EPPTB (100μM)	*32.24 ± 9.04*	*3.15 ± 0.52*	*38.42 ± 8.07*	*3.21 ± 0.43*	*6*
ketanserin (1μM)	*11.65 ± 4.02*	*3.05 ± 0.53*	*12.18 ± 1.68*	*3.182 ± 0.31*	*4*
methiothepin (1μM)	*10.54 ± 3.14*	*3.18 ± 0.52*	*17.89 ± 3.95*	*3.224 ± 0.41*	*4*
yohimbine (100nM)	*34.00 ± 4.21*	*2.94 ± 0.35*	*47.72 ± 8.11*	*3.30 ± 0.31*	*6*

SUMMARY OF CONTRACTILE RESPONSES OF THE MESENTERIC, CORONARY, AND RENAL ARTERIES TO TRACE AMINES

A summary of hypothesized mechanisms of action of trace amine (tyramine, synephrine and octopamine) on porcine arterial responses are shown below (Table 15). On the mesenteric artery, responses to trace amines were mediated via an indirect sympathomimetic and direct α_1-adrenoceptor mechanism. These direct effects were not affected by the β-adrenoceptor antagonist, propranolol (1 μM) or by inhibiting nitric oxide synthase with L-NNA.

On the coronary artery, responses to trace amines were complex and involved several mechanisms whose contributions differed for each amine. Endogenous noradrenaline release did not affect the contraction of coronary vessels to trace amines. Trace amines appeared to have almost no indirect sympathomimetic actions but elicit contraction via weak direct agonist actions on α_1-adrenoceptors and possibly TAAR receptors other than the TAAR-1 subtype such as TAAR 3, 4 and 6, commonly expressed in heart tissues (Chiellini et al., 2007). In addition, the contractile response to tyramine appeared to be modified by an inhibitory effect mediated via TAAR-1, which was not observed with synephrine or octopamine. However, there were no parallel shifts of the curves for all three amines in the presence of EPPTB in the coronary artery, which suggest a non-competitive blockade. Until there is a more specific antagonist developed for the other TAAR subtypes, EPPTB's blockade of trace amine activity in the coronary artery is non-specific

All three amines induced contraction of the renal artery, but their mechanisms of action were different (Table 15). Tyramine mediated contractile responses predominantly via an indirect sympathomimetic action. In noradrenaline-depleted tissues, the contractions to tyramine were not affected by the TAAR-1 antagonist EPPTB (100 nM or 100 μM). In contrast, the contractile effects to synephrine were more complex and involve a predominant contractile effect that was mediated via an indirectly acting sympathomimetic action and direct agonist effects on α_1-adrenoceptors in noradrenaline-depleted tissues. This overall contractile response to synephrine was simultaneously reduced by nitric oxide release and the stimulation of β-adrenoceptors and TAAR-1 receptors. Octopamine induces contraction of the renal artery via

complex mechanisms that were similar to those elicited by synephrine, but with less indirect sympathomimetic activity.

Table 15. Summary of the mechanism of actions of trace amines on the mesenteric, coronary and renal arteries. (✓), indicates trace amine activity at their respective receptors; (x) indicates no activity; and (*), represents a plausible mechanism of action

Types of Arteries	Trace amines	Mechanisms of action					
		Indirectly Acting Sympathomimetic (ISA)	α_1-adrenoceptor	β-adrenoceptor	Nitric Oxide Synthase	Trace Amine Associated Receptor 1 (TAAR-1)	Other TAAR-subtype
Mesenteric arteries	Tyramine	✓✓	✓	✗	✗	✗	✗
	Synephrine	✓	✓	✗	✗	✗	✗
	Octopamine	✓	✓	✗	✗	✗	✗
Coronary arteries	Tyramine	✗	✓	✗	✗	✓	*
	Synephrine	✗	✓	✗	✗	✓	*
	Octopamine	✗	✓	✗	✗	✓	*
Renal arteries	Tyramine	✓	✗	✗	✗	✗	*
	Synephrine	✓	✓	✓	✓	✓	*
	Octopamine	✗	✓	✓	✓	✓	*

STUDY 4: DIFFERENCES IN CORONARY ARTERY RESPONSES TO SYNTHETIC TRACE AMINES AND *C. AURANTIUM* EXTRACTS

A preliminary study was conducted to investigate the contractile effects of the porcine coronary artery to *C. aurantium* extracts and compare the responses to that produced following the addition of an equivalent standard mixture of the three trace amines. The trace amines found in *C. aurantium* standard reference materials were quantified using HPLC-UV-MS outlined in chapter 3.4. In *C. aurantium* extracts, synephrine was the predominant amine (38.03 ± 4.09 mg/g), whereas the amount of octopamine (0.35 ± 0.03 mg/g) and tyramine (1.90 ± 1.00 mg/g) was significantly lower than synephrine (Chapter 3.4, Table 20). In this experiment, a mixture of trace amine standards with equivalent amounts of synephrine, octopamine and tyramine (in the same ratio as *C. aurantium*) was made and added to coronary arteries in a dose-dependent manner.

Interestingly, the coronary arteries showed dose-dependent vasorelaxations to *C. aurantium* extracts (0.3 mg/mL to 10 mg/mL), which were attenuated to cause vasoconstrictions in the presence of the β-adrenoceptor antagonist, propranolol (1 μM) (Figure 30A). However, the trace amine mixture caused dose-dependent contractions similar to those found in study 2 of this chapter (Figure 30B).

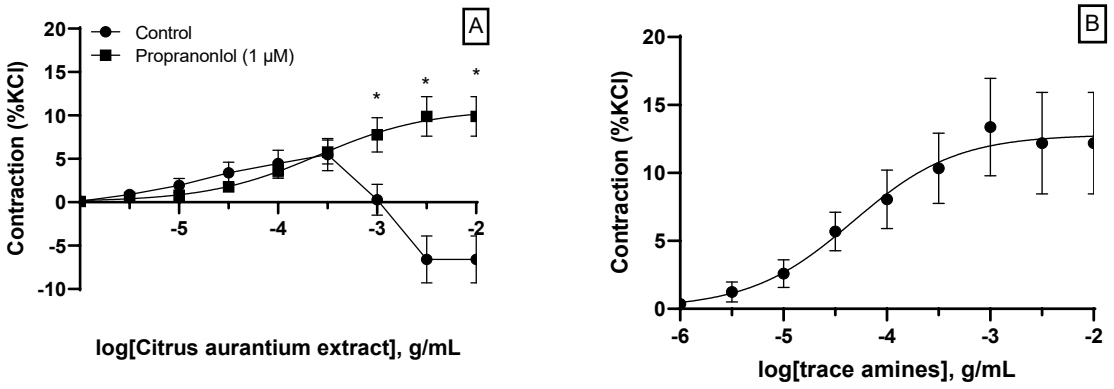


Figure 30*: Concentration-response curves to C. aurantium extracts (A) and trace amine mixture (B) in control tissues (●) and the presence of propranolol (1μM) (▪). Data are means ± sem values from n= 6 experiments, expressed as a percentage of the contractile response to potassium chloride (60 mM).*

To investigate any indirect contractile actions of the trace amines mediated by the neuronal release of noradrenaline, the tissues were depleted of noradrenaline using the protocol established in section 2.3 of this chapter. The vasorelaxant response to *C. aurantium* was reversed in noradrenaline-depleted coronary arteries, which produced small but significant contractile responses. The contractile response of the coronary artery to the trace amine mix appeared to have halved after endogenous noradrenaline-depletion (Figure 31). However, two-way ANOVA analysis showed that the difference between noradrenaline-intact controls and noradrenaline-depleted tissues were not significantly different.

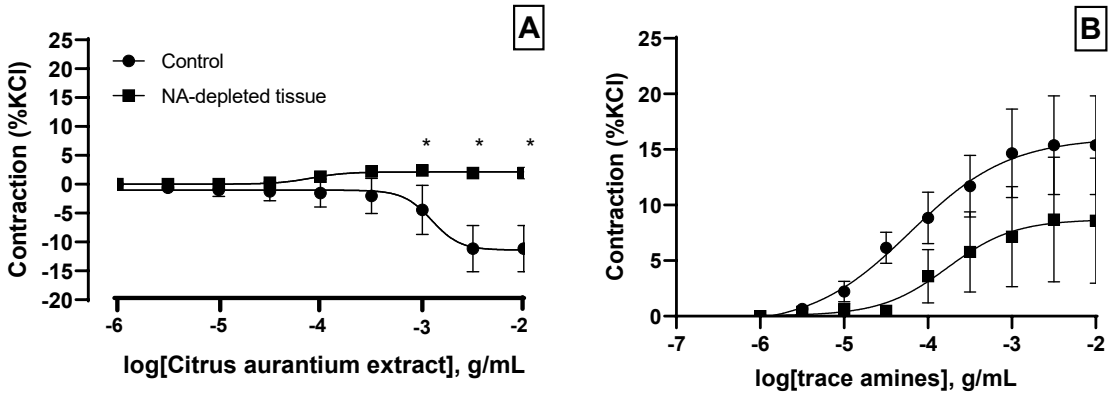


Figure 31*: Concentration-response curves to C. aurantium extracts (A) and trace amine mixture (B) in control tissues (●) and NA-depleted tissues (▪). Data are means ± sem values from n = 6 experiments, expressed as a percentage of the contractile response to potassium chloride (60 mM).*

In noradrenaline-depleted tissues, the addition of the α_1-adrenoceptor selective antagonist (prazosin, 1 μM) caused a 50% decrease in coronary arteries contractile response to *C. aurantium* and a 75% decrease in the contractile response to the trace amines (Figure 32AB, Table 16). On the other hand, propranolol (1 μM) failed to alter responses to both *C. aurantium* or trace amines (Figure 32CD, Table 16).

Table 16*: Effects of antagonists on the maximum responses and potency values of tyramine and synephrine in porcine coronary arteries. Paired parametric Student's t-test *vs. controls p < 0.05. **vs. controls p< 0.01*

Agonists and interventions	Controls		Treatment		n
	Max response (%KCl)	Potency (pEC50)	Max response (%KCl)	Potency (pEC50)	
Trace amine mix					
Tyramine pre-treatment (3 mM)	*16.08 ± 3.96*	*4.21 ± 0.39*	*8.71 ± 4.16*	*3.77 ± 0.34*	*6*
Propranolol (1 µM)	*10.29 ± 5.06*	*3.80 ± 0.43*	*11.04 ± 2.61*	*3.52 ± 0.57*	*4*
Prazosin (1 µM)	*20.62 ± 4.7*	*3.72 ± 0.52*	*6.21 ± 1.78**	*3.68 ± 0.51*	*4*
C. aurantium extract					
Tyramine pre-treatment (3 mM)	*(-)11.39 ± 4.3*	*Curve 1 (4.02 ± 0.26)* *Curve 2 (2.89 ± 0.68)*	*2.13 ± 0.41**	*4.09 ± 0.37**	*6*
Propranolol (1 µM)	*7.92 ± 2.32*	*4.82 ± 0.49*	*17.05 ± 7.43*	*3.89 ± 0.43*	*4*
Prazosin (1 µM)	*10.40 ± 2.89*	*5.16 ± 0.54*	*2.61 ± 1.63**	*5.16 ± 0.39*	*4*

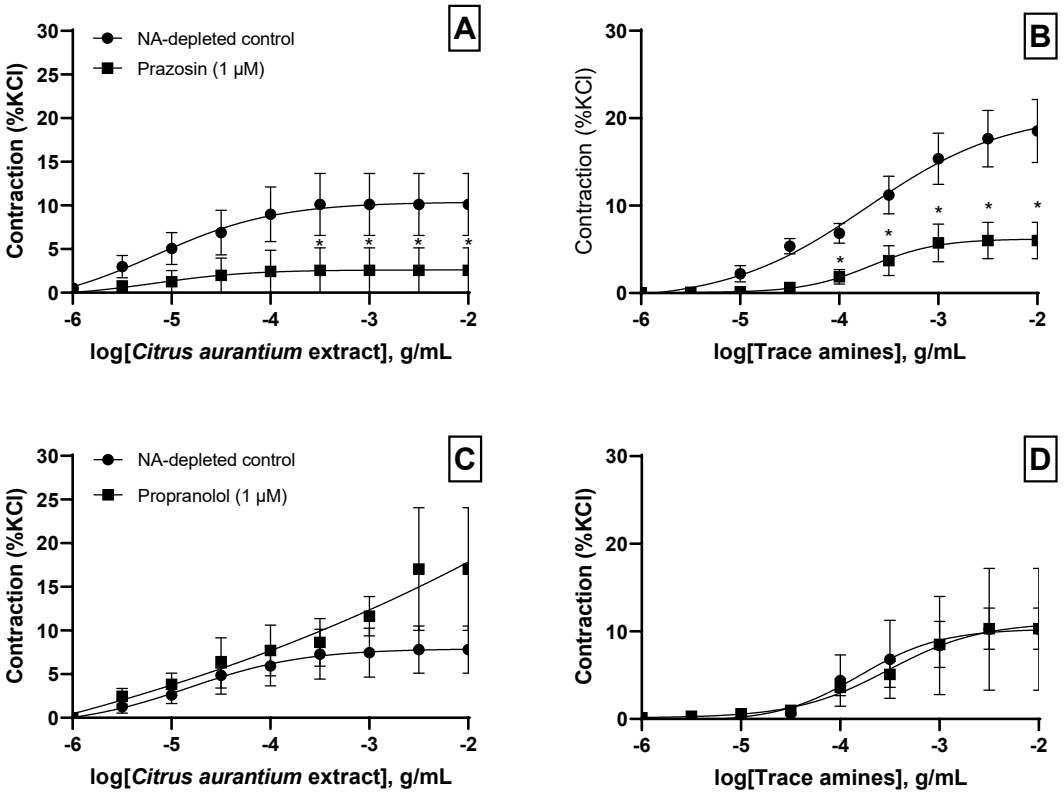


Figure 32*: Concentration-response curves to C. aurantium extracts and trace amine mix in the absence (●) and presence of prazosin (1 µM, ■; A and B) or propranolol (1 µM, ■; C and D) in NA-depleted coronary arterial rings. Data are means ± sem (n = 4) values expressed as a percentage of the contractile response to potassium chloride (60mM).*

2.5 DISCUSSION

All three trace amines contracted the mesenteric artery but were weaker and less potent than the biogenic amine noradrenaline. The potency order for the amines was noradrenaline > tyramine = synephrine = octopamine, where the differences between the three trace amines were not significantly different. Huang et al. (1995) reported that synephrine was incapable of producing contractions of isolated rat mesenteric arteries, but in this study, synephrine produced concentration-dependent vasoconstrictions of the porcine mesenteric artery, suggesting that species differences may occur. In the same study by Huang and colleagues, a *C. aurantium* extract caused dose-dependent vasoconstrictions of the rat mesenteric artery with a maximum value of 27 ± 3 % (% 60 mM KCl), a value similar to the maximum contractions observed in the responses to synephrine in this current study.

In contrast to this study's experiments on isolated mesenteric arteries, Anwar and colleagues (2012) showed tyramine failed to elicit a contractile response in perfused rat mesenteric beds rather than isolated mesenteric arteries This discrepancy between the reports may be a result of species dependency (rat vs pig). In the rat, adrenergic nerves were shown to be more densely expressed in the 2nd and 3rd branches of mesenteric arteries compared to the superior mesenteric artery (Yokomizo et al., 2015). However, no similar study on the distribution of nerve density has been reported on the mesenteric artery of pigs.

All three trace amines were weak agonists on the coronary artery and contracted the tissues with potencies similar to those seen on the mesenteric artery (i.e. pEC50 range 3.3 - 4.0). The observations for tyramine were consistent with previous studies on the porcine coronary artery (Herbert et al., 2008) and the rat aorta (Broadley et al., 2013; Hibino et al., 2009; Varma et al., 1995). However, unlike the mesenteric artery, the left anterior coronary artery contains a high proportion of β_2-adrenoceptors and relatively few α_1-adrenoceptors (Corr & Burnstock, 1991) and noradrenaline administered to control arteries caused vasorelaxation, which would be consistent with predominant β_2-adrenoceptor activation. In the presence of the β-adrenoceptor antagonist, propranolol (1 μM), the coronary artery responses to noradrenaline were reversed, and vasoconstrictions were observed. Hence, the constriction of the coronary smooth muscle in response to noradrenaline was caused by α_1-adrenoceptors, as evidenced

by the abolition of responses in the presence of the α_1-adrenoceptor antagonist prazosin. However, in contrast to noradrenaline, propranolol did not affect the responses to the trace amines, and even in the presence of prazosin, these amines could produce coronary artery vasoconstriction suggesting a non-adrenoceptor mechanism of action.

On the renal artery, contractions occurred with all three amines and their potencies were similar to the mesenteric and coronary arteries. However, the maximum contractile responses to synephrine in noradrenaline-intact renal arteries (80.26 ± 11.03 %KCl) were three times larger than on the other two arteries. The renal artery has been shown to express a highly complex network of nerves and adrenoceptors (Sata et al., 2018) and potentially a greater distribution in α_1-adrenoceptors that contributed to the large maximum contraction of synephrine. However, it may be possible that an additional reservoir of noradrenaline might be stored in perivascular adipose tissue surrounding the artery (Restini et al., 2018).

CONTRIBUTION OF INDIRECT SYMPATHOMIMETIC AGONIST ACTION TO ARTERIAL RESPONSES

To identify the ISA mechanisms involved in renal artery responses to trace amines, tissues were first depleted of noradrenaline stores by incubating with a high concentration of tyramine after washout vasoconstrictor responses to the amines were then examined. Tyramine is an indirectly acting sympathomimetic drug that releases noradrenaline from sympathetic nerves to induce autonomic responses on vascular tissues (Burn & Rand, 1958). Synephrine and octopamine have similar structures to tyramine and might be expected to act via a similar mechanism.

The depletion of noradrenaline from porcine mesenteric arteries almost abolished subsequent responses of tissues to tyramine, indicating that stores of noradrenaline were depleted. The mesenteric vasculature contains a dense neuronal network that redirects blood flow away from the intestine during sympathetic nervous system activation (Ceppa et al., 2003). Responses to exogenous noradrenaline were not affected by prior depletion of noradrenaline stores, demonstrating direct activation of α-adrenoceptors by noradrenaline caused vasoconstriction. In contrast, the responses to octopamine and synephrine were halved in noradrenaline-depleted tissues, suggesting that, like tyramine, the release of endogenous noradrenaline from sympathetic nerves contributes, at least partially, to the responses of these mesenteric vessels to these trace amines.

Different results were obtained in the coronary arteries where the three trace amines continued to elicit contractile responses after noradrenaline depletion. Responses to tyramine were almost identical in control and noradrenaline-depleted tissues; however, responses to octopamine and synephrine appeared to be reduced slightly, but the changes were small and not statistically significant. These results suggest that in the coronary artery, unlike the mesenteric artery, the release of endogenous noradrenaline from sympathetic nerves does not contribute significantly to the responses of coronary vessels to trace amines. This conclusion was further supported by the vasorelaxant responses of the coronary artery to noradrenaline in the absence of antagonists, making it impossible for the release of endogenous noradrenaline to cause the contractile responses to the trace amines.

In the renal arteries, however, the responses to tyramine were greatly reduced following depletion of tissue noradrenaline stores, with maximum responses reduced by 68%, whilst the maximum responses to synephrine were affected less and were reduced by only half that amount (34%). Synephrine has previously been shown to cause the release of [^{3}H]-noradrenaline from sympathetic nerves in the rat cerebral cortex (Kim et al., 2001) and this action appears to explain part of its effects on the renal artery, albeit with a weaker ISA action than tyramine.

In contrast, renal artery responses to octopamine were not altered by depletion of tissue noradrenaline stores, suggesting octopamine has a different mechanism of action in this tissue. Octopamine has been shown to release [^{3}H]-noradrenaline in rat vas deferens suggesting that it can act as an ISA in tissue with high sympathetic innervation (Schönfeld & Trendelenburg, 1989), but its action appears to be more direct in the rat aorta. This blood vessel would have little sympathetic innervation (Broadley et al., 2013). Therefore, it may be hypothesised that in the renal artery, indirect sympathomimetic effects predominantly mediated responses to tyramine, partially mediated responses to synephrine but were not involved in constrictor responses to octopamine.

In noradrenaline depleted tissues, there was a significant reduction in contractile responses to tyramine and synephrine in the mesenteric and renal arteries but not the coronary artery. Although this was an expected finding for tyramine, the indirect sympathomimetic behaviour of synephrine has not been reported before. So far, Kim et al. (2001) showed that naturally occurring R-(-)-synephrine increased spontaneous [^{3}H]noradrenaline release in a concentration dependant manner. Noradrenaline release was markedly inhibited by nisoxetine (a noradrenaline transporter blocker) but was not influenced by tetrodotoxin or the removal of extracellular calcium. These results suggested that the R-(-)-synephrine could act as a partial adrenergic agonist via noradrenaline release. On the other hand, octopamine was not affected by noradrenaline depletion across all three arteries, suggesting that it acts as a direct agonist on the vascular smooth muscle.

ROLE OF ADRENOCEPTORS IN THE DIRECT EFFECTS OF TRACE AMINES ON NORADRENALINE-DEPLETED TISSUES

Further studies were performed on depleted tissues to try and establish the mechanisms involved in the significant contractile responses remaining after noradrenaline depletion. Possible direct actions on renal artery adrenoceptors were investigated using prazosin and propranolol to selectively block α_1- and β-adrenoceptors, respectively.

In mesenteric arteries, the small contractile responses to the trace amines remaining after noradrenaline depletion were not affected by the β-adrenoceptor antagonist, propranolol, but were abolished in the presence of prazosin. This suggests that the amines lacked significant effects on vascular β-adrenoceptors in this tissue and that the residual contractile effects after noradrenaline depletion were mediated via α_1-adrenoceptor stimulation.

It has previously been reported that coronary artery responses to the trace amines, tyramine and β-phenylethylamine (β-PEA) were not affected by either prazosin or propranolol (Herbert et al., 2008), suggesting a lack of adrenoceptor involvement in responses. However, the coronary responses to synephrine and octopamine were not explored. In the present study, the maximum contractions of coronary arteries to all three trace amines were significantly reduced by prazosin, indicating a weak partial agonist action at the α_1-adrenoceptors of this tissue. These results were consistent with previous data using rat aorta where octopamine and synephrine activated α_1-adrenoceptors to cause contraction (Broadley et al., 2013; Brown et al., 1988; Hibino et al., 2009). However, the residual vasoconstrictions to these three amines in the presence of prazosin suggest another non-adrenergic receptor.

In noradrenaline-depleted renal arteries, the remaining responses to tyramine were not significantly affected by the presence of prazosin. On the other hand, the maximum contractile response to synephrine and octopamine were reduced in the presence of prazosin, with a small but significant shift of agonist curves. This was an unexpected outcome as prazosin is a competitive α_1-adrenoceptor antagonist, which indicated additional mechanisms activated by these amines, such as TAAR.

There was significant potentiation of vasoconstrictions to synephrine and octopamine but not tyramine in the renal arteries but not the mesenteric nor coronary arteries. β-adrenoceptors cause relaxation of vascular smooth muscle, and blockade of the receptors with propranolol increased responses to both synephrine and octopamine, demonstrating agonist actions at these receptors. A study by Varma et al. (1995) on the rat aorta has shown that after blocking α_1-adrenoceptors, synephrine and octopamine cause vasorelaxation of pre-contracted isolated rat aorta. It was suggested that in the absence of α-adrenoceptor blockade, the vasorelaxant activities on β-adrenoceptors of these amines was masked by their vasoconstrictor activities. Thus, synephrine and octopamine, but not tyramine, were direct agonists of both α- and β adrenoceptors on the renal artery. They cause an overall contraction due to their predominant α_1-adrenoceptor actions, while the β-adrenoceptor action depresses the overall contractile responses to the amines.

INFLUENCES OF NITRIC OXIDE ON CONTRACTILE RESPONSES TO TRACE AMINES

Nitic oxide appears to play a minimal role in the contractile responses to all three trace amines in the mesenteric and coronary arteries but not the renal artery. In the presence of NOS-inhibitor, L-NNA (100μM), there was a significant increase in renal contractile responses to synephrine and octopamine. Endothelial NOS (eNOS)-derived nitric oxide has been recognised to play essential roles in controlling renal hemodynamics (Toda & Okamura, 2011). In contrast, neuronal derived NOS (nNOS) is more abundant in the arterioles of the kidney. It may be possible that there was a larger distribution of nNOS present in the renal artery compared to the mesenteric or coronary artery. In denuded guinea pig aortae treated with the NOS inhibitor, N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), the contractile responses to both phenylephrine and β-phenylethylamine were enhanced. The researchers suggested that these two compounds could release nitric oxide from non-endothelial sites, presumably from the vascular smooth muscle tissue (Broadley & Broadley, 2019). It may be possible that synephrine and octopamine follow a similar pathway with NO release mediating vasodilation on the renal artery, which has an equal receptor distribution to the abdominal aorta (Sata et al., 2018).

ROLE OF TRACE AMINE-ASSOCIATED RECEPTORS (TAAR) IN ARTERIAL RESPONSES

The physiological effects of trace amine-associated receptors were first identified in the process of olfaction, but these receptors have since been identified in tissues throughout the body. The TAAR-1 subtype is unusual as it is not involved in olfaction but is involved in neuromodulation and is, therefore, a potential target for drug development in the treatment of psychiatric and neurodegenerative disorders(Lindemann et al., 2005). Stimulation of TAAR-1 causes activation of the G-protein alpha subunit (Gs) and subsequent increase in cellular cAMP concentration (Bunzow et al., 2001). The selective competitive antagonist for TAAR-1, EPPTB (RO-5212773), was first described by Bradaia et al. (2009). It has a high affinity for the mouse TAAR-1 (Ki = 0.9 nM) and was employed in the present study at a concentration of 100 nM.

The maximum responses to tyramine, but not octopamine or synephrine, appeared to be increased in the presence of 100 nM EPPTB, although the effect was not statistically significant. Had tyramine activated TAAR-1 receptors, enhanced responses would be expected since the receptor normally inhibits contraction. This would fit with the known activation of adenylyl cyclase following activation of this receptor and the inhibition of contraction by intracellular cAMP. It has been reported that tyramine was more potent at TAAR-1 than octopamine or synephrine (Borowsky et al., 2001; Wainscott et al., 2007). However, the effects of EPPTB were known to be species-dependent since the drug exhibited a high affinity for the mouse variant of TAAR-1 (Ki = 0.9 nM) but a much lower affinity for the rat (Ki = 942 nM) and human TAAR-1 (Ki > 5 μM) (Bradaia et al., 2009). The affinity of EPPTB at the porcine receptor is unknown, but this study examined a higher concentration of the antagonist that would block responses mediated via the low-affinity variant of this receptor. In the presence of the higher concentration of EPPTB (100 μM), contractile responses to all three trace amines were reduced by more than half. Whether this represents an action of these trace amines at the low-affinity TAAR-1 or a non-selective action at other TAAR receptors at higher drug concentrations is not known.

The role of TAAR was explored in the coronary and renal artery to investigate the residual contractile responses to the trace amines after the α_1-adrenoceptor blockade. In the presence of EPPTB (100 μM), the contractile responses to tyramine, synephrine and octopamine were reduced in the coronary artery by the higher concentration of the antagonist. It is highly likely that the antagonist was less selective at the higher concentrations and may block contractions induced by the other TAAR that is not TAAR-1. The TAAR-1 was hypothesised to be expressed on the porcine coronary artery. The residual responses to tyramine were resistant to a cocktail of inhibitors of the noradrenergic, dopaminergic and serotonergic pathways (Herbert et al., 2008). The current study showed that the TAAR-1 selective antagonist, EPPTB (100μM), could reduce the contractile responses of all three trace amines.

The kidney has been reported to express low TAAR-1, TAAR-3, TAAR-4 and TAAR-5 mRNA (Borowsky et al., 2001). So far, no information on whether trace amines tyramine, octopamine or synephrine could mediate a vascular response on the renal artery via TAARs. The selective TAAR-1 antagonist, EPPTB (100nM) or (100μM), failed to attenuate or enhance the contractile responses to tyramine on the renal artery. In noradrenaline-depleted renal arteries, the residual contractile responses to low concentration (10-100μM) of synephrine and octopamine but not tyramine were potentiated in the presence of EPPTB (100 μM). Once again, the change in contractile effects only occurred when treated with a higher concentration of antagonists. So, it may be possible that the contractile effects of the renal artery to the trace amines were mediated by a different TAAR-subtype.

ROLE OF 5-HYDROXYTRYPTAMINE (5-HT) AND OTHER POSSIBLE RECEPTORS IN MEDIATING CONTRACTILE RESPONSES TO TRACE AMINES

In some arteries, 5-HT activation is known to cause direct arterial constriction via 5-HT_{2A} and 5-$HT_{1B/1D}$ receptors (Watts et al., 2012). Additionally, synephrine has been suggested to mediate vasoconstriction of the rat aorta via 5-HT_{1D} and 5-HT_{2A} receptors (Hibino et al., 2009). Neither the non-selective 5HT receptor antagonist methiothepin (1 μM) nor the selective 5-HT2A-selective antagonist ketanserin (1 μM) had any effect in this study on the responses to any of the amines in porcine renal arteries. Several of the 5-HT antagonists, including ketanserin, used in the study by Hibino et al. (2009), have a high affinity for α-adrenoceptors, which may explain the disparity between these results and that of others. Indeed, the affinity of ketanserin at α_1-adrenoceptors and 5-HT_{2A} receptors was almost identical (about 1nM, (Hoyer et al., 1987)) and thus, this antagonist could possibly act via α_1-adrenoceptors and not via 5HT receptors as concluded in the Hibino study. However, this study reduced the possible interfering effects by incubating ketanserin in the presence of prazosin (1 μM). Alternatively, the rat aorta and porcine renal arteries may differ in their 5HT receptor populations. Either way, there was no evidence that any of the trace amines caused vasocontraction via serotonergic receptors in the porcine renal artery.

DIFFERENCES IN CORONARY ARTERY CONTRACTILE RESPONSES TO *C. AURANTIUM* EXTRACTS COMPARED TO AN EQUIVALENT MIXTURE OF TRACE AMINES

Due to potential contributions to contractile responses to the three trace amines of interest within *C. aurantium*-containing PWS, this study investigated whether a similar pattern of responses would be observed using *Citrus-aurantium* and a standard mixture of the three trace amines, synephrine, octopamine and tyramine in the ratio commonly found in *C. aurantium* (namely, 100:1:1).

Previous reports on the rat aortae showed dose-dependent contractions to the synephrine-containing plant extract, *Evodiae Fructus* derived from *Evodia rutaecarpa* of the same family (Rutaceae) *as* Citrus (Hibino et al., 2009a; Hibino et al., 2009b). The aortic contractions to *Evodiae Fructus* (1 µg/mL - 300 µg/mL) were attenuated in the presence of prazosin (300 pM – 3 nM) and were not affected by propranolol (1 µM). The study's authors attributed the contractile effects to the synephrine contained in the plant extract but did not disclose compositional details such as the concentration of synephrine present in *Evodiae*. In general, the levels of synephrine in the Citrus genus vary between 0.1%-2% synephrine and may be similar in this genus.

In this study, the contractile responses of the coronary artery to *C. aurantium* extracts were quantified and compared to the equivalent amounts of a standard mixture of the pure trace amines. A vasoconstrictor response was observed in the coronary response to *C. aurantium* extract (1 µg/mL – 300 µg/mL) *C. aurantium* extract, similar to Hibino et al. (2009a). However, contrary to Hibino et al. (2009), higher *C. aurantium* extract (300 µg/mL to 10 mg/mL) concentrations caused coronary artery vasorelaxations. The bell-shaped response curve (Figure 31A) suggested that endogenous noradrenaline was released at the higher concentration range, which showed similar responses to incremental additions of noradrenaline in Study 2. The vasorelaxation was subsequently reversed in the presence of the β-adrenoceptor antagonist, propranolol (1 µM), in noradrenaline-intact tissue, suggesting. However, the mixture of trace amines showed dose-dependent vasoconstrictions consistent with coronary arterial responses to synephrine. Therefore, the initial contractions to *C. aurantium* extracts were attributed to synephrine acting directly on α_1-adrenoceptors, whereas at higher

concentrations, other constituents than octopamine or tyramine in *C. aurantium,* mediated noradrenaline release to cause vasorelaxation.

In noradrenaline-depleted coronary artery rings, the responses to *C. aurantium* were reversed to cause dose-dependent contractions. Similar to the responses observed to synephrine in Study 2, the tissues were not affected by noradrenaline depletion. The vasoconstrictor effects of *C. aurantium* and trace amine mixture were attenuated in the presence of the α_1-adrenoceptor antagonist, prazosin (1μM) but were not affected by a β-adrenoceptor blockade by propranolol (1 μM). These results were consistent with previous data using rat aorta where octopamine and synephrine activated α_1-adrenoceptors to cause contraction (Broadley et al., 2013; Brown et al., 1988; Hibino et al., 2009).

This study made it clear that there are other compounds within *C. aurantium* than the three trace amines, which facilitate vasoactive effects. The trace amines had a roughly 100-fold higher concentration of synephrine relative to octopamine and tyramine, as did the plant extract. Therefore, it was unsurprising that the contractile responses to the trace amine mix were similar to those found to synephrine. However, *C. aurantium* contains other phenethylamine derivatives such as hordenine and *N*-methyltyramine (Pawar & Grundel, 2017; Pellati & Benvenuti, 2007), which have been shown to stimulate noradrenaline release in rat hearts (Camp, 1970) and inhibit α_2-adrenoceptors in mice (Koda et al., 1999). However, there have been limited studies on the effects of these two additional amines on the vasculature. Therefore, further investigations into the individual and synergistic effects of active alkaloids present in *C. aurantium* extracts are needed, especially in the presence of other stimulants found in PWS, such as caffeine.

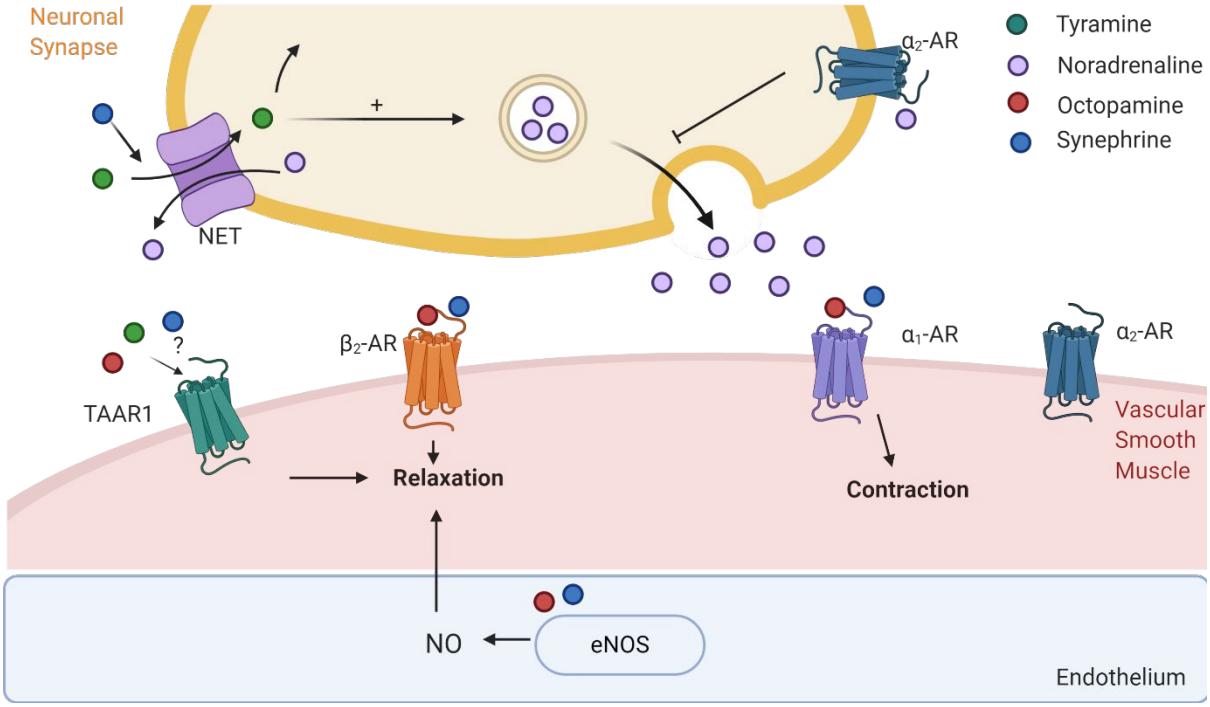


Figure 33 Diagram summarising possible mechanism of action of trace amines on the neuronal synapse, vascular smooth muscle, and endothelium of an artery. Noradrenaline transporter (NET); α_1-adrenoceptor (α_1-AR); α_2-adrenoceptor (α_2-AR); β_2-adrenoceptor (β_2-AR); Trace amine-associated receptor 1 (TAAR1). Arrows show the direction of signalling cascade; stimulation (+); potential role (?). Created by Author using BioRender

In summary, the trace amines, synephrine, octopamine, and tyramine found in *C. aurantium* elicit their effects via complex mechanisms of action that differed across the three selected arteries investigated in this study. Figure 33 provides an illustrative example of the various mechanisms involved. The trace amines have a relatively lower potency than the agonist noradrenaline. This study suggests that tyramine and synephrine cause contractile responses via an indirect sympathomimetic action in the mesenteric and renal artery but not the coronary artery. Synephrine and octopamine appear to be direct agonists on the α_1-adrenoceptors present in all three arteries but mediate vasorelaxation by β-adrenoceptors on the renal artery only. Tyramine and octopamine have been previously reported to be an agonist on TAAR-1, but this study suggests that synephrine was an additional ligand affected by TAAR-1 blockade. The contractile effects of these amines on the mesenteric and renal arteries may cause an overall increase in blood pressure. In contrast, the contractile effect on the coronary artery may suggest a risk of cardiac ischemia.

CHAPTER 3: ANALYTICAL INVESTIGATIONS INTO THE TRACE AMINE CONTENT IN PWS

3.1 INTRODUCTION

It was established in chapter 2 that synephrine, octopamine and tyramine caused dose-dependent vasoconstrictions (30μM – 3mM) in arteries that mediate blood pressure. The stimulatory effects of *C. aurantium*-listing PWS have been commonly associated with synephrine but not octopamine or tyramine. So far, only synephrine has an established daily consumption limit of 30 mg/day (Rebera, 2020), whereas no such limits are set for octopamine and tyramine in dietary products. However, octopamine is considered a banned stimulant in competitive sport, but synephrine and tyramine are legal despite having similar effects on the cardiovascular system (World Anti-doping Agency, 2015). While synephrine is the predominant trace amine in *C. aurantium* and therefore the contributions in activity from the other two may be of less importance, concerns regarding authenticity in labelling and composition prompted us to conduct a quantitative analysis on a pilot sample set of PWS. It is likely important to some consumers to know exactly which trace amines are in the *C. aurantium*-listing PWS they are consuming and in what quantities to avoid unwanted adverse effects of unintentional doping. Evidence of a lack of labelling authenticity, where it occurs, may prompt changes in future labelling requirements by regulators.

As mentioned in Chapter 1, when purchasing PWS, several barriers exist when determining the amounts of active constituents. Firstly, the labels of PWS often only list either the amount of plant extract or itemize them as part of a *proprietary blend* (Eudy et al., 2013). This is in accordance with FDA requirements which stipulate only that the total weight of the ingredients within the supplement are listed, but individual ingredient amounts do not need to be disclosed (FDA, 2015). Unfortunately, this does not provide the consumer with adequate information to determine whether their consumption is within the daily limit or whether constituents therein may produce undesirable interactions with other supplements or medications. For example, if an individual were to consume a *C. aurantium –listing* PWS with an unusually high tyramine content, in addition to monoamine oxidase inhibitors (MAOI), they could unknowingly be at high risk (Suzuki et al., 1979) of an adverse cardiovascular event.

Secondly, PWS may not list active ingredients or fail to list synthetic ingredients from a non-plant-based source. Synephrine in PWS could be either synthetically prepared or naturally derived from *C. aurantium* extracts (often standardised to contain 6 to 8% synephrine) (Bakhiya et al., 2017). However, studies have reported supplements listing plant extracts while instead containing non-plant derived amines such as methyl-synephrine (Cohen et al., 2017) and isopropyl-octopamine (Cohen et al., 2021; Pawar et al., 2020), which are not permitted for use in dietary supplements by the FDA. Hence, this presents a need for plant extract-containing PWS to be screened with a rapid analytical method to minimise the risks to consumers.

Thirdly, PWS often contain a variety of ingredients, but the actual amounts of active ingredients often do not match the product's labels (Desbrow et al., 2019; R. A. Jagim et al., 2019; Pawar et al., 2020). In addition to having lower quantities than expected, Jagim et al. (2019) found that some ingredients such as β-alanine or creatine in PWS do not meet the minimum amounts for ergogenic benefits. For *C. aurantium*-listing supplements specifically, Niemann and Gay (2003) found that synephrine levels did not match the labels of 48 dietary or weight-loss supplements. A recent FDA study showed that most synephrine in *C. aurantium*-labelled dietary supplements had quantities inconsistent with their labelled amounts (Pawar et al., 2020).

Finally, there is variability between active compounds in plant extracts, which depends on geographical factors, species, processing conditions and harvest times, some of which may be beyond the manufacturers' control. It is known that the content of active constituents in plant extracts from the same species can vary depending on several factors ranging from growth conditions to during the processing to extraction and packaging (Hamburger & Hostettmann, 1989). The origins of plant extracts used in PWS are commonly not disclosed, making it difficult to compare synephrine content directly. One approach to monitoring the authenticity and consistency of herbal constituents is to compare the ratios of amines in samples to the plant material (Liu et al., 2015). Thus far, this ratio comparison method has been used to authenticate the presence of citrus in traditional Chinese medicine and supplements (Jiang et al., 2010). However, these studies mainly focussed on identifying citrus flavonoids, such as hesperidin, nobiletin and tangeretin but not alkaloid amines, such as synephrine.

Hence, the ratio of the primary trace amines, synephrine, octopamine and tyramine in *C. aurantium* extracts can be utilised as a means of evaluating PWS authenticity.

Additionally, the ratio method could be further applied to the enantiomers of synephrine as a screen for authenticity in PWS. The *R*-(-)-synephrine (*l*-enantiomer) predominates in the immature fruits, rinds and leaves of *C. aurantium*. Similarly, the *S*-(+)-enantiomer (*d*-enantiomer) is found only in small quantities in ethanolic extracts of the fruit (Tanaka et al., 2019). The two enantiomers have been reported to have a percentage ratio of 92:8 (*R*-synephrine: *S*-synephrine) (Pellati et al., 2005; Tanaka et al., 2019). Therefore, PWS that show a racemic synephrine ratio could be identified as being from a synthetic source.

Combining the total quantities of trace amines, the ratio of trace amine and the ratio of synephrine enantiomers will give an insight into whether the quantities of amines included in a *C. aurantium*-listing pre-workout supplement could lead to vasoconstriction in the blood vessels investigated in the previous chapter. In this context, this chapter of the thesis will be subdivided to show the development, validation, and application of two distinct analytical methods to answer the aims of this thesis (Figure 34).

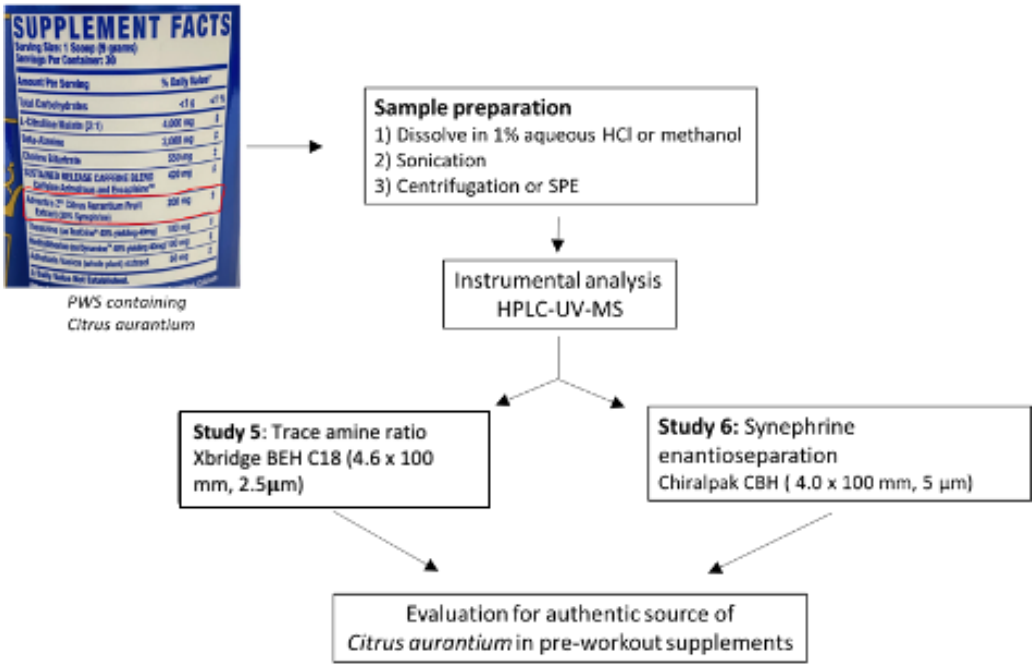


Figure 34 Workflow to quantify trace amines and synephrine enantiomers and to compare the ratio between *C. aurantium* plant material and pre-workout supplements

3.2 AIMS

The aim of Study 5 was to develop and validate a HPLC-UV-MS method to quantify the levels of trace amines in *C. aurantium* standard reference material and compare their relative ratio to that found in a sample of *C. aurantium-listing* pre-workout supplements.

The aim of Study 6 was to develop a direct, convenient, and accurate screening method suitable for separating and quantifying synephrine enantiomers in a convenience sample of *C. aurantium*-listing pre-workout supplements.

3.3 GENERAL MATERIALS AND METHODS

CHEMICALS AND SOLVENTS

(±)-*p*-Synephrine, (±)-*p*-octopamine hydrochloride, and (±)-*p*-tyramine were obtained from Sigma-Aldrich (Sydney, Australia). Hydrochloric acid (HCl), acetonitrile (CH_3CN), ammonium carbonate ($(NH_4)_2CO_3$) and 0.1 % ammonium hydroxide (NH_4OH) were from Merck (Sydney Australia). Ultrapure water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). Ammonium carbonate solution was prepared by dissolving anhydrous ammonium carbonate in purified water to obtain the desired molar concentration. The mobile phase at starting conditions was adjusted to the final pH with either 0.1 % HCl or 0.1% NH_4OH. All chemicals were of an analytical grade suitable for LC-MS.

INSTRUMENTATION

All experiments were performed 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 dual beam-UV/Vis Detector (Waters, Ireland) in series with an ACQUITY single quadrupole mass detector (QDa) (Figure 35). Empower V3 software was used for data acquisition and processing. All pH measurements were performed using an UltraBasic pH meter model UP10 (Denver Instrument, Norfolk, United Kingdom).

Figure 35: Representative Alliance e2695 set up paired to a UV/Vis detector and a QDa detector

PLANT MATERIAL AND PRE-WORKOUT SUPPLEMENT SELECTION

Standard reference materials (SRM) were kindly donated by the National Institute of Standards and Technology (NIST). The three *C. aurantium* SRMs have been certified and developed to represent natural (SRM 3258; *C. aurantium* fruit), ethanolic extract, which was evaporated to dryness (SRM 3259; ethanolic extract) and processed samples (SRM 3260; grounded tablets containing *C. aurantium*). These samples were stored in an opaque, vacuum-sealed packets at standard room temperature (Figure 36).

Figure 36: Photo of dry powdered bitter orange extract (SRM 3259) samples in vacuumed sealed packets

Twelve pre-workout supplements (Table 17) were purchased for this study. *C. aurantium* was listed on the labels of approximately 25% of all pre-workout supplements sold online and in local health outlets. Six supplements out of approximate twenty-five available *C. aurantium*-listed supplements were purchased online (bodybuilding.com), and five out of twenty were available from local dietary supplement shops (Gold Coast, Queensland, Australia). The products were selected by convenience sampling based on their labels listing the following: "*C. aurantium* extract" or "*bitter orange* extract" either as a specified ingredient or as a part of a proprietary blend. The matrix blank was a supplement that did not list *C. aurantium* or any target constituents on the label. The supplements were purchased in January of 2017 and were in date at the time of analysis.

Table 17: List of pre-workout supplements that label *C. aurantium*, caffeine and other stimulants. (N/A = where no caffeine or other stimulant information was provided on the label)

Pre-workout supplement code	Pre-workout supplement name	Serving size (g)	Preparation type	Labelled amount of *C. aurantium* extract (mg/serving)	Labelled amount of caffeine (mg/serving)	Other stimulants or potential stimulant-containing plant extract
PWS1	Switch Nutrition Power Switch	5	Dry powder	*C. aurantium* 15mg	Caffeine anhydrous (175 mg)	Kuding Extract (DYNAMINE®) (100 mg)
PWS2	GHOST Legend	12.7	Dry powder	*C. aurantium* (50mg)	Caffeine anhydrous (200mg); Di-caffeine malate (37mg)	Theobromine (50 mg); Olive Leaf extract (50mg); NADH 10% (2.5mg)
PWS3	Grenade 0.5 Calibre Pre-workout	9.7	Dry powder	*C. aurantium* (60mg)	Caffeine anhydrous (300 mg); Infinergy (DiCaffeine Malate, 50mg); PurCaf (Organic Caffeine;50mg)	Higenamine (50mg)
PWS4	Ronnie Coleman YeahBuddy	8	Dry powder	*C. aurantium* (200mg)	Caffeine anhydrous and Encapfeine (420mg)	Theacrine (100mg); Methyliberine (100mg); Adhatoda Vasica extract (50mg)
PWS5	Labrada Supercharge Pre-workout	27	Dry powder	*C. aurantium* extract (40mg)	Caffeine anhydrous (300 mg)	N/A
PWS6	PrimaForce Syneburn	0.25	Gel capsule	*C. aurantium* (10mg synephrine)	N/A	N/A
PWS7	BPI Sports PumpHD	10	Dry powder	*C. aurantium* (part of blend 500mg)	Theobroma cacao extract (part of a blend 500mg)	Green tea leaf extract, Cordyceps extract; Rhodiola root extract; Echinacea purpurea extract; Quercetin dihydrate
PWS8	BPM Labs The One	12	Dry powder	*C. aurantium* peel extract (part of blend 12g)	Caffeine (part of a blend 12g)	Nandina Domestica Extract; Puchong Tea Extract
PWS9	Panthera Pharmaceuticals KardioFire	10	Dry powder	*C. aurantium* (part of a blend 625mg)	Caffeine anhydrous (part of a blend 625 mg)	Raspberry Ketone (part of blend)
PWS10	Cellucor C4 Ultimate	19	Dry powder	*C. aurantium* fruit (part of a blend 805mg)	Caffeine anhydrous (300mg)	Theacrine; Toothed Clubmoss extract (part of blend)
PWS11	ErgoGenix Ergoblast	14	Dry powder	*C. aurantium* (part of a blend 1600mg)	Caffeine anhydrous (300mg)	N/A
PWS12	AllMax Nutrition Muscle Prime	19	Dry powder	Synephrine 20 mg, Octopamine 10 mg	Caffeine anhydrous 160mg)	American Ginseng (12mg); Cordyceps (10mg); Hawthorne Berry (8mg)

METHOD VALIDATION

Validation of both methods was important to show the reliability and repeatability of each analytical method. Specific regulatory requirements must be demonstrated in a quality assurance setting 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. The International Conference sets the validation parameters On Harmonisation Of Technical Requirements For Registration Of Pharmaceuticals For Human Use (ICH Expert Working Group, 1994) was the chosen guideline for this study.

SELECTIVITY, ACCURACY AND PRECISION

Selectivity is the ability to distinguish between the analyte being measured and other substances present. Selectivity was confirmed using UV-detection and QDa-mass spectrometry by incorporating a starting condition blank and a dietary supplement matrix blank (not containing any of the analytes being measured). Accuracy was determined by evaluating the closeness of values in spiked matrix blanks with 3 known concentrations of amine standards.

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

Precision (%RSD) = Standard deviation/ mean x 100

Intra-assay precision of the assay was determined by analysing three different concentrations (5, 50, and 100µg/mg) of the standards from each method in triplicate on the same day. These concentrations were selected to represent the lowest and highest concentration of amines anticipated to be found in the supplements. Inter-day precision was determined based on analysing these concentrations over three separate days.

LINEARITY

For each run, at least 6 standard concentrations were prepared and analysed with three independent injections. Concentrations ranged between 0.5 µg/mL – 200 µg/mL. Linear regressions were generated on 3 separate days using the standards to I.S peak area ratios by weighted ($1/x^2$) least-squares linear regression. The calibration curve equation and correlation coefficients (r^2) were calculated. The acceptance criteria for an acceptable calibration curve were a correlation coefficient (r^2) of 0.99 or better.

LIMITS OF DETECTION (LOD) AND LIMIT OF QUANTITATION (LOQ)

Determination of the limits of detection (LOD), limits of quantitation (LOQ), intra-assay and inter-assay precision were performed according to that 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 the 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.

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

$$S/N = \frac{2h}{hn}$$

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. 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 regression line to the noise and calculates the residual amount for each data point.

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:

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

The S/N was calculated for each analyte peak found with each subsequent analysis.

3.4 STUDY 5: TRACE AMINES AND THEIR RATIOS IN *C. AURANTIUM* EXTRACT AND *C. AURANTIUM*-LISTING PRE-WORKOUT SUPPLEMENTS

INTRODUCTION

Synephrine is the main adrenergic alkaloid found in *C. aurantium* fruits and extracts. Most commercial *C. aurantium* extracts are standardized to 6-8% of synephrine content (Avula et al., 2005). Although the active constituents in plant extracts vary depending on harvest times, geographic origin and extraction process, the trend of a naturally derived plant extract is the ratio of synephrine to octopamine and tyramine

A study by the NIST found an average of 71.9 mg/g of synephrine in dried extracts and 9.10 mg/g synephrine in dried fruit (Nelson et al., 2007; Santana et al., 2008). The other trace amines such as octopamine and tyramine have been reported to be present in small amounts, often 100-times lower than synephrine with a ratio of 1: 0.02: 0.02 (synephrine: octopamine: tyramine)(Pellati & Benvenuti, 2007; Sander et al., 2008). Therefore, a pre-workout supplement containing *C. aurantium* extract would be expected to have a similar synephrine, octopamine, and tyramine distribution.

Multiple analytical methods have been used to quantify synephrine, octopamine, tyramine and their associated enantiomers in dietary supplements, as identified in Chapter 1. The most common method of quantification is achieved by reversed-phased chromatography paired to a UV detector (HPLC-UV) (Nelson et al., 2007; Pellati & Benvenuti, 2007; Putzbach et al., 2007; Putzbach et al., 2007; Sander et al., 2008). However, as noted in Chapter 1, the multi-ingredient nature of PWS coupled with the polarity of the amines have resulted in some poor baseline separation between the trace amines in *C. aurantium*-containing supplements.

Under the more common acidic mobile phase conditions positively charged, protonated bases are subject to shorter retention times and poor peak shape. The mobile phase pH is an important factor in RP-HPLC since retention is based on polar interactions between the analyte and stationary phase. In the case of amine analytes, the uncharged species will predominate at a mobile phase with a pH above the amine's pKa, which leads to better retention of the analyte. However, mixed-mode retention

effects can occur with a higher pH mobile phase whereby residual silanol groups (Si-OH) of the silica gel of the stationary phase become negatively charged. Column manufacturers have minimised these effects through end-capping techniques, which decrease surface polarity. Furthermore, maintaining the integrity of the column and avoiding degradation of the stationary phase chemistry will also minimise silanol effects. To avoid mixed-mode retention effects, low pH is usually employed, which ensures silanol groups (average pKa < 7) are protonated and neutral.

Thus, for the RP-HPLC separation of amines, mobile pH decisions are often based on which effect is most likely to impact resolution. In many cases, ion-pairing reagents (IPR) such as the negatively charged sodium dodecyl sulphate (SDS) are employed, which bind to the charged analyte (in this case, positive amine), reducing the potential for interaction with the silanol. Under neutral pH conditions, these have been shown to improve chromatographic peak shape and retention of synephrine (Putzbach, Rimmer, Sharpless, & Sander, 2007). However, the use of IPRs is not without limitations, including incompatibility with MS detection, lowered selectivity, and decreased reproducibility.

An alternative to IPRs that has not yet been explored to separate these trace amines was to use a high-pH mobile phase (above pH 8). Traditionally, most silica-based LC columns are not compatible for use with the high-pH mobile phases (Espada & Rivera-Sagredo, 2003), but more pH-tolerant LC columns have been developed. Typically, ammonium bicarbonate and ammonium hydroxide can increase pH because they are volatile and compatible with mass spectrophotometric detection (Delatour & Leclercq, 2005). For this analysis, it was hypothesised that separating these trace amines could be achieved without using IPR by using a high-pH buffer, which would make it compatible with mass detection and employing a high-quality column with good stability at high pH.

At the time of this experiment, no other studies included the SRMs to quantify and compare the ratios of the trace amines in *C. aurantium* SRM to those found in PWS. Hence, this study aimed to develop a rapid quantitative HPLC-UV-QDa method that compares the ratio of trace amines between *C. aurantium* extracts and PWS. The values recovered from the developed method was used to determine whether the synephrine found was from a natural or synthetic source.

METHODS AND MATERIALS

HPLC-UV-QDA ANALYSIS

The column used was an XBridge BEH C18 (4.6 x 100 mm, 2.5 µm, Waters, Ireland) protected by an XBridge BEH C18 guard column (3.9 x 5 mm, 2.5 µm, Waters, Ireland). Empower V3 software was used for data acquisition and processing.

Three 10µL sample injections were used for each sample or standards replicate. The trace amine standards were in included in a 7-point linear dilution curve ranging from 1 µg/mL to 250 µg/mL. The composition of the mobile phase was: A (water + 5mM $(NH_4)_2CO_3$ + 0.2% NH_4OH) and B (acetonitrile + 5mM $(NH_4)_2CO_3$+ 0.2% NH_4OH). Both solvents were pH adjusted to a pH of 10. The flow rate was maintained at 0.8 mL/min while the column temperature was set to 42.5°C and sample temperature was set at 25°C. The analysis was performed using the following gradient elution: 1% B isocratic for 3 minutes, 1% to 15% B over 6 minutes with a 1-minute hold, 15% to 45% B over 2 minutes, before re-equilibrating to 1% B over 3 minutes. The total run time was 15 minutes with a 5-minute delay between 10 µL injections allowed for mobile phase and system pressure equilibration. UV detection was at 242 nm. Data acquisition with a QDa mass detector was performed in positive mode under the following conditions: total ion current (TIC) between mass-to-charge (*m/z*) 100-400; capillary voltage, 0.8 kV; cone voltage, 15 V. Single Ion Recording (SIR) was set for positive mode base peak of synephrine (150.2 *m/z*), octopamine (136.2 *m/z*) and tyramine (121.2 *m/z*).

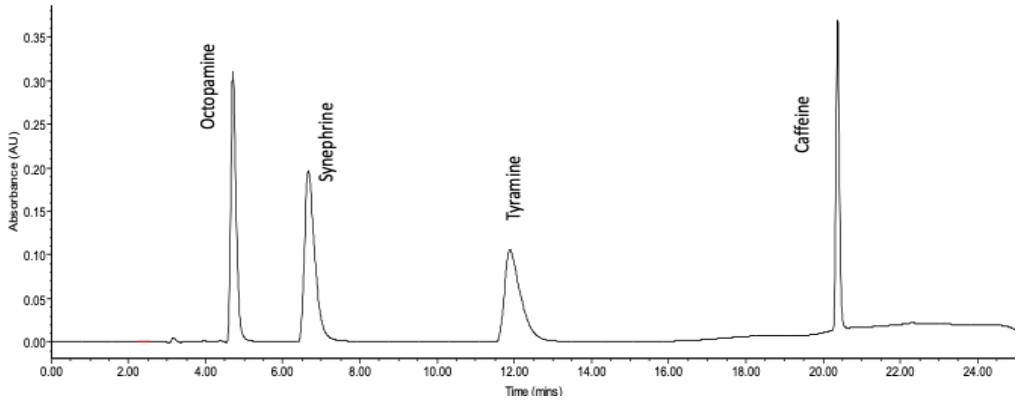


Figure 37 Representative UV chromatogram of 100µg/mL of octopamine, synephrine, tyramine and caffeine standards. The chromatographic peaks were observed with XBridge BEH C18 (4.6 x 100 mm, 2.5 µm, Waters, Ireland) under gradient conditions with a pH of 10.5. UV

STANDARD SOLUTIONS AND SAMPLE PREPARATION

Standard solutions of (±)-*p*-synephrine, *p*-tyramine and (±)-*p*-octopamine were dissolved in acidified water (H_2O + 2 drops of 0.1% HCl) to a concentration of 1 mg/mL. Stock solutions were stored at 4°C until analysis. Linear dilutions were prepared for each standard between 1 µg/mL to 250 µg/mL. Caffeine standards were prepared in similar method with a standard calibration curve ranging from 0.5 µg/mL to 200 µg/mL.

Dry SRMs, PWS or matrix blank were weighed (1 g), and 10 mL of acidified water (H_2O + 0.1% HCl) was added (Figure 38). The mixture was sonicated under ambient temperature for 30 minutes. The samples were then centrifuged at 3000 rpm for 20 minutes, and the supernatants were subjected to a 2-step solid-phase extraction (SPE) protocol with a 60 mg Oasis PRiME MCX cartridge (Waters, Ireland). The samples were loaded onto the cartridge, and non-polar impurities were washed with methanol. The polar analytes of interest were subsequently eluted with 1 mL methanol + 5% NH_4OH into a 2mL HPLC vial. The samples were evaporated to dryness, reconstituted with 1 mL of acidified water and stored at 4°C before analysis. Three replicates of each sample were prepared for HPLC-UV-QDa analysis.

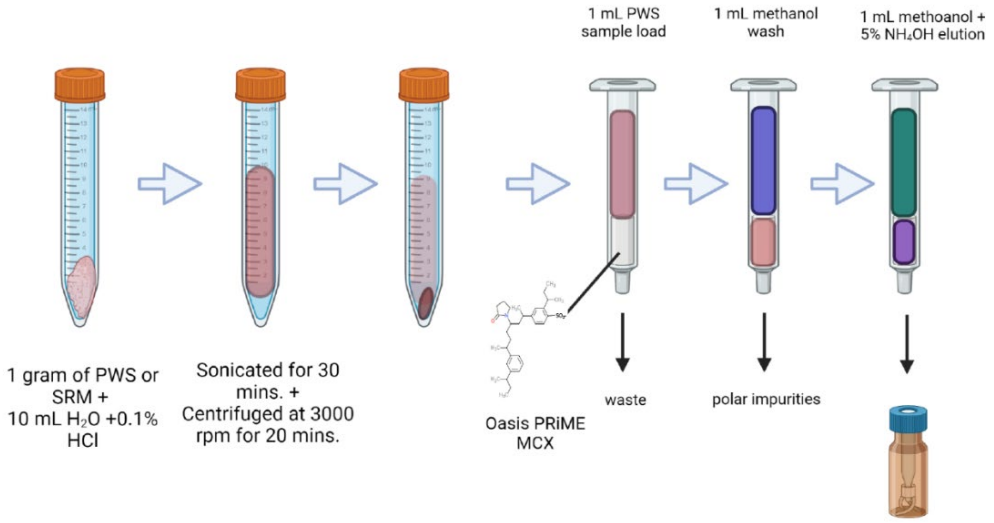


Figure 38: Representative diagram of the SPE procedure with the Oasis MCX cartridge

All PWS used in this study were dry powdered oral dosage form except for *Syneburn (PWS6)*. *PWS6* was a dry extract that was packed into a gel capsule. For this sample, the gel capsules were weighed to 1 gram and prepared in the same way as the abovementioned method.

EXTRACTION RECOVERIES AND MATRIX EFFECTS CALCULATION

Matrix effects can influence analysis performance for identification and quantification as suppression or enhancement of the analyte during the chromatographic method. To determine the cation exchange, SPE extraction efficiency, recovery and matrix effects were evaluated by mathematical equations adapted from Matuszewski et al. (2003) (Matuszewski et al., 2003). The recovery values were calculated as follows:

$$Recovery\ (\%) = \frac{Response\ of\ extracted\ sample\ (Pre\ SPE\ spike)}{Response\ of\ Post\ SPE\ spiked\ sample} \times\ 100$$

Matrix effects are important measures for interpreting the data when applying bioanalytical samples to solid-phase extraction (Matuszewski et al., 2003). The following equation calculated the matrix effects:

$$Matrix\ effects\ (\%) = \left(\left(\frac{Response\ of\ Post\ SPE\ spiked\ matrix\ blank}{Response\ of\ standards\ in\ solvent}\right) - 1\right) \times\ 100$$

RESULTS AND DISCUSSION

METHOD DEVELOPMENT

HPLC-UV-QDa conditions were optimised to detect and quantify the three main polar phenethylamines in *C. aurantium*, synephrine, octopamine and tyramine. Optimal chromatography was achieved with an XBridge BEH C18 column (pH range 1 to 12) with basic solvent conditions (0.2% NH_4OH) with ammonium carbonate buffer (5 mM; pH 10). Chromatographic peaks for the amines (Figure 39) were resolved, and the analysis was compatible with a mass detector. The amines eluted with a gradual increase in acetonitrile, with the elution order of octopamine, synephrine and tyramine at 2.45 minutes, 5.60 minutes, and 6.8 minutes, respectively.

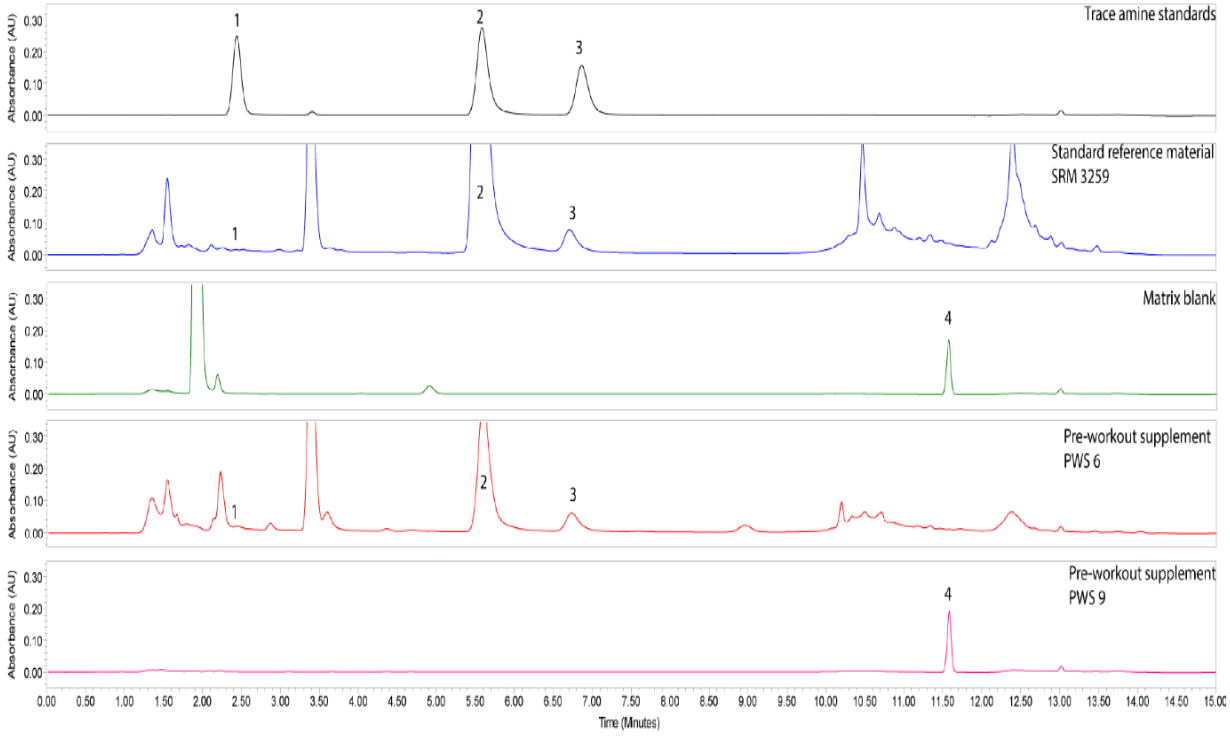


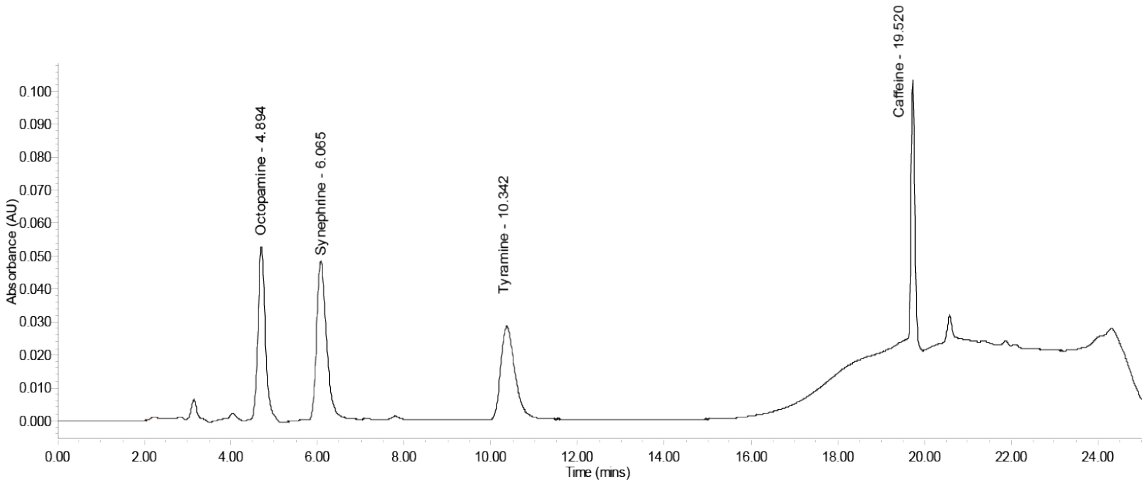
Figure 39: Representative chromatograms of trace amine standards, trace amines detected in standardised reference material (*C. aurantium* extract), pre-workout supplement matrix blank a pre-workout supplement (PWS6) containing all three amines, and a pre-workout supplement without any amines present. The compounds found were octopamine (1), synephrine (2), tyramine (3) and caffeine (4)

A preliminary method was developed prior to the start of this thesis that had shown that the XBridge BEH C18 column was more selective for the polar trace amines such as synephrine, octopamine and tyramine (Koh, 2017). Furthermore, acetonitrile as organic component of the mobile phase showed greater peak resolution compared to methanol. In this thesis, the mobile phase conditions were further investigated to separate these trace amines in varying basic pH. The polar trace amines were well separated at less basic conditions (pH $\leqslant$ 9 with ammonium acetate). Still, they had a significant shift in the baseline as the percentage of acetonitrile in the mobile phase increased.

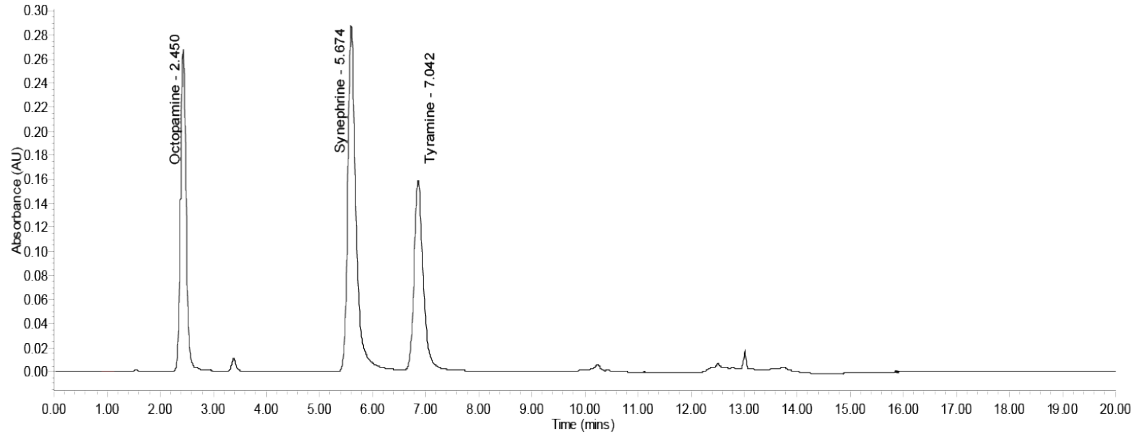
Additionally, the peaks for these amines were less resolved at pH ≤ 9, which is expected as the lower pH likely protonate the primary or secondary amines (pKa 9.7 – 10.5). Significant improvements were observed when the pH of the mobile phase was increased to 10 with a combination of ammonium carbonate with ammonium hydroxide (0.2%). However, this had suppressed the chromatographic peak for caffeine (Figure 40). At pH 11, the peaks for the trace amines had excellent baseline resolution. However, there was a noticeable artefact eluting at 2.22 mins. It may be hypothesised that this artefact is from an on-line *N*-nitrosation reaction between the ammonium hydroxide and acetonitrile, similar to that found by Myers et al. (2013). As the method aimed to quantify the trace amines found in *C. aurantium* extracts and pre-workout supplements that claimed to contain them, the ammonium carbonate buffer was used as a compromise. The advantage of ammonium carbonate and ammonium hydroxide over ion-pairing reagents was its compatibility with mass spectrometry, which improved selectivity (Tan & Fanaras, 2019).

SP: XBridge BEH C18 (4.6 x 100 mm, 2.5 µm)

MP: Acetonitrile+ Ammonium acetate (pH 8)



MP: Acetonitrile+ Ammonium carbonate + 0.2% NH_4OH (pH 10)



MP: Acetonitrile+ Ammonium hydroxide (pH 11)

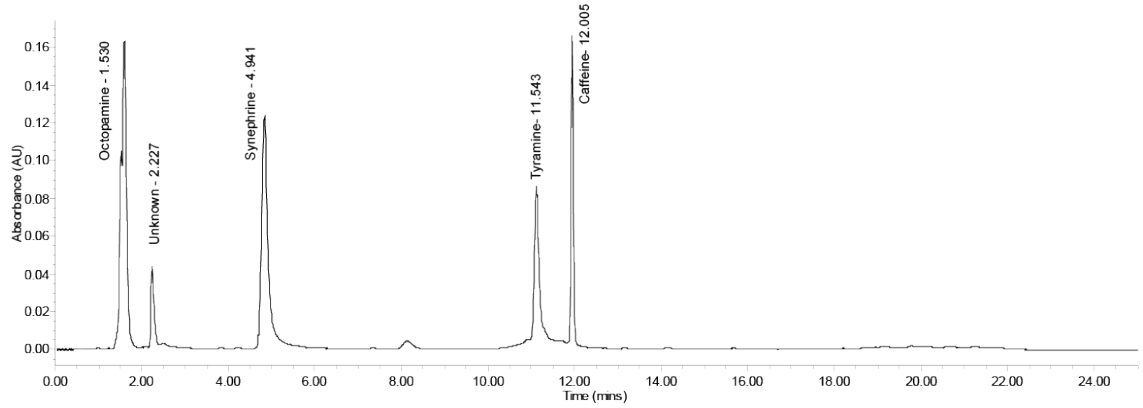


Figure 40 :Comparison between different pH modifiers in in the mobile phase of the method.

OPTIMISATION OF SAMPLE PREPARATION

In most analytical methods involving these amines in *C. aurantium* and citrus dietary supplements, extractions have either been performed with non-polar organic solvents, derivatization, or acidic conditions (Pellati & Benvenuti, 2007). During the optimization, standardised reference materials and PWS were prepared in either 10mL of water, methanol or acidified water (1%, 0.1M HCl) prior to a 30-minute sonication at ambient temperatures and centrifugation at 3000 rpm. Upon HPLC-UV analysis at 242 nm, the amines prepared in acidified water had the best separation with minimal matrix effects relative to water or methanol samples. The amines in the methanol samples eluted at the solvent front, whereas the amines prepared in water had a significant degree of peak tailing, suggesting an uneven distribution of charged and uncharged species of the amines interacting with the column material. The addition of 1% HCl (0.1M) would have encouraged the primary amines of tyramine and octopamine and the secondary amine of synephrine to be predominantly protonated.

Previous LC-MS studies on *C. aurantium* extracts did not use a clean-up procedure (Nelson et al., 2007; Pellati & Benvenuti, 2008). In this work, the use of an additional clean-up step was investigated with a solid-phase extraction to reduce the interference from the matrix. Hydrophilic-lipophilic balance reversed-phase sorbent (Oasis PRiME HLB, 60mg) and mixed-mode cation exchange for bases (Oasis PRiME MCX, 60mg) were evaluated. A standard mix of the three amines, standardised reference materials and pre-workout samples were prepared in acidified water.

The protocol of the HLB extraction was done in a sequence of loading 3 mL of sample, washing with 1 mL of 5% methanol and eluting with 1 mL of acetonitrile: methanol (90:10, v/v). The MCX extraction was prepared in a sequence of loading 3mL of the sample, washing the impurities with 1mL of methanol, before eluting with 1mL of 5% ammonium hydroxide in methanol. There were more significant retentions of the three amines in the MCX extraction than the HLB protocol but suppressed the signal intensity for caffeine. In contrast to a non-clean-up procedure, the amines were recovered with minimal matrix interferences with the MCX extraction. This improvement might be due to the alkaline conditions (5% NH_4OH) and the partial reversed-phase contribution from the MCX cartridge, which is consistent with the observations in a previous method of on *C. aurantium* products on gas

chromatography with a strong cation exchange clean-up protocol (Andrade et al., 2009).

EVALUATION OF RECOVERY VALUES AND MATRIX EFFECTS

The percentage recovery (%) correction factor was calculated and applied to the amine values recovered in all samples prepared with this protocol. To evaluate the influence of the matrix on the HPLC-UV-QDa procedure, a mix of amine standards (100 µg/mL) was spiked in the cleaned and non-cleaned matrix blanks as described in the Methods section above. The recoveries of the amines ranged from 88.6 -96.2% (Table 18), with tyramine having the lowest percentage of recovery (88.6%). It is possible that tyramine is not retained as it is the most polar (pKa = 10.5) among the three amines and would require a more alkaline condition to increase retention. Negative values were recovered for the matrix effects (%), which was suggested that the supplement matrix suppressed the responses of amine quantification. It was hypothesised that there might be interfering polar compounds within a pre-workout supplement matrix that suppressed the interaction of these amines to the cation exchange column of the SPE cartridge.

Table 18: Solid-phase extraction (SPE) recovery (%) and matrix effects (%) of p-(±)-synephrine, p-(±)-octopamine and p-tyramine in a supplement blank.

Trace amines	Average amount quantified (µg/mL)			Solid phase extraction recovery (%) ((A/B)*100)	Matrix effects (%) (((B/C) - 1)*100)
	Pre-SPE spike (100 µg/mL) (A)	Post-SPE spike (100 µg/mL) (B)	Standard mix (100 µg/mL) (C)		
Synephrine	95.00 ± 1.62	98.81 ± 0.59	103.51 ± 0.32	96.15	-4.55
Octopamine	86.49 ± 1.67	92.62 ± 1.83	101.24 ± 0.46	93.38	-8.51
Tyramine	84.28 ± 1.38	95.12 ± 1.41	100.60 ± 0.42	88.60	-5.46

An impurity was found to co-elute with synephrine at 5.5 minutes. This impurity was subsequently identified as nicotinamide by the QDa mass detector. The samples that were prepared and extracted with the cation exchange SPE cartridge (Waters PRiME

Oasis MCX, 60mg, 3cc) extracted vitamin B_3, which co-eluted with synephrine during the chromatographic optimisation process (Figure 41).

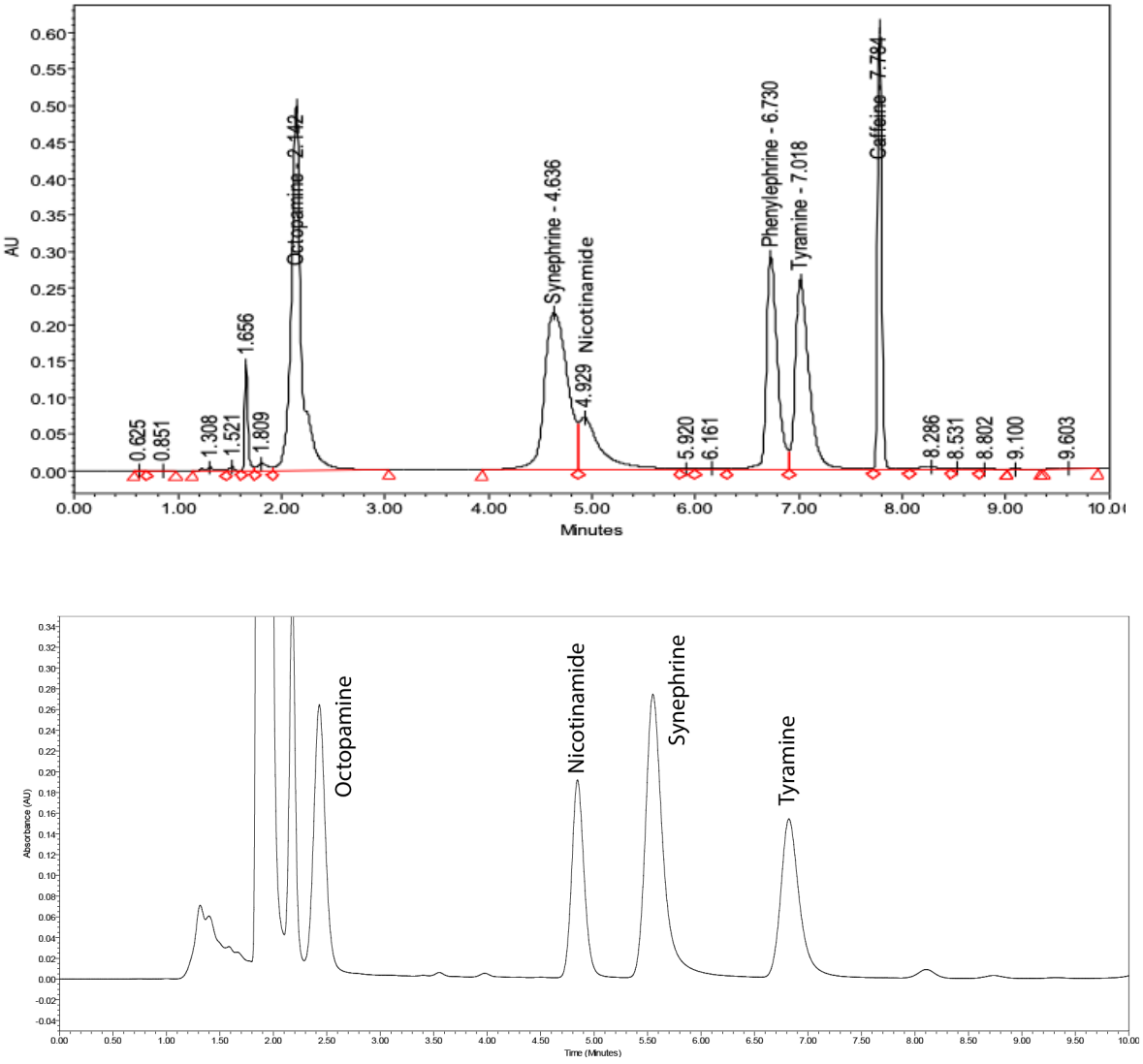


Figure 41: Spiked matrix blank of standard mix, phenylephrine and caffeine in matrix blank that showed co-elution of synephrine and nicotinamide (vitamin B3) (top) and the resolution between synephrine and nicotinamide (bottom).

Nicotinamide was identified with QDa mass fragments and cross-referenced with another method that used the same MCX cartridge (Hiki et al., 2016). This issue was resolved by adjusting the pH and concentration of ammonium carbonate. During the sample preparation optimisation, this study found that methanol extracts had lower selectivity for our amines of interest in SRMs, which was in line with previous findings by the NIST (Sander et al., 2008).

METHOD VALIDATION

LINEARITY, LIMITS OF DETECTION (LOD) AND LIMITS OF QUANTIFICATION (LOQ).

Linearity of the amine standards was assessed using the least-square regression method of dilutions for each standard solution. An intra-day 7-point standard curve for each analyte, the slope and *y*-intercept, correlation coefficient (r^2) and RSD % of the standard curve were determined (Table 19).

Optimal correlation coefficient was within the range of 1 µg/mL to 250 µg/mL, where: synephrine (r^2 = 0.998 ± 0.002); octopamine (r^2 = 0.999 ± 0.001); and tyramine (r^2 = 0.997 ± 0.003). Further analysis of trace amine standards of lower concentrations of 500 ng/mL and 100 ng/mL showed a reduction in signal detection, affecting the co-efficient linearity correlation. The calibration ranges for all three analytes were based on known quantities of these amines previously in *C. aurantium* fruits and dietary supplements (Pawar et al., 2020; Putzbach et al.,2007; Sander et al., 2008) while taking into consideration of this experiment's analytical LOD and LOQ. The LOD and LOQ are in the nanogram range for all three amines. The LOQ for synephrine was 0.454 µg/g, octopamine was 0.781 µg/g, and tyramine was 0.820 µg/g. The LOQ of this method was more sensitive than other LC-UV methods, which report amines in microgram levels (Roman et al., 2007). However, the current method is less sensitive than more sophisticated LC/MS/MS and LC-API-ES-MS (Putzbach, Rimmer, Sharpless, & Sander, 2007) that report picogram quantification levels. Therefore, this method showed good sensitivity whilst being cost-effective relative to the more expensive LC-MS/MS techniques.

Table 19: Calibration data for linearity of p-(±)-synephrine, p-(±)-octopamine and p-tyramine.

Trace amine standards	Calibration range (µg/mL)	Correlation coefficient (r^2)	Limit of detection (µg/g)	Limit of quantification (µg/g)
Synephrine	1.0 - 250	0.998 ± 0.002	0.136	0.454
Octopamine	1.0 - 250	0.999 ± 0.001	0.234	0.781
Tyramine	1.0 - 250	0.997 ± 0.003	0.246	0.820

SELECTIVITY, ACCURACY, AND PRECISION

The method's selectivity was demonstrated by injecting each of the reference standards to show resolution between all the standards and injecting the negative control pre-workout supplement (matrix blank) to show no interfering peaks above the limit of quantification (Figure 42).

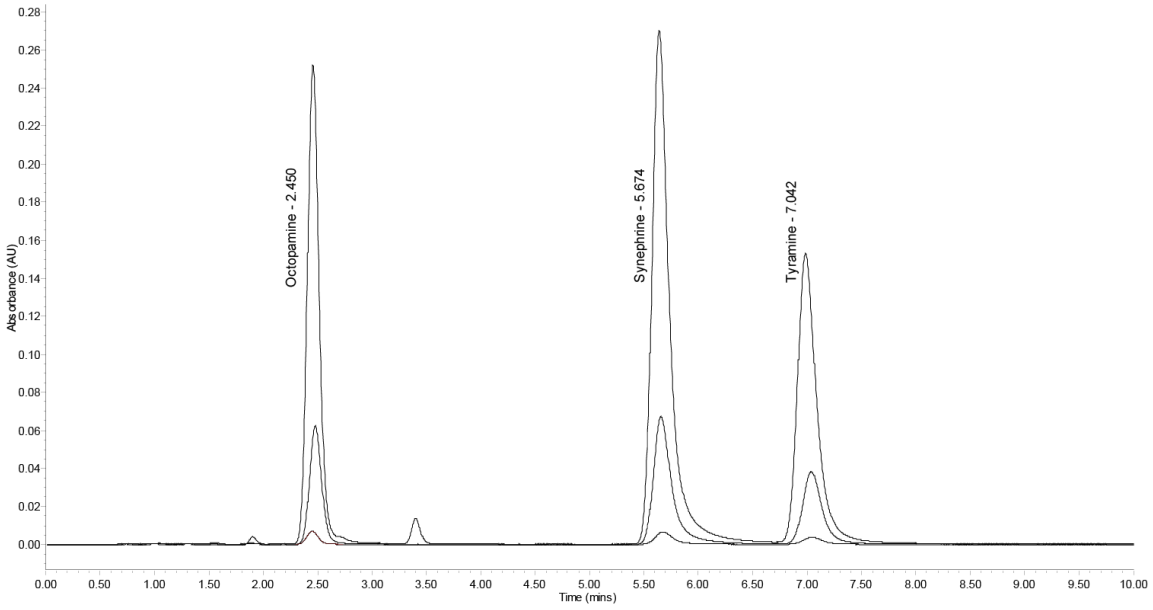


Figure 42: Spiked matrix blank containing three different levels of trace amines, 5 µg/mL, 50µg/mL and 200µg/mL.

The accuracy for the spiked samples varied from 86% to 106%, with their intermediate precision showing relative standard deviations ranging from 0.3% to 5.4% (Table 20).

Table 20: Accuracy and intermediate precision of synephrine (SYN), octopamine (OCT) and tyramine (TYR)

Standards concentration	Recovered concentration ± SEM (µg/g)			Accuracy (%)			Intermediate precision (%RSD)		
	SYN	OCT	TYR	SYN	OCT	TYR	SYN	OCT	TYR
5µg/mL	4.28 ± 0.11	4.97 ± 0.16	5.30 ± 0.15	85.68	99.48	106.05	4.46	5.44	4.96
50µg/mL	50.59 ± 0.47	48.76 ± 0.35	49.51 ± 0.11	101.17	97.49	99.03	1.6	1.26	0.4
200µg/mL	200.77 ± 0.51	200.26 ± 0.37	200.43 ± 0.42	100.39	100.13	100.21	0.44	0.32	0.36

The most variable of the three amines was octopamine at the lowest concentration range of 0.5 µg/mL, which had a %RSD at 5.4% but were within acceptable limits under the ICH and FDA guidelines. Lower analyte recoveries were observed in matrix blank spiked with low concentrations of trace amines. The selectivity for trace amines could be improved by increasing the pH to reduce the polarity of the amines prior to chromatographic separation. System suitability tests were performed to assess the reproducibility of the method. The test was conducted by injecting 10 µL of the standard mixture (100 µg/mL) for ten replicates. The %RSD for all three amines were 0.5%, which was acceptable as they were less than 1%, indicative of minimal variations between injections.

QDA ANALYSIS

QDa mass detection provides a rapid confirmatory advantage over conventional HPLC-detection methods. The mass chromatograms in Figure 43 show the target analytes distinguishable by the mass-to-charge ratio (*m/z*) ratios of the parent compound or major fragment: synephrine 150.2 (MW 167); octopamine 136.2 (MW 153), tyramine 138.2 (MW137). Accurate structure confirmation was achieved by analysis of the mass chromatograms through peak purity in which a clear, strong peak at the relevant m/z ratio was observed. The QDa-analysis enabled the identification of false positives for octopamine (6/15) and tyramine (2/15) found in PWS (Table 22).

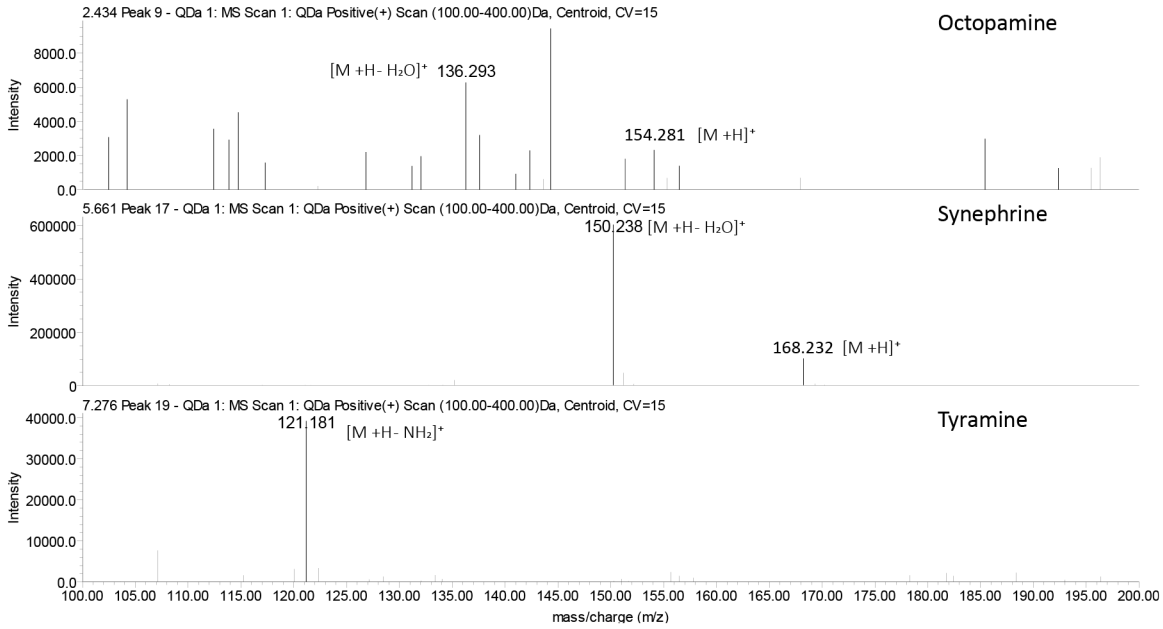


Figure 43. QDa-MS profiles for protonated amines found in *C. aurantium*-listing supplement, Ronnie Coleman's Yeah Buddy™. Octopamine, precursor molecule, m/z 154; Synephrine, precursor molecule, m/z 168; and tyramine, precursor molecule, m/z 138.

3-Dimensional chromatograph plots with mass to charge (*m/z)* on the Z-axis provides a convenient way to visualise separation and identification as shown by the plots of the standards, matrix blank and an example of PWS, Syneburn (Figure 44). Adducts of the various ingredients found in the plant material and pre-workout out supplement were also visualised in the 3-dimensional plots. Hence, it was advantageous to compare the mass signals of the trace amine standards to those found in the samples analysed.

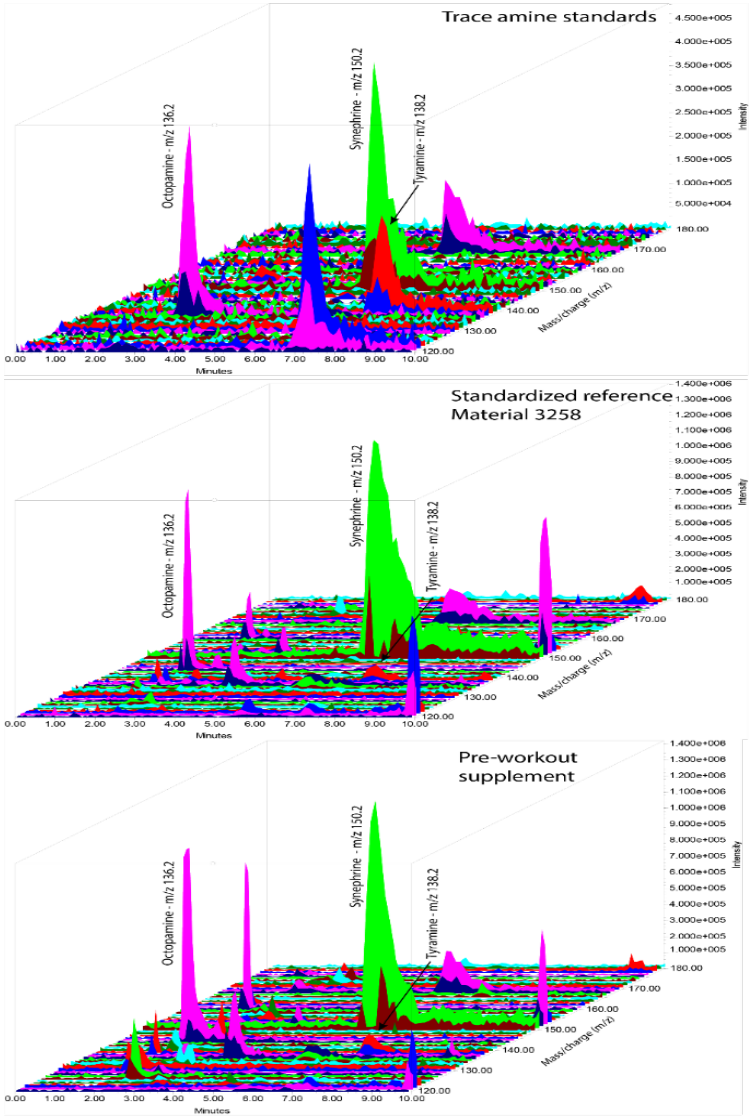


Figure 44: Representative 3D chromatogram plots of the trace amine standards (top), standardised reference material 3258 (*C. aurantium* fruit, middle), and pre-workout supplement (Syneburn; bottom).

QUANTIFICATION OF AMINES IN STANDARD REFERENCE MATERIAL (SRM) AND PRE-WORKOUT SUPPLEMENTS (PWS)

I) C. AURANTIUM SRMS

The trace amines quantified in the three SRMs certified in this analysis were compared to levels determined by the NIST (Table 21). In all three preparations, synephrine (5.28 – 38.03 mg/g) was 62-68% of the quoted values of the NIST; octopamine (0.14 – 0.35 mg/g) and tyramine (0.15-1.9 mg/g) were either similar or higher than that quoted by the NIST (Sander et al., 2008). In all three SRM preparations, synephrine was present in 100-fold higher concentrations than either octopamine or tyramine (Figure 45). This trend of trace amine relative quantities was consistent with previous analytical studies (Nelson et al., 2007; Pellati & Benvenuti, 2007; Putzbach et al., 2007).

Table 21: Comparison of octopamine (OCT), synephrine (SYN) and tyramine (TYR) quantities in standardised reference materials (SRMs) using current method and values certified by the National Institute of Standards and Technology (NIST). The values presented in the top three rows were obtained with the developed XBridge BEH C18 column whereas the bottom three rows represent data collected by Sander et al. (2008). *Standard deviation not provided due to limited data from original author.

	The current method (XBridge BEH C18)					
Standard reference material (SRM)	Average quantified (mg/g) ± SEM			Ratio of amines		
	OCT	SYN	TYR	OCT	SYN	TYR
C. aurantium fruits (SRM 3258)	0.138 ± 0.008	5.278 ± 1.071	0.314 ± 0.301	0.026	1	0.059
C. aurantium extract (SRM 3259)	0.352 ± 0.031	38.027 ± 4.085	1.901 ± 1.004	0.009	1	0.049
C. aurantium pill (SRM 3260)	0.159 ± 0.011	9.679 ± 2.045	0.149 ± 0.011	0.013	1	0.013
NIST Certified values (Sander et al., 2008)						
C. aurantium fruits (SRM 3258)	0.124 ± 0.016	9.10 ± 0.15	0.031*	0.014	1	0.003
C. aurantium extract (SRM 3259)	0.809 ± 0.051	71.90 ± 2.3	0.800 ± 0.067	0.011	1	0.011
C. aurantium pill (SRM 3260)	0.161 ± 0.022	18.19 ± 0.49	0.187 ± 0.022	0.009	1	0.01

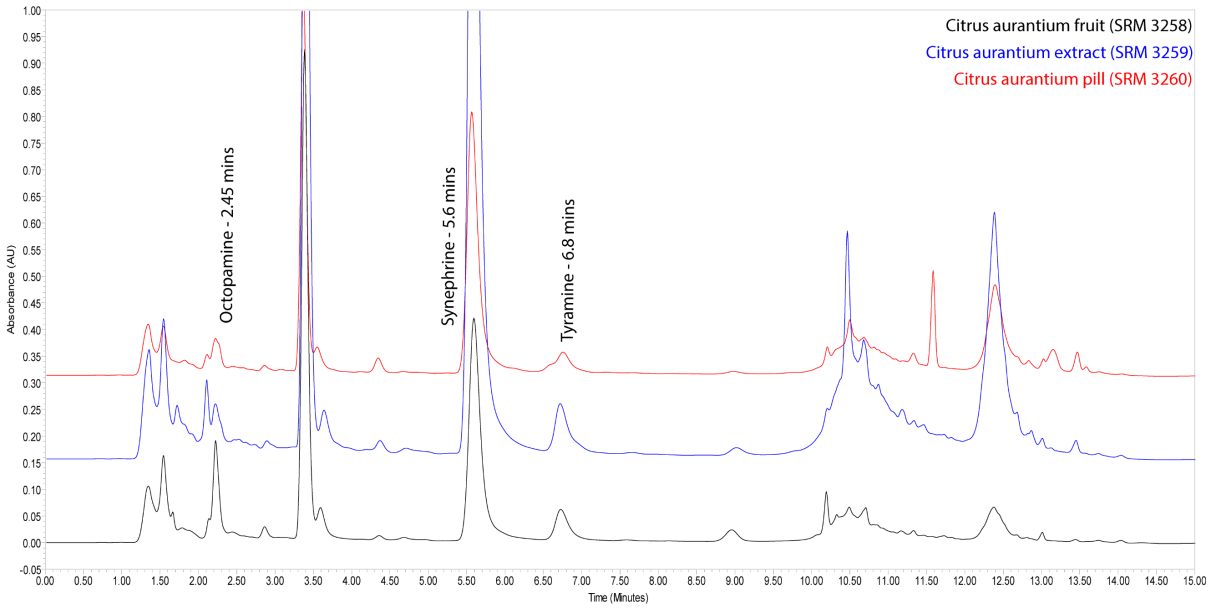


Figure 45: Overlapped chromatogram of the standardized reference materials, *C. aurantium* fruit (SRM 3258), *C. aurantium* extract (SRM 3259) and *C. aurantium* pill (SRM 3260).

There are two likely possibilities for the variability of amine concentrations in this analysis compared to their certified values. First, the current samples were extracted in a single cycle protocol compared to the double cycle extraction process used by the NIST for LC-MS, LC-MS/MS, or recent LC-MS/MS (Pawar et al., 2020) by the FDA. Sander et al. (2008) had previously outlined the challenges with different matrix types. SRM 3259 (dried ethanolic extract) appeared to dissolve thoroughly compared to botanical fruit sample (SRM 3258) or solid oral dosage form (SRM 3260), which required multiple solvent extractions with longer extraction times (Sander et al., 2008). In this analysis, a shorter single cycle extraction may have caused a lower yield of synephrine but did not significantly affect the yield of octopamine or tyramine in all three samples.

Secondly, it is noted that the standard reference materials were discontinued in 2014, and no further batches have been made. The stability of the amines in these SRMs have not been tested, and it is possible that some degradation of the amines might have occurred when subject to 1% aqueous hydrochloric acid during the sample preparation. However, this was unlikely as the FDA had similarly used the SRMs post-expiration date to show similar yields of synephrine to the NIST (Pawar et al., 2020).

In contrast to the dried powdered form of the botanical and dry oral dosage forms, the ethanolic extract (SRM 3259) had a dark solid appearance across three different

batches of the sample used. The combination of the solidification of the SRM and the shorter extraction times likely caused the variable quantities of the amines found in this study compared to the NIST (Putzbach et al., 2007). Importantly despite the variation in amounts, the relative ratio of trace amines was consistent.

The ratio method overcomes the quantitative discrepancies by converting the quantities of amines to a ratio rather than specific amounts. On average, our results showed the ratio across the three amines were (synephrine: octopamine: tyramine, 100:1:5). The NIST analysis data showed a ratio of 100:2:2 (Sander et al., 2008). In order to assess the accuracy of the pre-workout supplement labels, this study looked for similar ratios in which synephrine may be present at roughly 100-fold higher levels than either octopamine or tyramine. From this, it may be inferred that the supplement was likely to contain the trace amines derived from a *C. aurantium* source. This chromatographic ratio comparison technique has some limitations. Similar to the challenges in other herbal medicine analysis, the ratios of compounds may be affected by the instability of amines during the manufacturing process or during sample preparation (Jiang et al., 2010).

II) PRE-WORKOUT SAMPLE ANALYSIS

All supplements purchased for this study were marketed to increase “energy” and “sporting performance”. The optimised method was applied to quantify the trace amine composition of twelve PWS listed *C. aurantium* either with specific amounts or as part of a “proprietary blend” on their labels (Table 22).

Of the twelve PWS, only 42% (5/12) supplements showed trace amine ratios similar to that detected in the natural extracts of *C. aurantium* fruits. Of the remaining supplements tested, synephrine was detected in four supplements but with no detectable tyramine or octopamine, while the remainder had no detectable levels of synephrine. This suggests that for the majority of samples tested, the amines quantified were unlikely to originate from an authentic botanical source of *C. aurantium*. It should also be noted that for those supplements where synephrine alone was detected, the expected levels of octopamine and tyramine would still have been above this experiment’s LOD.

PWS that specifically listed *C. aurantium* on their labels (Table 22) contained all three trace amines and in a ratio consistent with that in the SRMs. The highest amount of synephrine per gram of sample quantified in this group of supplements was in *PWS6* (31.30 ± 1.68 g/g). However, when the serving size of *PWS6* was considered (0.25 g dry oral dosage form), it had a lower amount of synephrine than the labelled amount at 8.14 ± 0.44 mg/serving. On the other hand, *PWS4* appeared to have a low amount of synephrine (4,672.61± 149.78 µg/g) but had the highest synephrine amount (38.88 ± 3.26 mg/serving) when considering its single serving size of 8 grams/serving. The relevant authorities have set a maximum daily dose limit of 30 mg/day of synephrine and octopamine (Gill, 2015; Pawar & Grundel, 2017). Consumers of *PWS4 would likely* reach this limit in a single serving of the supplement, placing the consumer at an increased risk of adverse cardiovascular effects, especially if more than one serving is consumed per day. Nonetheless, these supplements had average amine ratios (100:1:3) consistent with a natural source of *C. aurantium*.

Table 22: Quantitative analysis (µg/g & mg/serving size) of octopamine (OCT), synephrine (SYN) and tyramine (TYR) from twelve pre-workout supplements and their labelled quantities of amines. The samples are arranged according to their labelled amount of *C. aurantium* extract, *C. aurantium* existing as a part of a proprietary blend, and specifically containing synephrine and octopamine (n=9). Values with a ND= not detected by UV and QDa mass detection and *ND= signal detected but rejected with QDa mass detection. <LOQ = data that was below the limit of quantification. LOD = 0.14 µg/g, LOQ = 0.45 µg/g

Pre-workout supplement code	Pre-workout supplement	Labelled amount of *C. aurantium* extract (mg)	Serving size (g)	Average quantified (µg/g)			Average quantified (mg/serving)			Average ratio of amines		
				SYN	OCT	TYR	SYN	OCT	TYR	SYN	OCT	TYR
PWS1	*Switch Nutrition Power Switch*	*C. aurantium* 15mg	5	N.D.*	N.D.*	N.D.	N.D.*	N.D.*	N.D.	0	0	0
PWS2	*GHOST Legend*	*C. aurantium* (50mg)	12.7	818.05 ± 9.13	9.55 ± 0.77	9.33 ± 9.33	10.81 ± 0.13	0.013 ± 0.01	0.14 ± 0.13	100	0.1	1.2
PWS3	*Grenade 0.5 Calibre Pre-workout*	*C. aurantium* (60mg)	9.7	1,172.09 ± 1.002	51.56 ± 25.20	22.95 ± 09.28	11.82 ± 1.51	0.54 ± 0.26	0.25 ± 0.10	100	4.5	2.1
PWS4	*Ronnie Coleman YeahBuddy*	*C. aurantium* (200mg)	8	4,672.61 ± 149.78	30.12 ± 9.0	47.32 ± 18.54	38.88 ± 3.26	0.26 ± 0.01	0.43 ± 0.17	100	0.7	1.1
PWS5	*Labrada Supercharge Pre-workout*	*C. aurantium* extract (40mg)	27	347.78 ± 391.51	5.12 ± 2.55	10.74 ± 10.04	9.77 ± 1.21	0.15 ± 0.07	0.33 ± 0.32	100	1.5	3.4
PWS6	*PrimaForce Syneburn*	*C. aurantium* (10mg synephrine)	0.25	31,306.54 ± 1675.38	103.66 ± 15.52	1,510.64 ± 524.62	8.14 ± 0.44	0.03 ± 0.014	0.43 ± 0.15	100	0.3	5.2
PWS7	*BPI Sports PumpHD*	*C. aurantium* (part of blend 500mg)	10	2.45 ± 1.22	N.D.*	<LOQ	0.03 ± 0.01	N.D.*	<LOQ	100	0	<LOQ
PWS8	*BPM labs The One*	*C. aurantium* peel extract (part of blend 12g)	12	592.15 ± 32.52	N.D.*	N.D.*	7.39 ± 0.41	N.D.*	N.D.*	100	0	0
PWS9	*Panthera Pharmaceuticals KardioFire*	*C. aurantium* (part of a blend 625mg)	10	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0	0	0

PWS10	*Cellucor C4 Ultimate*	*C. aurantium* fruit (part of a blend 805mg)	19	945.61 ± 40.13	N.D.*	N.D.*	18.69 ± 0.79	N.D.*	N.D.*	100	0	0
PWS11	*ErgoGenix Ergoblast*	*C. aurantium* (part of a blend 1600mg)	14	N.D.	N.D.*	N.D.	N.D.	N.D.*	N.D.	0	0	0
PWS12	AllMax Nutrition Muscle Prime	Synephrine 20 mg, Octopamine 10 mg	19	166.34 ± 166.34	N.D.*	8.12 ± 4.17	3.16 ± 3.15	N.D.*	0.15 ± 0.08	100	0	5.3

For those supplements that listed *C. aurantium* as a part of a *proprietary blend* (5/12), only three supplements (*PWS7, PWS8, and PWS10*) had synephrine only but not tyramine or octopamine. Synephrine was quantified in *PWS8* (592.15 ± 32.52 µg/g) and *PWS10* (945.61 ± 40.13 µg/g), but octopamine and tyramine were not detected despite predicted levels being well above the current limit of detection (e.g. 100-fold lower than synephrine). The absence of octopamine and tyramine suggests that synephrine may have been added from a source other than from a *C. aurantium*-source. In the case of *PWS7,* synephrine was found in low concentrations (2.45 ± 1.22 µg/g) and, although tyramine was detected*,* it was below the LOQ of 0.820 µg/g. Therefore, it is still possible that the synephrine found was from a *C. aurantium* source.

The complex and variable nature of the pre-workout supplement matrices may have led to signal suppression in some cases despite the sample clean up protocol (Matuszewski et al., 2003). *PWS12* was included for comparison since the label stated a specific amount of synephrine (20 mg/serving) and octopamine (10 mg/serving) but not the plant extract. However, this analysis showed that only synephrine (3.16 mg/serving) was present at concentrations lower than the claimed amount in only one of three replicate samples. No octopamine was detected in all replicates of the sample. The synephrine added in *PWS12* was likely from a synthetic source but with varying amounts found in each serving, similar to the variability of caffeine found in PWS (Desbrow et al., 2019).

A limitation of this pilot study was the relatively small sample size that may not represent all supplements labelled to contain bitter orange. However, previous studies found phenethylamines in *Acacia rigidula* dietary supplements (Pawar et al., 2014) or adulterants in plant food supplements (Paiga et al., 2017) cited a similar sample size to this study. Additionally, because of the 100-fold difference in concentration of synephrine and the other two amines, a more sensitive method that can quantify very low levels of octopamine and tyramine would be preferable to provide more insight into the authenticity of these PWS.

Pre-workout supplement formulations are highly variable between batches and brands. Stohs et al. (2012) had highlighted this issue surrounding poorly standardized or non-standardized *C. aurantium* extracts used in supplements (Stohs et al., 2012). The lack of information surrounding the manufacturer's source or standardization of synephrine makes it difficult to reproduce results with a high level of precision. An advantage of the ratio comparison method is that it provides authentication of a natural compound without comparing absolute amounts in the sample.

The chromatographic ratio comparison technique has some limitations. Similar to other herbal medicine analysis challenges, the ratios of compounds may be affected by the instability of amines during the manufacturing process or sample preparation (Jiang et al., 2010). The current study was optimised for the three main amines in *C. aurantium.* A direct comparison between their levels in supplement samples and the *C. aurantium* plant material could be made. The inclusion of other known adrenergic amines in *C. aurantium,* such as hordenine and *N*-methyl-tyramine could enhance the ratio profile of *C. aurantium* fruits and extracts.

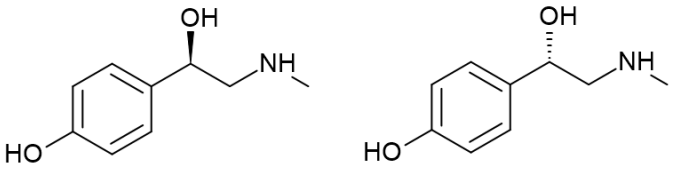
Additionally, this study was not optimized for other possible synthetic stimulants that have been previously found in *C. aurantium* labelled PWS, such as oxilofrine (methyl-synephrine; *m/z 182*) (Cohen et al., 2017) or deterenol (isopropyloctopamine; *m/z 196)* (Pawar et al., 2020). Upon further screening of the total ion chromatogram, the corresponding mass fragments of these two stimulants were absent in the samples used in this analysis. The targeted inclusion of these synthetic stimulants was beyond the scope of this study but would be useful for regular screening, especially since PWS regularly change formulations depending on the regulatory body where the supplement is manufactured. Nonetheless, the current results show clear inconsistencies between what was listed on the labels of PWS and levels of amines detected. Those pre-workouts that listed specific amounts of *C. aurantium* were most authentic, with the trace amine ratios consistent with that expected from a plant-derived source.

Strategies to aid consumers recognise authentic products is needed. For example, in the United States of America, a USP Dietary Supplement Verification Program has been set up, which offers supplement manufacturers the chance to have their products verified and include their symbol on the label (U. S. F. D. Administration, 2015). Although the service is offered worldwide, many manufacturers (including those in Australia) do not appear to partake of that service. In Australia, PWS is regulated by the Therapeutics Goods Administration ((FSANZ), 2015); however, the level of testing and quality assurance performed is low and Australian-made supplements are a very small proportion of supplements on the market. In those cases where *C. aurantium* was listed as part of a proprietary blend, or when pure synephrine appears unlikely the synephrine within originated from a natural source. The current results underscore the importance of developing a sensitive, accurate and rapid quantitative screening tool to detect discrepancies in the authenticity of origin of the key active components in commercially available PWS.

3.5 STUDY 6: SYNEPHRINE ENANTIOMERS FOUND IN *C. AURANTIUM*-LISTING PRE-WORKOUT SUPPLEMENTS

INTRODUCTION

Synephrine enantiomers (Figure 46), have been shown to elicit different pharmacological activity on α- and β-adrenoceptors (Brown et al., 1988; Stohs & Preuss, 2012). Natural plant extracts contain the *R*-(-)-synephrine (*l*-enantiomer) predominantly. PWS containing non-plant derived synephrine may contain higher levels of the more potent enantiomer. Reports have emerged showing discrepancies in PWS labelling potentially posing a higher risk to consumers. The two enantiomers have been reported to a percentage ratio of 92:8 (*R*-synephrine: *S*-synephrine) Hence, PWS that list *C. aurantium* as a source of synephrine should have a similar ratio of enantiomers. However, there have been no investigations into the quantification of synephrine enantiomers in *C. aurantium*-listing PWS.



R-(-)-synephrine *S*-(+)-synephrine

Figure 46 ***Chemical structure of R-(-)-synephrine, S-(+)-synephrine***

Synephrine enantioseparation is conducted either by direct or indirect methods on HPLC-UV. A direct enantioseparation method uses a chiral mobile phase additive or chiral stationary phase to separate the enantiomers of interest. *R*-(-)-synephrine was the only enantiomer found in various citrus fruits (0.07% - 0.40%), crude drugs (0.07%-0.40%) and Chinese medicines (0.03% - 0.11%). Pellati et al. (2002 & 2005) developed and validated a direct HPLC-UV method analysis of synephrine enantiomers in *C. aurantium* and *Evodia* species using a protein-based chiral stationary phase [11, 23]. *R*-(-)-synephrine was the only enantiomer isolated from fresh citrus fruits, while *S*-(+)-synephrine was detected and quantified in dry extracts of *C. aurantium*.

Indirect methods use derivatising agents, such as ion-pair reagents, which react with enantiomers during sample preparation to produce diastereoisomers, separated by conventional RP-HPLC (Gal & Brown, 1986; Tanaka et al., 2019). Tanaka and colleagues derivatised different sections of citrus fruit samples with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosil isothiocyanate (TAG-ITC) before HPLC with UV detection at 254 nm (Figure 47)

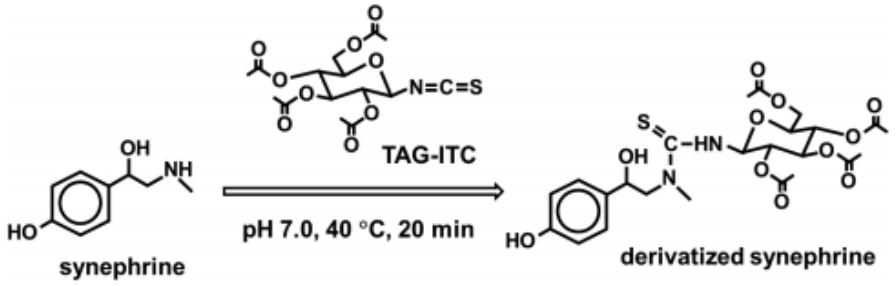


Figure 47: Reaction scheme of synephrine derivatization with TAG-ITC (Tanaka et al., 2019)

Only the *R*-(-)-synephrine enantiomer was found in the exocarp, mesocarp, endocarp and sarcocarp sections of *C. aurantium* at levels below 2.6 mg/g (Tanaka et al., 2019). *S*-(+)-synephrine was found in *Citrus unshiu* and citrus hybrids but were at levels below 1% that of the *R*-(-)-synephrine levels. Pellati et al. (2010) studied the racemization of synephrine enantiomers and found that it hardly occurs in nature. However, less than 10% of isomerization was possible in buffer solutions at pH values between 3 and 8 at 80°C after 48 hours. Similarly, Tanaka et al. (2019) observed racemization of *R*-synephrine after subjecting it to a high pH value of 9.6 and at 100°C Therefore, the presence of high levels of *S*-(+)-synephrine in commercially available PWS would be suggestive of a non-plant source.

Individual synephrine enantiomer standards are not commercially available, which presents difficulties for chiral separation analyses. The cost and analytical capabilities associated with the synthesis of enantiomers are prohibitive for many laboratories. Other issues arising from enantiomeric separation include column pre-conditioning and sample derivatisation, which can be expensive and time-consuming. Since the *R*-enantiomer of synephrine predominates and elutes before *S*-(+)-synephrine in natural extracts and plant material, the use of *C. aurantium* SRMs can present a rapid and

cost-effective alternative to screen for synephrine enantiomers without the need to synthesise pure synephrine enantiomers.

The aim of this study was to develop a direct, convenient, and accurate screening method suitable for the separation and quantification of synephrine enantiomers in *C. aurantium*-listing PWS.

METHODS AND MATERIALS

HPLC-UV-QDA ANALYSIS

Chiral analysis was carried out on a cellobiohydrolase (CBH) column (4.0 x 100 mm, 5 µm, Daicel Chiral Technologies Europe, France), coupled to a Chiral-CBH guard column (4.0 x 10 mm, 5 µm). Under isocratic conditions, the mobile phase was 5 mM ammonium acetate buffer (pH 7.0)-methanol (85:15, w/w). The flow rate was 0.6 mL/min. The column temperature was 25˚C. The injection volume was 10 µL. The UV detection was set at 225 nm, and the QDa mass detector was performed in positive mode under the following conditions: total ion current (TIC) between mass-to-charge (m/z) 100-400; capillary voltage, 0.8 kV; cone voltage, 15 V. Single Ion Recording (SIR) was set for positive mode base peak of synephrine (150.2 m/z). The total analysis time was 15 minutes. Three injections were performed for each sample. Quantification was performed by integrating the areas of the peaks due to synephrine enantiomers. Peak areas were used to calculate the amount of synephrine enantiomers present in the samples by applying the linear regression equation obtained from the external calibration. The method was validated for synephrine quantitation, according to the ICH guidelines in terms of its specificity, linearity, limits of detection (LOD) and limits of quantitation (LOQ), accuracy and precision.

ENANTIOMERIC COLUMN SELECTION

Chiral analysis was carried out on a cellobiohydrolase (CBH) column (100 mm x 4.0 mm, 5 µm, Daicel Chiral Technologies Europe, France), coupled to a Chiral-CBH guard column (10 mm x 4.0 mm, 5 µm). The column was selected because it was previously developed and validated for the enantioselective analysis of synephrine in *C. aurantium* plant material (Pellati et al., 2005) and the *Evodia* fruit (Pellati et al., 2006). The CBH stationary phase is a cellulase enzyme extracted from *Trichoderma reesei* (PDB accession code 1DY4; Figure 48) immobilised on 5 µm silica beads with an isoelectric point of 3.9 (Hermansson & Grahn, 1994). It contains multiple chiral centres and mechanism for ionic hydrophobic and hydrogen bonding (Fornstedt et al., 1997).

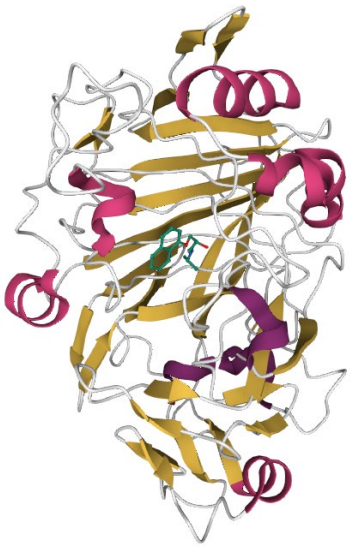


Figure 48: 3D structure of cellohydrobiohydrolase 1 (CBH1) from Trichoderma ressei (PDB: 1DY4) with a (+)-propranolol ligand

STANDARDS AND SAMPLE PREPARATION

Standard solutions of (±)-*p*-synephrine were dissolved in acidified water (H_2O + 2 drops of 0.1% HCl) to a 1 mg/mL concentration. Stock solutions were stored at 4 °C until analysis. Linear dilutions were prepared for each standard between 0.5 µg/mL to 200 µg/mL. Dried SRMs were weighed (0.5 g), and 5 mL of methanol was added. The mixture was sonicated under ambient temperature for 30 minutes. The samples were then centrifuged at 3000 rpm for 20 minutes, and the supernatants were extracted for a 1:5 dilution with aqueous ammonium acetate buffer (pH 7). The mixture was then eluted into a 2 mL HPLC vial (ThermoScientific, Australia) and stored at 4 °C before analysis. The method for PWS was similar as above, except 1 g of samples were weighed, and 10 mL of acidified water was added as the solvent prior to a 1: 5 dilution with aqueous ammonium acetate buffer (pH 7).

RESULTS AND DISCUSSION

METHOD DEVELOPMENT

OPTIMISATION OF CHROMATOGRAPHY

The direct enantioseparation was conducted on an enzymatic stationary phase, cellobiohydrolase immobilised onto 5 µm silica particles. The mobile phase was 15% methanol in an ammonium acetate buffer (pH 7.0, 5mM), and separation of enantiomers was achieved with an isocratic flow rate of 0.6 mL/min. The retention times for *R*-(-)-synephrine and *S*-(+)-synephrine was 7.9 mins and 8.9 mins, respectively (Figure 49).

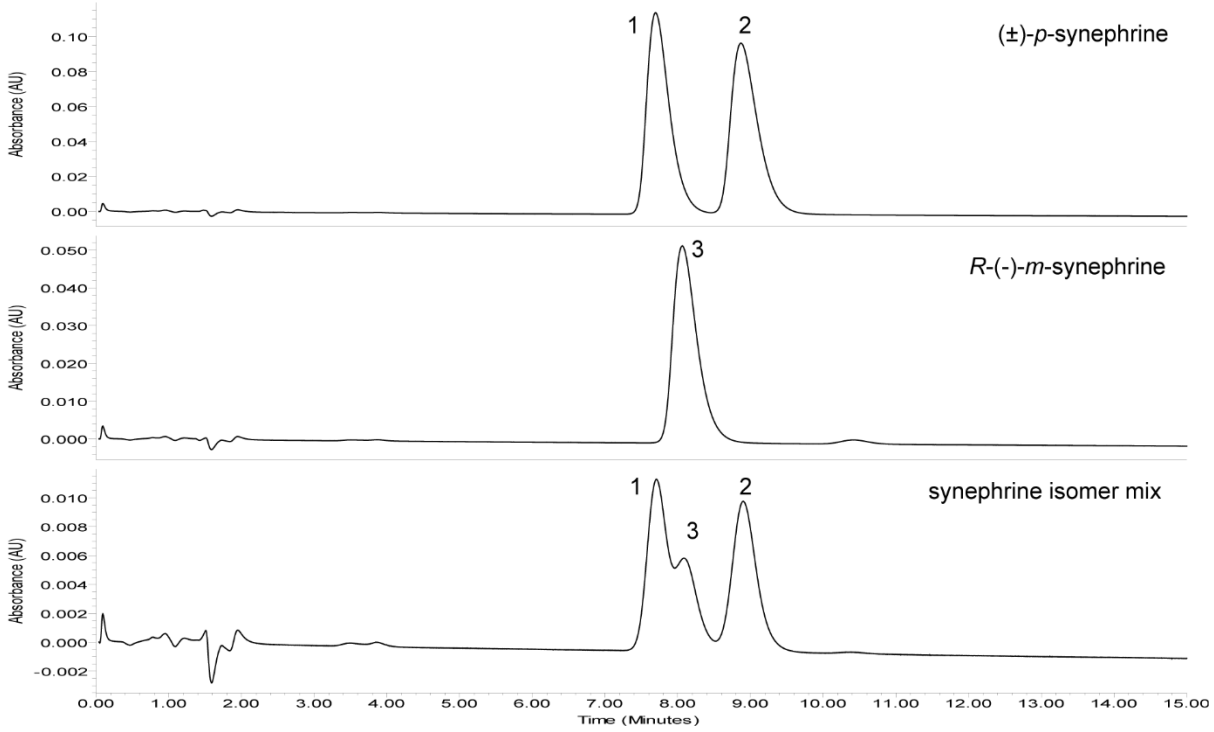


Figure 49: The separation of racemic-(±)-synephrine standard (100µg/mL); R-(-)-phenylephrine hydrocholoride standard (m-synephrine, 100µg/mL) and a combination of both synephrine isomer in a matrix blank (10µg/mL). The compounds found were (1) R-(-)-synephrine, (2) S-(+)-synephrine and (3) R-(-)-m-synephrine.

Previous studies have suggested that *m*-synephrine (phenylephrine) is present in *C. aurantium* (Allison et al., 2005). In this experiment, *m*-synephrine was investigated as a possible artefact that could interfere with detecting the target enantiomer analytes. *R*-(-)-Phenylephrine hydrochloride eluted between p-synephrine enantiomers and had a retention time closer to *R*-synephrine relative to *S*-synephrine (Figure 49). No traces of *m*-synephrine were found in *C. aurantium* extracts or PWS in this study. The current data clearly show that *m*-synephrine is not a naturally occurring phenylethylamine and is not expected to be found in either *C. aurantium* fruits or extracts.

In a preliminary study, a matrix blank was spiked with 100 µg/mL of (±)-synephrine, (±)-octopamine and tyramine to determine the relative retention times of the other trace amines found in *C. aurantium* (Figure 50). The trace amine mix had an elution order of the *R*- and *S*-synephrine enantiomers, followed by the first eluent of octopamine (E1-octopamine), the second eluent of octopamine (E2-octopamine), and tyramine.

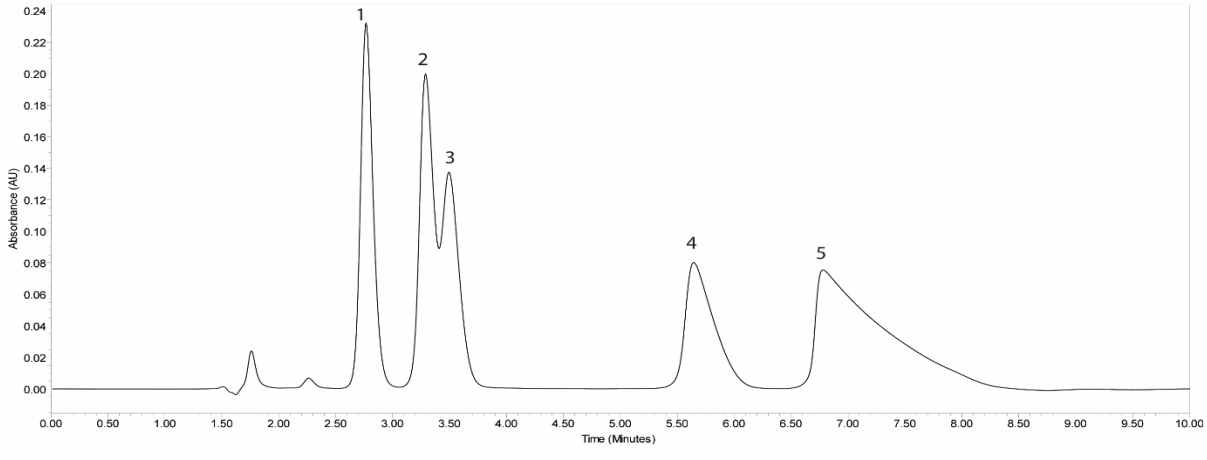


Figure 50: The separation of trace amine standards, (±)-synephrine (100µg/mL); (±)-octopamine hydrochloride (100µg/mL), and tyramine 100µg/mL in a matrix blank. The compounds found were (1) R-(-)-synephrine, (2) S-(+)-synephrine, (3) E1-octopamine, (4) E2-octopamine and (5) tyramine.

The cellobiohydrolase stationary phase was selected for its effectiveness in enantiomer separation of basic drugs (Fornstedt et al., 1997; Henriksson et al., 1996), amphetamine-like compounds (Bagnall et al., 2012), and was successful in separating synephrine enantiomers (Pellati et al., 2005; Pellati et al., 2010). However, the column is particularly sensitive to changes in pH, organic buffer content and temperature. The

optimisation of the HPLC conditions was carried out in accordance with the instructions provided by the column manufacturer. In this study, the concentration of the ammonium acetate buffer, the phase pH and the strength of organic solvent were the variables chosen for the optimisation.

The cellobiohydrolase stationary phase (pI = 3.9) was significantly influenced by the organic solvent and had a linear correlation to the decrease in resolution and the increase in elution strength. The stronger non-polar solvents of 2-propanol and acetonitrile (10%) had a lower peak resolution than methanol (10%) (Figure 51). However, the increase in methanol proportions resulted in a decreased peak separation. Hence, methanol (15%) was selected as the organic solvent due to its ability to resolve the enantiomers (R_s of 1.6).

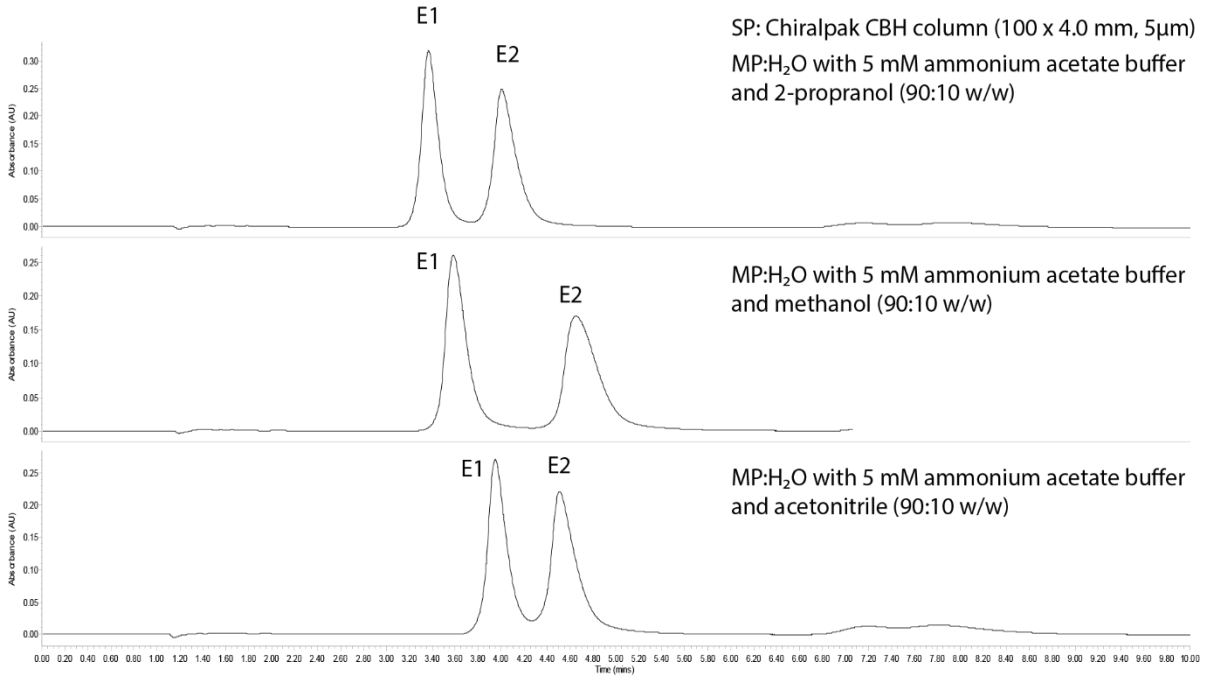


Figure 51: Representative chromatographs of different mobile phase conditions affecting synephrine enantiomer peak resolution

Enantiomeric peak resolution improved under neutral pH conditions. In the preliminary study, acetic acid (pH 5.0) showed poor resolution and baseline issues than ammonium acetate (pH 6.0). The peak resolution was further improved when adjusted to a pH of 7.0 with 0.1% ammonium hydroxide (NH_4OH) (Figure 52).

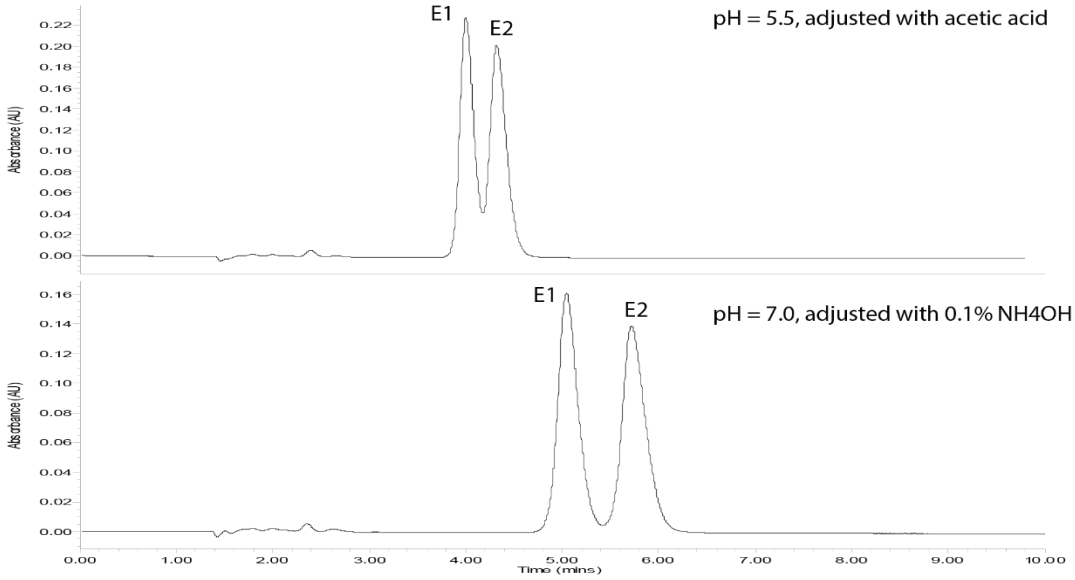


Figure 52: Improved baseline resolution with (±)-synephrine separation at higher pH

This may suggest a weak cation exchange between the charged, basic drug and the active site of cellobiohydrolase (Henriksson et al., 1996). A decrease in resolution was observed with incremental increases in temperature from 25ºC to 30ºC. The sensitive nature of the cellobiohydrolase particles within the column introduced a challenge to optimisation as the risk of column denaturation was possible when the organic content, pH and temperature were tested beyond its recommended limits.

OPTIMISATION OF SAMPLE PREPARATION

In a previous direct chiral separation, Pellati et al. (2005) prepared methanolic extractions of plant material before clean-up with solid-phase extraction (SPE) with strong ionic exchange properties (LiChrolut SCX, Merck) (Pellati et al., 2005). For the current experiment's optimisation, a notable baseline artefact and a decrease in enantiomer resolution were found when the cation exchange solid-phase extraction (Oasis PRiME MCX, Waters) was used. This interference may have been due to interactions between the SPE solvents and the cellobiohydrolase particles in the column. A more considerable drop in baseline and an artefact was observed in a hydrophilic, reversed-phase SPE (Oasis PRiME HLB, Waters), which suggested that solid phase-extraction was not suitable for the analysis due to the sensitive nature of the column. By incorporating a 1 in 50 sample dilution with starting solvent conditions, the optimised protocol demonstrated improved enantiomeric yield and reduced baseline issues (Figure 53).

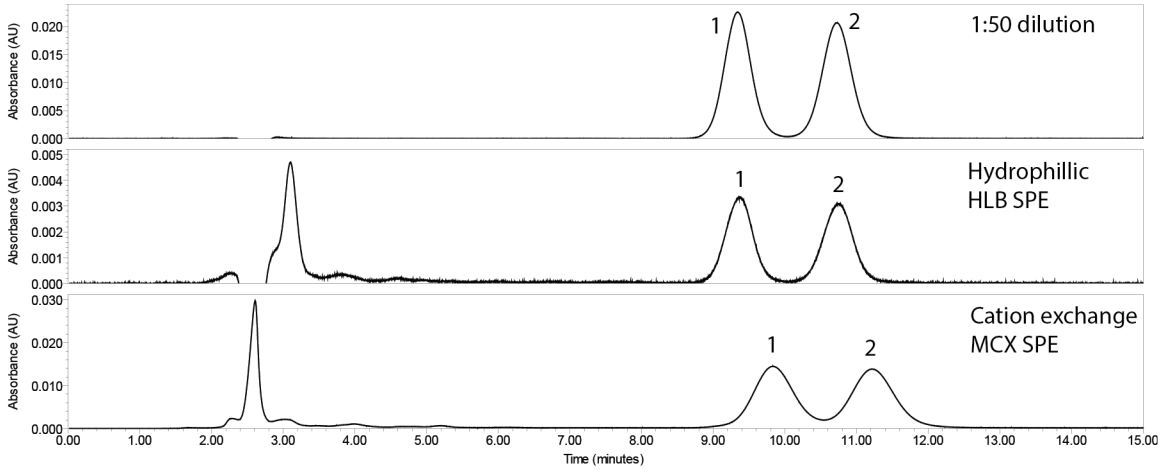


Figure 53: Representative chromatograms of (±)-synephrine standard when prepared as a 1:50 dilution, under hydrophilic solid-phase extraction (HLB) conditions and strong cation exchange solid phase extraction (MCX).

EVALUATION OF RECOVERY VALUES AND MATRIX EFFECTS

The percentage recovery (%) and correction factor were calculated and applied to the synephrine enantiomers in all samples prepared with this protocol. To evaluate the influence of the pre-workout supplement matrix on the HPLC-UV-QDa procedure, three levels of (±)-synephrine was spiked in matrix blanks that do not contain *C. aurantium* or synephrine (BodyScience ™ K-OS pre-workout). The average recoveries of the synephrine enantiomers were lower in the spiked matrix blank than when the standard was prepared in a solvent with starting HPLC condition (Table 23). There was a 20.4% signal suppression for synephrine enantiomers in pre-workout supplement matrix. PWS often contain a myriad of other ingredients. It may be hypothesised that the polar compounds within the pre-workout supplement matrix may interfere with the interaction of synephrine enantiomers to the cellobiohydrolase stationary phase.

Table 23: Recoveries and matrix effects (%) of racemic (±)-p-synephrine spiked in (A) matrix blank and (B) under starting solvent conditions.

Spiked (±)-*p*-Synephrine concentration	Amount recovered in matrix blank (µg/mL) (A)		Amount recovered in mobile phase solvent (µg/mL) (B)		Matrix effects (%) (([A/B]-1)*100)	
	(-)-SYN	(+)-SYN	(-)-SYN	(+)-SYN	(-)-SYN	(+)-SYN
50 µg/mL	37.72 ± 0.52	37.46 ± 0.51	49.62 ± 0.35	49.24 ± 0.34	-23.97	-23.92
100 µg/mL	81.15 ± 0.42	80.75 ± 0.40	95.32 ± 0.16	94.61 ± 0.15	-14.86	-14.66
150 µg/mL	112.94 ± 0.93	112.36 ± 0.98	145.58 ± 0.75	145.02 ± 0.75	-22.42	-22.52

METHOD VALIDATION

LINEARITY, LIMITS OF DETECTION AND LIMITS OF QUANTIFICATION

The linearity of the synephrine standard was assessed using the least-square regression method of dilutions for each standard solution. An intra-day 8-point standard curve for (±)- synephrine (0.5 µg/mL to 200 µg/mL), the slope and *y*-intercept, correlation coefficient (r^2) and RSD % of the standard curve were determined (Table 24). The increase in calibration concentration beyond 200 µg/mL or less than 0.5 µg/mL yielded a decrease in resolution and was not used in this analysis. The limit of quantification, 1.7 µg/mL (0.009 mM), was an order of magnitude higher than Pellati et al. (2005) and Tanaka et al. (2019) but was sufficiently sensitive for the determination of synephrine enantiomers in *C. aurantium* and pre-workout supplement samples.

Table 24: Calibration data for linearity of R-(-)-synephrine and S-(+)-synephrine.

Synephrine standards	***Calibration range (µg/mL)***	***Correlation coefficient (r^2)***	***Limit of detection (µg/mL)***	***Limit of quantification (µg/mL)***	***Retention time (min)***	***Theoretical plates (N)***	***Resolution (Rs)***	***Selectivity (α)***
R-(-)-synephrine	0.5 - 200	0.999 ± 0.001	0.580	1.757	7.85	2864	-	-
S-(+)-synephrine	0.5 - 200	0.999 ± 0.001	0.534	1.617	8.94	3006	1.605	1.302

SELECTIVITY, ACCURACY AND PRECISION

The method's selectivity was demonstrated by injecting each reference standard to show resolution between all the standards and injecting the negative control dietary supplement (matrix blank) to indicate no interfering peaks above the limit of quantification. The designation of the synephrine enantiomers in this study was based on the trend of *R*-(-)-synephrine enantiomer eluting before the *S*-(+)-counterpart (Gal & Brown, 1986; Kusu et al., 1996; Kusu et al., 1995; Lebedeva et al., 2014; Pellati et al., 2005; Pellati et al., 2002; Pellati et al., 2010; Tanaka et al., 2019; Zhang et al., 2018).

Henriksson et al. (1996) showed that the cellobiohydrolase enzyme had a greater affinity for *S*-(+)-enantiomers of positively charged beta-blockers, propranolol and atenolol (Henriksson et al., 1996) and therefore would elute with a longer retention time than *R*-(-)-synephrine. The researchers hypothesised that the hydroxyl group on the asymmetric carbon influences binding activity onto cellobiohydrolase protein. Indeed, a similar specificity may occur with the synephrine enantiomers in this study. The results here support existing literature, demonstrating that the *R*-synephrine is the predominant enantiomer in *C. aurantium* fruits and extracts. Hence, the denotation of the synephrine enantiomers in this work was based on the combined evidence from previous studies on the behaviour of cellobiohydrolase as a chiral stationary phase, others using enantiomeric standards and the current results for *C. aurantium* standard reference material.

The accuracy of the semi-quantitative method was evaluated by the recovery (%) of synephrine enantiomers based on the evaluation of concentrations found in racemic synephrine standards. The accuracy for the spiked samples varied from 94.6% to 99.2%, with their intermediate precision showing relative standard deviations ranging from 0.5% to 2.1% (Table 25). The analytical method for determining synephrine enantiomers can be considered precise and accurate within the broad concentration range investigated.

Table 25: Recovered concentration, accuracy and intermediate precision of R-(-)-synephrine ((-)-SYN)) and S-(+)-synephrine ((+)-SYN).

Standard concentration	Recovered concentration ± SEM (µg/mL)		Accuracy (%)		Intermediate precision (%RSD)	
	(-)-SYN	(+)-SYN	(-)-SYN	(+)-SYN	(-)-SYN	(+)-SYN
50 µg/mL	49.617 ± 0.346	49.242 ± 0.344	99.234	98.483	2.098	2.098
100 µg/mL	95.319 ± 0.164	94.614 ± 0.152	95.319	94.614	0.515	0.493
150 µg/mL	145.583 ± 0.753	145.017 ± 0.750	97.055	96.678	1.551	1.551

The method here is an adapted version of a direct HPLC-UV method analysis of synephrine enantiomers in *C. aurantium* (Pellati et al., 2005; Pellati et al., 2002) to include the use of a single quadrupole array mass detector. The current optimised method had a slightly lower resolution between the enantiomeric peaks. Still, the inclusion of the mass detection provided additional confirmation to the identification of the correct synephrine enantiomer. The optimised method has been applied to the *C. aurantium* standard reference materials (SRM) and PWS that list *C. aurantium*.

QDA-MS ANALYSIS

The single quadrupole (QDa) mass detection provides a rapid confirmatory advantage over conventional HPLC-detection methods. The mass chromatograms of synephrine enantiomers were distinguishable by the intensities of mass-to-charge ratio (*m/z*) of the parent ion $[M + H]^+$ (168.2) or the major fragment ion of synephrine $[M + H - H_2O]^+$ (150.2). The intensity of *R*-(-)-synephrine fragment ion was double that of *S*-(+)-synephrine (Figure 54).

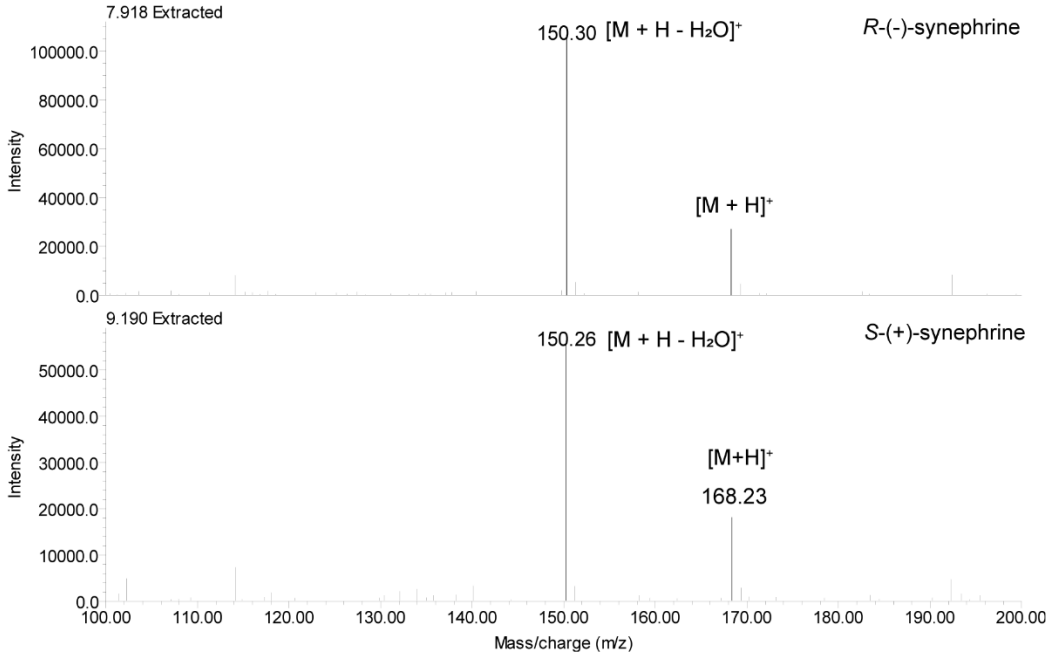


Figure 54: QDa-MS profiles for protonated synephrine found in racemic (±)-synephrine standard spiked in a matrix blank. Both synephrine enantiomers have a precursor ion of m/z 168 and a major fragment ion of m/z 150.

This correlates with the predominance of the R-(-)-synephrine enantiomer in plant material. Analysis of the QDa-results allowed identification of false positives for *R*-(-)-synephrine in two samples (2/12) and *S*-(+)-synephrine in five samples (5/12). In these instances, chromatographic peaks at the respective enantiomer retention times were examined in the mass spectrometry data, which did not show mass fragments corresponding to synephrine

Three-dimensional plots with *m/z* on the Z-axis are a convenient way to visualise separation and identification, as shown by the plots of the standards in a spiked matrix blank (A), *C. aurantium* standardised reference material (B) and two PWS (C & D) (Figure 55). High-resolution time of flight mass spectrometry (QTOF) systems often rely on high organic content in their mobile phases to increase their sensitivity (Bagnall

et al., 2012). However, a compact single quadrupole mass spectrometer (QDa) was more suitable to provide mass confirmation for this analysis. Currently, this is the first implementation of an on-line quadrupole mass spectrometer paired to a direct enantiomeric separation method.

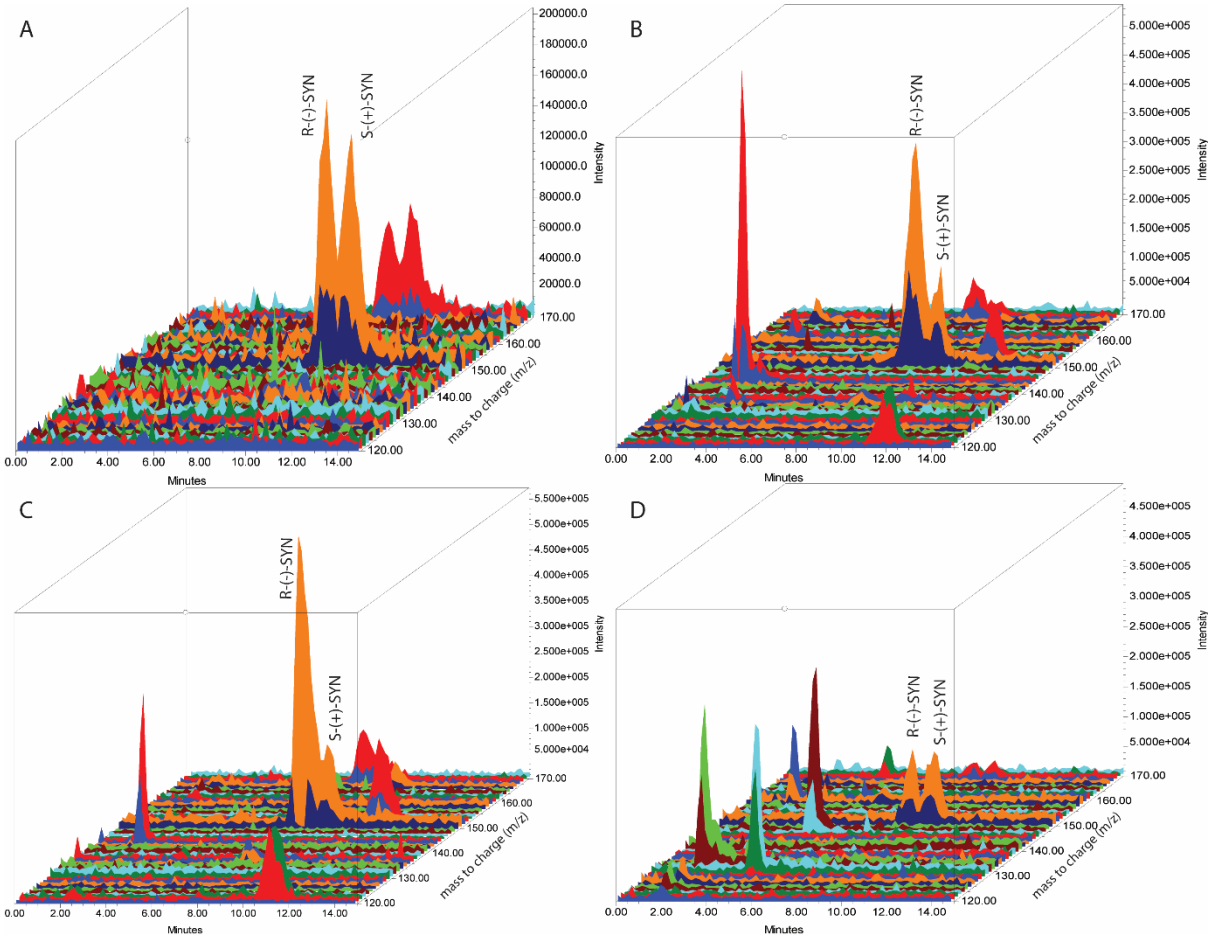


Figure 55: Representative 3D chromatogram plots of standard ±-synephrine in (A) matrix blank spiked with (±)-synephrine standard, (B) standardised reference material 3259 (*C. aurantium* extract, (C) pre-workout supplement showing a natural synephrine enantiomer ratio (PrimaForce Syneburn™), and (D) pre-workout supplement showing a racemic enantiomeric ratio (Allmax Nutrition MusclePrime™).

QUANTIFICATION OF SYNEPHRINE ENANTIOMERS IN STANDARD REFERENCE MATERIAL (SRM) AND PRE-WORKOUT SUPPLEMENTS (PWS)

I) C. AURANTIUM SRMS

In all three SRMs, *R*-(-)-synephrine was detected as the predominant enantiomer with an average ratio of 94:6 (*R*-(-)-synephrine: *S*-(+)-synephrine) (Table 26). An example of the product chromatograms from an SRM and PWS is illustrated in Figure 56. This pattern of *R*-(-)-synephrine existing as the dominant enantiomer (92 to 100%) was consistent with the observations on previous analysis of *C. aurantium* fruits and extracts (Pellati et al., 2005; Pellati et al., 2002; Tanaka et al., 2019). A similar trend was observed in other citrus species, where *S*-(+)-synephrine was significantly lower (Tanaka et al., 2019). However, it should be noted that extracts of the same species may vary in composition depending on the geographical origin, growth conditions, climate, soil quality, harvest season, drying process, powdering methods, extraction, and stabilisation procedures.

Table 26: Quantitative analysis (mg/g) of R-(-)-synephrine (R-(-)-SYN) and S-(+)-synephrine (S-(+)-SYN)) in standardised reference materials (SRMs) and their ratios (n=9).

Standard reference Material Samples	Amount of synephrine quantified (mg/g)		Total amount synephrine (mg/g)	Percentage ratio of Synephrine enantiomers (%) ((Enantiomer/ Total enantiomers) *100)	
	R-(-)-SYN	*S*-(+)-SYN		*R*-(-)-SYN	*S*-(+)-SYN
C. aurantium fruits (SRM 3258)	5.363 ± 0.226	0.344 ± 0.010	5.705	96	4.0
C. aurantium ethanolic extract (SRM 3259)	83.966 ± 4.621	6.187 ± 0.410	90.153	93	7.0
C. aurantium solid oral dosage (SRM 3260)	23.448 ± 0.332	2.149 ± 0.028	25.597	92	8.0

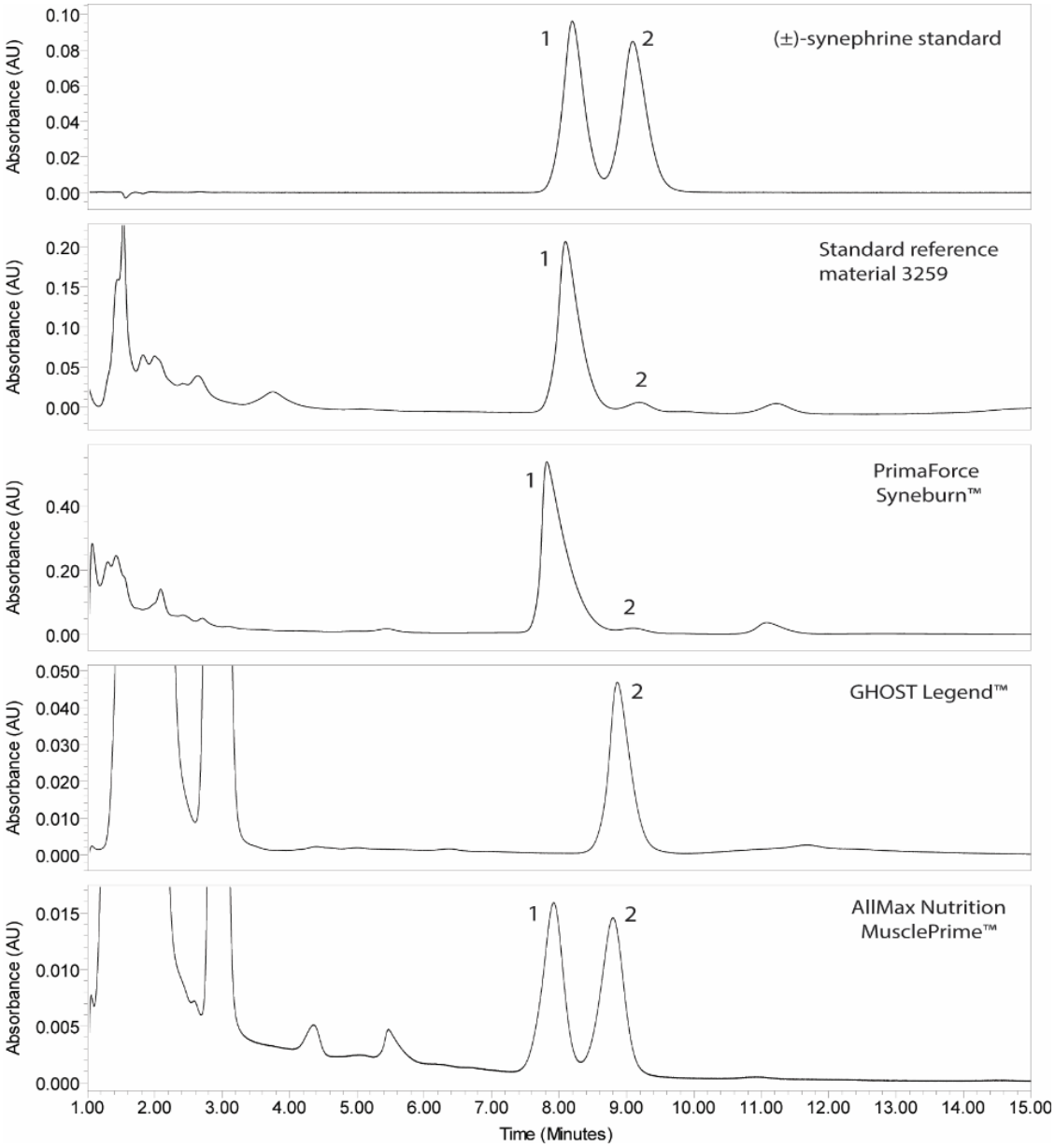


Figure 56: Representative chromatograms of ±-synephrine standard, synephrine enantiomers detected in standardised *C. aurantium* reference material (SRM3259), PrimaForce Syneburn™ containing a predominant R-(-)-synephrine, pre-workout supplement containing S-(+)-synephrine (GHOST Legend™) and a pre-workout supplement containing a racemic enantiomer profile (Allmax Nutrition MusclePrime™). The compounds found were R-(-)-synephrine (1) and S-(+)-synephrine (2).

II) PRE-WORKOUT SUPPLEMENT ANALYSIS

In the PWS tested, the amounts of total synephrine ranged from 0.03 mg/g – 91.19 mg/g. When the serving size of the supplements was considered, the quantity of synephrine ranged from 0.27 mg/serving – 39.31 mg/serving (Table 27). Of the twelve PWS tested, 42% (5/12) supplements had *R*-(-)-synephrine levels expected from a natural *C. aurantium* extract or fruit. Seven of the twelve samples had levels of synephrine enantiomers that were not expected from a natural source. For example, a racemic ratio was detected in four of these seven supplements. Neither synephrine enantiomer was detected in two samples while one showed detectable levels only for the *S*-(+)-enantiomer. These findings show that nearly half of the tested products does not appear to contain synephrine or were likely of *C. aurantium* origin.

The percentage ratio of the *R*-(-)-synephrine to *S*-(+)-synephrine in the five supplements expected to contain *C. aurantium* had an average of 91.4: 8.6, which was consistent to the values found in the SRMs. However, *PWS3* showed a lower amount of *R*-(-)-synephrine (71%), which may suggest racemic conversion during the manufacturing process. Synephrine enantiomers have been reported to have higher rates of racemisation from *R*-(-)-synephrine to *S*-(+)-synephrine under acidic (pH 1) and basic pH (pH 9 to 10) at high temperatures (80 ºC) (Pellati et al., 2010).

Table 27: Quantitative analysis (mg/g & mg/serving size) of R-(-)-synephrine (R-(-)-SYN) and S-(+)-synephrine (S-(+)-SYN)) from fifteen pre-workout supplements and their labelled quantities of amines. The samples are arranged according to their labelled amount of *C. aurantium* extract, *C. aurantium* existing as a part of a proprietary blend, other forms of Citrus, and specifically containing synephrine (n=9). Values with a ND= not detected by UV and QDa mass detection and *ND= signal detected but rejected with QDa mass detection. < LOQ = data that was below the limit of quantification of R-synephrine (1.76 µg/mL) or S-synephrine (1.62 µg/mL).

Pre-workout supplement code	Samples	Labelled description and amount of Citrus or synephrine (mg)	Serving size (g)	Amount of synephrine quantified (mg/g)		Total amount synephrine (mg/g)	Average amount of synephrine in a serve (mg/serving)		Total amount synephrine (mg/serving)	Percentage ratio of Synephrine enantiomers (%) ((Enantiomer/ Total enantiomers) *100)	
				R-(-)-SYN	S-(+)-SYN		R-(-)-SYN	S-(+)-SYN		R-(-)-SYN	S-(+)-SYN
PWS1	Switch Nutrition Power Switch	C. aurantium 15mg	5	N.D.*	N.D.*	0	N.D.*	N.D.*	0	0	0
PWS2	GHOST Legend	C. aurantium (50mg)	12.7	<LOQ	0.15 ± 0.01	0.15	<LOQ	1.99 ± 0.03	1.99	0	100
PWS3	Grenade 0.5 Calibre Pre-workout	C. aurantium (60mg)	9.7	0.33 ± 0.01	0.14 ± 0.01	0.47	3.21 ± 0.04	1.40 ± 0.05	4.61	71	30
PWS4	Ronnie Coleman YeahBuddy!	C. aurantium (200mg)	8	0.82 ± 0.07	0.92 ± 0.03	1.74	6.53 ± 0.55	7.35 ± 0.25	13.88	49	51
PWS5	Labrada SuperCharge Pre-workout	C. aurantium extract (40mg)	27	0.75 ± 0.09	N.D.*	0.75	20.19 ± 2.42	N.D.*	20.19	100	0
PWS6	PrimaForce Syneburn	C. aurantium (10mg synephrine)	0.25	87.83 ± 6.65	3.36 ± 0.08	91.19	21.96 ± 1.67	0.84 ± 0.02	22.80	86	14
PWS7	BPI Sports PumpHD	C. aurantium (part of blend 500mg)	10	0.03 ± 0.01	N.D.*	0.027	0.27 ± 0.01	N.D.*	0.27	100	0
PWS8	BPM labs The One: Black Label	C. aurantium peel extract (part of blend 12g)	12	2.40 ± 0.23	N.D.*	2.4	28.79 ± 2.72	0	28.79	100	0
PWS9	Panthera Pharmaceuticals KardioFire	C. aurantium (part of a blend 625mg)	10	N.D.*	N.D.*	0	N.D.*	N.D.*	0	0	0
PWS10	Cellucor C4 Ultimate	C. aurantium fruit (part of a blend 805mg)	19	0.07 ± 0.01	0.07 ± 0.01	0.137	1.23 ± 0.16	1.27 ± 0.01	2.5	48	52
PWS11	ErgoGenix Ergoblast	C. aurantium (part of a blend 1600mg)	14	2.57 ± 0.01	2.79 ± 0.08	5.36	36	39.06 ± 1.18	39.06	50	50
PWS12	AllMax Nutrition MusclePrime	Synephrine 20 mg	19	0.97 ± 0.07	1.10 ± 0.01	2.07	18.42 ± 1.28	20.89 ± 0.01	39.31	50	50

Racemic ratios of synephrine were detected in four supplements (*PWS4, PWS10, PWS11* and *PWS12*), with total synephrine ranging from 0.82 -2.57 mg/g. The synephrine labelled in *PWS4* was claimed to be from 200 mg of *C. aurantium,* but it was not clear if this was from a fruit, rind or an extract. In contrast, *PWS10* and *PWS11* had listed *C. aurantium* fruits as an ingredient in a proprietary blend. The results for these three supplements do not align with *p*-synephrine from a plant-based source. The racemic synephrine found in *PWS12* was expected as it listed pure synephrine (20mg/ serving) on its label. One pre-workout supplement (*PWS2*) had detectable levels of *S*-(+)-synephrine alone at 0.157 mg/g or 1.99 mg/serving. There are no records of *S*-(+)-synephrine existing as the sole enantiomer in nature. In dietary supplements, the *S*-(+)-synephrine enantiomer has been reported in a range of 0.006 -3.596 % but not as a racemic ratio or as a pure *S*-(+)-enantiomer (Pellati et al., 2005). Whilst some variation in the enantiomer ratio may be expected due to different harvest times or production techniques, this is unlikely to explain the differences observed here. Therefore, the synephrine found in these five supplements are not expected to be from a natural *C. aurantium* fruit or extract.

Some discrepancies were observed between the quantity of total synephrine found in samples tested in this study and that from samples from the same batch tested in the previous non-chiral analysis (Koh et al., 2021). Five supplements in this study had higher amounts of total synephrine, four supplements had lower amounts of synephrine, and three supplements were the same as the previous analysis. It can be hypothesised that these discrepancies were likely due to the non-homogenous preparation of the dry powdered samples during the manufacturing process. The variability of stimulant distribution may be more significant in supplements that list their ingredients as a part of a proprietary blend, similar to the variability of caffeine reported by Desbrow et al. (2019). However, this study did not find any trends that samples specifically listing *C. aurantium* as a stand-alone ingredient were more authentically labelled than those *C. aurantium* as part of a proprietary blend.

This pilot study was limited by a relatively small sample size which may not represent the large array of *C. aurantium*-listing PWS available. Since this was a convenience sample set for a pilot study further studies with a broader sample set would be desirable. However, PWS change formulations frequently and that the composition of some PWS can differ to their current version. As *C. aurantium* contains other biologically active amines such as octopamine, tyramine, hordenine or *N*-methyltyramine, the inclusion of these standards and their respective enantiomers could provide a more comprehensive fingerprint profile to elucidate the authenticity of *C. aurantium* use in PWS on a broader scale.

3.6 OVERALL DISCUSSION

Over the past decade, the consumption of dietary supplements and PWS has increased to improve overall health, improve performance, or gain an advantage in competitive sport. Since 2004, *C. aurantium* has been one of the more popular ingredients included in PWS. However, the authenticity of labelling in PWS has been brought into question and raises concerns for public health. For example, it is unclear whether the active compound in *C. aurantium*, synephrine, may be from an authentic plant source. The overall aim of the two studies in this chapter was to develop two HPLC-UV-MS methods which quantify the levels of trace amines and synephrine enantiomers in *C. aurantium* standard reference material and compare their relative ratio to that found in a sample of *C. aurantium-listing* PWS. Both methods aid in the assessment of labelling authenticity.

In the SRMs, the quantities of total synephrine were consistently higher in the enantiomeric separation with the Chiralpak CBH column than the ratio method with the XBridge BEH column (Table 28). The most significant difference of 52.13 mg/g was observed from the dry ethanolic extract of *C. aurantium* (SRM 3259), whereas the values for the dry powdered fruit (SRM 3258) were similar. Of these two methods, the Chiralpak CBH column showed synephrine values that most closely matched those quantified by the NIST (Sander et al., 2008).

The quantities of total synephrine were different between the two methods, with four pre-workout supplements (*PWS2, PWS3, PWS4,* and *PWS10*) having higher amounts of synephrine when quantified with the XBridge BEH C18 column (Table 28). On the other hand, six pre-workout supplements (*PWS5, PWS6, PWS7*, *PWS8, PWS11* and *PWS12*) had higher quantities of total synephrine when quantified with the Chiralpak CBH column. The most significant difference was found in *PWS11* that did not contain any trace of synephrine when quantified with the XBridge BEH C18 but had 39.06 mg/serving when quantified with the chiral column. Neither of the pre-workout supplements, *PWS1* and *PWS9,* contained synephrine, as confirmed by both methods.

Table 28: Comparison between total synephrine values and ratio between two chromatographic methods for pre-workout supplements. N.D. represents values that were not detected; N.D.* represents values that were found but were later rejected via QDa-MS. Higher synephrine values in the Chiralpak CBH column are shown in blue^.

Samples analysed	Average amount of synephrine (mg/ serving) ± SEM		Difference in total synephrine amount (mg/serving)	Percentage difference (%)
	XBridge BEH C18	Chiralpak CBH		
C. aurantium fruits (SRM 3258)	5.28 ± 1.07	5.71 ± 0.24	0.43^	8.09
C. aurantium extract (SRM 3259)	38.03 ± 4.09	90.15 ± 5.03	52.13^	137.08
C. aurantium pill (SRM 3260)	9.68 ± 2.05	25.60 ± 0.36	15.92^	164.46
PWS 1	N.D.*	N.D.	N.D.	N.D.
PWS 2	10.81 ± 0.13	1.99	8.82	-81.59
PWS 3	11.82 ± 1.51	4.61	7.21	-60.99
PWS 4	38.88 ± 3.26	13.88	25.00	-64.3
PWS 5	9.77 ± 1.21	20.19	10.42^	106.65
PWS 6	8.14 ± 0.44	22.8	14.66^	180.09
PWS 7	0.03 ± 0.01	0.27	0.24^	800
PWS 8	7.39 ± 0.41	28.79	21.4^	289.58
PWS 9	N.D.	N.D.	N.D.	N.D.
PWS 10	18.69 ± 0.79	2.5	16.19	-86.62
PWS 11	N.D.	39.06	39.06^	100
PWS 12	3.16 ± 3.15	39.31	36.15^	1143.98

There was no clear trend that would contribute to the differences in synephrine values between the two columns. It can be hypothesised that these discrepancies were likely due to the non-homogenous preparation of the dry powdered samples during the manufacturing process. The variability of stimulant distribution may be more significant in supplements that list their ingredients as a part of a proprietary blend (Desbrow et al., 2019). This observation was not unexpected, as Attipoe et al. (2016) showed that the levels of stimulants in nine dietary supplements varied significantly across a period of nine months. There were 30% variations in synephrine (8 -13 mg/day) and octopamine (7 – 16 mg/day) in two supplements that were declared to contain these two amines. However, trace amounts (≤ 0.2 mg) of undeclared synephrine and octopamine were detected in five out of nine samples. The current results highlight a potential problem to consumers of PWS that may ingest variable amounts of stimulants within a product. Hence, the relevant authorities should implement an appropriate screening method to monitor the levels of stimulants in a range of products.

The trace amine ratio method in study 1 was more sensitive with a LOD of 0.14 µg/mL than the chiral analysis (0.58 µg/mL). Despite having a similar column length, the particle size of the XBridge column (2.5 µm) was half the size of the chiral CBH column (5 µm). The smaller particle size increased the linear velocity of the mobile phase and the separation between the amines (Karger et al., 1974). However, particle size is negligible for the separation of enantiomers compared to the influence of column chemistry. Nonetheless, these two methods had LODs comparable to those from more costly UPLC-MS methods (0.01 µg/mL to 0.5 µg/mL) (Avula et al., 2019; Paiga et al., 2017; Pawar et al., 2020).

Only three of the twelve PWS (*PWS3*, *PWS5* and *PWS6*) tested were consistent with having a natural source of synephrine based on the ratio of trace amine and synephrine enantiomer. As expected, based on the ratio of trace amines, the pre-workout supplements (*PWS8*, *PWS11* and *PWS12*) had a racemic distribution for synephrine. However, there were discrepancies between the ratio of trace amine to the percentage of synephrine enantiomers in the two pre-workout supplements that were suggested to be from a natural source (*PWS1* and *PWS4)* and two pre-workout supplements that were suspected to be from a synthetic origin (*PWS7*

and *PWS8)*. The trace amine ratio of *PWS2* and *PWS4* were consistent with those of a natural plant extract but had had synephrine enantiomers that were not suggestive of a natural source. *PWS2* had only the S-enantiomer of synephrine, whereas *PWS4* had a racemic distribution of synephrine. On the other hand, *PWS7* and *PWS8* had only the R-enantiomer, consistent with a natural plant extract. Nonetheless, these results in this pilot study of supplements suggest that the ratio method consistently determined whether the PWS had trace amines from an authentic source.

Seven PWS had a comparable amount of *C. aurantium* on their label (Table 28). Most of these supplements (5/7) had higher levels of synephrine than is expected based on an estimate that the *C. aurantium* was standardised to 10% synephrine. The remaining two supplements had lower levels of synephrine than stated on the label. The labels of the pre-workout supplement do not disclose the origins of plant extracts as it is not a regulatory requirement (U. S. F. a. D. Administration, 2015). The variability of trace amines could affect the overall cardiovascular effects, especially when present in a multi-ingredient blend that contains other stimulants such as caffeine. Therefore, this study's developed methods could be a useful screening tool for the relevant authorities to determine the variability and authenticity of trace amines found in *C. aurantium*-listing PWS.

These studies focused on the three active amines found in *C. aurantium*, synephrine, octopamine and tyramine. *C. aurantium*, like other *Citrus* species, contains different classes of flavonoids (Bocco et al., 1998), and other adrenergic amines (*n*-methyltyramine and hordenine) have different chromatographic patterns that are species-specific (Avula et al., 2005). So far, no single method can detect both adrenergic amines and flavonoids due to polarity and chemical differences. However, no current studies show a comparison of synephrine enantiomers in PWS, let alone the ratio of amines to suggest authentic sources of plant extracts.

In summary, this chapter presents the development of two methods that aid in determining the quantities of trace amines and the authenticity of labelling in *C. aurantium*-listing PWS. The key findings of these two studies were:

- In all three SRM preparations, synephrine was present in 100-fold higher concentrations than octopamine and tyramine. Besides that, *R*-(-)-synephrine was the predominant enantiomer at a percentage ratio of 94:6 (*R*-(-)-synephrine: *S*-(+)-synephrine).
- In study 1, the trace amine ratios of five out of twelve PWS, only (42%) were similar to the natural extracts of *C. aurantium*. However, synephrine was detected in four of the remaining seven, but neither octopamine nor tyramine was detected. The three remaining supplements did not have detectable levels of all three trace amines. Hence, this suggested that a minority of supplements had synephrine from an authentic plant extract source.
- In study 2, five out of twelve (42%) supplements had *R*-(-)-synephrine levels expected from a natural *C. aurantium* extract or fruit. A racemic ratio of synephrine enantiomers was detected in four of the remaining seven supplements. Two samples did not contain synephrine, while one showed detectable levels only for the *S*-(+)-enantiomer. Hence, these seven of the twelve samples had levels of synephrine enantiomers that were not expected from a natural source.

The advantage of these two methods was their compatibility with mass spectrometry, which gives a confirmatory advantage to HPLC methods that used an ion-pairing reagent (Di Lorenzo et al., 2014; Pellati & Benvenuti, 2007; Roman et al., 2007; Schaneberg & Khan, 2004; Tang et al., 2006; Viana et al., 2013). Additionally, the two methods described in this chapter used standardised reference materials developed and certified by the NIST to assess the reliability of the methods for their application to quantify the amines found in PWS. Besides that, the chiral separation method was developed without synthesising synephrine enantiomers or involving extensive derivatization, which could be of value to the industry by decreasing increased development and operational costs.

CHAPTER 4: GENERAL DISCUSSION

This work aimed to investigate the vascular effects of trace amines found in *C. aurantium*-listing PWS and to elucidate differences in their mechanisms of action in mediating their effects in different arteries. Previous research on the vascular effects of these trace amines has been conducted mostly on rat aortae, and this work expanded investigations to include responses of arteries such as the inferior mesenteric and renal arteries. The mechanisms of action of the trace amines, tyramine, octopamine and synephrine were investigated using functional assays on the respective blood vessels in the presence of various antagonists and the absence of endogenous noradrenaline. Furthermore, as PWS are often consumed for exercise and constriction of the coronary arteries can lead to ischemia and cardiovascular damage, this study investigated the effects of the trace amines on porcine left anterior descending coronary arteries.

In addition, a further aim of this work was to develop analytical HPLC methods to quantify the amount of trace amines found in *C. aurantium*-listing PWS to assess their authenticity insofar as they are derived from *C. aurantium*. To achieve this aim, a simple, accurate and rapid HPLC-UV-MS method was developed to determine the ratio of synephrine, octopamine and tyramine in standardised reference materials of *C. aurantium,* which could *then be compared to that found in C. aurantium*-listing PWS. An additional chiral HPLC-UV-MS method was developed to determine the ratio of synephrine enantiomers to assess whether or not the synephrine in PWS samples were likely to have derived from an authentic botanical source.

This discussion focuses on synthesising the findings from the individual studies and determining whether the amount of trace amines quantified in *C. aurantium*-listing PWS could mediate vascular responses.

p-Synephrine is the main adrenergic alkaloid in *C. aurantium* and has been correlated to adverse cardiovascular reports (Bakhiya et al., 2017; Hansen et al., 2013; Pellati & Benvenuti, 2007). In Chapter 2, it was determined that all three trace amines were similarly potent in their vasocontractile effects on the porcine mesenteric, coronary and renal arteries, but their potencies were two orders of magnitude lower than that of noradrenaline. The potencies of synephrine and octopamine on these arteries were similar to those characterised in the rat aortae (Brown et al., 1988; Hibino et al., 2009). However, the magnitude of responses to the trace amines varied depending on the artery. Synephrine and octopamine caused larger contractions than tyramine in the renal and mesenteric arteries, while the maximum responses of the coronary artery to the three trace amines were similar. The inferior mesenteric artery is connected to a large vascular bed, and the renal artery is involved in diuresis, thus having a greater role in mediating blood pressure. On the other hand, although important for the blood flow to the cardiac muscle, the coronary artery has a relatively smaller vascular bed, but alterations in blood flow will influence overall cardiac output.

Tyramine is the most researched of the three trace amines and is a vasoconstrictor on isolated blood vessels. Tyramine dose-dependently induced vasoconstrictions on isolated rat aortae (Broadley et al., 2013; Chan & Chow, 1976; Fehler et al., 2010; Krishnamurty & Grollman, 1972) and coronary arteries of pigs (Herbert et al., 2008). The established mechanism of action of tyramine is via an indirectly acting sympathomimetic action that releases neuronal noradrenaline to interact with post-junctional adrenoceptors on the vascular smooth muscle cells of arteries (Goldstein, 2008). In the current studies, the contractile responses to synephrine and octopamine were attenuated in noradrenaline-depleted mesenteric and renal arteries but not the coronary arteries, suggesting that these amines have an indirect sympathomimetic activity, similar to tyramine in the mesenteric and renal vessels. This observation was not unexpected since Broadley (2010) had identified that trace amines were indeed capable of eliciting both direct and indirect sympathomimetic effects on the vasculature. Synephrine and octopamine share a hydroxyl (OH) group at the β-carbon, similar to ephedrine – a feature that promotes indirect sympathomimetic activity (Ma et al., 2007). Ephedrine has been shown to be a direct and indirect-

acting sympathomimetic (Liles et al., 2006). This correlates well with the results described in Chapter 2, which showed synephrine and octopamine had a mixed indirectly acting sympathomimetic and direct agonist activity on the α_1-adrenoceptor evidenced by the attenuation of contractile response in the presence of prazosin (1 μM).

Perivascular adipose tissue (PVAT) is an ectopic fat that surrounds most large blood vessels such as the aorta or the vasculature of specific organs such as the epicardium or the kidneys (Britton and Fox, 2011). Anatomically, perivascular adipose tissue is contiguous with the adventitial layer of the blood vessel wall and was previously regarded as scaffolding for organ-specific blood vessels. However, seminal studies on the rat aorta performed 20 years ago first demonstrated an increased vasoconstrictor response to tyramine in intact aortas compared to PVAT-free aortas (Soltis, 1991). The contractile effects of tyramine were abolished in the presence of the α-adrenoceptor antagonist, phentolamine, suggesting that the presence of PVAT contributed to the indirect sympathomimetic effects of tyramine on the vasculature (Soltis, 1991). Therefore, PVAT may be an underappreciated source of vasodilatory mediator release stimulated by trace amines such as tyramine.

The composition of perivascular adipose tissue varies by blood vessel type. Resistance vessels contain predominantly white adipose tissue, whereas large vessels are characterised by brown and white adipose tissues (Hildebrand, 2018). However, the properties of PVAT may vary in different vascular beds and different animal species. PVAT has been reported to have anticontractile effects on the aortas of rats (Gao, 2007; Soltis, 1991) and humans (Greenstein, 2009) by stimulating adipokine-mediated nitric oxide release, but this did not occur in the coronary arteries of dogs (Payne et al., 2008). However, not much is known about the effects of PVAT on the vasculature of pigs.

Ayla-Lopez et al. (2014) hypothesised that renal perivascular adipose tissue (RPVAT) of rats acts as an additional reservoir of noradrenaline, evidenced by the presence of tyrosine hydroxylase, aromatic amino acid decarboxylase (AAD) and dopamine β-hydroxylase (DBH) in perivascular adiposities. Furthermore, the

ability of tyramine to elicit dose-dependent vasoconstrictions on denervated rat renal arteries suggests that noradrenaline was not derived from neuronal synapses (Figure 57). Interestingly, uptake of tyramine into the perivascular adipose tissue could result in converting tyramine to octopamine via DBH, further enhancing the contractile effects on the artery.

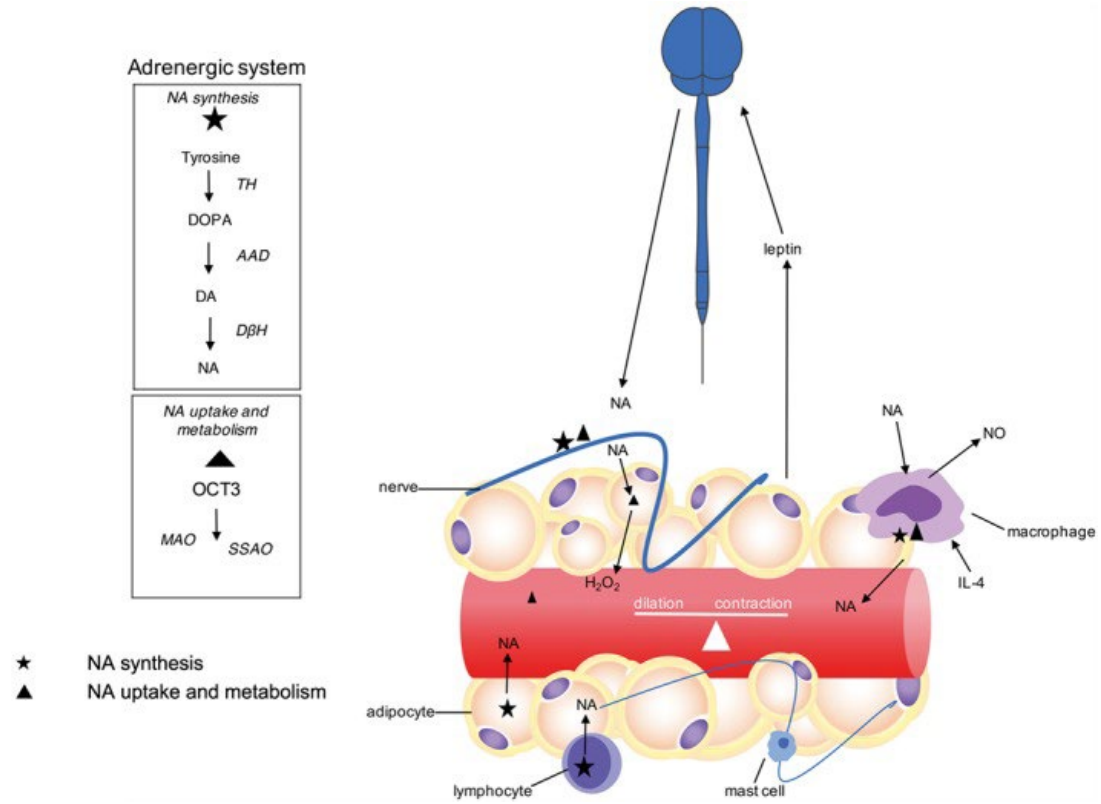


Figure 57 Diagram of the adrenergic system in perivascular adipose tissue (PVAT). Adipocytes, lymphocytes, macrophages, mast cells and nerves comprise the adrenergic system within PVAT. Amino acid decarboxylase (ADD); dopamine β-hydroxylase (DBH); dopamine (DA); 3,4-dihydroxyphenylalanine (DOPA); tyrosine hydroxylase (TH). Original diagram from Ayala-Lopez et al. (2014)

Obesity is associated with inflammation of the adipose tissues (Engin, 2017). The cellular mechanisms underlying these changes are poorly understood but involve alterations in adipokine secretion by cells within adipose tissue and proinflammatory responses, which contribute to endothelial dysfunction (Wilmalasundera et al., 2003), increased oxidative stress (Salgado-Somoza et al., 2010) and vascular stiffness (Tsopifos et al., 2007). These collectively would contribute to hypertension, which may be exacerbated in combination with the consumption of *C. aurantium* PWS.

In this study, the contractile responses to tyramine were completely abolished in noradrenaline depleted mesenteric arteries but not the coronary or the renal arteries. The residual contractile response in noradrenaline-depleted renal arteries to tyramine was not affected by prazosin, similar to the findings by Restini et al. (2018). Additionally, there appeared to be enhanced contractile responses to synephrine and octopamine in noradrenaline-depleted renal arteries treated with the β-adrenoceptor antagonist, propranolol (1 μM). It may be hypothesised that the noradrenaline release from PVAT in response to synephrine or octopamine facilitated additional vascular smooth muscle contractions. However, this may be limited to the differences between species in white adipose tissue, brown adipose tissue, and PVAT distribution.

Trace amine-associated receptor 1 (TAAR1) has been suggested to mediate vasoconstrictions in porcine coronary arteries (Herbert et al., 2008) and rat aortae (Broadley et al., 2013; Fehler et al., 2010). Although the rat aorta contains both the TAAR1 protein and receptor mRNA (Fehler et al., 2010), not much is known about the distribution of TAAR1 in other vascular tissues or different species. From these studies, TAAR1 played a minimal functional role in mediating trace amine contraction as the only known antagonist, EPPTB, failed to alter arterial contractions to all three trace amines. However, at high concentrations of EPPTB (100 μM), there was small but significant potentiation of the renal and coronary artery contractions to synephrine and octopamine. Unlike previous studies, this finding suggests that TAAR activation could lead to vasodilatory effects. However, it is not clear whether the high concentrations of EPPTB would had inhibited a different subtype of the TAAR family, which is yet to be elucidated in porcine vasculature.

Vascular smooth muscle cell contractions can be mediated by non-adrenergic systems such as neuropeptide Y (NPY), a 36-amino acid residue peptide initially isolated from the porcine brain (Tatemoto et al., 1982). NPY occurs in high concentrations in the cardiovascular system, and NPY-immunoreactive sympathetic nerve fibres densely innervate the blood vessels, being more numerous around arteries than around the corresponding veins. In these sympathetic fibres, NPY co-exists with noradrenaline (Chen et al., 1997).

Tyramine has been shown to induce the release of NPY in rabbit ileal segments (Cheng & Shen, 1986). However, tyramine was shown to cause noradrenaline release but not NPY on isolated splenic arteries of pigs (Lundberg et al., 1989). There are no current reports on the possibility for either synephrine or octopamine to show a similar NPY-release mechanism. Thus far, it remains unlikely that the contractile effects observed across the porcine mesenteric, coronary, or renal artery were mediated by the indirect release of NPY.

The stimulatory effects of *C. aurantium*-listing PWS have been commonly associated with synephrine but not octopamine or tyramine. The serving size of powdered PWS varied between brands, but this study had shown a broad range of synephrine amounts found in the twelve supplements (0.03 – 39 mg/serving). So far, the only synephrine has an established daily consumption limit of 30 mg/day in Australia (Rebera, 2020), 25 mg/day in Germany (BfR, 2013) or 40 mg/day in Canada (2013), and most of the supplements investigated in this study were within this daily limit. Although present in *C. aurantium,* there are no such daily maximum limits for octopamine and tyramine in dietary supplements. Nonetheless, octopamine is banned in competitive sport, while synephrine and tyramine are acceptable in competitive sport despite having similar effects on the cardiovascular system (World Anti-doping Agency, 2015). Despite these regulations, PWS consumers in North America have been reported to ingest multiple servings of PWS and occasionally more than once a day, which exceeds the daily limits in multiple countries (Jagim et al., 2019). Furthermore, this does not consider the additive stimulatory effects from the other ingredients such as caffeine, typically found in PWS, often with amounts that do not match their respective labels (Desbrow et al., 2018).

The quantification of these amines alone may not be sufficient to explain the observed effects in humans. The nanomolar plasma concentrations of these amines have been reported (D'Andrea et al.,2017; Haller et al. 2005), but a comprehensive comparison in plasma concentrations of trace amines before and after *C. aurantium* or trace amine-containing foods have not been investigated. Haller et al. (2005) reported that orally ingested synephrine (47 mg) in the form of a *C. aurantium* extract had a peak plasma concentration of 2.85 ng/mL and

caused an increase in heart rate in 10 healthy subjects. However, the change of plasma synephrine from baselines was not reported, and it was not possible to evaluate whether the increase in plasma synephrine mediated the increase in heart rate.

A separate study by Thevis et al. observed the urine concentrations of synephrine and octopamine after oral ingestion of 150 mg of pure synephrine (*Sympatol)* or octopamine (*Norphen retard).* Both drugs had their peak urine concentrations at 5 hours post-ingestion, with the highest concentration for synephrine was 65 µg/mL and octopamine was 140 µg/mL. However, cardiovascular responses such as heart rate or blood pressure were not recorded in this study. Furthermore, this study was limited to observing the urine concentration in one healthy subject. Therefore, clinical investigations into the cardiovascular effects of orally ingested synephrine should consider quantifying the test sample (e.g. supplement or drug), baseline subject sample concentrations (e.g. urine, plasma or serum), and post-ingestion plasma concentrations. This approach would be especially important in establishing a possible correlation between the concentration of *C. aurantium*-containing products and cardiovascular events.

The current labelling laws surrounding botanical ingredients in Australia, especially those in PWS, do not apply to PWS manufactured overseas (TGA). An issue faced by regulators such as the *Therapeutic Goods Administration of Australia* is the large volume and range of PWS products that are not only sold in stores but are also available to consumers directly from online vendors. Hence, there is a need for a rapid, effective analytical method that can be used as a screening tool for authenticity testing, such as those developed in Chapter 3.

In this study (Chapter 3), only three out of twelve *C. aurantium*-listing PWS contained synephrine that were likely derived from authentic botanical sources. The advantage of the two assay methods was their compatibility with mass spectrometry, which gives a confirmatory advantage to HPLC methods that used an ion-pairing reagent (Di Lorenzo et al., 2014; Pellati & Benvenuti, 2007; Roman et al., 2007; Schaneberg & Khan, 2004; Tang et al., 2006; Viana et al., 2013). Additionally, this study used standardised reference materials developed and

certified by the NIST to assess the reliability of the methods for their application to quantify the amines found in PWS. Given the increasing use and ubiquitous nature of these supplements in the community, these methods can provide a fast and convenient screening for authenticity in *C. aurantium*-listing PWS.

LIMITATIONS AND FUTURE DIRECTION

The functional assays used in this study were limited to isolated arterial tissues. As the three arteries selected in this study were important in mediating blood pressure regulation, it cannot be dismissed that the trace amines selected in this study could mediate contractile responses in the venous circulatory system and cardiac tissues. The main functions of the systemic venous system are to act as a conduit to return blood to the heart from the periphery and serve as a reservoir (Funk et al., 2013). Although veins are more compliant to diameter changes than arteries, changes in venous return affect overall cardiac output.

The investigations to elucidate a potential role of TAAR1 were limited to the only known available selective TAAR1 antagonist, EPPTB. Considerable efforts by Roche Pharma have been made to identify TAAR1 antagonists, where over 700,000 compounds were screened based on their capacity to inhibit cAMP production in cells that expressed a chimeric human/rat TAAR1 (Bradaia et al., 2009). However, the affinity of EPPTB was shown to be species-dependent, with good selectivity for mouse TAAR1 (Ki = 0.9 nM), and lower affinity for rat TAAR1 (Ki = 942 nM) and even lower for human TAAR1 (Ki > 5 μM) (Bradaia et al., 2009). Furthermore, EPPTB has been reported to have low bioavailability, limiting its use *in vivo* (Berry et al., 2017). Although the TAAR1 sequence has been identified in the pig genome (Li et al., 2017), its functional distribution in the vascular system of the pig is not known. Furthermore, there is a paucity of data on the functional role of the other TAAR subtypes on the vasculature or whether EPPTB has affinities for these receptors.

Similarly, the α-adrenoceptor distribution in the arteries of pigs is not as well described as rodents or humans (Akinaga et al., 2019; Rudner et al., 1999), yet the porcine vascular system is comparable to humans in anatomy and physiology (Tsang et al., 2016). It has been assumed that porcine tissues share similar

receptor pharmacology to humans based on how pig vasculature has been used as a suitable replacement for cardiovascular surgeries (Patelis et al., 2017). Nonetheless, future studies can be directed to comparing the functional adrenoceptor sequences present in pigs and humans to determine sequence homology to identify any unique differences, particularly around the orthosteric binding site, which may lead to differences in binding affinity toward the trace amines and ultimately different responses.

There is an overall paucity of receptor binding studies for these trace amines with adrenergic receptors, limiting a complete understanding of their pharmacology. As mentioned in Chapter 1, the three trace amines share a similar β-phenylethylamine backbone with substitutions on the ethyl-bridge and amino group, resulting in small but significant differences in their respective mechanisms of action observed in Chapter 2. Molecular-level studies may shed light on the direct impact of these relatively minor structural differences. Future studies on the binding affinity of the trace amines in the binding sites of the post-junctional adrenoceptors may shed further light on this knowledge gap. Computational molecular modelling approaches on adrenergic receptors has been limited by a lack of known X-ray crystal structures since membrane proteins are notoriously difficult to crystalise. Most studies to date were based on the β_2-adrenoceptor model deposited in the Protein Data Bank (https://www.rcsb.org), although in 2019, the structure of an α_2-adrenoceptor (PDB ID: 6KUX) has become available. Relevant to this work, where interactions with the α_1-adrenoceptor subtypes are of interest, a homology model of the α_1-adrenoceptor can be produced using the available 3D α_2-adrenoceptor model for subsequent molecular docking or molecular dynamics binding studies. These studies may reveal important binding site interactions that explain the amines' relative binding affinities and, therefore, aid in elucidating differences in the pharmacological behaviour of the trace amines.

A similar *in silico* approach could be applied to the binding sites of the various TAAR subtypes since in vitro studies are limited by a lack of pharmacological probes to differentiate the subtypes. Whilst no crystal structure for TAAR have been produced, one homology model for TAAR1 has been described based on the 3D template of a related GPCR, in which the binding modes of 42 lead compounds were compared using molecular docking at the orthosteric site (Lam et al., 2015). The objective of that study was to identify unique compounds that had an affinity to TAAR1. The authors found ten analogues of the two most potent agonists and discovered novel ligands that have not yet been described. This approached could be similarly applied to compare the binding modes of the trace amines of interest.

The analytical studies were limited to a small sample size which may not represent the large array of *C. aurantium*-listing PWS available but were comparable to previous analytical methods with similar sample sizes (Putzbach et al., 2007; Roman et al., 2007; Venhuis et al., 2014). Since this was a convenience sample for a pilot study, further studies with a larger sample would support the results identified herein. Additionally, because of the 100-fold difference in concentration of synephrine compared to the other two trace amines, more sensitive methods such as UPLC be better equipped to discern those PWS with very low levels of the amines. This, however, is not such an issue because the risk of cardiovascular events is associated with PWS containing higher rather than lower levels of trace amines. *C. aurantium* contains other biologically active amines such as hordenine or *N*-methyltyramine; therefore, including these amines and their respective enantiomers could provide a more comprehensive fingerprint profile to elucidate the authenticity of *C. aurantium* use in PWS on a broader scale.

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RECOMMENDATIONS FOR CONSUMERS AND REGULATORS OF PRE-WORKOUT SUPPLEMENTS

According to Sports Integrity Australia, PWS is generally not advised to be taken by athletes (Sports Integrity Australia, 2021). A framework was developed by the Australian Institute of Sport (AIS) and the Australasian College of Sport and Exercise Physician (ACSEP) that recommends that the consumer should check for the associated risks of particular supplements/ingredients listed on the TGA's website (https://www.tga.gov.au/current-year-alerts) or for approved Australian-made supplements tested by an independent laboratory, Human and Supplement Testing Australia (HASTA) (https://hasta.org.au/certified/).

The problem with placing the onus on consumers to check ingredient safety is that the labels either do not list specific ingredients, do not list amounts, do not contain the ingredients listed on the labels, or contain adulterants (including banned stimulants) not listed on the labels. This situation can lead to problems and potentially places consumers at risk (Cohen et al., 2017; Cohen et al., 2018; Martínez-Sanz et al., 2017; Pawar et al., 2020; White, 2020). The potent and designer stimulants and their associated derivatives may be the most commonly implicated to provide additional stimulatory and adverse effects beyond caffeine. Due to the reactive rather than proactive nature of dietary supplement regulation in Australia, various novel stimulants are likely to be present given that earlier versions are banned, such as the case of Ephedra or DMAA discussed in Chapter 1. The results from this study show a snapshot of a small subset of PWS that list *C. aurantium* on its label, where only three supplements out of twelve were found to contain authentic sources of synephrine. Therefore, regulators should utilise rapid detection methods to screen incoming PWS into Australia for any potential adulterants. Furthermore, the TGA should adopt more comprehensive labelling requirements, catering to listing stimulants and their amounts as a separate category. For example, a table of the 'total stimulant content', separate from the nutritional facts to avoid unintentional adverse health effects in consumers.

CONCLUDING REMARKS

The aims and research questions presented in this thesis were addressed by investigating the mechanisms of action of the trace amines, synephrine, octopamine and tyramine in *C. aurantium*-listing PWS. Furthermore, two HPLC-UV-MS methods were developed to determine the quantity of these trace amines and evaluate whether their trace amine or synephrine enantiomer ratio was comparable to those found in actual *C. aurantium* extracts.

In summary, all three amines had similar potencies for the pharmacological effects of these trace amines, but the efficacies differed depending on the arterial tissue location. This study has contributed to the current body of knowledge of the specific mechanisms of action of synephrine and octopamine by showing that these two amines mediated contractile responses by a complex mechanism of indirect sympathomimetic and partial direct agonists on the α_1-adrenoceptors on arterial tissues. Furthermore, this study demonstrated that the trace amines mediate vasoconstrictions on the epicardial coronary arteries, whereas endogenous noradrenaline would normally cause vasorelaxation. Moreover, this study postulates a minor role for TAAR1 in mediating contractions to synephrine and octopamine.

In the analytical studies, two HPLC-UV-MS methods were developed, validated, and capable of quantifying the amounts of trace amines in *C. aurantium*-listing PWS and, importantly, able to assess the authenticity of *C. aurantium-containing PWS*. In all three SRM preparations, synephrine was the predominant amine at 100-fold higher concentrations than octopamine and tyramine. In addition, *R*-(-)-synephrine was the predominant enantiomer at an average percentage ratio of 94:6 (*R*-(-)-synephrine: *S*-(+)-synephrine). Overall, only three out of twelve *C. aurantium*-listing PWS had the expected ratios of trace amines and synephrine enantiomers to suggest they were from authentic botanical sources. Seven of the nine remaining supplements had trace amine or synephrine enantiomer ratios that did not suggest that it was from an authentic source. In the remaining supplements, neither trace amines nor synephrine enantiomers were detected.

This thesis's findings highlight a level of unnecessary risk when consuming *C. aurantium*-listing pre-workout supplements, which could be extrapolated to pre-workout supplements in general. Although there might be some evidence of exercise performance, most of the current literature is preliminary. Studies that have evaluated ingredient safety in humans have concluded that short term consumption may be safe, but long-term effects have yet to be investigated. Individuals with a pre-existing health condition may be at risk of exacerbating cardiovascular complications given the vasoconstrictor properties of synephrine and octopamine, which may be more pronounced with other stimulants such as caffeine. Besides that, the analysis of a small number of supplements in this pilot study suggested a great degree of variability between stimulant content in pre-workout supplements. Therefore, further investigations and regulations into stimulatory pre-workout supplements should be conducted to avoid unintentional adverse cardiovascular events.

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APPENDICES

Appendix Table 1: Adverse reports of dietary supplements that include C. aurantium in the Australian Database of Adverse Event Notifications (DAEN) from 2010 to 2021 (Data available on https://www.tga.gov.au/database-adverse-event-notifications-daen).

Case number	Report entry date	Age (yrs)	Gender	Medicines reported as being taken	MedDRA reaction terms
520898	1/3/21	32	F	Thermonuke Pink Ops (Not on ARTG) (Alanine; Caffeine; Taurine; *C. aurantium*; Withania somnifera; Acetyl tyrosine; Carnitine; Aframomum melegueta; Other unspecified ingredient; Theanine; N,N-dimethyltyramine) - Suspected	Blood creatine phosphokinase increased C-reactive protein abnormal Liver function test abnormal Red blood cell sedimentation rate abnormal Rhabdomyolysis
515923	5/1/21	60	F	Fit Affinity Fat Burner - Not on ARTG (Capsicum; Caffeine; Taurine; cocoa powder; *C. aurantium*; Withania somnifera; Riboflavin; Green Tea Extract (Camellia Sinensis); BIOPERINE Black Pepper Extract (Piper nigrum); Theanine; Raspberry Ketone; Coleus Forksohlii Roots Extract) - Suspected	Blood caffeine increased Hypokalaemia Malaise Ventricular tachycardia Vomiting
515861	4/1/21	55	F	Fatblaster Max (Cyanocobalamin; Nicotinamide; pyridoxine hydrochloride; riboflavine; Eleutherococcus senticosus; Fucus vesiculosus; *C. aurantium*; Ilex paraguariensis; Thiamine nitrate; Paullinia cupana; chromic chloride hexahydrate; Garcinia quaesita; Camellia sinensis; Coffea canephora; Plectranthus barbatus; Chromium picolinate; Plantago afra) - Suspected	Asthenia Cardiac discomfort Chest discomfort Feeling hot Hyperhidrosis Syncope
515004	19/12/20	49	F	Transform HCG Weight Loss (Not on ARTG) (Cyanocobalamin; Ornithine; iodine; bromine; Arginine; Ascorbic acid; Fucus vesiculosus; *C. aurantium*; Brassica oleracea; Honey; human chorionic gonadotrophin; Camellia sinensis; asparagus; Carnitine; Bladderwrack) - Suspected	Diarrhoea Dizziness Headache Malaise Menstrual disorder Pyrexia
512294	17/11/20	-	F	Lean Envy - Weight Slimming Kit (Not on ARTG) (Choline bitartrate; Glutamine; Glycine; Myrrh; Linoleic acid; Alanine; Ascorbic acid; Caffeine; Isoleucine; berberine chloride; Theobroma cacao; *C. aurantium*; Valine; Capsicum frutescens; Paullinia cupana; Piper nigrum; Ginger Powder; hydrolysed collagen; Camellia sinensis; Chromium picolinate; Leucine; alpha lipoic acid; Garcinia gummi-gutta; Resveratrol; Carnitine; Other unspecified ingredient) - Suspected	Asthenia Diarrhoea Headache Nausea Product complaint
511046	3/11/20	-	M	BergaMet Pro+ AUST L 253193 and 342506 (Ascorbic acid; *C. aurantium*; Olea europaea; Piper nigrum; Chromium picolinate; alpha lipoic acid; zinc citrate dihydrate) - Suspected	Diarrhoea Drug ineffective Product advertising issue
482570	23/11/19	53	F	Garcinia 8300+ Complex - AUST L 297445 (*C. aurantium*; chromic chloride hexahydrate; Chromium picolinate; Garcinia gummi-gutta;	Anxiety Confusional state Fatigue

				Caralluma adscendens var. fimbriata) - Suspected	Memory impairment
460217	21/3/19	22	M	No Fat Shredded beyond belief - Not on the ARTG (Black Pepper Oil; Caffeine; Linum usitatissimum; Capsicum annuum; *C. aurantium*; Salvia hispanica; hydrolysed milk protein; soy phosphatidylserine-enriched soy lecithin powder; Green Tea Extract (Camellia Sinensis); Other unspecified ingredient) - Suspected Manshake - Not on the ARTG (Guar Gum; Ascorbic acid; Magnesium oxide; folic acid; riboflavine; maize starch; potassium iodide; Calcium phosphate; cocoa powder; Thiamine; Pyridoxine; Vitamin A; Xanthan gum; zinc; Whey protein; Lactobacillus acidophilus; coffee; beetroot; maltodextrin; Sucralose; Bifidobacterium lactis; Niacinamide Free Flowing; Lactobacillus rhamnosus; Bacillus coagulans; Green Tea Extract (Camellia Sinensis); Vitamin E; Psyllium husk; Sweet potato; Vitamin D) - Not suspected Exforge (Valsartan; amlodipine besilate) - Not suspected Panadol Extra (Paracetamol; Caffeine) - Not suspected Tradename not specified (Aspirin) - Not suspected Tradename not specified (Amitriptyline) - Not suspected Tradename not specified (magnesium) - Not suspected	
457097	13/2/19	55	F	Fatblaster Max (Cyanocobalamin; Nicotinamide; pyridoxine hydrochloride; riboflavine; Eleutherococcus senticosus; Fucus vesiculosus; *C. aurantium*; Ilex paraguariensis; Thiamine nitrate; Paullinia cupana; chromic chloride hexahydrate; Garcinia quaesita; Camellia sinensis; Coffea canephora; Plectranthus barbatus; Chromium picolinate; Plantago afra) - Suspected Ventolin (Salbutamol) - Not suspected Desfax (Desvenlafaxine) - Not suspected	Seizure
456209	1/2/19	34	F	Fatblaster Max (Cyanocobalamin; Nicotinamide; pyridoxine hydrochloride; riboflavine; Eleutherococcus senticosus; Fucus vesiculosus; *C. aurantium*; Ilex paraguariensis; Thiamine nitrate; Paullinia cupana; chromic chloride hexahydrate; Garcinia quaesita; Camellia sinensis; Coffea canephora; Plectranthus barbatus; Chromium picolinate; Plantago afra) - Suspected FatBlaster Clinical (Moringa oleifera; Murraya koenigii; Curcuma longa) - Suspected	Diarrhoea Decreased appetite Dizziness Dyspnoea Headache Insomnia Nausea Thirst
455940	30/1/19	53	F	Fatblaster Max (Cyanocobalamin; Nicotinamide; pyridoxine hydrochloride; riboflavine; Eleutherococcus senticosus; Fucus vesiculosus; *C. aurantium*; Ilex paraguariensis; Thiamine nitrate; Paullinia	Pruritus

				cupana; chromic chloride hexahydrate; Garcinia quaesita; Camellia sinensis; Coffea canephora; Plectranthus barbatus; Chromium picolinate; Plantago afra) - Suspected Olmesartan (Olmesartan medoxomil) - Not suspected	Retching
436259	17/6/18	62	F	Tradename not specified (Paeonia lactiflora; Glycyrrhiza uralensis; Ligusticum striatum; Ophiopogon japonicus; Paeonia suffruticosa; *C. aurantium*; Cyperus rotundus; Albizia julibrissin; Prunus persica; Carthamus tinctorius; Schisandra chinensis; Angelica sinensis; Paeonia officinalis; Corydalis yanhusuo) - Suspected	Disorientation Eyelid disorder
428939	9/3/18	78	F	Metabolift Fat Burner AUST L 288805 (Camellia sinensis; Capsicum annuum; Choline bitartrate; Chromium picolinate; *C. aurantium*; Coffea arabica; Fucus vesiculosus; Garcinia quaesita; Gymnema sylvestre; Inositol; pyridoxine hydrochloride) - Suspected Tradename not specified (levothyroxine sodium) - Not suspected	
418375	18/9/17	70	F	Fatblaster Max (Cyanocobalamin; Nicotinamide; pyridoxine hydrochloride; riboflavine; Eleutherococcus senticosus; Fucus vesiculosus; *C. aurantium*; Ilex paraguariensis; Thiamine nitrate; Paullinia cupana; chromic chloride hexahydrate; Garcinia quaesita; Camellia sinensis; Coffea canephora; Plectranthus barbatus; Chromium picolinate; Plantago afra) - Suspected	Mouth ulceration Abdominal pain Dizziness Eructation Erythema Feeling hot Vomiting
408975	15/5/17	46	F	Fatblaster Max (Cyanocobalamin; Nicotinamide; pyridoxine hydrochloride; riboflavine; Eleutherococcus senticosus; Fucus vesiculosus; *C. aurantium*; Ilex paraguariensis; Thiamine nitrate; Paullinia cupana; chromic chloride hexahydrate; Garcinia quaesita; Camellia sinensis; Coffea canephora; Plectranthus barbatus; Chromium picolinate; Plantago afra) - Suspected Thyroxine (levothyroxine sodium) - Not suspected	Chest discomfort Palpitations
389868	10/6/16	-	F	Burn with Svetol (AUST L198697) (Camellia sinensis; *C. aurantium*; Coffea canephora; Garcinia quaesita; Paullinia cupana; Piper nigrum; Plectranthus barbatus; potassium iodide; Tyrosine) - Suspected	Dyspnoea Hyperhidrosis Myalgia
388179	17/5/16	-	F	Fatblaster Max (Cyanocobalamin; Nicotinamide; pyridoxine hydrochloride; riboflavine; Eleutherococcus senticosus; Fucus vesiculosus; *C. aurantium*; Ilex paraguariensis; Thiamine nitrate; Paullinia cupana; chromic chloride hexahydrate; Garcinia quaesita; Camellia sinensis; Coffea canephora; Plectranthus barbatus; Chromium picolinate; Plantago afra) - Suspected	Diarrhoea Vomiting
387734	10/5/16	-	F	Fatblaster Max (Cyanocobalamin; Nicotinamide; pyridoxine hydrochloride; riboflavine; Eleutherococcus senticosus; Fucus vesiculosus; *C. aurantium*; Ilex paraguariensis; Thiamine nitrate; Paullinia cupana; chromic chloride hexahydrate; Garcinia quaesita; Camellia sinensis; Coffea canephora; Plectranthus barbatus; Chromium picolinate; Plantago afra) - Suspected	Pain

387741	10/5/16	-	-	Fatblaster Max (Cyanocobalamin; Nicotinamide; pyridoxine hydrochloride; riboflavine; Eleutherococcus senticosus; Fucus vesiculosus; *C. aurantium*; Ilex paraguariensis; Thiamine nitrate; Paullinia cupana; chromic chloride hexahydrate; Garcinia quaesita; Camellia sinensis; Coffea canephora; Plectranthus barbatus; Chromium picolinate; Plantago afra) - Suspected	Vomiting
387739	10/5/16	-	F	Fatblaster Max (Cyanocobalamin; Nicotinamide; pyridoxine hydrochloride; riboflavine; Eleutherococcus senticosus; Fucus vesiculosus; *C. aurantium*; Ilex paraguariensis; Thiamine nitrate; Paullinia cupana; chromic chloride hexahydrate; Garcinia quaesita; Camellia sinensis; Coffea canephora; Plectranthus barbatus; Chromium picolinate; Plantago afra) - Suspected	Headache Nausea
387740	10/5/16	62	M	Fatblaster Max (Cyanocobalamin; Nicotinamide; pyridoxine hydrochloride; riboflavine; Eleutherococcus senticosus; Fucus vesiculosus; *C. aurantium*; Ilex paraguariensis; Thiamine nitrate; Paullinia cupana; chromic chloride hexahydrate; Garcinia quaesita; Camellia sinensis; Coffea canephora; Plectranthus barbatus; Chromium picolinate; Plantago afra) - Suspected	Abdominal pain Constipation
387735	10/5/16	41	F	Fatblaster Max (Cyanocobalamin; Nicotinamide; pyridoxine hydrochloride; riboflavine; Eleutherococcus senticosus; Fucus vesiculosus; *C. aurantium*; Ilex paraguariensis; Thiamine nitrate; Paullinia cupana; chromic chloride hexahydrate; Garcinia quaesita; Camellia sinensis; Coffea canephora; Plectranthus barbatus; Chromium picolinate; Plantago afra) - Suspected	Abdominal pain upper Flatulence
387732	10/5/16	37	M	Fatblaster Max (Cyanocobalamin; Nicotinamide; pyridoxine hydrochloride; riboflavine; Eleutherococcus senticosus; Fucus vesiculosus; *C. aurantium*; Ilex paraguariensis; Thiamine nitrate; Paullinia cupana; chromic chloride hexahydrate; Garcinia quaesita; Camellia sinensis; Coffea canephora; Plectranthus barbatus; Chromium picolinate; Plantago afra) - Suspected	Insomnia Nausea Palpitations
337191	25/3/14	20	F	Lipo 6 Black (*C. aurantium*; Coffea arabica; Geranium Oil; Nicotinic acid; Piper nigrum; Plectranthus barbatus; Tetradium ruticarpum; Theobroma cacao) - Suspected	Syncope
321472	14/6/13	57	F	Fatblaster Max (Cyanocobalamin; Nicotinamide; pyridoxine hydrochloride; riboflavine; Eleutherococcus senticosus; Fucus vesiculosus; *C. aurantium*; Ilex paraguariensis; Thiamine nitrate; Paullinia cupana; chromic chloride hexahydrate; Garcinia quaesita; Camellia sinensis; Coffea canephora; Plectranthus barbatus; Chromium picolinate; Plantago afra) - Suspected Seroquel (Quetiapine; Quetiapine fumarate) - Not suspected Dexamfetamine Sulphate (dexamfetamine sulfate) - Not suspected Efexor (Venlafaxine hydrochloride) - Not suspected	Flatulence Thinking abnormal
316225	25/3/13	33	M	Lipo 6 Black (*C. aurantium*; Coffea arabica; Geranium Oil; Nicotinic acid; Piper nigrum; Plectranthus barbatus; Tetradium ruticarpum; Theobroma cacao) - Suspected	Chest pain Gastritis
282761	13/5/11	-	F	Tradename not specified (Product not coded) - Suspected	Dermatitis psoriasiform

Neways Ming Vmm (Camellia sinensis; Chromium picolinate; *C. aurantium*; Cyanocobalamin; Panax ginseng; Paullinia cupana; potassium iodide; Psyllium Husk Powder; pyridoxine hydrochloride; Riboflavin; Thiamine nitrate; Zingiber officinale) - Suspected	Eosinophil count increased
Garlic (Allium macrostemon) - Suspected	Rash
	Rash pruritic

Appendix Table 2: 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.