UTS UNIVERSITY OF TECHNOLOGY SYDNEY

A FORENSIC INVESTIGATION INTO THE PRESENCE OF PHOSPHODIESTERASE 5 INHIBITORS AS ADULTERANTS IN HERBAL REMEDIES

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Thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy (Science)

under the supervision of

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September 2020

CERTIFICATE OF ORIGINAL AUTHORSHIP

I, Ahmad Yusri Mohd Yusop declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy (Science), in the Faculty of Science at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

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ABSTRACT

The proliferation of herbal-based male sexual performance products, particularly those adulterated with phosphodiesterase 5 (PDE5) inhibitors, has sparked grave public health and food safety concerns. The advent of their unapproved analogues presents an additional challenge to forensic drug testing laboratories, as these adulterants may evade detection during routine screening. A comprehensive strategy is warranted to address these problems and protect consumers' health and well-being. This study investigated the presence of PDE5 inhibitors as adulterants in herbal remedies, using a two-tier screening strategy of rapid qualitative assay and confirmatory analytical analysis.

A bioactivity-based PDE5 inhibition assay was established using fluorescein-labelled cyclic-3',5'-guanosine monophosphate as substrates to PDE5 enzyme. The PDE5 inhibitions, measured using a fluorescence polarisation technique, was applied to 50 herbal-based food samples. The results were in agreement with the confirmatory analytical analysis for all food products, except for the instant coffee premix samples, postulated due to the presence of caffeine. The assay, nevertheless, exhibited a promising potential to rapidly screen PDE5 inhibitors in various types of food products, except those containing naturally-occurring phosphodiesterase inhibitors.

A confirmatory liquid chromatography-high-resolution mass spectrometry (LC-HRMS) analysis was developed using 23 target analytes; selected to represent different groups of PDE5 inhibitors, based on their structural similarities. The targeted analysis was primarily optimised to mitigate the matrix effect (ME), via chromatographic separation, sample extraction, and sample dilution. The insignificant ME percentages, within -9.2%–8.8% for all target analytes in food and pharmaceutical matrices, were evidenced with satisfactory validation results; notably, the accuracy was within 77.4%–124.7%. The development, optimisation, and validation of the targeted analysis provided a solid foundation for suspected-target and non-targeted screenings. The suspected-target screening employed a library comprising 95 PDE5 inhibitors, providing extended coverage of known analytes. Contrarily, the non-targeted screening adopted top-down and bottom-up approaches to flag novel PDE5 inhibitors analogues based on common fragmentation patterns of target analytes.

The confirmatory LC-HRMS analysis was applied to 50 herbal-based food samples and 52 herbal-based pharmaceutical samples. The targeted analysis and the suspected-target screening identified 11 target analytes and detected five suspected analytes, respectively, from 74 adulterated samples. The non-targeted screening returned insignificant signals, indicating the absence of potentially novel analogues. Some of these samples contained up to five different PDE5 inhibitors and quantified at supratherapeutic level, making them unsafe for consumption. The comprehensive strategies provide a superior approach to curb the widespread adulteration of herbal remedies, thus, safeguarding the public's health.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my principal supervisor, Professor Shanlin Fu. He has patiently guided me throughout my PhD journey and shared his knowledge while entrusting me to take charge of my research. I attribute the completion of this research to his guidance, support, and encouragement. He is my mentor, and I am forever in debt to him.

I also wish to express my sincere gratitude to my co-supervisor, Dr Linda Xiao, for her consistent support and guidance during the running of this research. She continuously encouraged me and was always willing and keen to assist in any way she could throughout the research project.

My sincere appreciation goes to all the academic and professional staff who have supported me throughout my time at UTS. Thanks to Dr Ronald Shimmon for helping me in the laboratories as well as the training on NMR spectroscopy, and to Dr Verena Taudte and Dr Dayanne Bordin for the technical support with LC-MS/MS instruments. I also very much valued the feedback I have received from the forensic science and toxicology research group to improve my research. To Professor Claude Roux and the Centre for Forensic Science, thank you for providing financial support, particularly with the attendance of conferences.

I extend my warmest thanks to my family and friends for their moral support, which made my journey endurable. Finally, I am grateful to the Ministry of Health Malaysia for the Study Leave with Full Pay and Federal Training Reward that helped me to achieve my dream.

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List of abbreviations and symbols

%	percentage
%RSD	percentage of relative standard deviation
-	minus
+	plus
$[M+H]^+$	protonated molecule
<	less than
>	more than
±	plus, or minus
×	times
°C	degree Celsius
μ	average
μg	microgram
μL	microlitre
μm	micrometre
μM	micromolar
^{13}C	carbon-13
^{1}H	hydrogen-1 or proton
AMP	5’-adenosine monophosphate
AOAC	The Association of Official Agricultural Chemists
BPC	base peak chromatogram
brs	broad singlet
Ca^{2+}	calcium
cAMP	3',5'-cyclic adenosine monophosphate

Cap	capsule
$CDCl_3$	deuterated chloroform
CE	collision energy
cGMP	3’,5’-cyclic guanosine monophosphate
CI	chemical ionisation
CID	collision-induced dissociation
COOH	carboxyl
CRM	certified reference material
CWG	chewing gum
CYP3A4	cytochrome P3A4
d	doublet
D&S	dilute and shoot
Da	Dalton
DAD	diode array detector
dd	doublet of doublet
DDA	data-dependent acquisition
DMSO	dimethyl sulfoxide
ED	erectile dysfunction
EFS	electrical field stimulation
EIC	extracted ion chromatogram
Eq.	Equation
ESI	electrospray ionisation
eV	electronvolt
FAM-cGMP	fluorescein-labelled cyclic-3’,5’-guanosine monophosphate
FAM-GMP	fluorescein-labelled 5’-guanosine monophosphate

Fig.	Figure
FP	fluorescence polarisation
FP_{POS}	fluorescence polarisation (positive control)
FP_{SPL}	fluorescence polarisation (sample)
FP_{SUB}	fluorescence polarisation (substrate control)
FT-ICR	Fourier transform-ion cyclotron resonance
FTIR	Fourier transform infrared
g	gravity
g	gram
GC	guanylyl cyclase
GC-MS	gas chromatography-mass spectrometry
GC-QQQ-MS	gas chromatography-triple quadrupole mass spectrometry
GMP	5’-guanosine monophosphate
GTP	guanosine triphosphate
HCD	hard candy
HNY	honey
HPLC	high-performance liquid chromatography
HPTLC	high-performance thin-layer chromatography
HRMS	high-resolution mass spectrometry
Hz	hertz
$I_{\parallel}$	parallel emission light intensities
$I_{\perp}$	perpendicular emission light intensities
IC_{50}	half-maximal inhibitory concentration
ICH	The International Conference on Harmonisation
ICP	instant coffee premix

IR	infrared
IS	internal standard
IT	ion trap
IUPAC	The International Union of Pure and Applied Chemistry
J	coupling constants
JLY	jelly
L	litre
LC	liquid chromatography
LC-DAD	liquid chromatography-diode array detection
LC-HRMS	liquid chromatography-high-resolution mass spectrometry
LC-MS	liquid chromatography-mass spectrometry
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LC-QTOF-MS	liquid chromatography-quadrupole time-of-flight mass spectrometry
LC-UV	liquid chromatography-ultraviolet
LLE	liquid-liquid extraction
LOD	limit of detection
Log P	logarithm of the partition coefficient
Log_{10}	logarithm with base 10
LOQ	limit of quantification
m	multiplet
M	molar mass
m/z	mass-to-charge ratio
MDMA	3,4-methylenedioxymethamphetamine
ME	matrix effect

mg	milligram
MHz	megahertz
min	minute
mL	millilitre
mm	millimetre
mM	millimolar
mP	millipolarisation
MRM	multiple reaction monitoring
MS	mass spectrometry
ms	millisecond
MS/MS	tandem mass spectrometry
MΩ-cm	megaohm-centimetre
NA	not applicable
ND	not detected
ng	nanogram
NH_2	amino
NIR	near-infrared
nM	nanomolar
nm	nanometre
NMR	nuclear magnetic resonance
NO	nitric oxide
P	phosphorylation
PCDL	personal compound database and library
PDA	photodiode array
PDE	phosphodiesterase

PDE5	phosphodiesterase 5
PDM	powdered drink mix
pg	picogram
pH	power of hydrogen
pK_a	negative log of acid dissociation constant
PKG	3’,5’-cyclic guanosine monophosphate-dependent protein kinase
ppm	parts per million
psi	pounds per square inch
psig	pounds per square inch gauge
PTFE	polytetrafluoroethylene
Pty Ltd.	Proprietary Limited
Q	single quadrupole
q	quartet
QC	quality control
QQQ	triple quadrupole
QTOF	quadrupole time-of-flight
QTOF-MS	quadrupole time-of-flight mass spectrometry
QuEChERS	quick, easy, cheap, effective, rugged, and safe
R^2	coefficient of determination (curve)
r^2	coefficient of determination (linear)
RE	extraction recovery
RT	retention time
s	singlet
SD	standard deviation

sec	second
SERS	surface-enhanced Raman spectroscopy
SPE	solid-phase extraction
SPR	supratherapeutic
SUB	subtherapeutic
t	triplet
Tab	tablet
THE	therapeutic
$T_{inhibition}$	threshold value of phosphodiesterase 5 inhibition
TLC	thin-layer chromatography
T_{max}	the amount of time that a drug is present at the maximum concentration in serum
TOF	time-of-flight
TRC	trace
U	unit
USFDA	The United States Food and Drug Administration
UV	ultraviolet
UV-Vis	ultraviolet-visible
V	volt
v/v	volume/volume
WHO	The World Health Organization
Zn^{2+}	zinc
α	alpha
δ	chemical shifts
σ	standard deviation

List of peer-reviewed journal publications

1. Mohd Yusop AY, Xiao L, Fu S. Determination of phosphodiesterase 5 (PDE5) inhibitors in instant coffee premixes using liquid chromatography-high-resolution mass spectrometry (LC-HRMS). *Talanta* 2019;204:36-43. doi:https://doi.org/10.1016/j.talanta.2019.05.078 — Chapter 4
2. Mohd Yusop AY, Xiao L, Fu S. Data on the optimisation and validation of a liquid chromatography-high-resolution mass spectrometry (LC-HRMS) to establish the presence of phosphodiesterase 5 (PDE5) inhibitors in instant coffee premixes. *Data Brief* 2019;25:104234. doi:https://doi.org/10.1016/j.dib.2019.104234 — Chapter 4
3. Mohd Yusop AY, Xiao L, Fu S. Suspected-target and non-targeted screenings of phosphodiesterase 5 inhibitors in herbal remedies by liquid chromatography-quadrupole time-of-flight mass spectrometry. *Drug Test Anal* 2020. doi:https://doi.org/10.1002/dta.2861 — Chapter 6
4. Mohd Yusop AY, Xiao L, Fu S. Fluorescence polarisation for high-throughput screening of adulterated food products via phosphodiesterase 5 (PDE5) inhibition assay. *Drug Test Anal* 2020. doi: https://doi.org/10.1002/dta.2926 — Chapter 7

List of manuscripts submitted to peer-reviewed journal publications

1. Mohd Yusop AY, Xiao L, Fu S. Comparison of sample extraction techniques for the determination of erectile dysfunction drugs as adulterants in selected food products. *Manuscript submitted for publication* 2020.	Chapter 3
2. Mohd Yusop AY, Xiao L, Fu S. Isolation and identification of an isomeric sildenafil analogue as an adulterant in an instant coffee premix. *Manuscript submitted for publication* 2020.	Chapter 5

List of peer-reviewed conference proceedings

(presenting author underlined)

1. Mohd Yusop AY, Xiao L, Fu S. Mitigating matrix effects: an evaluation of sample extraction techniques for forensic investigations of synthetic aphrodisiacs illegally added into consumable products. *The 56th Annual Meeting of The International Association of Forensic Toxicologists.* Ghent, Belgium. 26–30 August 2018.
2. Mohd Yusop AY, Xiao L, Fu S. Strategies to overcome matrix effect for reliable determination of sexual enhancing drugs found as adulterants in premixed coffee. *The Australian and New Zealand Forensic Science Society 24th International Symposium.* Perth, Australia. 9–13 September 2018.
3. Mohd Yusop AY, Xiao L, Fu S. Adulterant detection in herbal dietary supplements marketed to enhance male sexual performance. *The Forensic and Clinical Toxicology Association Inc. 10th Conference.* Adelaide, Australia. 16–19 June 2019.
4. Mohd Yusop AY, Xiao L, Fu S. Application of liquid chromatography-high resolution mass spectrometry (LC-HRMS) to determine male sexual stimulant in selected food matrices. *The 57th Annual Meeting of The International Association of Forensic Toxicologists.* Birmingham, United Kingdom. 2–6 September 2019.

5. Mohd Yusop AY, Xiao L, Fu S. Safeguarding food safety: rapid screening of phosphodiesterase 5 (PDE5) inhibitors as adulterants in selected food matrices using enzyme assay. *The 55th Congress of the European Societies of Toxicology (EUROTOX 2019).* Helsinki, Finland. 8–11 September 2019.

CHAPTER 1

Introduction

1.1 BACKGROUND

Across the globe, people consume a wide range of healthcare products to treat minor ailments, prevent illnesses, and boost their health and well-being [1]. Plant-based products or herbal remedies have conquered a significant share of this market, with annual sales grossing several billion dollars worldwide [2]. The World Health Organization (WHO) estimated that at least 80% of the population in developing countries still depend on herbal remedies to meet their healthcare needs. In recent years, herbal remedies have also gained public acceptance and are widely consumed in developed countries [3].

Herbal remedies, in general, are widely perceived as being healthy and safe compared to modern medicines [4]. These perceptions are often instilled onto consumers through various catchphrases such as all-natural, certified organic, and chemical-free. Moreover, the popularity of herbal remedies is currently thriving through the rapid expansion of online shopping platforms and market globalisation, where manufacturers, retailers, and sellers alike market and advertise their products. Unfortunately, more often than not, the advertisements of these products are found to be misleading and deceptive, with dubious and unproven claims over their efficacy and safety [5].

This lucrative market has also instigated fraudulent manufacturers to deliberately adulterate the herbal remedies, particularly with pharmaceutical drugs, to deliver immediate pharmacological effects as claimed by their labels [6]. Male sexual performance products, purportedly made of herbal aphrodisiacs, such as *Panax ginseng*, *Eurycoma longifolia*, and *Lepidium meyenii*, to name a few, are among the most prevalent [7]. These products are frequently found to be adulterated with approved phosphodiesterase 5 (PDE5) inhibitors, such as sildenafil, vardenafil, and tadalafil [8]. Worse, the adulterated herbal remedies may as well contain analogues of the approved drugs, which are usually undetected, as they are not included in the routine screening procedure applied by forensic drug testing laboratories [9]; and thus, slip past into the market.

An analogue of PDE5 inhibitors is often synthesised by minor modifications to the parent structure of the approved drugs, inevitably altering its physical and chemical properties [10]. Clinical studies have shown that the approved PDE5 inhibitors may produce common side effects such as flushing, headache, dyspepsia, and abnormal vision, among others. Furthermore, they may also cause severe and life-threatening drug-drug interactions in patients on nitrates or α-blockers [11,12]. Therefore, their structurally modified analogues may pose unknown adverse events which could be more hazardous than responses recorded in previous studies [13,14]. They can also be too potent or too toxic for human consumption [15-17].

Conventionally, herbal remedies are marketed in pharmaceutical dosage forms such as tablets and capsules. In recent years, however, the trend has shifted towards food products as they are not as heavily regulated compared to those in pharmaceutical dosage forms [18]. These food products are conveniently available through drugstores, supermarkets, grocery stores, herbal shops, gyms, online shopping platforms, and black markets [19,20]. The definition and classification of herbal remedies, at present, are not standardised internationally, resulting in confusions among consumers and difficulties among drug control authorities across the borders. They may be classified as foods, functional foods, dietary supplements, or medicines depending on the regulations and legislation of each country [3]. Herbal remedies referred to in this study may include those in pharmaceutical dosage forms or food products, labelled to contain at least one herbal ingredient.

The widespread adulteration of herbal remedies has sparked elevated public health and food safety concerns, as consumers are often unaware of the risks associated with the consumption of such products [20]. The continuous emergence of novel PDE5 inhibitors analogues remains a challenge for forensic drug testing laboratories. Furthermore, complex matrices, as herbal remedies are, may hinder the accurate and precise determination of PDE5 inhibitors and their analogues. Currently, there is limited literature addressing the presence of PDE5 inhibitors and their analogues, particularly in herbal-based food products, with scarce information on matrix-specific validation. Therefore, there is an urgent need to address these problems to protect consumers from short- and long-term health issues, which could lead to life-threatening crises.

1.2 AIM AND RESEARCH OBJECTIVES

This study aims to investigate the presence of PDE5 inhibitors and their analogues as adulterants in herbal remedies. To achieve the aim, comprehensive analytical strategies using a bioactivity-based screening assay and a universal liquid chromatography-high-resolution mass spectrometry (LC-HRMS)-based method, were developed, optimised, and validated for an extensive range of known and potentially novel PDE5 inhibitors. A two-tier screening strategy via rapid qualitative assay and confirmatory analytical analysis would be valuable for routine casework to curb the widespread adulteration of herbal remedies, particularly in different types of food and pharmaceutical matrices.

The research specific objectives were to:

1. Develop, optimise, and validate an LC-HRMS-based analytical method that is capable of accurately detecting, identifying, and quantifying PDE5 inhibitors and their analogues in herbal remedies.
2. Screen PDE5 inhibitors and their analogues in herbal remedies using suspected-target and non-targeted strategies via the data-dependent acquisition of an LC-HRMS.
3. Establish a bioactivity-based PDE5 inhibition assay using fluorescence polarisation technique to rapidly screen PDE5 inhibitors and their analogues in selected food products.

1.3 REFERENCES

1. Mohd Yusop AY, Xiao L, Fu S. Suspected-target and non-targeted screenings of phosphodiesterase 5 inhibitors in herbal remedies by liquid chromatography-quadrupole time-of-flight mass spectrometry. *Drug Test Anal* 2020. doi:https://doi.org/10.1002/dta.2861
2. Muschietti L, Redko F, Ulloa J. Adulterants in selected dietary supplements and their detection methods. *Drug Test Anal* 2020. doi:https://doi.org/10.1002/dta.2806
3. Ekor M. The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. *Front Pharmacol* 2014;4:177. doi:https://doi.org/10.3389/fphar.2013.00177
4. Žuntar I, Krivohlavek A, Kosić-Vukšić J, Granato D, Bursać Kovačević D, Putnik P. Pharmacological and toxicological health risk of food (herbal) supplements adulterated with erectile dysfunction medications. *Curr Opin Food Sci* 2018;24:9-15. doi:https://doi.org/10.1016/j.cofs.2018.10.012
5. Ismail SF, Abd Ghani Azmi IM, Daud M, Abd Jalil J, Safuan S. Regulatory control of herbal and traditional medicines in Malaysia: issues and concerns. *Int J Bus Soc* 2020;21(S1):192-204.
6. Liu Y, Lu F. Adulterated pharmaceutical chemicals in botanical dietary supplements: novel screening approaches. *Rev Anal Chem* 2017;36(3):20160032. doi:https://doi.org/10.1515/revac-2016-0032
7. Srivatsav A, Balasubramanian A, Pathak UI, et al. Efficacy and safety of common ingredients in aphrodisiacs used for erectile dysfunction: a review. *Sex Med Rev* 2020. doi:https://doi.org/10.1016/j.sxmr.2020.01.001

8. Kee CL, Ge X, Gilard V, Malet-Martino M, Low MY. A review of synthetic phosphodiesterase type 5 inhibitors (PDE-5i) found as adulterants in dietary supplements. *J Pharm Biomed Anal* 2018;147:250-277. doi:https://doi.org/10.1016/j.jpba.2017.07.031

9. Gerssen A, Bovee THF, van Ginkel LA, van Iersel MLPS, Hoogenboom RLAP. Food and feed safety: cases and approaches to identify the responsible toxins and toxicants. *Food Control* 2019;98:9-18. doi:https://doi.org/10.1016/j.foodcont.2018.10.028

10. Skalicka-Wozniak K, Georgiev MI, Orhan IE. Adulteration of herbal sexual enhancers and slimmers: the wish for better sexual well-being and perfect body can be risky. *Food Chem Toxicol* 2017;108:355-364. doi:https://doi.org/10.1016/j.fct.2016.06.018

11. Ventimiglia E, Capogrosso P, Montorsi F, Salonia A. The safety of phosphodiesterase type 5 inhibitors for erectile dysfunction. *Expert Opin Drug Saf* 2016;15(2):141-152. doi:https://doi.org/10.1517/14740338.2016.1131818

12. Ekar T, Kreft S. Common risks of adulterated and mislabeled herbal preparations. *Food Chem Toxicol* 2019;123:288-297. doi:https://doi.org/10.1016/j.fct.2018.10.043

13. Poon WT, Lam YH, Lai CK, Chan AY, Mak TW. Analogues of erectile dysfunction drugs: an under-recognised threat. *Hong Kong Med J* 2007;13(5):359-363.

14. Bakota EL, Kelly AT, Walterscheid JP, Phatak DR. A case report of fatal desmethyl carbodenafil toxicity. *J Anal Toxicol* 2017;41(3):250-255. doi:https://doi.org/10.1093/jat/bkw128

15. Kee C-L, Ge X, Low M-Y, Koh H-L. Structural elucidation of a new sildenafil analogue using high-resolution Orbitrap mass spectrometry. *Rapid Commun Mass Spectrom* 2013;27(12):1380-1384. doi:https://doi.org/10.1002/rcm.6590

16. Patel DN, Li L, Kee C-L, Ge X, Low M-Y, Koh H-L. Screening of synthetic PDE-5 inhibitors and their analogues as adulterants: analytical techniques and challenges. *J Pharm Biomed Anal* 2014;87:176-190. doi:https://doi.org/10.1016/j.jpba.2013.04.037

17. Sakamoto M, Suzuki J, Saito Y, et al. Structural characterization of dimethyldithiodenafil and dimethylthiocarbodenafil, analogs of sildenafil. 2018;148:136-141. doi:https://doi.org/10.1016/j.jpba.2017.09.025

18. da Justa Neves DB, Caldas ED. Dietary supplements: international legal framework and adulteration profiles, and characteristics of products on the Brazilian clandestine market. *Regul Toxicol Pharmacol* 2015;73(1):93-104. doi:https://doi.org/10.1016/j.yrtph.2015.06.013

19. Gilard V, Balayssac S, Tinaugus A, Martins N, Martino R, Malet-Martino M. Detection, identification and quantification by ^{1}H NMR of adulterants in 150 herbal dietary supplements marketed for improving sexual performance. *J Pharm Biomed Anal* 2015;102:476-493. doi:https://doi.org/10.1016/j.jpba.2014.10.011

20. Rocha T, Amaral JS, Oliveira MBPP. Adulteration of dietary supplements by the illegal addition of synthetic drugs: a review. *Compr Rev Food Sci Food Saf* 2016;15(1):43-62. doi:https://doi.org/10.1111/1541-4337.12173

CHAPTER 2

Literature review

2.1 PHOSPHODIESTERASE ENZYME

In 1971, Earl Sutherland was awarded the Nobel Prize in Physiology or Medicine for his ground-breaking discovery of 3',5'-cyclic adenosine monophosphate (cAMP) [1]. He and his colleague were the first to isolate a compound known as cAMP from liver homogenates in 1958. Their study identified an enzyme capable of inhibiting the effect of cAMP in various tissues. They proposed the enzyme, which was experimentally observed to be activated by magnesium ions and inhibited by caffeine, as a phosphodiesterase (PDE) [2]. A few years later, Ashman and his colleagues identified another similar compound known as 3',5'-cyclic guanosine monophosphate (cGMP) in urine [3]. Both cAMP and cGMP in Fig. 2.1 are presently known as the first intracellular second messenger, an integral component in intracellular signalling [4].

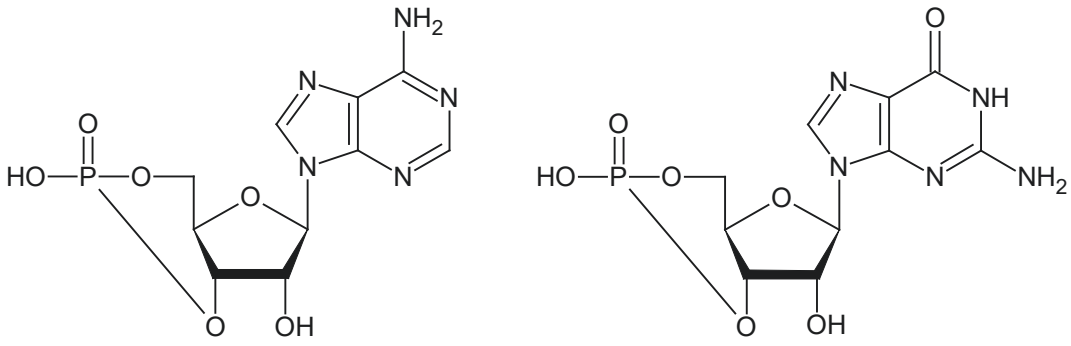


Fig. 2.1: Structure of 3',5'-cyclic adenosine monophosphate (cAMP) and 3',5'-cyclic guanosine monophosphate (cGMP).

In general, the cyclic nucleotide PDEs are a group of enzymes responsible for breaking the phosphodiester bond of either cAMP or cGMP to produce the inactive 5'-adenosine monophosphate (AMP) or 5'-guanosine monophosphate (GMP), respectively [5,6]. PDEs are mainly involved in the regulation of vascular smooth muscle and cell proliferation, facilitation of insulin signalling, transduction of photoresponse signal, activation of the immune system, and regulation of cardiac contractility, to name a few [7]. Initially, the PDEs were classified as those having a selective activity towards cAMP, cGMP, or activated by calcium-calmodulin [8]. PDEs are currently classified into 11 families based on their structural characteristics, substrate specificity, kinetic properties, and sensitivity to endogenous regulators and inhibitors [9]. Table 2.1 summarises the PDE families based on their tissue distributions in human. PDEs have three variants according to their substrate specificity. Class I PDEs (PDE4, PDE7, and PDE8) are cAMP-specific, while class II PDEs (PDE5, PDE6, and PDE9) are cGMP-specific. Finally, class III PDEs (PDE1, PDE2, PDE3, PDE10, and PDE11) hydrolyse both cAMP and cGMP with varying degrees depending on the isoform. To date, 21 different PDE-encoding genes in human have been reported in the literature [10,11].

Table 2.1: Tissue distribution of phosphodiesterase (PDE) enzymes found in human.

Family	Gene numbers	Tissue distribution
PDE1	3	Blood vessels, vascular tissue, heart, lung, testis, platelets, lymphocytes, brain, and smooth muscle.
PDE2	1	Heart, brain, platelets, adrenal glomerulosa cells, endothelial cells, macrophages, lung, and liver.
PDE3	2	Platelets, kidney, vascular smooth muscle, heart, oocyte, adipocytes, hepatocytes, developing sperm, B cells, T-lymphocytes, macrophages, lung, liver, platelets, and adipocytes.
PDE4	4	Brain, smooth muscles, inflammatory cells, immune system, keratinocytes, Sertoli cells, kidney, liver, heart, lung, endothelial cells, and immunocytes.
PDE5	1	Aortic smooth muscle cells, heart, placenta, skeletal muscle, pancreas, brain, liver, lung, platelets, corpus cavernosum, retina, and endothelial cells.
PDE6	3	Lung, retina, and pineal gland.
PDE7	2	Cardiac myocytes, B-lymphocytes, brain, heart, skeletal muscle, pancreas, kidney, and T-lymphocytes.
PDE8	2	Testis, eye, liver, skeletal muscle, heart, kidney, ovary, brain, T-lymphocytes, and thyroid.
PDE9	1	Kidney, brain, heart, liver, and lung.
PDE10	1	Brain, pineal gland, thyroid, and testis.
PDE11	1	Brain, prostate, testis, pituitary gland, liver, skeletal muscle, and heart.

Adapted from [6,12]

Since the discovery of PDEs, they have been the primary choice for potential drug development to treat various diseases and disorders. Among the most notable examples are PDE3 inhibitors for the treatment of heart failure and peripheral vascular disease, and PDE4 inhibitors for the treatment of inflammatory disorders, such as asthma and chronic obstructive pulmonary disease. Perhaps, the most widely published research is the selective inhibition of PDE5 for the treatment of erectile dysfunction (ED) [13].

2.2 PHOSPHODIESTERASE 5 ENZYME

The PDE5 enzyme was discovered in 1976 by Lincoln and his colleagues [14]. It was described as having a highly selective cGMP-binding and cGMP-hydrolysing activity in platelets [15]. PDE5 is expressed predominantly in smooth muscles, primarily located in the heart, pancreas, brain, liver, lungs, and penis [6,12]. At present, only one PDE5 gene, PDE5A, has been identified. Further studies revealed the existence of three spliced PDE5A versions: PDE5A1, PDE5A2, and PDE5A3 [16].

Fig. 2.2 illustrates the structure of PDE5 enzyme, which is a homodimer, where each monomeric unit consists of a regulatory and a catalytic domain [17]. The regulatory domain is located towards the amino-end (NH_2) and consists of two allosteric binding sites, namely the GAF-A and the GAF-B [18]. The allosteric binding of cGMP occurs at GAF-A, prompting the PDE5 phosphorylation, and consequently enhancing the affinity of the catalytic domain to cGMP or inhibitors. PDE5 dimerisation occurs at GAF-B which inhibits the binding of cGMP to GAF-A and sequestrates the phosphorylation site [19]. Located towards the carboxyl-end (COOH) is the catalytic domain, composed of two zinc (Zn^{2+}) binding sites that are instrumental in the catalysis process. The catalytic activity of PDE5 is believed to be sustained by the concentration of Zn^{2+} [18]. Potential compounds targeting to inhibit the PDE5 will be bound exclusively to the catalytic domain [20].

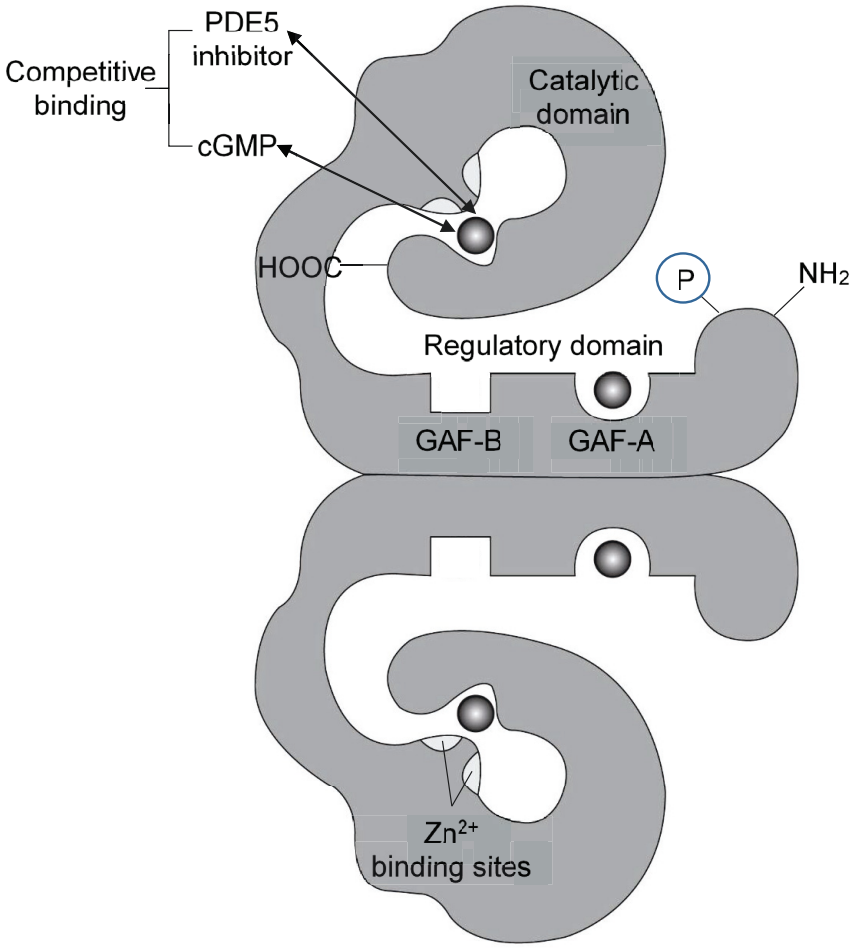


Fig. 2.2: Phosphodiesterase 5 (PDE5) enzyme molecular structure. Adapted from [21,22]. (Abbreviations: cGMP, 3',5'-cyclic guanosine monophosphate; COOH, carboxyl; P, phosphorylation; NH_2, amino; Zn^{2+}, zinc)

PDE5 has long been documented and recognised for its role in the management of smooth muscle contraction through the regulation of cGMP. This role is particularly evident in the lungs and penis. Notably, the inhibition of PDE5 in the corpus cavernosum of the penis is the most commercially successful breakthrough compared to the inhibition of any other PDEs families. The advance is attributed to the highly selective and highly potent compound that acts as a competitive inhibitor to cGMP in the treatment of ED. Since then, the PDE5 inhibitor has been widely studied to treat diseases of various aetiology [7,8].

2.3 PHOSPHODIESTERASE INHIBITORS

For centuries, the Chinese have been using *Panax ginseng* for different medicinal purposes, particularly those involving digestive, heart, and lung diseases [23]. This ancient Chinese herb is one of the examples of naturally occurring PDE inhibitor [24,25]. Then, in 1886, Henry Salter came up with the idea that drinking a strong cup of coffee on an empty stomach could improve breathing, notably in asthmatic patients [26]. At that time, the actual mechanism of action was yet to be known. The bronchodilating effect of coffee was later found to be generated by caffeine [18]. These findings and subsequent findings from many other studies demonstrated the inhibitory effects of caffeine on PDEs. Further investigations suggested that caffeine is a non-selective PDE inhibitor [27].

Since the discovery of PDEs, pharmacologists and medicinal chemists had synthesised and evaluated a few compounds that can mimic the effects of cAMP and cGMP [28-31]. Most of these compounds are non-selective PDE inhibitors with a low affinity towards competitive inhibition of PDEs and inhibit both cAMP and cGMP. Among the earliest and well-established non-selective PDE inhibitors include caffeine [2], theophylline [32], and 3-isobutyl-1-methylxanthine [33]. However, most of these non-selective PDE inhibitors exhibit major disadvantages by having a narrow therapeutic window and undesirable side effects [34]. Therefore, only a limited number of approved non-selective PDE inhibitors is available, as the interest in the PDEs drug discovery has shifted towards selective PDE inhibition [9].

Knowledge of specific PDE distribution at the cellular and subcellular levels in human tissue provides the idea of selective regulation of various cellular functions [4]. The significant role of PDEs in intracellular signalling makes them an ideal preference for new therapeutic agents [7,11]. In-depth evidence in this area of research has produced novel therapeutic agents which are potent and selective towards PDE inhibition. Thus, it is possible to target specific functions and pathological conditions while minimising the occurrences of undesirable side effects [7]. At present, the United States Food and Drug Administration (USFDA) has only approved selective PDE3, PDE4, PDE5, and PDE10 inhibitors as therapeutic agents [9].

2.4 PHOSPHODIESTERASE 5 INHIBITORS

2.4.1 Background

Before the commercial success of sildenafil, a compound previously labelled as M&B29948 was synthesised in 1974 [35] and demonstrated a cGMP-specific PDE inhibition in rat mast cells, bovine coronary artery, and human lung [36]. M&B29948, presently known as zaprinast, was extensively researched and studied to determine various functional features of the PDE5 [27]. Unfortunately, due to lack of safety profile and low therapeutic efficacy, the development of zaprinast as a therapeutic agent had to be discontinued [37]. On the positive side, this discovery led to the initiation of numerous research programmes to develop novel and selective PDE5 inhibitors to treat an array of diseases [27].

One of these research programmes was undertaken by Pfizer research laboratories in Kent, England, which aimed to develop a novel anti-hypertensive agent [27]. Eventually, a derivative of zaprinast labelled as UK-92480 was synthesised and submitted to clinical trials for the indication of angina pectoris [21]. Phase 1 clinical trials for UK-92480, or presently known as sildenafil, was less than a success. It has little to no significant improvement in patients with angina pectoris over the current nitrate therapy [27]. Some side effects were reported such as muscle pain, headaches, indigestion, and flushing. Surprisingly, penile erection was the most notable side effect following an initial dose of sildenafil [21].

During the same period, several published studies revealed the role of nitric oxide (NO) in the mechanism of penile erection. Ignarro and colleagues demonstrated that electrical field stimulation (EFS) induced the relaxation of isolated strips of rabbit corpus cavernosum. The relaxation occurs with the endogenous formation and release of NO, nitrite, and cGMP [38]. Seminal work by Rajfer and colleagues likewise revealed the same findings using human strips of corpus cavernosum. These findings acknowledged the importance of zaprinast in enhancing the EFS-stimulated and NO-stimulated relaxation of human penile tissue [39]. Several other findings in the same field [40,41] suggested that inhibition of PDE5 may be beneficial for the treatment of ED.

2.4.2 Approved PDE5 inhibitors

Based on the commonly reported side-effect of penile erection and several published studies on its possible mechanism, Pfizer quickly shifted their sildenafil research focus from angina pectoris to ED [27]. In late 1993, sildenafil was submitted to its first clinical trial for the treatment of ED [21]. It was a success, where a single dose of sildenafil positively enhanced erectile responses to sexual stimulation and was well tolerated. In pharmacological views, sildenafil has shown to be a successful oral agent by showcasing suitable pharmacokinetic and pharmacodynamic properties [42]. This initial success led to a full-fledged development programme where sildenafil was proven to be effective in just about all types of patients with ED [43-45]. After 21 separate clinical trials with more than 4500 participants, sildenafil was approved by the USFDA for the treatment of ED in March 1998 [21]. Sildenafil, marketed by Pfizer under the trade name Viagra®, is the first commercially available selective PDE5 inhibitor for clinical

use [8]. More than one million patients have been prescribed with Viagra® in the United States alone within a few weeks after its approval [21].

Although Viagra® enjoys a sizeable market share, some disadvantages such as visual disturbances due to weak inhibition of PDE6 as well as the relatively short duration of action have led to the research and development of newer agents in its class [46]. Five years later, in 2003, USFDA approved vardenafil and tadalafil for the treatment of ED. Both of these newer agents exert some slightly different pharmacological properties that may be beneficial in the management of ED [47].

Marketed as Levitra®, vardenafil is the second USFDA-approved PDE5 inhibitor that is superior in its potency and selectivity compared to sildenafil. Vardenafil is approximately ten-fold more potent than sildenafil in its inhibitory activity against PDE5 [48]. Therefore, to achieve an equivalent effect of penile erection, only a small dose of vardenafil is required, which translates into the marketed dosage strength of 5 to 20 mg against sildenafil of 25 to 100 mg [49]. Vardenafil also has the shortest onset of action, approximately 15 minutes post-dose compared to the 30 minutes of sildenafil [50,51].

Tadalafil is famously nicknamed as the weekend pills because it has the most prolonged duration of action compared to the first two PDE5 inhibitors. The USFDA approved it under the trade name Cialis® several months after vardenafil's approval [46]. The duration of action for tadalafil is up to 36 hours after the initial dose [52,53] compared to approximately 12 hours for sildenafil and vardenafil [50,51]. Although the prolonged duration of action might be superior,

especially for increasing sexual impulsiveness, there is also a strong probability for side-effects to occur due to longer exposure in the systemic circulation [54].

Avanafil, marketed under the trade name Stendra®, is the newest addition to the PDE5 inhibitors family, approved by the USFDA in 2012 [55,56]. Avanafil, dubbed as the second-generation PDE5 inhibitor, offers increased selectivity for the PDE5 compared to the first-generation ED drugs. Avanafil has a rapid onset of action within 15 minutes, which makes it a superior choice compared to the first-generation PDE5 inhibitors. Nevertheless, vardenafil and tadalafil may also work as fast as avanafil while delivering a longer duration of action [50,51]. Table 2.4.2 summarises the pharmacokinetic and pharmacodynamic properties of all USFDA-approved PDE5 inhibitors. Outside the United States, three additional PDE5 inhibitors were approved for ED, i.e. udenafil, mirodenafil, and lodenafil carbonate [57].

Table 2.4.2: Pharmacokinetic and pharmacodynamic properties of approved phosphodiesterase 5 (PDE5) inhibitors.

Parameters	Sildenafil (Viagra®)	Vardenafil (Levitra®)	Tadalafil (Cialis®)	Avanafil (Stendra®)
Available doses (mg)	25, 50, 100	5, 10, 20	5, 10, 20	50, 100, 200
Onset of action (min)	30–60	15–30	15–45	15
T_{max} (min)	60	60	120	30–45
Duration of action (hour)	12	12	36	6
Half-life (hour)	4	4–5	17.5	3–5
Metabolism	CYP3A4	CYP3A4	CYP3A4	CYP3A4
Elimination	80% faeces 13% urine	91–95% faeces 2–6% urine	61% faeces 36% urine	62% faeces 21% urine
Mean IC_{50} for PDE5 (nM)	1.6	0.1	4.0	5.2
Other PDE inhibition	PDE6	PDE1, PDE6	PDE11	PDE6

Adapted from [50,51,55]. (Abbreviation: CYP3A4, cytochrome P3A4)

Considering the expressions of PDE5 in other tissues such as brain, heart, and lungs, its selective inhibition may be beneficial for an array of diseases and disorders. To date, apart from being approved for the treatment of ED, sildenafil and tadalafil were subsequently approved by the USFDA for the treatment of pulmonary arterial hypertension, marketed as Revatio® and Adcirca®, respectively [9].

2.4.3 Mechanism of action

Full understanding of the mechanism of penile erection is crucial to explain how PDE5 inhibitors work their wonders. Generally, the smooth muscle lining the penis, known as corpus cavernosum, must be in a relaxed state for an erection [54,58]. Fig. 2.4.3 illustrates the mechanism of action of PDE5 inhibitors for the treatment of ED.

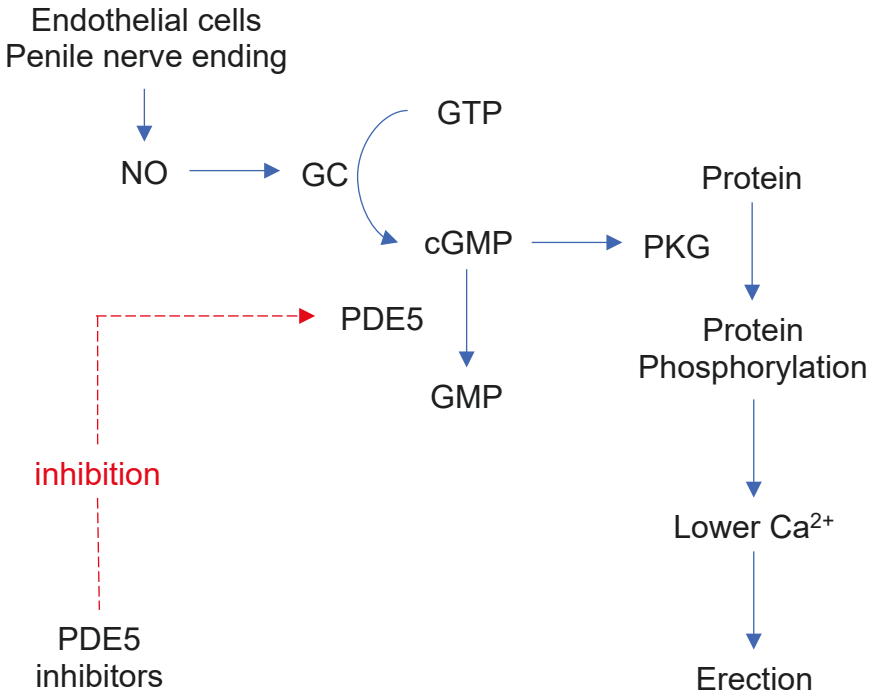


Fig. 2.4.3: Mechanism of action of phosphodiesterase 5 (PDE5) inhibitors for the treatment of erectile dysfunction. Adapted from [22,59]. (Abbreviations: NO, nitric oxide; GC, guanylyl cyclase; GTP, guanosine triphosphate; cGMP, 3',5'-cyclic guanosine monophosphate; GMP, 5'-guanosine monophosphate; PKG, cGMP-dependent protein kinase; Ca^{2+}, calcium)

During sexual stimulation, the endothelial cells and penile nerve ending release NO straight into the penis [60]. NO then diffuses into the cytoplasm of the smooth muscle cells and binds to the intracellular soluble guanylyl cyclase (GC), inducing its conformational change. As a result, guanosine triphosphate (GTP) is

converted into cGMP via catalytic pathway [22,61]. The boost of intracellular cGMP level activates the cGMP-dependent protein kinase (PKG), which in turn causes the reduction of intracellular calcium (Ca^{2+}) levels through the phosphorylation of specific proteins [62]. In normal circumstances, these series of events relax the arterial and trabecular smooth muscle. Consequently, more blood will fill the sinusoidal spaces of the corpus cavernosum and corpus spongiosum as a result of arterial dilatation, and at the same time limiting blood flow out of the penis through venous constriction, which ultimately leads to an erection [63,64].

PDE5 enzyme, which acts through a negative feedback control mechanism in the corpus cavernosum, degrades cGMP to the inactive GMP, resulting in penile detumescence [65,66]. This degradation occurs within the catalytic domain of PDE5 with the help of Zn^{2+} [22]. PDE5 inhibitors, which generally synthesise to mimic the structure of cGMP, competitively bind to the PDE5 enzyme, subsequently decreasing the cGMP degradation, enhancing the effects of NO. This sequence of events maintain the cGMP level, and consequently, prolong an erection [46].

2.4.4 Chemical structure

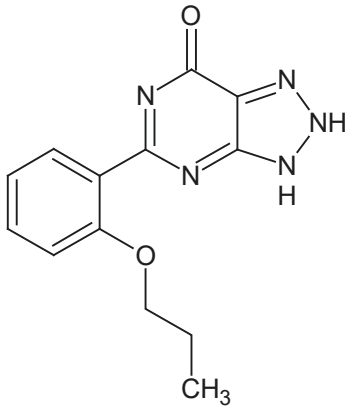
All USFDA-approved PDE5 inhibitors are structurally designed to mimic the purine ring of the cGMP substrate, which act competitively with cGMP to access the catalytic domain of PDE5 [67]. As mentioned earlier, sildenafil was discovered based on the derivatisation of zaprinast. The core template of zaprinast was modified to produce a pyrazolopyrimidine-7-one based compound which is more

potent in inhibiting PDE5 [68]. As seen in Fig. 2.4.4, the pyrazolopyrimidine-7-one core of sildenafil closely resembles the purine structure of cGMP [69].

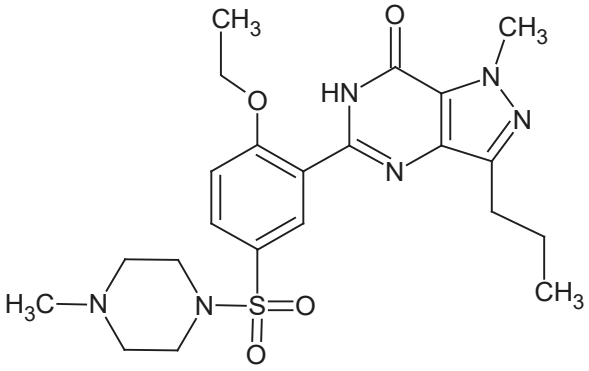
Under different circumstances, vardenafil adopts the imidazotriazine-4-one ring system, which is hypothesised to circumvent the xanthine oxidase metabolism [60]. Vardenafil has a similar molecular structure with sildenafil, as shown in Fig. 2.4.4. However, the rearrangement of one nitrogen atom within the heterocyclic core of vardenafil leads to the enhancement of its potency towards PDE5 [17].

The molecular structure of tadalafil completely differs from that of sildenafil and vardenafil, which gives rise to its distinct binding affinity [17]. Tadalafil was synthesised based on the tetrahydro-β-carboline core structure [70], and the hydantoin ring of sildenafil was modified to produce the diketopiperazine ring [71,72]. To date, tadalafil is the most successful synthetic compound to adopt such core structure [73].

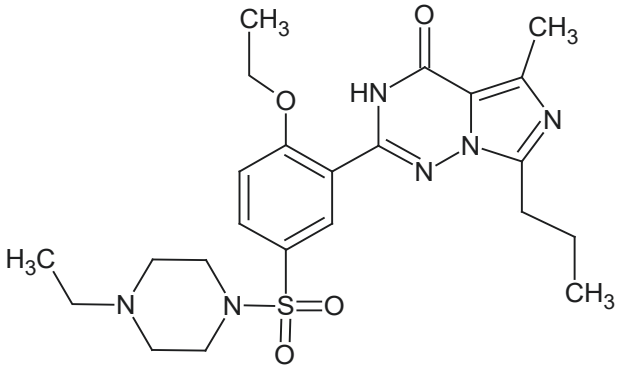
The highly selective PDE5 inhibitor, avanafil, adopts a unique structure derived from diaminopyrimidine [74,75]. Avanafil possesses a distinct structure compared to the average (nucleo) base/sugar/phosphate diester model of all the first-generation PDE5 inhibitors. Theoretically, avanafil can bind to the catalytic domain of PDE5 regardless of the spatial orientation of the molecule. This significant feature plays a focal role in increasing the affinity of avanafil towards PDE5 as well as improving its clinical efficacy [76,77].



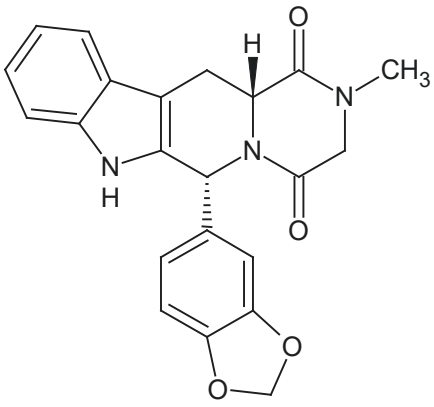
Zaprinast



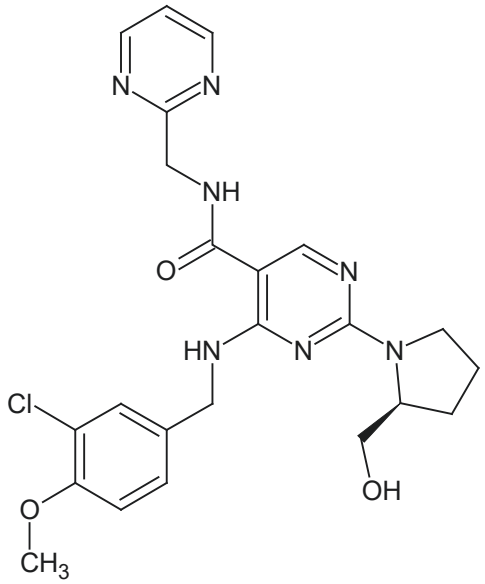
Sildenafil



Vardenafil



Tadalafil



Avanafil

Fig. 2.4.4: Chemical structures of zaprinast and approved phosphodiesterase 5 (PDE5) inhibitors.

2.4.5 Safety profiles

The USFDA-approved PDE5 inhibitors are proven to be successful for the treatment of ED, well-tolerated, and have a low incidence of severe side effects. Nevertheless, the most common side-effects shared among the PDE5 inhibitors include dyspepsia, headache, back pain, nasal congestion, rhinitis, flushing, and myalgia [9,78]. All PDE5 inhibitors vary in terms of their potency and selectivity towards the 11 PDE families, which translated into varying efficacy and safety profiles [79]. Table 2.4.5 summarises the selectivity of all USFDA-approved PDE5 inhibitors expressed as fold differences versus PDE5, whereby the lower the value, the more significant the inhibition towards a given PDE. The inhibition of PDE1, PDE6, and PDE11 families are commonly associated with the clinically relevant side-effects of these inhibitors [78].

Table 2.4.5: Selectivity of approved phosphodiesterase 5 (PDE5) inhibitors (fold differences versus PDE5 enzyme) for clinically relevant phosphodiesterase (PDE) family.

PDE Family	Sildenafil	Vardenafil	Tadalafil	Avanafil
PDE1	375	1012	10500	10192
PDE5	1	1	1	1
PDE6	16	21	550	121
PDE11	4875	5952	25	>19231

Adapted from [78,80]

As highlighted in Table 2.4.5, sildenafil has a weaker selectivity towards PDE1 compared to the other PDE5 inhibitors. Inhibition of PDE1 by sildenafil is of clinical importance as PDE1 is primarily expressed in the myocardial cells, vascular smooth muscle cells, and brain, which may induce tachycardia, flushing, and vasodilation [79,81]. Sildenafil and vardenafil weakly inhibit PDE6, which is primarily expressed in the retina, resulting in incidences of visual disturbance

[82,83]. These visual-related side effects are usually reversible, notably changes in colour perception, such as blue-tinged vision, and changes in brightness perception, such as increased sensitivity to light [84,85]. The inhibition of PDE11, primarily expressed in the skeletal muscle tissues, has often been associated with an increased incidence of myalgia and lower back pain [86]. These muscle-related side-effects are commonly observed with tadalafil as it has weak selectivity towards PDE11 [87]. In contrast, the high selectivity of avanafil towards PDE5 limits the prevalence of its potential side-effects compared to the first-generation drugs in its class [77].

Concurrent administration of all PDE5 inhibitors with nitrates, such as nitroglycerin, isosorbide mononitrate, and glyceryl trinitrate, is strictly contraindicated. These PDE5 inhibitors may intensify the hypotensive effect of the nitrates, which could be life-threatening [78]. Patients may also be at risk of a sudden drop in blood pressure when taking the PDE5 inhibitors concurrently with α-blockers, such as doxazosin and terazosin [51]. All PDE5 inhibitors are primarily metabolised via the cytochrome P3A4 (CYP3A4) pathway; thus, any compound that inhibits or induces the CYP3A4 may interfere with the elimination and systemic exposure of these ED drugs [77,79]. Therefore, the PDE5 inhibitors should be used with caution when they are taken concurrently with established CYP3A4 inhibitors, such as ketoconazole, itraconazole, erythromycin, clarithromycin, ritonavir, saquinavir, and grapefruit juice, as well as established CYP3A4 inducers, such as rifampicin, carbamazepine, and phenytoin [77,88].

Elderly males aged 65 years and older have higher sildenafil and vardenafil plasma concentrations compared to younger males aged 18 to 45 years [89]. Therefore, initiating a lower dose for the elderly populations when prescribing sildenafil or vardenafil is recommended [46]. In contrast, there is no clinically significant effect of age with tadalafil and avanafil; thus, no dose adjustment is required [77,90]. Equally important, the selection of suitable PDE5 inhibitors and dose adjustment is also necessary for patients with renal or hepatic impairment [46,77].

2.5 ADULTERATION OF HERBAL REMEDIES

2.5.1 Background

Over the past decade, the consumption of health products, particularly those of herbal remedies, increased exponentially[91,92]. The World Health Organization (WHO) defines herbal remedies as a finished and labelled product, containing below or above-ground parts of plants or other plant material, or a combination of both, for its main ingredients. It can either be in a raw state or as a plant preparation with added excipients [93]. The organisation also estimated that nearly 80% of the world's population depends on herbal remedies for their primary health care needs [94].

People have used herbal remedies since ancient times to prevent diseases and maintain health, as well as to relieve and cure various ailments. In recent years, herbs have been incorporated into pharmaceuticals, dietary supplements, nutraceuticals, health products, homoeopathic medicines, foods, and cosmetics [95]. Consumers have always perceived herbal remedies as safe, effective, and free from side effects since they originate from natural sources [96]. Additionally, the demand from consumers to achieve specific health and body goals have further escalated the market share of herbal remedies. These goals include the desire for enhanced sexual performance for men and a perfect slim body for women [97].

Unfortunately, the vast market of herbal remedies attracts unscrupulous and greedy manufacturers, who adulterate their products to produce immediate and enhanced effects, which may pose a significant risk to consumers' health and

well-being [96]. Furthermore, aggressive advertising strategies on the Internet, in particular, social networking media, have often presented consumers with misleading testimonials and false claims concerning herbal remedies [98,99]. In general, adulteration of herbal remedies can be defined as the addition of impure, extraneous, improper, or inferior substances, either partly or entirely. This fraudulent practice can either be accidental or intentional [100]. In recent years, several studies have reported intentional adulteration of herbal remedies with approved or unapproved pharmaceutical ingredients. This trend is of serious food safety and public health concerns, as consumers are unaware of the risks associated with the consumption of such products [95]. Among the most prevalent ones are products that claim to enhance male sexual performance [101,102].

2.5.2 Male sexual performance products

Herbal aphrodisiacs are valued since ancient times for their ability to enhance male sexual performance. Notable examples include *Tribulus terrestris*, *Lepidium meyenii*, *Panax ginseng*, and *Eurycoma Longifolia*, among others. Although most of these herbs have shown their potential for sexual enhancement in animal models, the evidence of their efficacy in human remains scarce [103,104].

The commercial success of Viagra® has since led to the massive influx of herbal remedies labelled to contain herbal aphrodisiacs with claims to enhance male sexual performance into the market. These products are often marketed as herbal dietary supplements in pharmaceutical dosage forms and advertised as all-natural, without any side-effects [97]. However, in recent years, this trend has

shifted towards food products as they are not as heavily regulated as those in pharmaceutical dosage forms [95]. These food products can be easily purchased through supermarkets, convenience stores, herbal shops, restaurants, stalls, and various online shopping platforms [95]. Unfortunately, most of these products tend to be adulterated with PDE5 inhibitors and their analogues.

Herbal remedies claiming to enhance male sexual performance are not only used by ED patients but are also used for recreational purposes by males without ED. Furthermore, it is sometimes taken concurrently with alcohol and illicit drugs such as alkyl nitrites (poppers), cocaine, ketamine, methamphetamine, and 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) [95,105]. The consumption of male sexual enhancement products has often been linked to risky sexual behaviours which may increase the incidence of sexually transmitted diseases [105]. Recreational use of herbal remedies adulterated with PDE5 inhibitors, in particular, may cause irreversible damage to the corpus cavernosum as demonstrated in animal models [106].

2.5.3 PDE5 inhibitors and their analogues

The main PDE5 inhibitors found as adulterants in herbal remedies are sildenafil, vardenafil, and tadalafil [101]. However, since 2003, a steady stream of adulteration patterns is observed, after an analogue of sildenafil was detected for the first time in beverages believed to contain herbs and marketed for ED [107]. Numerous PDE5 inhibitors analogues, including those derived from vardenafil and tadalafil, were later found as adulterants [108]. Minor modifications to the parent structure of the approved PDE5 inhibitors often resulted in novel

analogues [102]. Variations or rearrangements of one or more atoms, including addition or elimination of functional groups or sub-structures, often alter the physical and chemical properties of these analogues [109]. Based on these findings, PDE5 inhibitors analogues are often utilised as adulterants to avoid detection from relevant authorities; and thus, escape the consequences of law enforcement [110]. Besides, the incorporation of these analogues into complex matrices, such as food products, often hinders their detection using routine targeted screening procedures [92].

Unapproved PDE5 inhibitors analogues can either maintain the similar pharmacological efficacy of the approved drugs or assume slightly or entirely different pharmacological properties. However, one of the substantial public health concerns revolves around their safety and toxicological profiles, which are often unknown. Theoretically, any changes to the molecular structure will influence the absorption, distribution, metabolism, and excretion of these analogues, which may subsequently result in unknown side effects [97]. For example, a sildenafil analogue, namely propoxyphenyl-thiohydroxyhomosildenafil, is ten-fold more potent in inhibiting PDE5 compared to sildenafil [111]. Therefore, at the same dose, the analogue is more likely to cause severe side effects compared to sildenafil. Another PDE5 inhibitor analogue, i.e. acetildenafil, has been reported causing ataxia, a side effect that was never documented for PDE5 inhibitors before [109].

To date, more than 90 unapproved PDE5 inhibitors analogues have been detected and reported as adulterants in various products [112]. This number is still on the rise as there are endless possibilities of synthesising novel analogues. Analogues of sildenafil are more frequently detected compared to those of vardenafil and tadalafil. One of the factors that contribute to this trend is the cheap and readily available raw materials required to synthesise the sildenafil analogues [98]. Furthermore, the synthesis steps are easily accessible from the Pfizer patent literature that could yield hundreds of active analogues [113].

As mentioned previously, tadalafil has the advantage over other inhibitors owing to its longer duration of action, providing extended time for sexual impulsiveness. Furthermore, the number of steps required to synthesise a tadalafil analogue are relatively short [114]. However, piperonal, which is crucial for tadalafil synthesis as well as its analogues [115], is heavily controlled by the United Nations' International Narcotics Control Board. It is listed in the red list of the precursors and chemicals frequently used in the illicit manufacture of narcotic drugs and psychotropic substances [116]. Therefore, despite the advantages of tadalafil, its analogues are often less detected than those of sildenafil. Vardenafil analogues are the least detected due to their insignificant pharmacological advantages over sildenafil and tadalafil [98,114]. To date, there are no reported adulteration cases with avanafil or its analogues.

In general, PDE5 inhibitors analogues can be categorised based on their structural similarities. The main categories include those derived from the core structures of sildenafil, vardenafil, and tadalafil, while those with novel structures

are categorised into one miscellaneous category. Fig. 2.5.3A shows the chemical structures of sildenafil group of analogues, classified into (a) sulphonamide-bonded, (b) acetyl-bonded, (c) carbonyl/thiocarbonyl-bonded, and (d) other sildenafil-related analogues. Sildenafil analogues are often synthesised with modifications at position X_1 and R_1, where X_1 can either be an oxygen or sulphur atom, while R_1 can either be ethoxyphenyl or propoxyphenyl functional group. Replacement of oxygen atom by sulphur at position X_1 changes the pyrazolopyrimidine-7-one core into a pyrazolopyrimidine-7-thione core, typically observed for the sulphonamide-bonded and carbonyl/thiocarbonyl-bonded analogues.

Similarly, position X_2 can either be occupied by an oxygen or sulphur atom to produce carbonyl or thiocarbonyl analogue, respectively. Variations at position R_1 typically resulted in ethoxyphenyl- and propoxyphenyl-linked compounds only observed for the sulphonamide-bonded and acetyl-bonded analogues. Sildenafil analogues can be further sub-categorised based on atom found at position X_1 or X_2, and the functional group found at position R_1. Position R_2 may vary significantly with many different structures discovered over time.

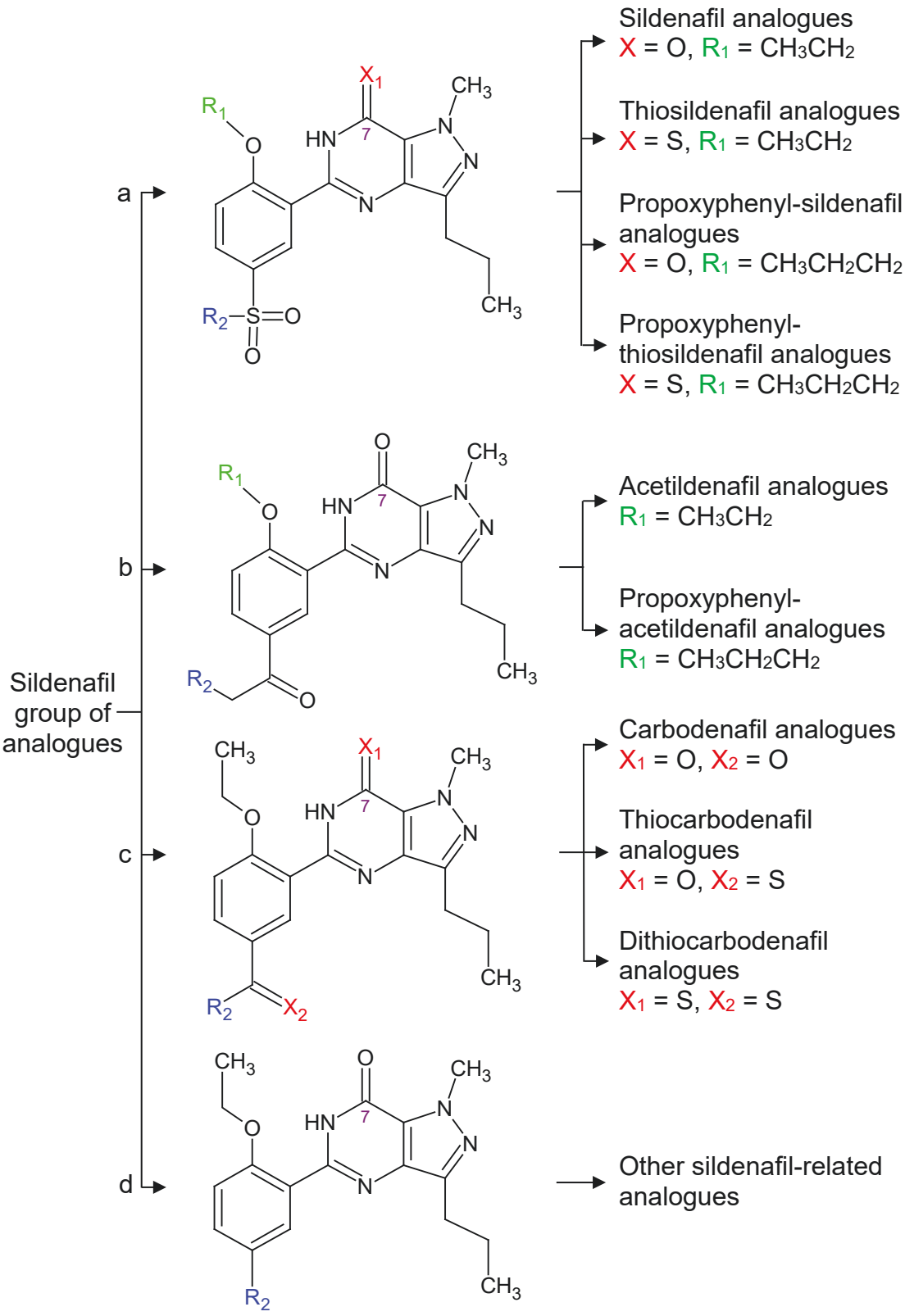


Fig. 2.5.3A: Chemical structures of sildenafil group of analogues with (a) sulphonamide-bonded analogues, (b) acetyl-bonded analogues, (c) carbonyl/thiocarbonyl-bonded analogues, and (d) other sildenafil-related analogues.

Fig. 2.5.3B shows the chemical structures of vardenafil group of analogues, classified into (a) vardenafil analogues and (b) thiovardenafil analogues. Substitution of an oxygen atom with sulphur at position X converts the imidazotriazine-4-one core of vardenafil analogue into an imidazotriazine-4-thione core of thiovardenafil analogue. Vardenafil has a similar structure to that of sildenafil. Therefore, similar modification patterns of analogues can be predicted, with most variations observed at position R.

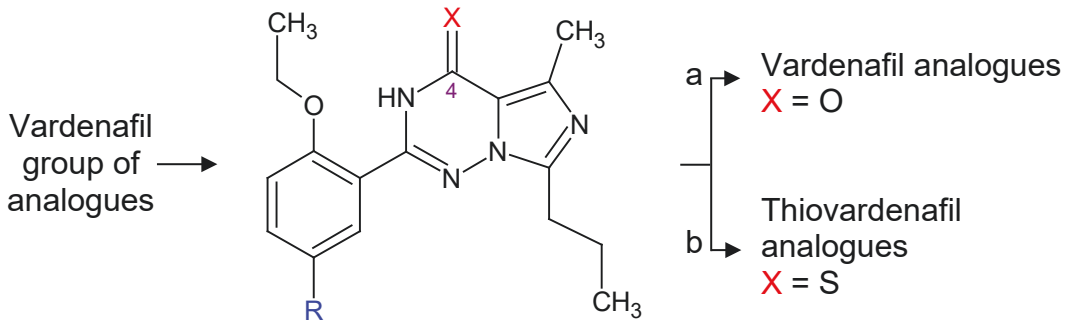


Fig. 2.5.3B: Chemical structures of vardenafil group of analogues with (a) vardenafil analogues and (b) thiovardenafil analogues.

Tadalafil, in general, consists of two chiral centres at positions 6 and 12a of the tetrahydro-β-carboline core [108]. Therefore, theoretically, tadalafil and its analogues may exist in different spatial arrangements resulting in four stereoisomers. However, to date, only two types of stereoisomers have been observed for tadalafil analogues, detected and elucidated as adulterants. Fig. 2.5.3C shows the chemical structures of tadalafil group of analogues, classified into (a) cis-oriented with diketopiperazine ring, (b) cis-oriented without diketopiperazine ring, (c) trans-oriented with diketopiperazine ring, and (d) trans-oriented without diketopiperazine ring. Variations at position R typically resulted in various tadalafil analogues, with or without the diketopiperazine ring.

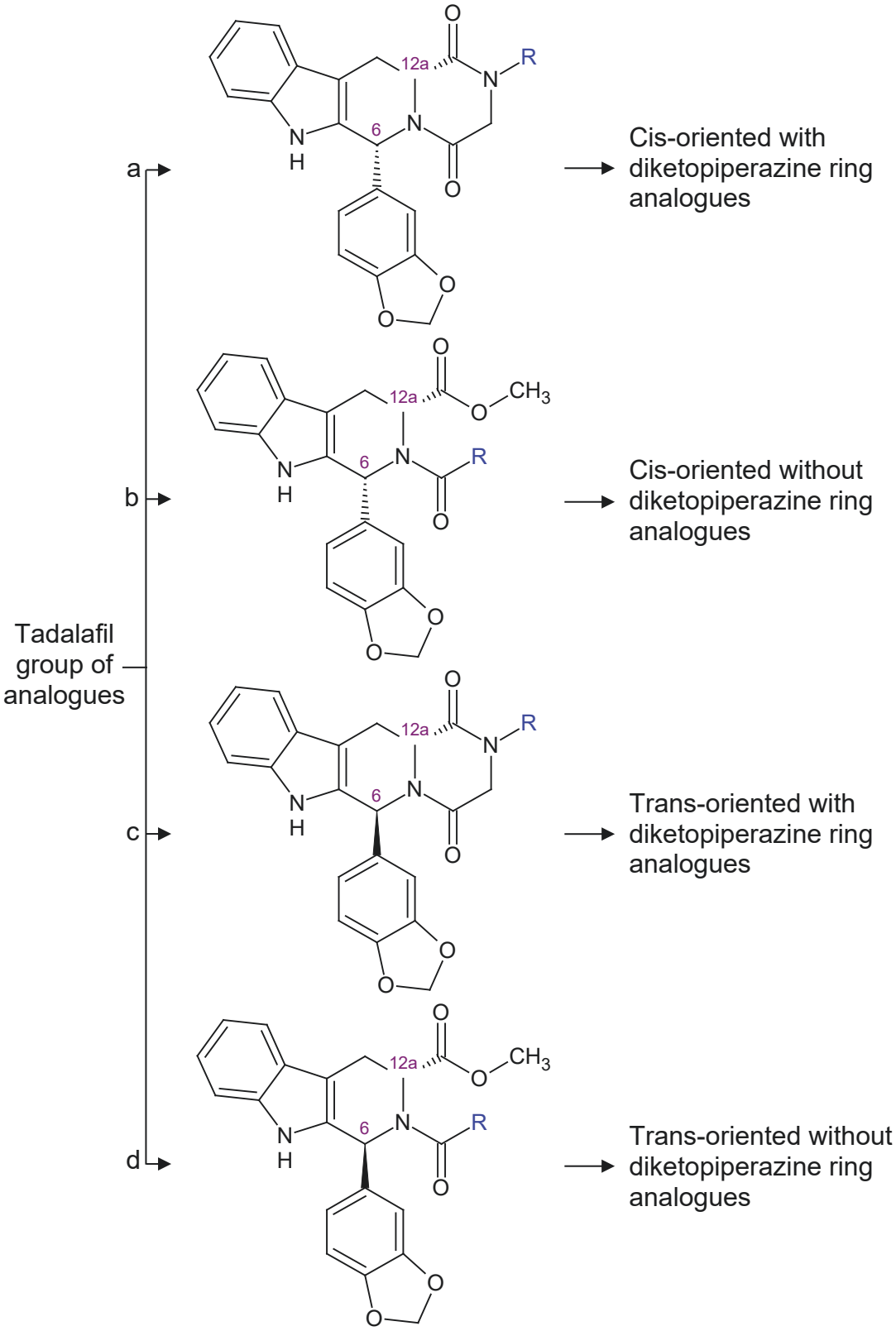


Fig. 2.5.3C: Chemical structures of tadalafil group of analogues with (a) cis-oriented with diketopiperazine ring analogues, (b) cis-oriented without diketopiperazine ring analogues, (c) trans-oriented with diketopiperazine ring analogues, and (d) trans-oriented without diketopiperazine ring analogues.

2.6 ANALYSIS OF PDE5 INHIBITORS

2.6.1 Background and challenges

The detection, identification, and, the eventual quantification of PDE5 inhibitors, particularly their novel analogues, can be demanding and time-consuming. Furthermore, the complexity of herbal remedies, which typically contain multiple ingredients in different types of matrices, presents an additional challenge to forensic drug testing laboratories [98]. Lately, the unscrupulous manufacturers have been finding ways to conceal these adulterants within complex matrices such as food products, which may hinder detection, and thus, circumvent the law.

Various studies have often highlighted the high levels of PDE5 inhibitors adulterated into herbal remedies, particularly those in pharmaceutical dosage forms [117-119]. However, the same findings may not be applicable for food products due to the substantial amount of matrix components relative to the adulterants. The screening procedures should, therefore, be sensitive enough to detect these adulterants and, at the same time, specific enough to confirm their identity. The method should demonstrate that the adulterants are not falsely or wrongly identified; that is, the matrices do not hinder their detection and affect their quantification.

Generally, it is a common practice to discard the capsule shells of herbal remedies during sample preparation procedures, as demonstrated by various studies [117,118,120-127]. Other studies had also discarded the sugar coatings of tablet [128-130] and the softgel shells of softgel capsule [131] before sample homogenisation and analysis. In these studies, only the samples' contents were

analysed. Unexpectedly, in recent years, several cases have revealed the concealment of PDE5 inhibitors within capsule shells [132,133] and softgel shells [134] of herbal remedies. The contents of these samples initially showed inconclusive evidence of adulteration. For instance, there were no traces of adulterants detected from the capsule contents in one case [132], and only after further analyses, two other cases found trace levels of adulterants in the capsule [133] and softgel [134] contents. These recent findings demonstrated novel adulteration strategies, instead of the usual incorporation of adulterants into capsules or softgels content. Critical assessments of specific matrices should, therefore, be carried out before the development of a method for the analysis of PDE5 inhibitors and their analogues. Preferably, each product should be analysed in its entirety, rather than focusing solely on its contents.

The risk assessment due to the consumption of adulterated herbal remedies is only possible if the PDE5 inhibitors and their analogues can be accurately quantified, particularly those at supratherapeutic levels. Sometimes, quantification at trace levels could indicate illegal manufacturing activities, usually hidden behind a legitimate production facility. However, most of the times, this could also be the results of poor manufacturing practices that led to cross-contamination along the production line, which eventually passed to the final products [135]. Furthermore, variations between different batches of the same product are common. Some unscrupulous manufacturers may also adulterate selective batches of the same product, primarily to gain initial consumers' trust. These strategies are usually adopted to circumvent the law and find loopholes to defend their case during criminal prosecutions. Other manufacturers may

deliberately add multiple PDE5 inhibitors in trace amount into their products just enough to produce the claimed therapeutic effects, and, at the same time, evade detection by relevant authorities [112,135].

Consequently, the evidence determined from a comprehensive analytical strategy, produced by a forensic drug testing laboratory, is crucial for the drug control authority and law enforcement agency to curb the widespread adulteration of herbal remedies, particularly those with PDE5 inhibitors and their analogues. Depending on the severity of the reported cases or potential adverse events, this information may also be immediately disseminated to the public, providing a warning and alert on specific adulterated products. The findings may then be incorporated into a classified intelligence database, establishing a foundation for conducting enforcement activities such as surveillance and eventual raids on the individuals or premises that sell, supply, distribute, store, or manufacture the adulterated products. Finally, the analytical data gathered by the forensic drug testing laboratories may be presented during criminal prosecutions to indict any individuals or companies according to the existing laws and regulations. Clear and definite evidence from accurate and precise analytical strategies performed by the forensic drug testing laboratories may bring the perpetrators to justice, and if found guilty, be subjected to penalties, including imprisonment specified by the law.

2.6.2 Sample extraction

The selection of sample extraction technique is crucial in any chemical analysis, as it is the most critical and time-consuming stage. The determination of PDE5 inhibitors and their analogues as adulterants in herbal remedies, particularly those in pharmaceutical dosage forms, is frequently discussed in the literature. In most of these studies, the samples were extracted using dilute and shoot (D&S) technique with either acetonitrile [124] or methanol [136], including their combination with ultrapure water [117,137]. The D&S technique is typically initiated with the homogenisation of a sample, followed by mixing through shaking, vortexing, or sonicating. The next steps usually involved centrifugation, filtration, and dilution. The D&S is indubitably quick and straightforward, where no sample clean-up is required. However, it is also prone to co-extract the matrix components which may interfere with the determination of PDE5 inhibitors. Thus far, only a small number of studies employed other extraction techniques such as solid-phase extraction (SPE) [138,139], liquid-liquid extraction (LLE) [118,140,141], and official quick, easy, cheap, effective, rugged, and safe (QuEChERS) with or without dispersive SPE clean-up [131,142].

2.6.3 Techniques used for the detection and identification of PDE5 inhibitors

Several non-instrumental and instrumental techniques have been proposed to determine PDE5 inhibitors and their analogues in herbal remedies [98,108,143]. However, most of the published studies only focused on herbal remedies in pharmaceutical dosage forms such as capsules and tablets, rather than food products. Only a couple of studies exclusively included food products into their

screening, identification, and quantification of PDE5 inhibitors and their analogues [112,142]. Other studies [144-146] had utilised them as supplementary samples, with little to no information on matrix-specific validation.

Just a few non-instrumental techniques were established for the detection of PDE5 inhibitors and their analogues in adulterated or counterfeit products. For instance, a colour-test was developed to identify the functional groups of sildenafil such as sulphonamide and phenyl ether. Although the colour change of the certified reference material (CRM) seemed promising, the detection of sildenafil in finished products was inconclusive due to interference from the excipients. The test was primarily developed to tackle the problems of counterfeiting, which is widespread with Viagra® tablets [147].

Immunoassay had also been established to screen PDE5 inhibitors and their analogues as adulterants in herbal remedies. Seminal work by Guo et al. [148] had developed an immunoassay using a group-specific monoclonal antibody, based on vardenafil chromophore structure. Since then, other immunoassay-based methods were developed for different PDE5 inhibitors such as sildenafil and its analogues [149] and tadalafil and its analogues [150]. Immunoassay methods are proven to be cost-effective for preliminary screening of PDE5 inhibitors and their analogues compared to other methods which typically require expensive and advanced equipment. However, the results can sometimes be inconclusive depending on the types of PDE5 inhibitors, and thus, necessitated an additional confirmatory analysis [95].

Recently, a study has proposed a broad-based screening of PDE5 inhibitors and their analogues in herbal remedies using PDE5 inhibition assay [151]. The fluorescence intensity of tetramethyl rhodamine-labelled cGMP in the presence of zirconyl chloride octahydrate as a quenching agent was measured to determine the presence of PDE5 inhibitors. The assay, however, was not validated using real samples. Furthermore, it may not be well-suited for high-throughput screening as the fluorescence intensity should be measured at seven points over a length of time to distinguish the adulterated herbal remedies.

Thin-layer chromatography (TLC) has often been utilised to analyse different constituents of herbal remedies before more advanced chromatographic techniques are available. Even today, TLC-based methods are still recommended by various pharmacopoeias to analyse products of plant origin. The technique is not only valuable in the quality control of herbal remedies but also useful to determine potential adulteration and contamination of similar products [152]. Several studies have demonstrated the applicability of TLC to identify PDE5 inhibitors and their analogues as adulterants in various matrices. For instance, Cai et al. [153] proposed the identification of eight PDE5 inhibitors in herbal remedies using a simple TLC method. An enhanced version of TLC, i.e. high-performance TLC (HPTLC), with improved accuracy and reproducibility [154], was reported to identify PDE5 inhibitors and their analogues in herbal remedies and food products [146,155,156]. Although conventional TLC provides qualitative and semi-quantitative data of the analyte of interest, it has poor reproducibility, resolution, and sensitivity [157]. Moreover, the availability of

CRMs is crucial for identification [153], and without them, the method might not be fit to screen novel PDE5 inhibitors analogues.

Vibrational spectroscopic methods, such as infrared (IR), near-infrared (NIR), and Raman spectroscopy, have recently attracted considerable attention as they are simple, rapid, and require minimal or no sample preparation. Furthermore, these techniques have been explored for their portability and high throughput potential, which are beneficial, particularly for on-site field screening [158]. To date, the analysis of PDE5 inhibitors using vibrational spectroscopy methods was successfully explored to detect counterfeit Viagra® and Cialis® tablets [159,160]. Additionally, different techniques of Fourier transform IR (FTIR) spectroscopy employing either attenuated total reflectance [161,162] or potassium bromide disc [163,164] are frequently utilised as a complementary technique to identify novel PDE5 inhibitors analogues. Recently, a surface-enhanced Raman spectroscopy (SERS), in combination with TLC, was proposed to detect PDE5 inhibitors and their analogues as adulterants in herbal remedies [165,166]. These techniques, however, have a limited application due to the lack of selectivity, particularly for the identification of adulterants from complex matrices such as herbal remedies.

Nuclear magnetic resonance (NMR) spectroscopy is a robust, non-destructive, and highly reproducible technique with options for quantitative analysis. It also requires minimal sample preparation [167]. To date, NMR has continued to be the most powerful technique, often employed to elucidate the molecular structure of PDE5 inhibitors, particularly those of novel analogues. Indeed, NMR

unequivocally elucidated the structure of almost all novel PDE5 inhibitors analogues in health and dietary supplements, from the initially discovered homosildenafil [107] up until the latest discovery of N-hydroxyethyl dithiodesethylcarbodenafil [168].

In an innovative approach, Gillard et al. [145] have demonstrated ^{1}H NMR as a first-line method for rapid screening of PDE5 inhibitors and their analogues found as adulterants in herbal remedies. A total of 150 herbal remedies that claimed to enhance male sexual performance were collected and analysed. As a result, 92 samples were found to be adulterated with PDE5 inhibitors and their analogues. Out of these, 33 samples were adulterated with more than one PDE5 inhibitors, and 12 samples exceeded the maximum dose recommended for sildenafil, vardenafil, and tadalafil. The identification and quantification were accomplished through the ^{1}H NMR technique, complemented by direct infusion mass spectrometry (MS) for chemical structures confirmation. Although NMR has proven to be a powerful tool for structural elucidation of novel PDE5 inhibitors analogues, it is less sensitive, and often requires a significant amount of sample compared to other analytical techniques [169].

Gas chromatography, coupled to MS (GC-MS) is often considered as the gold standard for drug analysis. The technique, however, is rarely used to determine PDE5 inhibitors and their analogues, particularly in adulterated products. The application of GC-MS is limited to highly volatile and thermally stable compounds [170]. Unfortunately, PDE5 inhibitors and their analogues are thermally unstable, and their derivatisation using standard silylation reagents has proved to be

challenging [171,172]. Nevertheless, Man et al. [173] have demonstrated the applicability of GC-MS using a short (10 metre) capillary column to identify sildenafil, vardenafil, and tadalafil as adulterants in herbal remedies and food products. The method demonstrated a good chromatographic separation within a short run time, thus, eliminating the complicated derivatisation and hydrolysis steps. Following this, a recent study reported the identification of sildenafil and five of its analogues in herbal remedies using GC-triple quadrupole-MS (GC-QQQ-MS). The MS was operated in multiple reaction monitoring (MRM) using two ionisation techniques. Both electron ionisation and chemical ionisation (CI) techniques showed satisfactory separation, sensitivity, and selectivity. The soft ionisation of the CI technique, however, is superior, producing high selectivity and sensitivity due to minimal matrix interferences [174].

Liquid chromatography (LC), in general, is widely used for qualitative and quantitative analysis of PDE5 inhibitors and their analogues. LC coupled with conventional detectors, such as ultraviolet-visible (UV-Vis) [175], photodiode array (PDA) [141], and fluorescence [176], have been utilised to determine PDE5 inhibitors and their analogues in various matrices. More frequently, LC coupled with MS detection, particularly in tandem mode, has demonstrated to be an indispensable tool in the analysis of PDE5 inhibitors due to its superior specificity, sensitivity, and the ability to separate multiple analytes from complex matrices. Several MS systems have been utilised depending on the extent of the required information and expected specificity level [135]. The use of low-resolution MS, which measures the nominal mass, has frequently been reported in the analysis

of PDE5 inhibitors and their analogues, including single quadrupole (Q) [125], triple quadrupole (QQQ) [57], and ion trap (IT) [141].

However, high-resolution MS (HRMS) has proven to be superior, as it delivers full-spectral information for both MS and tandem MS modes simultaneously, with excellent mass resolution and accuracy, on top of isotopic reliability [177]. These analytical advances have recently gained massive attention as the full-spectral information offers promising potential for suspected-target and non-targeted screenings, in addition to the conventional targeted analysis [178,179]. To date, all available HRMS techniques have been used to analyse PDE5 inhibitors and their analogues in adulterated products, specifically Fourier transform-ion cyclotron resonance (FT-ICR) [138], single-stage Orbitrap [140], and time-of-flight (TOF) [180]. Besides, several hyphenated HRMS techniques, i.e. quadrupole TOF (QTOF) [181] and quadrupole Orbitrap [121], have been successfully utilised with similar aims.

2.7 REFERENCES

1. Blumenthal SA. Earl Sutherland (1915-1974) [corrected] and the discovery of cyclic AMP. *Perspect Biol Med* 2012;55(2):236-249. doi:https://doi.org/10.1353/pbm.2012.0017
2. Sutherland EW, Rall TW. Fractionation and characterization of a cyclic adenine ribonucleotide formed by tissue particles. *J Biol Chem* 1958;232(2):1077-1091.
3. Ashman DF, Lipton R, Melicow MM, Price TD. Isolation of adenosine 3′,5′-monophosphate and guanosine 3′,5′-monophosphate from rat urine. *Biochem Biophys Res Commun* 1963;11(4):330-334. doi:https://doi.org/10.1016/0006-291X(63)90566-7
4. Lugnier C. Cyclic nucleotide phosphodiesterase (PDE) superfamily: a new target for the development of specific therapeutic agents. *Pharmacol Ther* 2006;109(3):366-398. doi:https://doi.org/10.1016/j.pharmthera.2005.07.003
5. Ahmad F, Murata T, Shimizu K, Degerman E, Maurice D, Manganiello V. Cyclic nucleotide phosphodiesterases: important signaling modulators and therapeutic targets. *Oral Dis* 2015;21(1):e25-e50. doi:https://doi.org/10.1111/odi.12275
6. Shafiee-Nick R, Afshari AR, Mousavi SH, et al. A comprehensive review on the potential therapeutic benefits of phosphodiesterase inhibitors on cardiovascular diseases. *Biomed Pharmacother* 2017;94:541-556. doi:https://doi.org/10.1016/j.biopha.2017.07.084

7. Bender AT, Beavo JA. Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. *Pharmacol Rev* 2006;58(3):488-520. doi:https://doi.org/10.1124/pr.58.3.5

8. Kass DA, Champion HC, Beavo JA. Phosphodiesterase type 5: expanding roles in cardiovascular regulation. *Circ Res* 2007;101(11):1084-1095. doi:https://doi.org/10.1161/circresaha.107.162511

9. Baillie GS, Tejeda GS, Kelly MP. Therapeutic targeting of 3′,5′-cyclic nucleotide phosphodiesterases: inhibition and beyond. *Nat Rev Drug Discov* 2019;18(10):770-796. doi:https://doi.org/10.1038/s41573-019-0033-4

10. Bobin P, Belacel-Ouari M, Bedioune I, et al. Cyclic nucleotide phosphodiesterases in heart and vessels: a therapeutic perspective. *Arch Cardiovasc Dis Suppl* 2016;109(6–7):431-443. doi:https://doi.org/10.1016/j.acvd.2016.02.004

11. Azevedo MF, Faucz FR, Bimpaki E, et al. Clinical and molecular genetics of the phosphodiesterases (PDEs). *Endocr Rev* 2014;35(2):195-233. doi:https://doi.org/10.1210/er.2013-1053

12. Keravis T, Lugnier C. Cyclic nucleotide phosphodiesterase (PDE) isozymes as targets of the intracellular signalling network: benefits of PDE inhibitors in various diseases and perspectives for future therapeutic developments. *Br J Pharmacol* 2012;165(5):1288-1305. doi:https://doi.org/10.1111/j.1476-5381.2011.01729.x

13. Manganiello VC, Fontana J, Degerman E, Ahmad F. Cyclic nucleotide phosphodiesterases. In: Shapiro SD, ed. *Encyclopedia of respiratory medicine*. Oxford: Academic Press; 2006:589-594.

14. Lincoln TM, Hall CL, Park CR, Corbin JD. Guanosine 3':5'-cyclic monophosphate binding proteins in rat tissues. *Proc Natl Acad Sci USA* 1976;73(8):2559-2563. doi:https://doi.org/10.1073/pnas.73.8.2559

15. Hamet P, Coquil JF. Cyclic GMP binding and cyclic GMP phosphodiesterase in rat platelets. *J Cyclic Nucleotide Res* 1978;4(4):281-290.

16. Zuo H, Cattani-Cavalieri I, Musheshe N, Nikolaev VO, Schmidt M. Phosphodiesterases as therapeutic targets for respiratory diseases. *Pharmacol Ther* 2019;197:225-242. doi:https://doi.org/10.1016/j.pharmthera.2019.02.002

17. Rotella DP. 2.23 - Phosphodiesterases. In: Triggle DJ, ed. *Comprehensive medicinal chemistry II.* Oxford: Elsevier; 2007:919-957.

18. Puzzo D, Sapienza S, Arancio O, Palmeri A. Role of phosphodiesterase 5 in synaptic plasticity and memory. *Neuropsychiatr Dis Treat* 2008;4(2):371-387. doi:https://doi.org/10.2147/ndt.s2447

19. Mostafa T. Oral phosphodiesterase-5 inhibitors and sperm functions. *Int J Impot Res* 2008;20(6):530-536. doi:https://doi.org/10.1038/ijir.2008.29

20. Carson CC, Lue TF. Phosphodiesterase type 5 inhibitors for erectile dysfunction. *BJU Int* 2005;96(3):257-280. doi:https://doi.org/10.1111/j.1464-410X.2005.05614.x

21. Ghofrani HA, Osterloh IH, Grimminger F. Sildenafil: from angina to erectile dysfunction to pulmonary hypertension and beyond. *Nat Rev Drug Discov* 2006;5(8):689-702. doi:https://doi.org/10.1038/nrd2030

22. Corbin JD. Mechanisms of action of PDE5 inhibition in erectile dysfunction. *Int J Impot Res* 2004;16(S1):S4-S7. doi:https://doi.org/10.1038/sj.ijir.3901205

23. Shergis JL, Di YM, Zhang AL, et al. Therapeutic potential of *Panax ginseng* and ginsenosides in the treatment of chronic obstructive pulmonary disease. *Complement Ther Med* 2014;22(5):944-953. doi:https://doi.org/10.1016/j.ctim.2014.08.006

24. Lü J-M, Yao Q, Chen C. Ginseng compounds: an update on their molecular mechanisms and medical applications. *Curr Vasc Pharmacol* 2009;7(3):293-302. doi:https://doi.org/10.2174/157016109788340767

25. Kang YJ, Sohn JT, Chang KC. Relaxation of canine corporal smooth muscle relaxation by ginsenoside saponin Rg_3 is independent from eNOS activation. *Life Sci* 2005;77(1):74-84. doi:https://doi.org/10.1016/j.lfs.2004.12.016

26. Salter HH. *On asthma: its pathology and treatment.* London: Churchill; 1868.

27. Boswell-Smith V, Spina D, Page CP. Phosphodiesterase inhibitors. *Br J Pharmacol* 2006;147(S1):S252-S257. doi:https://doi.org/10.1038/sj.bjp.0706495

28. Berndt SF, Schulz H-U, Stock K. Influence of papaverine derivatives on phosphodiesterase activity, cyclic 3′,5′-AMP levels and relaxing effect on rabbit ileum. *Naunyn Schmiedebergs Arch Pharmacol* 1976;294(3):271-275. doi:https://doi.org/10.1007/bf00508395

29. Lippmann W. Inhibition of beef heart and rat brain nucleoside-3′:5′-monophosphate phosphodiesterase by 3β-14-dihydroxy-21-oxo-23-desoxo-5-β-card-20(22)-enolide 3-acetate (AY-17,605) and structurally related compounds. *Experientia* 1974;30(3):237-239. doi:https://doi.org/10.1007/bf01934800

30. Meyer RB, Miller JP. Analogs of cyclic AMP and cyclic GMP: general methods of synthesis and the relationship of structure to enzymic activity. *Life Sci* 1974;14(6):1019-1040. doi:https://doi.org/10.1016/0024-3205(74)90228-8

31. Weinryb I, Chasin M, Free CA, et al. Effects of therapeutic agents on cyclic AMP metabolism in vitro. *J Pharm Sci* 1972;61(10):1556-1567. doi:https://doi.org/10.1002/jps.2600611003

32. Butcher RW, Sutherland EW. Adenosine 3',5'-phosphate in biological materials. I. Purification and properties of cyclic 3',5'-nucleotide phosphodiesterase and use of this enzyme to characterize adenosine 3',5'-phosphate in human urine. *J Biol Chem* 1962;237:1244-1250.

33. Chasin M, Harris DN. Inhibitory and activators of cyclic nucleotide phosphodiesterase. *Adv Cyclic Nucleotide Res* 1976;7:225-264.

34. Maurice DH, Ke H, Ahmad F, Wang Y, Chung J, Manganiello VC. Advances in targeting cyclic nucleotide phosphodiesterases. *Nat Rev Drug Discov* 2014;13(4):290-314. doi:https://doi.org/10.1038/nrd4228

35. Broughton BJ, Chaplen P, Knowles P, et al. New inhibitor of reagin-mediated anaphylaxis. *Nature* 1974;251(5476):650-652. doi:https://doi.org/10.1038/251650a0

36. Gibson A. Phosphodiesterase 5 inhibitors and nitrergic transmission-from zaprinast to sildenafil. *Eur J Pharmacol* 2001;411(1–2):1-10. doi:https://doi.org/10.1016/S0014-2999(00)00824-4

37. Schudt C, Hatzelmann A, Beume R, Tenor H. Phosphodiesterase inhibitors: history of pharmacology. In: Francis S, Conti M, Houslay M, eds. *Phosphodiesterases as drug targets. Handbook of experimental pharmacology.* Berlin: Springer; 2011:1-46.

38. Ignarro LJ, Bush PA, Buga GM, Wood KS, Fukuto JM, Rajfer J. Nitric oxide and cyclic GMP formation upon electrical field stimulation cause relaxation of corpus cavernosum smooth muscle. *Biochem Biophys Res Commun* 1990;170(2):843-850. doi:https://doi.org/10.1016/0006-291X(90)92168-Y

39. Rajfer J, Aronson WJ, Bush PA, Dorey FJ, Ignarro LJ. Nitric oxide as a mediator of relaxation of the corpus cavernosum in response to nonadrenergic, noncholinergic neurotransmission. *N Engl J Med* 1992;326(2):90-94. doi:https://doi.org/10.1056/nejm199201093260203

40. Bush PA, Aronson WJ, Buga GM, Rajfer J, Ignarro LJ. Nitric oxide is a potent relaxant of human and rabbit corpus cavernosum. *J Urol* 1992;147(6):1650-1655. doi:https://doi.org/10.1016/S0022-5347(17)37671-1

41. Azadzoi KM, Kim N, Brown ML, Goldstein I, Cohen RA, Saenz de Tejada I. Endothelium-derived nitric oxide and cyclooxygenase products modulate corpus cavernosum smooth muscle tone. *J Urol* 1992;147(1):220-225. doi:https://doi.org/10.1016/S0022-5347(17)37201-4

42. Boolell M, Allen MJ, Ballard SA, et al. Sildenafil: an orally active type 5 cyclic GMP-specific phosphodiesterase inhibitor for the treatment of penile erectile dysfunction. *Int J Impot Res* 1996;8(2):47-52.

43. Dinsmore WW, Hodges M, Hargreaves C, Osterloh IH, Smith MD, Rosen RC. Sildenafil citrate (Viagra) in erectile dysfunction: near normalization in men with broad-spectrum erectile dysfunction compared with age-matched healthy control subjects. *Urology* 1999;53(4):800-805. doi:https://doi.org/10.1016/S0090-4295(98)00586-X

44. Montorsi F, McDermott TE, Morgan R, et al. Efficacy and safety of fixed-dose oral sildenafil in the treatment of erectile dysfunction of various etiologies. *Urology* 1999;53(5):1011-1018. doi:https://doi.org/10.1016/S0090-4295(98)00643-8

45. Goldstein I, Lue TF, Padma-Nathan H, Rosen RC, Steers WD, Wicker PA. Oral sildenafil in the treatment of erectile dysfunction. Sildenafil Study Group. *N Engl J Med* 1998;338(20):1397-1404. doi:https://doi.org/10.1056/nejm199805143382001

46. Huang SA, Lie JD. Phosphodiesterase-5 (PDE_5) inhibitors in the management of erectile dysfunction. *Pharm Ther* 2013;38(7):407-419.

47. Ferguson III JE, Carson III CC. Phosphodiesterase type 5 inhibitors as a treatment for erectile dysfunction: current information and new horizons. *Arab J Urol* 2013;11(3):222-229. doi:https://doi.org/10.1016/j.aju.2013.07.009

48. Saenz de Tejada I, Angulo J, Cuevas P, et al. The phosphodiesterase inhibitory selectivity and the in vitro and in vivo potency of the new PDE5 inhibitor vardenafil. *Int J Impot Res* 2001;13(5):282-290. doi:https://doi.org/10.1038/sj.ijir.3900726

49. Corbin JD, Beasley A, Blount MA, Francis SH. Vardenafil: structural basis for higher potency over sildenafil in inhibiting cGMP-specific phosphodiesterase-5 (PDE5). *Neurochem Int* 2004;45(6):859-863. doi:https://doi.org/10.1016/j.neuint.2004.03.016

50. Katz EG, Tan RB, Rittenberg D, Hellstrom WJ. Avanafil for erectile dysfunction in elderly and younger adults: differential pharmacology and clinical utility. *Ther Clin Risk Manag* 2014;10:701-711. doi:https://doi.org/10.2147/tcrm.s57610

51. Zurawin JL, Stewart CA, Anaissie JE, Yafi FA, Hellstrom WJ. Avanafil for the treatment of erectile dysfunction. *Expert Rev Clin Pharmacol* 2016;9(9):1163-1170. doi:https://doi.org/10.1080/17512433.2016.1212655

52. Young JM, Feldman RA, Auerbach SM, et al. Tadalafil improved erectile function at twenty-four and thirty-six hours after dosing in men with erectile dysfunction: US trial. *J Androl* 2005;26(3):310-318. doi:https://doi.org/10.2164/jandrol.04126

53. Porst H, Padma-Nathan H, Giuliano F, Anglin G, Varanese L, Rosen R. Efficacy of tadalafil for the treatment of erectile dysfunction at 24 and 36 hours after dosing: a randomized controlled trial. 2003;62(1):121-125. doi:https://doi.org/10.1016/S0090-4295(03)00359-5

54. Campbell HE. Clinical monograph for drug formulary review: erectile dysfunction agents. *J Manag Care Spec Pharm* 2005;11(2):151-171. doi:https://doi.org/10.18553/jmcp.2005.11.2.151

55. Bruzziches R, Francomano D, Gareri P, Lenzi A, Aversa A. An update on pharmacological treatment of erectile dysfunction with phosphodiesterase type 5 inhibitors. *Expert Opin Pharmacother* 2013;14(10):1333-1344. doi:https://doi.org/10.1517/14656566.2013.799665

56. Traynor K. FDA approves new ED remedy. *Am J Health Syst Pharm* 2012;69(11):906. doi:https://doi.org/10.2146/news120037

57. Lee JH, Park HN, Park OR, Kim NS, Park S-K, Kang H. Screening of illegal sexual enhancement supplements and counterfeit drugs sold in the online and offline markets between 2014 and 2017. *Forensic Sci Int* 2019;298:10-19. doi:https://doi.org/10.1016/j.forsciint.2019.02.014

58. Rashid A. The efficacy and safety of PDE5 inhibitors. *Clin Cornerstone* 2005;7(1):47-55. doi:https://doi.org/10.1016/S1098-3597(05)80048-1

59. Rosen RC, Kostis JB. Overview of phosphodiesterase 5 inhibition in erectile dysfunction. *Am J Cardiol* 2003;92(9):9-18. doi:https://doi.org/10.1016/S0002-9149(03)00824-5

60. Rotella DP. Phosphodiesterase 5 inhibitors: current status and potential applications. *Nat Rev Drug Discov* 2002;1(9):674-682. doi:https://doi.org/10.1038/nrd893

61. Burnett AL. Phosphodiesterase 5 mechanisms and therapeutic applications. *Am J Cardiol* 2005;96(12, Supplement 2):29-31. doi:https://doi.org/10.1016/j.amjcard.2005.07.008

62. Corbin JD, Francis SH, Webb DJ. Phosphodiesterase type 5 as a pharmacologic target in erectile dysfunction. *Urology* 2002;60(2):4-11. doi:https://doi.org/10.1016/S0090-4295(02)01686-2

63. Andersson KE, Wagner G. Physiology of penile erection. *Physiol Rev* 1995;75(1):191-236. doi:https://doi.org/10.1152/physrev.1995.75.1.191

64. Dean J, de Boer B-J, Graziottin A, Hatzichristou D, Heaton J, Tailor A. The role of erection hardness in determining erectile dysfunction (ED) treatment outcome. *Eur Urol Suppl* 2006;5(13):767-772. doi:https://doi.org/10.1016/j.eursup.2006.06.004

65. Peak TC, Yafi FA, Sangkum P, Hellstrom WJ. Emerging drugs for the treatment of erectile dysfunction. *Expert Opin Emerg Drugs* 2015;20(2):263-275. doi:https://doi.org/10.1517/14728214.2015.1021682

66. Francis SH, Blount MA, Corbin JD. Mammalian cyclic nucleotide phosphodiesterases: molecular mechanisms and physiological functions. *Physiol Rev* 2011;91(2):651-690. doi:https://doi.org/10.1152/physrev.00030.2010

67. Francis SH, Sekhar KR, Ke H, Corbin JD. Inhibition of cyclic nucleotide phosphodiesterases by methylxanthines and related compounds. In: *Methylxanthines.* Berlin: Springer; 2011:93-133.

68. Terrett NK, Bell AS, Brown D, Ellis P. Sildenafil (VIAGRA™), a potent and selective inhibitor of type 5 cGMP phosphodiesterase with utility for the treatment of male erectile dysfunction. 1996;6(15):1819-1824. doi:https://doi.org/10.1016/0960-894X(96)00323-X

69. Baumann M, Baxendale IR. An overview of the synthetic routes to the best selling drugs containing 6-membered heterocycles. *Beilstein J Org Chem* 2013;9:2265-2319. doi:https://doi.org/10.3762/bjoc.9.265

70. Dunn PJ. Synthesis of commercial phosphodiesterase (V) inhibitors. *Org Process Res Dev* 2005;9(1):88-97. doi:https://doi.org/10.1021/op040019c

71. Coward RM, Carson CC. Tadalafil in the treatment of erectile dysfunction. *Ther Clin Risk Manag* 2008;4(6):1315-1330. doi:https://doi.org/10.2147/TCRM.S3336

72. Washington SL, 3rd, Shindel AW. A once-daily dose of tadalafil for erectile dysfunction: compliance and efficacy. *Drug Des Devel Ther* 2010;4:159-171. doi:https://doi.org/10.2147/DDDT.S9067

73. Laine A, Lood C, Koskinen A. Pharmacological importance of optically active tetrahydro-β-carbolines and synthetic approaches to create the C1 stereocenter. *Molecules* 2014;19(2):1544-1567. doi:https://doi.org/10.3390/molecules19021544

74. Corona G, Rastrelli G, Burri A, Jannini EA, Maggi M. The safety and efficacy of Avanafil, a new 2nd generation PDE5i: comprehensive review and meta-analysis. *Expert Opin Drug Saf* 2016;15(2):237-247. doi:https://doi.org/10.1517/14740338.2016.1130126

75. Kyle JA, Brown DA, Hill JK. Avanafil for erectile dysfunction. *Ann Pharmacother* 2013;47(10):1312-1320. doi:https://doi.org/10.1177/1060028013501989

76. Kedia GT, Uckert S, Assadi-Pour F, Kuczyk MA, Albrecht K. Avanafil for the treatment of erectile dysfunction: initial data and clinical key properties. *Ther Adv Urol* 2013;5(1):35-41. doi:https://doi.org/10.1177/1756287212466282

77. Boeri L, Capogrosso P, Ventimiglia E, et al. Avanafil - a further step to tailoring patient needs and expectations. *Expert Rev Clin Pharmacol* 2016;9(9):1171-1181. doi:https://doi.org/10.1080/17512433.2016.1195261

78. Ventimiglia E, Capogrosso P, Montorsi F, Salonia A. The safety of phosphodiesterase type 5 inhibitors for erectile dysfunction. *Expert Opin Drug Saf* 2016;15(2):141-152. doi:https://doi.org/10.1517/14740338.2016.1131818

79. Mehrotra N, Gupta M, Kovar A, Meibohm B. The role of pharmacokinetics and pharmacodynamics in phosphodiesterase-5 inhibitor therapy. *Int J Impot Res* 2007;19(3):253-264. doi:https://doi.org/10.1038/sj.ijir.3901522

80. Wang R, Burnett AL, Heller WH, et al. Selectivity of avanafil, a PDE5 inhibitor for the treatment of erectile dysfunction: implications for clinical safety and improved tolerability. *J Sex Med* 2012;9(8):2122-2129. doi:https://doi.org/10.1111/j.1743-6109.2012.02822.x

81. Bischoff E. Potency, selectivity, and consequences of nonselectivity of PDE inhibition. *Int J Impot Res* 2004;16(S1):S11-S14. doi:https://doi.org/10.1038/sj.ijir.3901208

82. Laties A, Sharlip I. Ocular safety in patients using sildenafil citrate therapy for erectile dysfunction. *J Sex Med* 2006;3(1):12-27. doi:https://doi.org/10.1111/j.1743-6109.2005.00194.x

83. Roessler G, Vobig M, Walter P, Mazinani BA. Ocular side effects of Levitra® (vardenafil) - results of a double-blind crossover study in healthy male subjects. *Drug Des Devel Ther* 2019;13:37-43. doi:https://doi.org/10.2147/DDDT.S186633

84. Kouvelas D, Goulas A, Papazisis G, Sardeli C, Pourzitaki C. PDE5 inhibitors: in vitro and in vivo pharmacological profile. *Curr Pharm Des* 2009;15(30):3464-3475. doi:https://doi.org/10.2174/138161209789206971

85. Azzouni F, Abu samra K. Are phosphodiesterase type 5 inhibitors associated with vision-threatening adverse events? A critical analysis and review of the literature. *J Sex Med* 2011;8(10):2894-2903. doi:https://doi.org/10.1111/j.1743-6109.2011.02382.x

86. Seftel AD, Farber J, Fletcher J, et al. A three-part study to investigate the incidence and potential etiologies of tadalafil-associated back pain or myalgia. *Int J Impot Res* 2005;17(5):455-461. doi:https://doi.org/10.1038/sj.ijir.3901374

87. Yuan J, Zhang R, Yang Z, et al. Comparative effectiveness and safety of oral phosphodiesterase type 5 inhibitors for erectile dysfunction: a systematic review and network meta-analysis. *Eur Urol* 2013;63(5):902-912. doi:https://doi.org/10.1016/j.eururo.2013.01.012

88. Schwartz BG, Kloner RA. Drug interactions with phosphodiesterase-5 inhibitors used for the treatment of erectile dysfunction or pulmonary hypertension. *Circulation* 2010;122(1):88-95. doi:https://doi.org/10.1161/circulationaha.110.944603

89. Corona G, Mondaini N, Ungar A, Razzoli E, Rossi A, Fusco F. Phosphodiesterase type 5 (PDE5) inhibitors in erectile dysfunction: the proper drug for the proper patient. *J Sex Med* 2011;8(12):3418-3432. doi:https://doi.org/10.1111/j.1743-6109.2011.02473.x

90. Meuleman EJ. Review of tadalafil in the treatment of erectile dysfunction. *Expert Opin Pharmacother* 2003;4(11):2049-2056. doi:https://doi.org/10.1517/14656566.4.11.2049

91. da Justa Neves DB, Caldas ED. Dietary supplements: international legal framework and adulteration profiles, and characteristics of products on the Brazilian clandestine market. *Regul Toxicol Pharmacol* 2015;73(1):93-104. doi:https://doi.org/10.1016/j.yrtph.2015.06.013

92. Fibigr J, Šatínský D, Solich P. Current trends in the analysis and quality control of food supplements based on plant extracts. *Anal Chim Acta* 2018;1036:1-15. doi:https://doi.org/10.1016/j.aca.2018.08.017

93. *General guidelines for methodologies on research and evaluation of traditional medicine.* Geneva: World Health Organization; 2000.

94. Mohammad Azmin SNH, Abdul Manan Z, Wan Alwi SR, Chua LS, Mustaffa AA, Yunus NA. Herbal processing and extraction technologies. *Sep Puriv Ref* 2016;45(4):305-320. doi:https://doi.org/10.1080/15422119.2016.1145395

95. Rocha T, Amaral JS, Oliveira MBPP. Adulteration of dietary supplements by the illegal addition of synthetic drugs: a review. *Compr Rev Food Sci Food Saf* 2016;15(1):43-62. doi:https://doi.org/10.1111/1541-4337.12173

96. Primpray V, Chailapakul O, Tokeshi M, Rojanarata T, Laiwattanapaisal W. A paper-based analytical device coupled with electrochemical detection for the determination of dexamethasone and prednisolone in adulterated traditional medicines. *Anal Chim Acta* 2019;1078:16-23. doi:https://doi.org/10.1016/j.aca.2019.05.072

97. Skalicka-Wozniak K, Georgiev MI, Orhan IE. Adulteration of herbal sexual enhancers and slimmers: the wish for better sexual well-being and perfect body can be risky. *Food Chem Toxicol* 2017;108:355-364. doi:https://doi.org/10.1016/j.fct.2016.06.018

98. Patel DN, Li L, Kee C-L, Ge X, Low M-Y, Koh H-L. Screening of synthetic PDE-5 inhibitors and their analogues as adulterants: analytical techniques and challenges. *J Pharm Biomed Anal* 2014;87:176-190. doi:https://doi.org/10.1016/j.jpba.2013.04.037

99. Ismail SF, Abd Ghani Azmi IM, Daud M, Abd Jalil J, Safuan S. Regulatory control of herbal and traditional medicines in Malaysia: issues and concerns. *Int J Bus Soc* 2020;21(S1):192-204.

100. Miller LG, Hume A, Harris FIM, et al. Adulteration: its various meanings. *Pharmacotherapy* 2001;21(6):770-771. doi:https://doi.org/10.1592/phco.21.7.770.34576

101. Ekar T, Kreft S. Common risks of adulterated and mislabeled herbal preparations. *Food Chem Toxicol* 2019;123:288-297. doi:https://doi.org/10.1016/j.fct.2018.10.043

102. Cheng WC, Ng CS, Poon NL. Herbal medicines and phytopharmaceuticals - contaminations. In: Saukko PJ, Houck MM, eds. *Encyclopedia of forensic sciences.* Waltham: Academic Press; 2013:280-288.

103. Melnyk JP, Marcone MF. Aphrodisiacs from plant and animal sources - a review of current scientific literature. *Food Res Int* 2011;44(4):840-850. doi:https://doi.org/10.1016/j.foodres.2011.02.043

104. Drewes SE, George J, Khan F. Recent findings on natural products with erectile-dysfunction activity. *Phytochemistry* 2003;62(7):1019-1025. doi:https://doi.org/10.1016/S0031-9422(02)00621-0

105. Harte CB, Meston CM. Recreational use of erectile dysfunction medications in undergraduate men in the United States: characteristics and associated risk factors. *Arch Sex Behav* 2011;40(3):597-606. doi:https://doi.org/10.1007/s10508-010-9619-y

106. Simsek A, Tugcu V, Erturkuner P, Alkan F, Ozbek E, Tasci AI. Effects of the recreational use of PDE5 inhibitors on the corpus cavernosum of young, healthy rats. *Int Urol Nephrol* 2014;46(10):1889-1893. doi:https://doi.org/10.1007/s11255-014-0746-6

107. Shin MH, Hong MK, Kim WS, Lee YJ, Jeoung YC. Identification of a new analogue of sildenafil added illegally to a functional food marketed for penile erectile dysfunction. *Food Addit Contam* 2003;20(9):793-796. doi:https://doi.org/10.1080/0265203031000121455

108. Kee CL, Ge X, Gilard V, Malet-Martino M, Low MY. A review of synthetic phosphodiesterase type 5 inhibitors (PDE-5i) found as adulterants in dietary supplements. *J Pharm Biomed Anal* 2018;147:250-277. doi:https://doi.org/10.1016/j.jpba.2017.07.031

109. Poon WT, Lam YH, Lai CK, Chan AY, Mak TW. Analogues of erectile dysfunction drugs: an under-recognised threat. *Hong Kong Med J* 2007;13(5):359-363.

110. Sze-Yin Oh S, Zou P, Low M-Y, Koh H-L. Detection of sildenafil analogues in herbal products for erectile dysfunction. *J Toxicol Environ Health A* 2006;69(21):1951-1958. doi:https://doi.org/10.1080/15287390600751355

111. Balayssac S, Gilard V, Zedde C, Martino R, Malet-Martino M. Analysis of herbal dietary supplements for sexual performance enhancement: first characterization of propoxyphenyl-thiohydroxyhomosildenafil and identification of sildenafil, thiosildenafil, phentolamine and tetrahydropalmatine as adulterants. *J Pharm Biomed Anal* 2012;63:135-150. doi:https://doi.org/10.1016/j.jpba.2012.01.035

112. Mohd Yusop AY, Xiao L, Fu S. Determination of phosphodiesterase 5 (PDE5) inhibitors in instant coffee premixes using liquid chromatography-high-resolution mass spectrometry (LC-HRMS). *Talanta* 2019;204:36-43. doi:https://doi.org/10.1016/j.talanta.2019.05.078

113. Bell AS, Brown D, Terrett NK. Pyrazolopyrimidinone antianginal agents. In. United States: Google Patents; 1993.

114. Venhuis BJ, de Kaste D. Towards a decade of detecting new analogues of sildenafil, tadalafil and vardenafil in food supplements: a history, analytical aspects and health risks. *J Pharm Biomed Anal* 2012;69:196-208. doi:https://doi.org/10.1016/j.jpba.2012.02.014

115. Gouda MA. Overview of the synthetic routes to tadalafil and its analogues. *Synth Commun* 2017;47(24):2269-2304. doi:https://doi.org/10.1080/00397911.2017.1380834

116. List of precursors and chemicals frequently used in the illicit manufacture of narcotic drugs and psychotropic substances under international control. In. *Annex to the annual information on substances frequently used in the illicit manufacture of narcotic drugs and psychotropic substances (Form D)*. 16 ed. Vienna, Austria: International Narcotics Control Board; 2018.

117. Jiru M, Stranska-Zachariasova M, Dzuman Z, et al. Analysis of phosphodiesterase type 5 inhibitors as possible adulterants of botanical-based dietary supplements: extensive survey of preparations available at the Czech market. *J Pharm Biomed Anal* 2019;164:713-724. doi:https://doi.org/10.1016/j.jpba.2018.11.007

118. Kim EH, Seo HS, Ki NY, et al. Reliable screening and confirmation of 156 multi-class illegal adulterants in dietary supplements based on extracted common ion chromatograms by ultra-high-performance liquid chromatography-quadrupole/time of flight-mass spectrometry. *J Chromatogr A* 2017;1491:43-56. doi:https://doi.org/10.1016/j.chroma.2017.02.032

119. Campbell N, Clark JP, Stecher VJ, et al. Adulteration of purported herbal and natural sexual performance enhancement dietary supplements with synthetic phosphodiesterase type 5 inhibitors. *J Sex Med* 2013;10(7):1842-1849. doi:https://doi.org/10.1111/jsm.12172

120. Wang X-B, Zheng J, Li J-J, et al. Simultaneous analysis of 23 illegal adulterated aphrodisiac chemical ingredients in health foods and Chinese traditional patent medicines by ultrahigh performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry. *J Food Drug Anal* 2018;26(3):1138-1153. doi:https://doi.org/10.1016/j.jfda.2018.02.003

121. Shi F, Guo C, Gong L, et al. Application of a high resolution benchtop quadrupole-Orbitrap mass spectrometry for the rapid screening, confirmation and quantification of illegal adulterated phosphodiesterase-5 inhibitors in herbal medicines and dietary supplements. *J Chromatogr A* 2014;1344:91-98. doi:https://doi.org/10.1016/j.chroma.2013.12.030

122. Ren Y, Wu C, Zhang J. Simultaneous screening and determination of 18 illegal adulterants in herbal medicines and health foods for male sexual potency by ultra-fast liquid chromatography-electrospray ionization tandem mass spectrometry. *J Sep Sci* 2012;35(21):2847-2857. doi:https://doi.org/10.1002/jssc.201200280

123. Liang Q, Qu J, Luo G, Wang Y. Rapid and reliable determination of illegal adulterant in herbal medicines and dietary supplements by LC/MS/MS. *J Pharm Biomed Anal* 2006;40(2):305-311. doi:https://doi.org/10.1016/j.jpba.2005.07.035

124. Zou P, Oh SS-Y, Hou P, Low M-Y, Koh H-L. Simultaneous determination of synthetic phosphodiesterase-5 inhibitors found in a dietary supplement and pre-mixed bulk powders for dietary supplements using high-performance liquid chromatography with diode array detection and liquid chromatography–electrospray ionization tandem mass spectrometry. *J Chromatogr A* 2006;1104(1-2):113-122. doi:https://doi.org/10.1016/j.chroma.2005.11.103

125. Gratz SR, Flurer CL, Wolnik KA. Analysis of undeclared synthetic phosphodiesterase-5 inhibitors in dietary supplements and herbal matrices by LC-ESI-MS and LC-UV. *J Pharm Biomed Anal* 2004;36(3):525-533. doi:https://doi.org/10.1016/j.jpba.2004.07.004

126. Wang Z, Wu C, Wang G, Zhang Q, Zhang J. Novel strategy for the determination of illegal adulterants in health foods and herbal medicines using high-performance liquid chromatography with high-resolution mass spectrometry. *J Sep Sci* 2015;38(6):925-935. doi:https://doi.org/10.1002/jssc.201401134

127. Biesterbos JWH, Sijm DTHM, van Dam R, Mol HGJ. A health risk for consumers: the presence of adulterated food supplements in the Netherlands. *Food Addit Contam Part A* 2019;36(9):1273-1288. doi:https://doi.org/10.1080/19440049.2019.1633020

128. Zhang Y, Huang Z, Ding L, Yan H, Wang M, Zhu S. Simultaneous determination of yohimbine, sildenafil, vardenafil and tadalafil in dietary supplements using high-performance liquid chromatography-tandem mass spectrometry. *J Sep Sci* 2010;33(14):2109-2114. doi:https://doi.org/10.1002/jssc.200900841

129. Damiano F, Silva C, Gregori A, et al. Analysis of illicit dietary supplements sold in the Italian market: identification of a sildenafil thioderivative as adulterant using UPLC–TOF/MS and GC/MS. *Sci Justice* 2014;54(3):228-237. doi:https://doi.org/10.1016/j.scijus.2014.02.009

130. Lee S, Ji D, Park M, Chung KH. Development of a comprehensive spectral library of sildenafil and related active analogues using LC-QTOF-MS and its application for screening counterfeit pharmaceuticals. *Forensic Sci Int* 2015;257:182-188. doi:https://doi.org/10.1016/j.forsciint.2015.08.016

131. Vaclavik L, Krynitsky AJ, Rader JI. Targeted analysis of multiple pharmaceuticals, plant toxins and other secondary metabolites in herbal dietary supplements by ultra-high performance liquid chromatography-quadrupole-orbital ion trap mass spectrometry. *Anal Chim Acta* 2014;810:45-60. doi:https://doi.org/10.1016/j.aca.2013.12.006

132. Venhuis BJ, Tan J, Vredenbregt MJ, Ge X, Low M-Y, de Kaste D. Capsule shells adulterated with tadalafil. *Forensic Sci Int* 2012;214(1):e20-e22. doi:https://doi.org/10.1016/j.forsciint.2011.07.013

133. Lanzarotta A, Crowe JB, Witkowski M, Gamble BM. A multidisciplinary approach for the analysis of an adulterated dietary supplement where the active pharmaceutical ingredient was embedded in the capsule shell. *J Pharm Biomed Anal* 2012;67-68:22-27. doi:https://doi.org/10.1016/j.jpba.2012.04.023

134. Doi T, Takahashi K, Yamazaki M, et al. Characterization of a new illicit phosphodiesterase-type-5 inhibitor identified in the softgel shell of a dietary supplement. *J Pharm Biomed Anal* 2018;161:61-65. doi:https://doi.org/10.1016/j.jpba.2018.08.031

135. Vaclavik L, Krynitsky AJ, Rader JI. Mass spectrometric analysis of pharmaceutical adulterants in products labeled as botanical dietary supplements or herbal remedies: a review. *Anal Bioanal Chem* 2014;406(27):6767-6790. doi:https://doi.org/10.1007/s00216-014-8159-z

136. Shi S, Wu Y, Zhou M, Cheng Q. Simultaneous analysis of 31 anti-impotence compounds potentially illegally added to herbal-based dietary supplements by ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry. *J Chromatogr B* 2020;1144:122077. doi:https://doi.org/10.1016/j.jchromb.2020.122077

137. Jeong JH, Lee JH, Kim HJ, et al. LC-ESI-MS/MS analysis of phosphodiesterase-5 inhibitors and their analogues in foods and dietary supplements in Korea. *Food Addit Contam Part B Surveill* 2016;9(1):1-8. doi:https://doi.org/10.1080/19393210.2014.968220

138. Gratz SR, Gamble BM, Flurer RA. Accurate mass measurement using Fourier transform ion cyclotron resonance mass spectrometry for structure elucidation of designer drug analogs of tadalafil, vardenafil and sildenafil in herbal and pharmaceutical matrices. *Rapid Commun Mass Spectrom* 2006;20(15):2317-2327. doi:https://doi.org/10.1002/rcm.2594

139. Lucas D. Sample prep for trace analysis of adulterants in erectile dysfunction dietary supplements. 2014. https://www.agilent.com/cs/library/applications/5991-3638EN.pdf. Accessed 20 May 2020.

140. Strano-Rossi S, Odoardi S, Castrignano E, Serpelloni G, Chiarotti M. Liquid chromatography-high resolution mass spectrometry (LC-HRMS) determination of stimulants, anorectic drugs and phosphodiesterase 5 inhibitors (PDE5I) in food supplements. *J Pharm Biomed Anal* 2015;106:144-152. doi:https://doi.org/10.1016/j.jpba.2014.06.011

141. Park SR, Lee JG, Roh SH, et al. Determination of PDE-5 inhibitors and appetite suppressants in adulterated dietary supplements using LC/PDA and LC/MS. *Food Addit Contam Part B Surveill* 2012;5(1):29-32. doi:https://doi.org/10.1080/19393210.2012.656706

142. Ding B, Wang Z, Xie J, Zeng G, Chen W. Phosphodiesterase-5 inhibitors in Chinese tonic liquors by liquid chromatography coupled with quadrupole time of flight mass spectrometry. *Food Addit Contam Part B Surveill* 2018;11(3):214-222. doi:https://doi.org/10.1080/19393210.2018.1488769

143. Mohd Yusop AY, Xiao L, Fu S. Fluorescence polarisation for high-throughput screening of adulterated food products via phosphodiesterase 5 (PDE5) inhibition assay. *Drug Test Anal* 2020. doi:https://doi.org/10.1002/dta.2926

144. Ozturk Er E, Ozbek B, Bakirdere S. Accurate and sensitive determination of sildenafil, tadalafil, vardenafil, and avanafil in illicit erectile dysfunction medications and human urine by LC with quadrupole-TOF-MS/MS and their behaviors in simulated gastric conditions. *J Sep Sci* 2019;42(2):475-483. doi:https://doi.org/10.1002/jssc.201800734

145. Gilard V, Balayssac S, Tinaugus A, Martins N, Martino R, Malet-Martino M. Detection, identification and quantification by ^{1}H NMR of adulterants in 150 herbal dietary supplements marketed for improving sexual performance. *J Pharm Biomed Anal* 2015;102:476-493. doi:https://doi.org/10.1016/j.jpba.2014.10.011

146. Do TT, Theocharis G, Reich E. Simultaneous detection of three phosphodiesterase type 5 inhibitors and eight of their analogs in lifestyle products and screening for adulterants by high-performance thin-layer chromatography. *J AOAC Int* 2015;98(5):1226-1233. doi:https://doi.org/10.5740/jaoacint.14-285

147. Awofisayo OS, Awofisayo OA, Eyen N. Development of basic tests for sildenafil citrate and sildenafil citrate tablet. *J Clin Med Res* 2010;2(11):175-179.

148. Guo J-B, Xu Y, Huang Z-B, He Q-H, Liu S-W. Development of an immunoassay for rapid screening of vardenafil and its potential analogues in herbal products based on a group specific monoclonal antibody. *Anal Chim Acta* 2010;658(2):197-203. doi:https://doi.org/10.1016/j.aca.2009.11.021

149. Guo J, Liu W, Lan X, Chen H, Xiao Z. Development and evaluation of an immunochromatographic strip for rapid screening of sildenafil-type compounds as illegal additives in functional foods. *Food Addit Contam Part A* 2016;33(7):1095-1104. doi:https://doi.org/10.1080/19440049.2016.1203072

150. He F, Zou T, Yang J, et al. Development of a skeleton-specific antibody and Au nanoparticle-based immunochromatographic sensor for simultaneous detection of various tadalafil adulterants in health food. *Food Agr Immunol* 2019;30(1):349-368. doi:https://doi.org/10.1080/09540105.2019.1585417

151. Santillo MF, Mapa MST. Phosphodiesterase (PDE5) inhibition assay for rapid detection of erectile dysfunction drugs and analogs in sexual enhancement products. *Drug Test Anal* 2018;10(8):1315-1322. doi:https://doi.org/10.1002/dta.2375

152. Liang YZ, Xie P, Chan K. Quality control of herbal medicines. *J Chromatogr B* 2004;812(1-2):53-70. doi:https://doi.org/10.1016/j.jchromb.2004.08.041

153. Cai Y, Cai T-G, Shi Y, et al. Simultaneous determination of eight PDE5-Is potentially adulterated in herbal dietary supplements with TLC and HPLC-PDA-MS methods. *J Liq Chromatogr Relat Technol* 2010;33(13):1287-1306. doi:https://doi.org/10.1080/10826076.2010.488979

154. Attimarad M, Ahmed KKM, Aldhubaib BE, Harsha S. High-performance thin layer chromatography: A powerful analytical technique in pharmaceutical drug discovery. 2011;2(2):71-75. doi:https://doi.org/10.4103/2229-4708.84436

155. Abourashed E, Abdel-Kader M, Habib A-A. HPTLC determination of sildenafil in pharmaceutical products and aphrodisiac herbal preparations. *JPC-J Planar Chromat* 2005;18(105):372-376. doi:https://doi.org/10.1556/jpc.18.2005.5.7

156. Reddy TS, Reddy AS, Devi PS. Quantitative determination of sildenafil citrate in herbal medicinal formulations by high-performance thin-layer chromatography. *JPC-J Planar Chromat* 2006;19(112):427-431. doi:https://doi.org/10.1556/jpc.19.2006.6.4

157. Tistaert C, Dejaegher B, Heyden YV. Chromatographic separation techniques and data handling methods for herbal fingerprints: a review. *Anal Chim Acta* 2011;690(2):148-161. doi:https://doi.org/10.1016/j.aca.2011.02.023

158. Liu Y, Lu F. Adulterated pharmaceutical chemicals in botanical dietary supplements: novel screening approaches. *Rev Anal Chem* 2017;36(3):20160032. doi:https://doi.org/10.1515/revac-2016-0032

159. Sacré P-Y, Deconinck E, De Beer T, et al. Comparison and combination of spectroscopic techniques for the detection of counterfeit medicines. *J Pharm Biomed Anal* 2010;53(3):445-453. doi:https://doi.org/10.1016/j.jpba.2010.05.012

160. Custers D, Cauwenbergh T, Bothy JL, et al. ATR-FTIR spectroscopy and chemometrics: an interesting tool to discriminate and characterize counterfeit medicines. *J Pharm Biomed Anal* 2015;112:181-189. doi:https://doi.org/10.1016/j.jpba.2014.11.007

161. Lee JH, Park HN, Jung A, et al. Isolation and characterisation of a novel sildenafil analogue adulterant, desmethylpiperazinyl propoxysildenafil, in a dietary supplement. 2018;58(6):447-454. doi:https://doi.org/10.1016/j.scijus.2018.07.003

162. Kee CL, Low MY, Ge X. Isolation and characterization of a novel dithio-carbodenafil analogue from a health supplement. *J Pharm Biomed Anal* 2017;137:132-138. doi:https://doi.org/10.1016/j.jpba.2017.01.010

163. Huang Y-C, Lee H-C, Lin Y-L, Li C-Y, Tsai C-F, Cheng H-F. Separation and identification of a novel tadalafil analogue adulterant in a dietary supplement. 2016;33(2):179-185. doi:https://doi.org/10.1080/19440049.2015.1125531

164. Gu Y, Hu Q, Sun J, et al. Isolation and identification of a new sildenafil analogue, hydroxycarbodenafil, found as an adulterant in a health supplement. *J Pharm Biomed Anal* 2020;185:113222. doi:https://doi.org/10.1016/j.jpba.2020.113222

165. Minh DTC, Thi LA, Huyen NTT, Van Vu L, Anh NTK, Ha PTT. Detection of sildenafil adulterated in herbal products using thin layer chromatography combined with surface enhanced Raman spectroscopy: “double coffee-ring effect” based enhancement. *J Pharm Biomed Anal* 2019;174:340-347. doi:https://doi.org/10.1016/j.jpba.2019.05.043

166. Hu X, Fang G, Han A, Fu Y, Tong R, Wang S. Rapid detection of six phosphodiesterase type 5 enzyme inhibitors in healthcare products using thin-layer chromatography and surface enhanced Raman spectroscopy combined with BP neural network. *J Sep Sci* 2017;40(11):2506-2514. doi:https://doi.org/10.1002/jssc.201700024

167. Martino R, Malet-Martino M, Gilard V, Balayssac S. Counterfeit drugs: analytical techniques for their identification. *Anal Bioanal Chem* 2010;398(1):77-92. doi:https://doi.org/10.1007/s00216-010-3748-y

168. Kee C-L, Ge X, Tran C-S, Bui Q-D, Cheah N-P, Low M-Y. Isolation and characterization of N-hydroxyethyl dithio-desethyl carbodenafil from a health supplement. *J Pharm Biomed Anal* 2020;189:113431. doi:https://doi.org/10.1016/j.jpba.2020.113431

169. Silva Elipe MV. Advantages and disadvantages of nuclear magnetic resonance spectroscopy as a hyphenated technique. *Anal Chim Acta* 2003;497(1–2):1-25. doi:https://doi.org/10.1016/j.aca.2003.08.048

170. Muschietti L, Redko F, Ulloa J. Adulterants in selected dietary supplements and their detection methods. *Drug Test Anal* 2020. doi:https://doi.org/10.1002/dta.2806

171. Pyo J-S, Lee H-S, Park Y-J, et al. Determination of the PDE-5 inhibitors and their analogues by GC-MS and TMS derivatization. *Mass Spectrom Lett* 2012;3(1):15-17. doi:https://doi.org/10.5478/MSL.2012.3.1.015

172. Saisho K, Scott KS, Morimoto S, Nakahara Y. Hair analysis for pharmaceutical drugs. II. Effective extraction and determination of sildenafil (Viagra®) and its N-desmethyl metabolite in rat and human hair by GC-MS. *Biol Pharm Bull* 2001;24(12):1384-1388. doi:https://doi.org/10.1248/bpb.24.1384

173. Man CN, Nor NM, Lajis R, Harn GL. Identification of sildenafil, tadalafil and vardenafil by gas chromatography-mass spectrometry on short capillary column. 2009;1216(47):8426-8430. doi:https://doi.org/10.1016/j.chroma.2009.10.016

174. Mokhtar SU, Chin ST, Kee CL, Low MY, Drummer OH, Marriott PJ. Rapid determination of sildenafil and its analogues in dietary supplements using gas chromatography-triple quadrupole mass spectrometry. *J Pharm Biomed Anal* 2016;121:188-196. doi:https://doi.org/10.1016/j.jpba.2016.01.034

175. Fejos I, Neumajer G, Beni S, Jankovics P. Qualitative and quantitative analysis of PDE-5 inhibitors in counterfeit medicines and dietary supplements by HPLC-UV using sildenafil as a sole reference. *J Pharm Biomed Anal* 2014;98:327-333. doi:https://doi.org/10.1016/j.jpba.2014.06.010

176. Farthing CA, Farthing DE, Koka S, et al. A simple and sensitive HPLC fluorescence method for determination of tadalafil in mouse plasma. *J Chromatogr B* 2010;878(28):2891-2895. doi:https://doi.org/10.1016/j.jchromb.2010.07.022

177. Xian F, Hendrickson CL, Marshall AG. High Resolution Mass Spectrometry. *Anal Chem* 2012;84(2):708-719. doi:https://doi.org/10.1021/ac203191t

178. Pasin D, Cawley A, Bidny S, Fu S. Current applications of high-resolution mass spectrometry for the analysis of new psychoactive substances: a critical review. *Anal Bioanal Chem* 2017;409(25):5821-5836. doi:https://doi.org/10.1007/s00216-017-0441-4

179. Álvarez-Ruiz R, Picó Y. Sequential window acquisition of all theoretical fragments versus information dependent acquisition for suspected-screening of pharmaceuticals in sediments and mussels by ultra-high pressure liquid chromatography-quadrupole time-of-flight-mass spectrometry. *J Chromatogr A* 2019;1595:81-90. doi:https://doi.org/10.1016/j.chroma.2019.02.041

180. Savaliya AA, Shah RP, Prasad B, Singh S. Screening of Indian aphrodisiac ayurvedic/herbal healthcare products for adulteration with sildenafil, tadalafil and/or vardenafil using LC/PDA and extracted ion LC-MS/TOF. *J Pharm Biomed Anal* 2010;52(3):406-409. doi:https://doi.org/10.1016/j.jpba.2009.05.021

181. Bortolini C, Pivato A, Bogialli S, Pastore P. "One-shot" analysis of PDE-5 inhibitors and analogues in counterfeit herbal natural products using an LC-DAD-QTOF system. *Anal Bioanal Chem* 2015;407(20):6207-6216. doi:https://doi.org/10.1007/s00216-015-8801-4

CHAPTER 3

Comparison of sample extraction techniques for the determination of erectile dysfunction drugs as adulterants in selected food products

3.1 FOREWORD

The following manuscript, in Chapter 3, has been submitted for publication. The selection of a suitable sample extraction technique is crucial in analytical chemistry. Ideally, the sample extraction should be able to tackle complex and variable matrices while also be compatible with the analytes of interest. It should equally possess the selectivity and sensitivity required for the intended application. At present, the determination of phosphodiesterase 5 (PDE5) inhibitors and their analogues as adulterants in herbal remedies, particularly those in pharmaceutical dosage forms, is frequently discussed in the literature. However, the presence of these adulterants in herbal-based food products is rarely addressed, with limited information on their sample extraction optimisation and matrix-specific validation. This chapter compares four conventional extraction techniques, i.e. dilute and shoot, solid-phase extraction, liquid-liquid extraction, and modified quick, easy, cheap, effective, rugged, and safe (QuEChERS), for the determination of PDE5 inhibitors and their analogues as adulterants in five selected food matrices. These matrices comprised powdered drink mix, honey, jelly, hard candy, and chewing gum. The best extraction procedure, based on the matrix effect and extraction recovery performance, was validated for each matrix, and subsequently applied to analyse 25 food samples that claimed to enhance male sexual performance. The manuscript highlighted

the importance of mitigating the matrix effect, particularly from complex matrices such as foods, for accurate and precise multi-analyte analysis. Mr Ahmad Yusri Mohd Yusop, Dr Linda Xiao, and Professor Shanlin Fu authored the manuscript. Mohd Yusop AY performed the experimental work, data analysis, and initial draft preparation including supplementary data with manuscript edits provided by Xiao L and Fu S.

3.2 ABSTRACT

The presence of erectile dysfunction (ED) drugs in adulterated dietary supplements, mainly in pharmaceutical dosage forms, is frequently addressed in the literature. Little attention is given to food products despite their increasing adulteration trend. To address this knowledge gap, four conventional extraction techniques, i.e. dilute and shoot (D&S); liquid-liquid extraction (LLE); solid-phase extraction (SPE); and modified quick, easy, cheap, effective, rugged, and safe (QuEChERS) were compared to determine ED drugs in powdered drink mix (PDM), honey, jelly, hard candy, and chewing gum. The matrix effect (ME) and extraction recovery (RE) of 23 targeted phosphodiesterase 5 (PDE5) inhibitors were assessed for each extraction technique using liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-QTOF-MS). The D&S, LLE, and SPE resulted in moderate MEs within -48.1%–41.7% for PDM at 1:2 matrix dilution, while the modified QuEChERS exhibited insignificant ME within -8.7%–4.0%. Poor REs were evident for several target analytes using the LLE and SPE. Indeed, four target analytes were not detected in different food matrices using the SPE. The modified QuEChERS provided complete coverage of target analytes with acceptable RE within 75.5%–123.9%, except for carbodenafil in the PDM matrix. Based on the ME and RE performance, the modified QuEChERS was validated at 1:10 matrix dilution to analyse 25 food samples that claimed to enhance male sexual performance. The method exhibited good specificity and linearity, with a limit of detection of <70 ng/mL and limit of quantification of 80 ng/mL. Similarly, the accuracy and precision were satisfactory within 77.4%–122.0% and <16.7%RSD, respectively. The suspected-target and non-targeted screenings of the LC-QTOF-MS identified and detected ten PDE5 inhibitors from

24 food samples. To our best knowledge, this is the first study to compare different extraction techniques for an accurate and precise determination of ED drugs in selected food products.

3.3 INTRODUCTION

Erectile dysfunction (ED) drugs are currently in high demand due to the immense success of sildenafil, vardenafil, and tadalafil [1]. Unfortunately, these phosphodiesterase 5 (PDE5) inhibitors are often intentionally added into various dietary supplements to deliver desired efficacy, despite their health risks to consumers [2]. Worse, they usually contain analogues of the approved drugs [3,4], presenting even significant health and life-threatening risks, attributed by their unknown safety and toxicological profiles [5,6]. Lately, these unscrupulous manufacturers have been finding ways to conceal the adulterants within complex matrices such as food products which may hinder detection, and thus, circumvent the law.

Liquid chromatography (LC) coupled to mass spectrometry (MS), particularly in tandem mode is commonly used to determine ED drugs in adulterated products due to its superior specificity, sensitivity, and the ability to separate multiple analytes from complex matrices [7]. The electrospray ionisation (ESI) technique in positive mode indubitably identified the PDE5 inhibitors due to their physical and chemical properties [8]. However, this technique has many drawbacks, mostly caused by matrix effect (ME), leading to various errors, especially in quantification [9]. Several strategies have been proposed to mitigate the ME, covering three broad categories of (1) sample extraction, (2) ionisation technique, and (3) chromatographic separation [10].

The determination of PDE5 inhibitors as adulterants in dietary supplements, particularly those in pharmaceutical dosage forms, is frequently discussed in the literature. In most of these studies, the samples were extracted using dilute and shoot (D&S) technique with either acetonitrile [11] or methanol [12], including their combination with ultrapure water [13,14]. The D&S is undoubtedly straightforward, simple, and quick; where no sample clean-up is required [7]. Unfortunately, it is also prone to co-extract the matrix components which may interfere with the determination of PDE5 inhibitors.

Only a small number of studies employed other extraction techniques such as liquid-liquid extraction (LLE) [15-17], solid-phase extraction (SPE) [18,19], and official quick, easy, cheap, effective, rugged, and safe (QuEChERS) [20,21]. LLE and SPE often produced clean extracts with effective removal of matrix components. Their method development, however, can be lengthy and complicated, requiring each step of the extraction to be optimised separately. Furthermore, the final procedure is time-consuming, particularly when a pre-concentration step, is incorporated into the methodology [9,22]. The official QuEChERS method is simple; initially developed and validated for recovering pesticides residues in fruits and vegetables. Although QuEChERS is a relatively new technique, it has attracted considerable attention and widely adopted due to its flexibility to extract analytes from different matrices [23].

To our best knowledge, just a few studies focused exclusively on the determination of PDE5 inhibitors in food products, for instance, Chinese tonic liquor [21] and instant coffee premix [24]. Whereas, other studies [25-27]

included them as supplementary samples, with little to no information on matrix-specific validation, particularly on ionisation suppression or enhancement.

The primary objective of this study is to compare the performance of four conventional extraction techniques, namely, D&S, LLE, SPE, and modified QuEChERS for the determination of ED drugs as adulterants in selected food products. The ME and extraction recovery (RE) of 23 targeted PDE5 inhibitors were evaluated in positive ESI mode of a liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-QTOF-MS). The best extraction procedure was validated for each matrix and subsequently applied to analyse 25 food samples that claimed to enhance male sexual performance. To our best knowledge, this is the first study to compare different extraction techniques for an accurate and precise determination of ED drugs, especially from complex matrices such as food products.

3.4 MATERIALS AND METHODS

3.4.1 Chemicals and reagents

A total of 23 PDE5 inhibitors' certified reference materials (CRMs) were purchased from TLC Pharmaceutical Standards Ltd. (Aurora, Ontario, Canada). They were as follows: (1) desmethylcarbodenafil, (2) carbodenafil, (3) N-desethylacetildenafil, (4) acetildenafil, (5) hydroxyvardenafil, (6) dimethylacetildenafil, (7) vardenafil, (8) sildenafil, (9) homosildenafil, (10) dimethylsildenafil, (11) propoxyphenyl-hydroxyhomosildenafil, (12) udenafil, (13) propoxyphenyl-sildenafil, (14) hydroxythiovardenafil, (15) tadalafil, (16) mirodenafil, (17) mutaprodenafil, (18) thiosildenafil, (19) thiohomosildenafil, (20) dithiodesmethylcarbodenafil, (21) thiodimethylsildenafil, (22) propoxyphenyl-thiohydroxyhomosildenafil, and (23) propoxyphenyl-thiodimethylsildenafil. The deuterated internal standard (IS) of sildenafil-d8 was procured from Sapphire Bioscience Pty Ltd. (Redfern, NSW, Australia).

Chem-Supply Pty Ltd. (Gillman, SA, Australia) supplied the methanol and acetonitrile of LC-MS grade; while the vendor for ethyl acetate and formic acid of LC-MS grade, chloroform of HPLC grade, ethanol of gradient grade, and ammonium formate and sodium hydroxide of analytical grade was Sigma Aldrich Pty Ltd. (Castle Hill, NSW, Australia). A Sartorius arium® pro ultrapure water system (Goettingen, Germany) dispensed the ultrapure water (18.2 MΩ-cm). Agilent Technologies Australia Pty Ltd. (Mulgrave, VIC, Australia) and LECO Australia Pty Ltd. (Castle Hill, NSW, Australia), respectively provided the Bond Elut C18 (500 mg, 3 mL) SPE cartridge and the QuEChERS extraction salt (EN 15662).

3.4.2 Standard solution preparation

The stock solution of each CRM and IS was prepared in methanol at 1 mg/mL and stored in the dark at 4°C. A mixture of all CRMs was freshly prepared for each analysis from the stock solutions by further dilution in methanol to produce 25 µg/mL of working solution.

3.4.3 Sample collection and storage

In total, 25 distinct food samples obtained from Malaysia (23 samples) and Australia (two samples) were in the form of powdered drink mix (PDM, 16 samples), honey (HNY, four samples), jelly (JLY, two samples), hard candy (HCD, two samples), and chewing gum (CWG, one sample). These products were selected based on their brand names, label claims, images, herbal ingredients, or advertising materials with associations of male sexual performance. The Pharmacy Enforcement Division, Ministry of Health Malaysia, kindly donated most of these samples, which were confiscated by the pharmacy enforcement officers at the international airport (three samples) and international seaport (six samples), as well as from routine market surveillance activities (12 samples). The remainder of the samples were purchased from various online retail stores based in Malaysia and Australia (two samples each).

The samples were kept in separate plastic zip-lock bags and stored in an airtight container in the dark. Blank matrices of each food product, free from any analyte of interests, were sourced from a local supermarket in Australia and used to compare the different extraction techniques as well as the analytical method

validation. Table 3.4.3 outlines the compositions of each food product used as the blank matrix based on the products' label.

Table 3.4.3: Contents of the blank food matrices based on the products' label.

No.	Matrix	Listed ingredients on the label
1	Powdered drink mix (PDM)	Citric acid, calcium phosphate, maltodextrin, ascorbic acid, natural and artificial flavour, blue 1, tocopherol, and preservatives.
2	Honey (HNY)	100% pure Australian eucalyptus and ground flora honey.
3	Jelly (JLY)	Water, sugar, gelling agents, acidity regulators, anthocyanins, and natural flavours.
4	Hard candy (HCD)	Sugar, glucose syrup, water, natural herbs extract, natural and artificial flavour, caramel colouring, and menthol.
5	Chewing gum (CWG)	Sorbitol, gum base, humectant, mannitol, flavour, sweetener, emulsifier, antioxidant, and phenylalanine.

3.4.4 Sample extraction procedures

The initial weight of each blank matrix or sample was recorded based on the recommended intake specified on its label. PDM and HNY were taken directly from their sachets; while JLY, HCD, and CWG were initially homogenised with mortar and pestle before the sample extraction procedures.

Dilute and shoot (D&S)

100 mg of the blank matrix was weighed in a polypropylene tube and then extracted with 5 mL of methanol by 1-min vortex mixing, 20-min sonication, and 5-min centrifugation at 2500 × *g*, successively. The upper solution was then filtered using a 0.22 mm PTFE syringe filter and diluted for ME assessment. The

RE evaluation was omitted since no phase transfer occurred when a D&S procedure was employed.

Liquid-liquid extraction (LLE)

The blank matrices were initially pre-treated in an aqueous phase for the LLE procedure. First, 100 mg of the blank matrix was weighed in a polypropylene tube and then added with 4.75 mL of ultrapure water. Next, the blank matrix was spiked with either 250 µL of methanol for post-extraction spiked matrix or 250 µL of working solution and IS for pre-extraction spiked matrix; with subsequent vortex mixing for 1 min, sonication for 20 min, and centrifugation for 5 min at 2500 × *g*. The pH of the mixture was then adjusted to 10.0 ± 0.1 with 1M sodium hydroxide solution and filtered using a 0.22 mm PTFE syringe filter for the LLE procedure.

For LLE, 5mL of chloroform, ethyl acetate, and ethanol (3:1:1, v/v/v) was added to a polypropylene tube containing 1 mL of the pre-treated blank matrix solution; followed by 5-min vortex mixing and 5-min centrifugation at 2500 × *g*, successively. Next, 4 ml of the organic layer was pipetted out and transferred into another polypropylene tube. The LLE was repeated for the second time, with the organic layer combined with that of the first extraction. The combined organic phase was then dried under a gentle stream of nitrogen gas; reconstituted in 800 µL of methanol; and subsequently, filtered and diluted for ME and RE assessment.

Solid-phase extraction (SPE)

The blank matrices were pre-treated in the same manner as described in the LLE procedure. The SPE employed an Agilent Technologies Bond Elut C18 (500 mg, 3 mL) SPE cartridge. The cartridge was initially conditioned with 3 mL of methanol followed by equilibration with 3 mL of ultrapure water. After loading 1 mL of the pre-treated blank matrix solution, 3 mL of 5% methanol in ultrapure water was used to wash out the matrix components. The retained components were then eluted with 3 mL of methanol and 3 mL of acetonitrile, successively. The eluate was dried under a gentle stream of nitrogen gas; reconstituted in 1 mL of methanol; and subsequently, filtered and diluted for ME and RE assessment.

Modified quick, easy, cheap, effective, rugged, and safe (QuEChERS)

Initially, 100 mg of the blank matrix was weighed in a polypropylene tube and then added with 2.5 mL of acetonitrile and 2.25 mL methanol. Next, the blank matrix was spiked with either 250 µL of methanol for post-extraction spiked matrix or 250 µL of working solution and IS for pre-extraction spiked matrix; with subsequent vortex mixing for 1 min, sonication for 20 min, and centrifugation for 5 min at 2500 × *g*. The resulting solution was then transferred into another polypropylene tube prefilled with half a sachet of the QuEChERS extraction salt (2 g magnesium sulphate, 0.5 g sodium chloride, 0.5 g trisodium citrate dihydrate, and 0.25 g disodium hydrogen citrate sesquihydrate) for the extraction procedure with 1-min vortex mixing and 5-min centrifugation at 2500 × *g*, successively. The upper layer was then filtered and diluted for ME and RE assessment.

3.4.5 LC-QTOF-MS conditions and data analysis

An Agilent Technologies (Santa Clara, CA, USA) 1290 Infinity II LC system coupled to an Agilent Technologies 6510 QTOF-MS, was used in this study following previous methodology [24]. In brief, the chromatographic separation was carried out using a reverse-phase high-performance LC column from Merck KGaA (Darmstadt, Germany) Chromolith® High-Resolution RP-18 end-capped (100 × 4.6 mm, 2.0 µm). The injection volume was set at 5 µL with the column, and autosampler compartment temperatures maintained at 20°C and 10°C, respectively. The mobile phases, consisted of 10 mM ammonium formate in ultrapure water (solvent A) and acetonitrile (solvent B), were acidified with 0.1% v/v of formic acid. The gradient elution was set as follows: 5% B for 0–1 min, 5%–25% B for 1–2 min, 25%–50% B for 2–32 min, 50%–95% B for 32–33 min, and 95% B for 33–34 min at 0.4 mL/min. The elution was immediately returned to the initial gradient at 34.01 min for 6 min at 1 mL/min with post-run equilibration kept at 0.4 mL/min, 5 min before the next injection.

The QTOF-MS was operated in positive ESI mode with the following experimental parameters: 300°C for gas temperature, 12 L/min for drying gas flow, 32 psig for nebuliser pressure, 3500 V for capillary voltage, 175 V for fragmentor voltage, 65 V for skimmer voltage, and 750 V for OCT 1 RF Vpp. Simultaneous MS and tandem MS experiments within a mass-to-charge range of *m/z* 100–1100 were performed using auto MS/MS mode. The collision-induced dissociation experiments were conducted at fixed collision energies (CEs) of 10, 20, and 40 eV in a separate scan using nitrogen as the collision gas. The reference mass solution, containing purine (*m/z* 121.050873) and hexakis (1H, 1H, 3H-

tetrafluoropropoxy) phosphazine (*m/z* 922.009798), was continually infused at a steady pressure of 5 psig throughout the chromatographic run.

All qualitative and quantitative data were processed with suspected-target and non-targeted screenings as well as targeted analysis workflow, developed previously [24,28], using Agilent Technologies Mass Hunter workstation software version B.07.00, Mass Hunter qualitative analysis software version B.07.00, and personal compound database and library (PCDL) manager software version B.04.00. The suspected-target screening employed a PCDL library of 95 PDE5 inhibitors and their analogues, including the 23 target analytes. The non-targeted screening via top-down and bottom-up approaches were utilised to flag novel analogues of PDE5 inhibitors based on common fragmentation patterns of target analytes. The physical and chemical properties of each target analyte were calculated using ChemAxon Ltd. (Budapest, Hungary) MarvinSketch software version 18.8.0. All other calculations were done using Microsoft (Redmond, WA, USA) Excel 2016 (Microsoft Office).

3.4.6 Comparison of sample extraction techniques

The sample extraction techniques were evaluated based on the ME and RE of each target analyte at low (0.1 µg/mL); medium (0.4 µg/mL); and high (1 µg/mL) quality control (QC) levels, each analysed in triplicate, following the recommended procedures [9,29]. Each blank matrix was assessed by preparing three sets of standards as follows: (1) standards in neat solution (methanol); (2) post-extraction spiked matrix (matrix-matched standards); and (3) pre-extraction spiked matrix. Additionally, a deuterated IS of sildenafil-d8 was spiked at 0.25

µg/mL into each set of standards to compensate for any possible volume variation during the extraction process.

The ME was evaluated based on the post-extraction addition method, by comparing the slopes of the post-extraction spiked matrix versus those of the standards in neat solution, expressed in Eq. 3.4.6A. The calibration curves were constructed using the QC analytes' concentrations at three levels of matrix dilutions of 1:2, 1:10, and 1:100. The percentage of ME was then categorised for each target analyte following the set criteria of insignificant (0% to ±10%), acceptable (±10% to ±20%), moderate (±20% to ±50%), and severe (less than -50% or more than +50%), where a positive value indicates ionisation enhancement. In contrast, a negative value indicates ionisation suppression.

$$ME\ (\%) = \left[\frac{Slope_{post\text{-}extraction\ spiked\ matrix}}{Slope_{standards\ in\ neat\ solution}} - 1\right] \times 100 \qquad \text{(Eq. 3.4.6A)}$$

The RE was determined by comparing the peak areas of the protonated molecule ($[M+H]^+$) precursor ion from the pre-extraction spiked matrix versus those of the post-extraction spiked matrix at the same QC level, expressed in Eq. 3.4.6B. The mean RE percentages at low, medium, and high QC levels were then categorised as follows: acceptable (±25%), moderate (±25% to ±75%), and poor (less than -75% or more than +75%).

$$RE\ (\%) = \frac{Peak\ area_{pre\text{-}extraction\ spiked\ matrix}}{Peak\ area_{post\text{-}extraction\ spiked\ matrix}} \times 100 \qquad \text{(Eq. 3.4.6B)}$$

3.4.7 Analytical method validation

The analytical method was validated for specificity, linearity, limit of detection (LOD), and limit of quantification (LOQ), according to the established guideline [30]. The accuracy and precision were evaluated as well for each target analyte in each of the blank matrices, at low, medium, and high QC levels, following the recommended procedures [29]. All validation parameters were analysed in triplicate.

The specificity of each target analyte was determined based on the chromatographic separation, the high-resolution mass of the ($[M+H]^+$), and the extent of matrix interferences. The presence of two product ions, corresponding to each of the target analytes, was then established from the tandem MS experiment. Furthermore, the average intensity ratio between the first and the second product ion at average CEs was compared to those obtained from the matrix-matched standards within ±30%, hence, confirming the identity of the target analytes.

The linearity was determined based on the coefficient of determination (r^2) of external calibration curves, constructed using the peak areas of the $[M+H]^+$ precursor ion, versus their concentrations within the expected range of target analytes in adulterated products. The regression equation was then used to calculate the QC analytes and samples concentrations.

The LOD was determined experimentally by gradually reducing the concentration of target analytes by 10 ng/mL, starting from 100 ng/mL and down to 10 ng/mL. The LOD was then established at the lowest concentration of target analyte that can be reliably identified with a signal-to-noise ratio of >3. The LOQ was set at the lowest concentration of the external calibration curve with acceptable accuracy and precision, together with a signal-to-noise ratio of >10.

The accuracy and precision were established at three QC levels using the post-extraction spiked matrix. The observed concentration of target analyte versus the expected concentration was expressed as a percentage of accuracy with an acceptable value of ±25%. Precision was determined at intra-day for repeatability and inter-day for intermediate precision. The peak areas of the $[M+H]^+$ precursor ion were then expressed as a percentage of relative standard deviation (%RSD) with an acceptable value of <20%RSD.

3.5 RESULTS AND DISCUSSION

3.5.1 Method development and optimisation

The chromatographic separation and MS conditions were developed and optimised following the previous literature, together with the extraction procedures for D&S and modified QuEChERS [31]. The LLE and SPE procedures were additionally established to compare different sample extraction techniques, as outlined in this study. Initially, several published LLE [15-17] and SPE [18,19] procedures were replicated to assess their suitability and performance for the selected matrices. However, they often led to non-detection, as well as severe and moderate MEs for several target analytes. Furthermore, one of the LLE procedures had resulted in the formation of a thick layer of emulsion that hinders the organic phase recovery. As these procedures were developed for adulterated products in pharmaceutical dosage forms, they may not be compatible with complex matrices such as food products. Both of the extraction techniques were, therefore, developed accordingly, with the best overall procedures utilised in the final comparison study.

The physical and chemical properties of each target analyte were initially assessed before the selection of appropriate chemicals and procedures for the different extraction techniques. For instance, the presence of multiple basic amine groups within all PDE5 inhibitors necessitated a pH adjustment, as these analytes may exist in both neutral and ionised forms. Inevitably, it is crucial to suppress the ionisation of each target analyte in the initial aqueous phase, particularly for LLE, to facilitate its transfer into the organic phase. The aqueous phase was, therefore, assessed at pH 9, 10, and 11 based on the predicted pK_a

of target analytes. The best RE was achieved for most target analytes at pH 10 and subsequently used for the pre-treatment step.

The LLE was assessed using different types or combinations of organic solvents by varying their volume and number of repeated extractions. The SPE, in contrast, was evaluated with different types of sorbent such as silica-based (reversed-phase; C8 and C18) and polymeric-based (mixed-mode; weak and strong cation exchange), each with different bed weight and tube volume. The solvents for each SPE step were carefully selected based on the sorbent type, and the volume was optimised to achieve the best possible RE.

3.5.2 Comparison of sample extraction techniques

The determination of multiple PDE5 inhibitors with diverse chemical structures from complex matrices such as food products can be a challenging task. The distinct physical and chemical properties of each PDE5 inhibitor may additionally hinder the extraction efficiency. In this study, 23 target analytes were selected to represent different groups of PDE5 inhibitors based on their structural similarity. The ME and RE of these analytes in five different blank food matrices, i.e. PDM, HNY, JLY, HCD, and CWG, were compared using four conventional extraction techniques, namely, D&S, LLE, SPE, and modified QuEChERS. The relationship between matrix dilution and ionisation suppression or enhancement was also investigated at 1:2, 1:10, and 1:100 while maintaining the target analytes concentration at three QC levels.

The ME of each extraction technique at three levels of matrix dilutions is summarised in Fig. 3.5.2A and detailed in Tables 3.8A–C (supplementary data). Moderate ME, mostly ionisation suppressions, were evidenced for PDM at 1:2 matrix dilution using D&S, LLE, and SPE techniques within -48.1%–41.7%. Notably, the SPE led to the non-detection of one target analyte in the PDM matrix. These results revealed that PDM is the most challenging matrix among the food matrices; additionally exhibiting moderate- ionisation suppression between -33.9% and -21.6% for 12 target analytes, and ionisation enhancement of 41.7% for one target analyte using the SPE, followed by D&S, and to a lesser extend LLE at 1:2 matrix dilution. Contrarily, the modified QuEChERS resulted in insignificant ME within -8.7%–4.0% for all target analytes in the PDM matrix at the same dilution level. In summary, at least one target analyte in all of the food matrices exhibited acceptable ME at 1:2 dilution using either D&S, LLE, SPE, or modified QuEChERS, except those in HCD matrix. The HCD has shown to produce minimal matrix interferences which were substantiated through insignificant ME within -5.8%–8.8% of all target analytes, utilising different extraction techniques; even at the lowest level of matrix dilution.

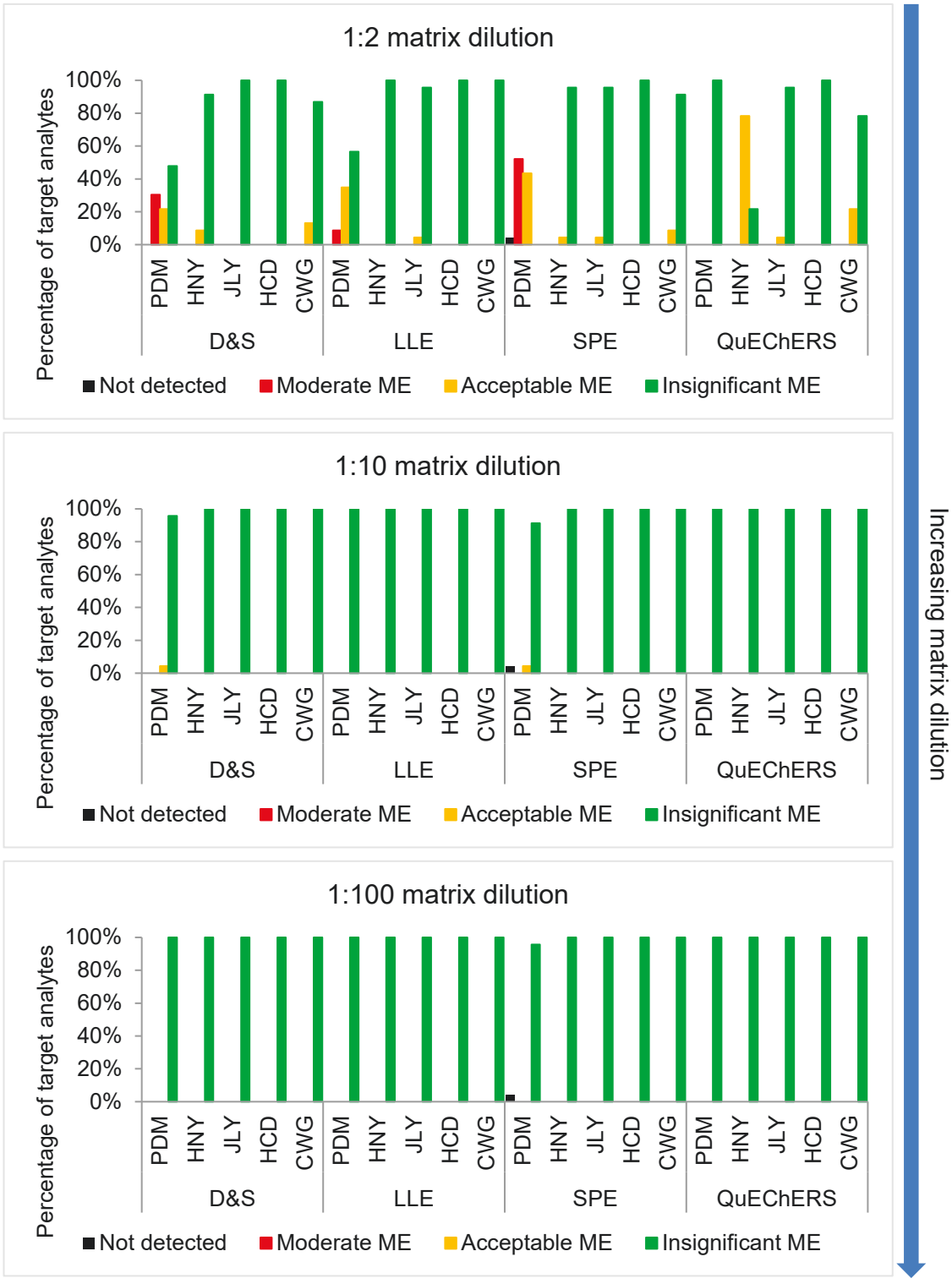


Fig. 3.5.2A: The matrix effect (ME) of dilute and shoot (D&S); liquid-liquid extraction (LLE); solid-phase extraction (SPE); and modified quick, easy, cheap, effective, rugged, and safe (QuEChERS) at 1:2, 1:10, and 1:100 matrix dilutions. Note: ME category: insignificant (0% to ±10%), acceptable (±10% to ±20%), moderate (±20% to ±50%), and severe (less than 50% or more than +50%) (Abbreviations: PDM, powdered drink mix; HNY, honey; JLY, jelly; HCD, hard candy; CWG, chewing gum)

The ME was minimised to insignificant percentages with increasing matrix dilutions from 1:2 to 1:100. These results demonstrated that matrix dilution is an effective strategy to mitigate the ME for each of the food matrices, regardless of the extraction technique used. However, higher levels of matrix dilutions may reduce the overall analytical method sensitivity, and therefore, should be optimised according to the ionisation efficiency of each target analyte to achieve accurate and precise quantification [32].

The non-detection of one target analyte, namely, mutaprodenafil in the PDM matrix using SPE was still prominent even at the highest level of 1:100 matrix dilution. These results could be attributed to severe ionisation suppression, leading to the non-detection of mutaprodenafil. Severe ionisation suppression in positive ESI mode was previously linked to (1) the competition in the sprayed solution between the matrix components and the analyte for access to the droplet surface during gas-phase emission, (2) matrix interferences competing for the available charge, (3) matrix components binding to an analyte or causing the analyte to co-precipitate, or (4) analyte ions which may be neutralised through gas-phase acid/base reactions [9]. Furthermore, SPE had previously shown to cause severe ionisation suppression due to the pre-concentration of the sample matrix [33].

Fig. 3.5.2B summarises the REs of LLE, SPE, and modified QuEChERS, while Tables 3.8D–F (supplementary data) provide the detailed results. LLE and SPE work through a similar principle, where analytes are partitioned between two different phases. A sample, usually prepared into an aqueous phase, is

partitioned using and an immiscible organic solvent for LLE and a solid sorbent for SPE. Therefore, comparable REs could be predicted using both LLE and SPE techniques. Specific target analytes, particularly those with sulphur-containing pyrazolopyrimidine-7-thione and imidazotriazine-4-thione, as well as acetyl-bonded pyrazolopyrimidine-7-one, often resulted in poor REs due to the greater selectivity of LLE and SPE. Nevertheless, the RE of LLE was slightly superior to SPE as it provided complete coverage of target analytes in each of the food matrices. SPE, in contrast, resulted in non-detection of N-desethylacetildenafil in HNY, JLY, HCD, and CWG; dimethylacetildenafil and udenafil in CWG; and mutaprodenafil in PDM, at different QC levels. Co-elution and, subsequently, pre-concentrated matrix components could have triggered these outcomes using the SPE. Still, the LLE and SPE have shown to produce acceptable RE for target analytes with Log P values of <2.0. These results suggest that SPE or LLE may not be the best choice to extract multiple ionisable analytes such as PDE5 inhibitors from the food matrices due to their diverse physical and chemical properties.

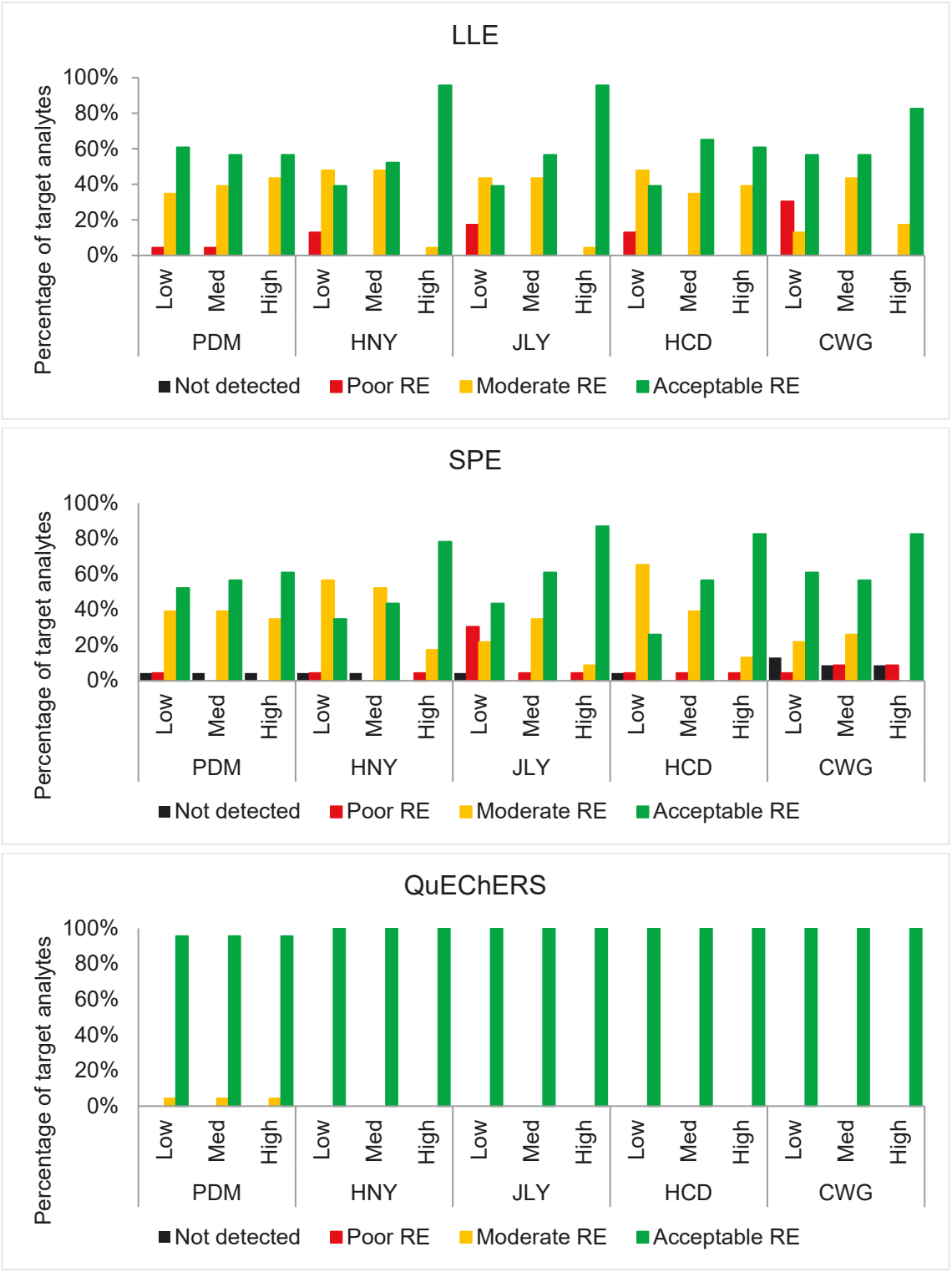


Fig. 3.5.2B: The extraction recovery (RE) of liquid-liquid extraction (LLE); solid-phase extraction (SPE); and modified quick, easy, cheap, effective, rugged, and safe (QuEChERS) at low (0.1 µg/mL); medium (Med, 0.4 µg/mL); and high (1 µg/mL) quality control levels. Note: RE category: acceptable (±25%), moderate (±25% to ±75%), and poor (less than -75% or more than +75%) (Abbreviations: PDM, powdered drink mix; HNY, honey; JLY, jelly; HCD, hard candy; CWG, chewing gum)

The modified QuEChERS procedure, on the other hand, resulted in acceptable RE for all target analytes in each of the food matrices within 75.5%–123.9%, except for carbodenafil in the PDM matrix at low (43.5%), medium (54.3%), and high (49.7%) QC levels. The modified QuEChERS is superior compared to D&S, LLE, and SPE; substantiated by acceptable and insignificant MEs, even at the lowest level of matrix dilution, as well as exhibiting acceptable RE for almost all target analytes in the selected food matrices. The procedure was, therefore, chosen as the standard extraction technique at 1:10 matrix dilution. Subsequently, it was validated for the determination of PDE5 inhibitors in each of the selected matrices.

3.5.3 Analytical method validation

The specificity, linearity, and sensitivity results are shown in Table 3.8G (supplementary data). The optimised chromatographic separation and the high-resolution MS data of the $[M+H]^+$ precursor ion established the specificity of each target analyte, while their identities were confirmed by the presence of two product ions from the tandem MS experiments. Interferences and carry-over effects, particularly from the extracted blank matrices, were not observed at the retention time of target analytes and in subsequent analysis, respectively. The linearity of each target analyte was verified by r^2 of >0.9960 within the selected range of 0.08–1.2 µg/mL. The LOD was established experimentally between 10 and 70 ng/mL, while the LOQ was set at 80 ng/mL for all target analytes.

The accuracy, repeatability, and intermediate precision results are presented in Tables 3.8H–J (supplementary data), respectively. The accuracy was satisfactory within 77.4%–122.0% for all target analytes at low, medium, and high QC levels. Similarly, the precision was acceptable with the %RSD of <16.7%. The repeatability and intermediate precision for all matrices were calculated within 0.1%–9.5% and 0.1%–16.7% of RSD, respectively, at all QC levels.

3.5.4 Determination of erectile dysfunction drugs in food samples

Table 3.5.4 compiles the analysis results of the 25 food samples. The suspected-target screening matched 24 samples with ten PDE5 inhibitors from the PCDL library. The tandem MS and retention time matching subsequently confirmed the identity of eight target analytes from 23 samples. The remaining two suspected analytes, i.e. propoxyphenyl-dimethylsildenafil and nortadalafil, were detected from samples HNY004 and HCD002, respectively. Sample HNY004 comprised other target analytes such as propoxyphenyl-thiodimethylsildenafil, thiodimethylsildenafil, and dimethylsildenafil, while sample HCD002 contained only one suspected analyte. The interrogations of MS and tandem MS data using top-down and bottom-up approaches of the non-targeted screening returned insignificant signals, indicating the absence of potentially novel PDE5 inhibitors analogue from all 25 samples, thus, confirming the PDM009 as the only non-adulterated sample.

Table 3.5.4: The identification of target analytes and the detection of suspected analytes in 25 food samples.

Sample	Target analytes identified / *suspected analytes detected (average weight per recommended intake in mg - quantification level)	Total average weight per recommended intake in mg	Total quantification level
PDM001	1. Sildenafil (0.34 - SUB) 2. Tadalafil (87.67 - SPR)	88.01	SPR
PDM002	1. Propoxyphenyl-thiohydroxyhomosildenafil (2.17 - SUB)	2.17	SUB
PDM003	1. Tadalafil (80.93 - SPR) 2. Thiodimethylsildenafil (1.08 - TRC) 3. Thiosildenafil (7.46 - SUB)	89.47	SPR
PDM004	Tadalafil (31.12 - SPR)	31.12	SPR
PDM005	1. Tadalafil (103.90 - SPR) 2. Thiosildenafil (<LOQ)	103.90	SPR
PDM006	1. Propoxyphenyl-thiohydroxyhomosildenafil (0.50 - TRC) 2. Thiodimethylsildenafil (0.43 - TRC) 3. Thiosildenafil (4.64 - SUB)	5.57	SUB
PDM007	1. Dimethylsildenafil (0.53 - SUB) 2. Sildenafil (0.13 - TRC) 3. Thiodimethylsildenafil (68.35 - THE) 4. Thiosildenafil (10.07 - SUB)	79.08	THE
PDM008	1. Tadalafil (38.54 - SPR)	38.54	SPR
PDM009	Not detected	NA	NA
PDM010	1. Dimethylsildenafil (0.27 - TRC) 2. Tadalafil (20.87 - SPR) 3. Thiodimethylsildenafil (33.86 - THE) 4. Thiosildenafil (6.03 - SUB) 5. Sildenafil (<LOQ)	61.03	SPR

PDM011	1. Tadalafil (9.63 - THE) 2. Thiodimethylsildenafil (24.60 - SUB) 3. Thiosildenafil (<LOQ)	34.23	THE
PDM012	1. Dimethylsildenafil (0.12 - TRC) 2. Tadalafil (28.89 - SPR) 3. Thiodimethylsildenafil (13.68 - SUB) 4. Thiosildenafil (0.20 - SUB) 5. Sildenafil (<LOQ)	42.89	SPR
PDM013	1. Sildenafil (4.60 - SUB) 2. Tadalafil (23.53 - SPR)	28.13	SPR
PDM014	1. Sildenafil (33.70 - THE) 2. Tadalafil (18.49 - THE)	52.19	THE
PDM015	1. Thiodimethylsildenafil (55.32 - THE) 2. Dimethylsildenafil (<LOQ)	55.32	THE
PDM016	1. Tadalafil (79.39 - SPR)	79.39	SPR
HNY001	1. Sildenafil (2.18 - TRC) 2. Thiosildenafil (36.17 - THE)	38.35	THE
HNY002	1. Sildenafil (0.93 - TRC) 2. Thiosildenafil (60.93 - THE)	61.86	THE
HNY003	1. Tadalafil (3.70 - SUB)	3.70	SUB
HNY004	1. Propoxyphenyl-thiodimethylsildenafil (33.36 - THE) 2. Thiodimethylsildenafil (3.85 - SUB) 3. Dimethylsildenafil (<LOQ) 4. Propoxyphenyl-dimethylsildenafil*	37.21	THE
JLY001	1. Vardenafil (17.86 - THE)	17.86	THE
JLY002	1. Sildenafil (148.37 - SPR)	148.37	SPR
HCD001	1. Tadalafil (49.84 - SPR)	49.84	SPR
HCD002	1. Nortadalafil*	NA	NA
CWG001	1. Sildenafil (0.32 - SUB) 2. Thiosildenafil (1.50 - SUB)	1.82	SUB

(Abbreviations: PDM, powdered drink mix; HNY, honey; JLY, jelly; HCD, hard candy; CWG, chewing gum; LOQ, limit of quantification; TRC, trace; SUB, subtherapeutic; THE, therapeutic; SPR, supratherapeutic; NA, not applicable)

Fig. 3.5.4 summarises the identification of target analytes and the detection of suspected analytes in all of the food samples. The majority of the adulterants were those of the approved PDE5 inhibitors such as sildenafil and tadalafil. Top of the list is tadalafil, identified in five samples as a sole adulterant, and also in combination with other PDE5 inhibitors in another eight samples. Sildenafil was identified in ten samples, mostly in combination with other PDE5 inhibitors. This study also identified other PDE5 inhibitors as follows: thiosildenafil (ten samples); thiodimethylsildenafil (eight samples); dimethylsildenafil (five samples); propoxyphenyl-thiohydroxyhomosildenafil (two samples); and propoxyphenyl-thiodimethylsildenafil and vardenafil (one sample each) with up to five different adulterants per sample.

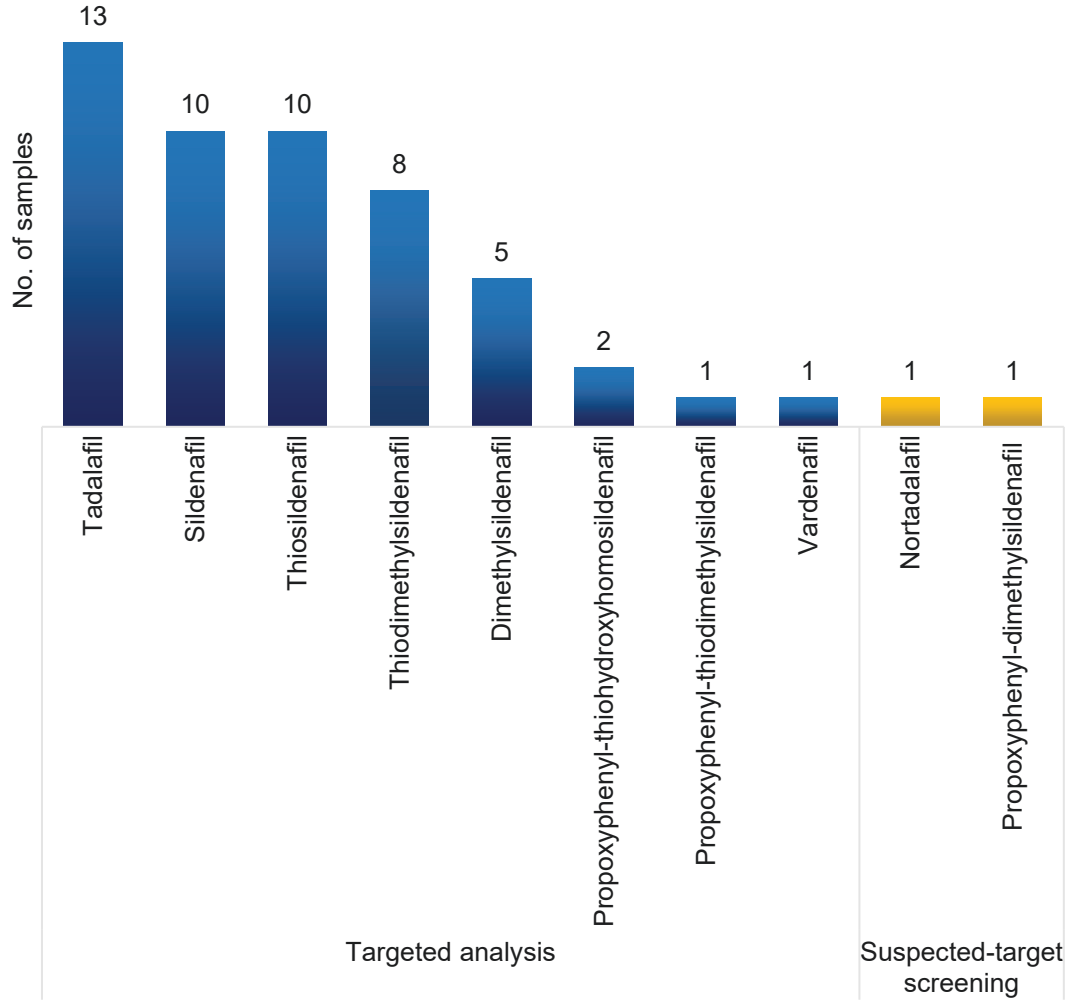


Fig. 3.5.4: Results summary of erectile dysfunction drugs in adulterated food samples; with identification of target analytes and detection of suspected analytes.

Briefly, only 23 samples were quantified, excluding samples HCD002 and PDM009. The target analytes were quantified from 1.82 to 148.37 mg per recommended intake, as specified on the products' labels. The quantification levels were divided into subtherapeutic, therapeutic, and supratherapeutic based on the recommended dose of the approved PDE5 inhibitors (i.e. 25–100 mg for sildenafil and 5–20 mg for vardenafil and tadalafil) [34]. In summary, four samples were quantified at subtherapeutic level, while another eight and 11 samples were quantified at therapeutic and supratherapeutic levels, respectively. The

supratherapeutic levels of tadalafil in ten samples were of grave concern, especially for sample PDM005, quantified at 103.90 mg per sachet, which exceeded five times of tadalafil maximum daily dose. Tadalafil possesses the longest duration of action among all of the approved PDE5 inhibitors [35]. Therefore, there is a strong probability of developing delayed side effects due to extended exposure of tadalafil in the systemic circulation [36]. At supratherapeutic level, tadalafil might lead to an even higher incidence of side effects, posing severe health and life-threatening risks to consumers.

3.6 CONCLUSION

In the present work, four conventional extraction techniques, i.e. D&S, LLE, SPE, and QuEChERS, were compared for an accurate and precise determination of ED drugs in selected food matrices. Each of the extraction techniques was assessed based on the ME and RE performance. The modified QuEChERS provided complete coverage of target analytes, as well as exhibiting insignificant ME and satisfactory RE for almost all target analytes in the selected food matrices. The modified QuEChERS was subsequently validated for each of the food matrices and applied to determine ED drugs in 25 food samples claiming to enhance male sexual performance. The suspected-target and non-targeted screenings, together with targeted analysis of an LC-QTOF-MS, revealed 24 adulterated food samples with 11 of them quantified at supratherapeutic levels. The comprehensive strategies discussed in this study would be beneficial to curb the widespread adulteration of PDE5 inhibitors in food products, and more importantly, to safeguard the consumers from potentially short- and long-term health problems which could lead to life-threatening crises.

3.7 REFERENCES

1. Muschietti L, Redko F, Ulloa J. Adulterants in selected dietary supplements and their detection methods. *Drug Test Anal* 2020. doi:https://doi.org/10.1002/dta.2806
2. Fibigr J, Šatínský D, Solich P. Current trends in the analysis and quality control of food supplements based on plant extracts. *Anal Chim Acta* 2018;1036:1-15. doi:https://doi.org/10.1016/j.aca.2018.08.017
3. Kee C-L, Ge X, Tran C-S, Bui Q-D, Cheah N-P, Low M-Y. Isolation and characterization of N-hydroxyethyl dithio-desethyl carbodenafil from a health supplement. *J Pharm Biomed Anal* 2020;189:113431. doi:https://doi.org/10.1016/j.jpba.2020.113431
4. Gu Y, Hu Q, Sun J, et al. Isolation and identification of a new sildenafil analogue, hydroxycarbodenafil, found as an adulterant in a health supplement. *J Pharm Biomed Anal* 2020;185:113222. doi:https://doi.org/10.1016/j.jpba.2020.113222
5. Skalicka-Wozniak K, Georgiev MI, Orhan IE. Adulteration of herbal sexual enhancers and slimmers: the wish for better sexual well-being and perfect body can be risky. *Food Chem Toxicol* 2017;108:355-364. doi:https://doi.org/10.1016/j.fct.2016.06.018
6. Ekar T, Kreft S. Common risks of adulterated and mislabeled herbal preparations. *Food Chem Toxicol* 2019;123:288-297. doi:https://doi.org/10.1016/j.fct.2018.10.043

7. Vaclavik L, Krynitsky AJ, Rader JI. Mass spectrometric analysis of pharmaceutical adulterants in products labeled as botanical dietary supplements or herbal remedies: a review. *Anal Bioanal Chem* 2014;406(27):6767-6790. doi:https://doi.org/10.1007/s00216-014-8159-z

8. Smyth WF, Rodriguez V. Recent studies of the electrospray ionisation behaviour of selected drugs and their application in capillary electrophoresis–mass spectrometry and liquid chromatography–mass spectrometry. *J Chromatogr A* 2007;1159(1):159-174. doi:https://doi.org/10.1016/j.chroma.2007.05.003

9. Furey A, Moriarty M, Bane V, Kinsella B, Lehane M. Ion suppression; a critical review on causes, evaluation, prevention and applications. *Talanta* 2013;115:104-122. doi:https://doi.org/10.1016/j.talanta.2013.03.048

10. Kruve A, Rebane R, Kipper K, et al. Tutorial review on validation of liquid chromatography-mass spectrometry methods: part I. *Anal Chim Acta* 2015;870:29-44. doi:https://doi.org/10.1016/j.aca.2015.02.017

11. Zou P, Oh SS-Y, Hou P, Low M-Y, Koh H-L. Simultaneous determination of synthetic phosphodiesterase-5 inhibitors found in a dietary supplement and pre-mixed bulk powders for dietary supplements using high-performance liquid chromatography with diode array detection and liquid chromatography–electrospray ionization tandem mass spectrometry. *J Chromatogr A* 2006;1104(1-2):113-122. doi:https://doi.org/10.1016/j.chroma.2005.11.103

12. Shi S, Wu Y, Zhou M, Cheng Q. Simultaneous analysis of 31 anti-impotence compounds potentially illegally added to herbal-based dietary supplements by ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry. *J Chromatogr B* 2020;1144:122077. doi:https://doi.org/10.1016/j.jchromb.2020.122077

13. Jiru M, Stranska-Zachariasova M, Dzuman Z, et al. Analysis of phosphodiesterase type 5 inhibitors as possible adulterants of botanical-based dietary supplements: extensive survey of preparations available at the Czech market. *J Pharm Biomed Anal* 2019;164:713-724. doi:https://doi.org/10.1016/j.jpba.2018.11.007

14. Jeong JH, Lee JH, Kim HJ, et al. LC-ESI-MS/MS analysis of phosphodiesterase-5 inhibitors and their analogues in foods and dietary supplements in Korea. *Food Addit Contam Part B Surveill* 2016;9(1):1-8. doi:https://doi.org/10.1080/19393210.2014.968220

15. Strano-Rossi S, Odoardi S, Castrignano E, Serpelloni G, Chiarotti M. Liquid chromatography-high resolution mass spectrometry (LC-HRMS) determination of stimulants, anorectic drugs and phosphodiesterase 5 inhibitors (PDE5I) in food supplements. *J Pharm Biomed Anal* 2015;106:144-152. doi:https://doi.org/10.1016/j.jpba.2014.06.011

16. Kim EH, Seo HS, Ki NY, et al. Reliable screening and confirmation of 156 multi-class illegal adulterants in dietary supplements based on extracted common ion chromatograms by ultra-high-performance liquid chromatography-quadrupole/time of flight-mass spectrometry. *J Chromatogr A* 2017;1491:43-56. doi:https://doi.org/10.1016/j.chroma.2017.02.032

17. Park SR, Lee JG, Roh SH, et al. Determination of PDE-5 inhibitors and appetite suppressants in adulterated dietary supplements using LC/PDA and LC/MS. *Food Addit Contam Part B Surveill* 2012;5(1):29-32. doi:https://doi.org/10.1080/19393210.2012.656706

18. Gratz SR, Gamble BM, Flurer RA. Accurate mass measurement using Fourier transform ion cyclotron resonance mass spectrometry for structure elucidation of designer drug analogs of tadalafil, vardenafil and sildenafil in herbal and pharmaceutical matrices. *Rapid Commun Mass Spectrom* 2006;20(15):2317-2327. doi:https://doi.org/10.1002/rcm.2594

19. Lucas D. Sample prep for trace analysis of adulterants in erectile dysfunction dietary supplements. 2014. https://www.agilent.com/cs/library/applications/5991-3638EN.pdf. Accessed 20 May 2020.

20. Vaclavik L, Krynitsky AJ, Rader JI. Targeted analysis of multiple pharmaceuticals, plant toxins and other secondary metabolites in herbal dietary supplements by ultra-high performance liquid chromatography-quadrupole-orbital ion trap mass spectrometry. *Anal Chim Acta* 2014;810:45-60. doi:https://doi.org/10.1016/j.aca.2013.12.006

21. Ding B, Wang Z, Xie J, Zeng G, Chen W. Phosphodiesterase-5 inhibitors in Chinese tonic liquors by liquid chromatography coupled with quadrupole time of flight mass spectrometry. *Food Addit Contam Part B Surveill* 2018;11(3):214-222. doi:https://doi.org/10.1080/19393210.2018.1488769

22. Trufelli H, Palma P, Famiglini G, Cappiello A. An overview of matrix effects in liquid chromatography-mass spectrometry. *Mass Spectrom Rev* 2011;30(3):491-509. doi:https://doi.org/10.1002/mas.20298

23. Perestrelo R, Silva P, Porto-Figueira P, et al. QuEChERS - fundamentals, relevant improvements, applications and future trends. *Anal Chim Acta* 2019;1070:1-28. doi:https://doi.org/10.1016/j.aca.2019.02.036

24. Mohd Yusop AY, Xiao L, Fu S. Determination of phosphodiesterase 5 (PDE5) inhibitors in instant coffee premixes using liquid chromatography-high-resolution mass spectrometry (LC-HRMS). *Talanta* 2019;204:36-43. doi:https://doi.org/10.1016/j.talanta.2019.05.078

25. Ozturk Er E, Ozbek B, Bakirdere S. Accurate and sensitive determination of sildenafil, tadalafil, vardenafil, and avanafil in illicit erectile dysfunction medications and human urine by LC with quadrupole-TOF-MS/MS and their behaviors in simulated gastric conditions. *J Sep Sci* 2019;42(2):475-483. doi:https://doi.org/10.1002/jssc.201800734

26. Gilard V, Balayssac S, Tinaugus A, Martins N, Martino R, Malet-Martino M. Detection, identification and quantification by ^{1}H NMR of adulterants in 150 herbal dietary supplements marketed for improving sexual performance. *J Pharm Biomed Anal* 2015;102:476-493. doi:https://doi.org/10.1016/j.jpba.2014.10.011

27. Do TT, Theocharis G, Reich E. Simultaneous detection of three phosphodiesterase type 5 inhibitors and eight of their analogs in lifestyle products and screening for adulterants by high-performance thin-layer chromatography. *J AOAC Int* 2015;98(5):1226-1233. doi:https://doi.org/10.5740/jaoacint.14-285

28. Mohd Yusop AY, Xiao L, Fu S. Suspected-target and non-targeted screenings of phosphodiesterase 5 inhibitors in herbal remedies by liquid chromatography-quadrupole time-of-flight mass spectrometry. *Drug Test Anal* 2020. doi:https://doi.org/10.1002/dta.2861

29. Kruve A, Rebane R, Kipper K, et al. Tutorial review on validation of liquid chromatography-mass spectrometry methods: part II. *Anal Chim Acta* 2015;870:8-28. doi:https://doi.org/10.1016/j.aca.2015.02.016

30. ICH. ICH harmonized tripartite guideline: validation of analytical procedures: text and methodology Q2(R1). In: International Conference on Harmonization of technical requirements for registration of pharmaceuticals for human use; 2005.

31. Mohd Yusop AY, Xiao L, Fu S. Data on the optimisation and validation of a liquid chromatography-high-resolution mass spectrometry (LC-HRMS) to establish the presence of phosphodiesterase 5 (PDE5) inhibitors in instant coffee premixes. *Data Brief* 2019;25:104234. doi:https://doi.org/10.1016/j.dib.2019.104234

32. Yang P, Chang JS, Wong JW, et al. Effect of Sample Dilution on Matrix Effects in Pesticide Analysis of Several Matrices by Liquid Chromatography–High-Resolution Mass Spectrometry. *J Agric Food Chem* 2015;63(21):5169-5177. doi:https://doi.org/10.1021/jf505168v

33. Dams R, Huestis MA, Lambert WE, Murphy CM. Matrix effect in bio-analysis of illicit drugs with LC-MS/MS: influence of ionization type, sample preparation, and biofluid. *J Am Soc Mass Spectrom* 2003;14(11):1290-1294. doi:https://doi.org/10.1016/S1044-0305(03)00574-9

34. Huang SA, Lie JD. Phosphodiesterase-5 (PDE_5) inhibitors in the management of erectile dysfunction. *Pharm Ther* 2013;38(7):407-419.

35. Zurawin JL, Stewart CA, Anaissie JE, Yafi FA, Hellstrom WJ. Avanafil for the treatment of erectile dysfunction. *Expert Rev Clin Pharmacol* 2016;9(9):1163-1170. doi:https://doi.org/10.1080/17512433.2016.1212655

36. Campbell HE. Clinical monograph for drug formulary review: erectile dysfunction agents. *J Manag Care Spec Pharm* 2005;11(2):151-171. doi:https://doi.org/10.18553/jmcp.2005.11.2.151

Table 3.8A: Matrix effect (ME) of 23 targeted phosphodiesterase 5 (PDE5) inhibitors using dilute and shoot (D&S), liquid-liquid extraction (LLE), solid-phase extraction (SPE), and modified quick, easy, cheap, effective, rugged, and safe (QuEChERS) technique at 1:2 matrix dilution.

No.	Target analytes	ME (%) (n = 9) at 1:2 matrix dilution									
		D&S					LLE				
		PDM	HNY	JLY	HCD	CWG	PDM	HNY	JLY	HCD	CWG
1	Desmethylcarbodenafil	-8.2	4.8	-1.8	6.9	5.4	-2.1	0.0	3.3	-3.1	4.8
2	Carbodenafil	-48.1	1.8	-2.9	3.9	0.2	-2.3	1.8	7.0	-3.7	2.4
3	N-desethylacetildenafil	-14.0	-0.7	-4.6	5.4	4.3	-10.4	1.6	4.8	-3.2	0.5
4	Acetildenafil	-9.7	-0.9	0.1	-4.3	1.5	22.6	1.1	11.7	0.1	0.1
5	Hydroxyvardenafil	-7.5	8.4	0.1	5.2	5.2	19.9	-1.0	3.1	-4.5	2.4
6	Dimethylacetildenafil	-11.4	3.6	-2.3	-0.7	10.3	21.8	5.8	-6.4	-0.1	0.0
7	Vardenafil	-7.4	10.3	-0.9	7.4	7.6	-6.8	2.2	5.5	-2.4	0.7
8	Sildenafil	-8.8	5.3	-3.9	2.5	0.1	-6.7	0.2	2.3	-2.4	-2.4
9	Homosildenafil	-7.8	6.6	-3.4	3.7	1.9	-8.0	2.5	2.4	-0.6	-0.7
10	Dimethylsildenafil	-8.7	6.7	-0.6	7.1	-0.3	-6.7	1.2	3.8	-2.0	-1.3
11	Propoxyphenyl-hydroxyhomosildenafil	-7.7	7.8	-2.2	5.4	-4.3	-9.3	2.2	3.4	-3.4	-6.6
12	Udenafil	-8.4	2.5	-3.9	4.8	0.9	-7.7	2.5	7.6	-2.6	-1.4
13	Propoxyphenyl-sildenafil	-6.5	7.1	-3.5	3.8	1.2	-7.2	3.5	2.4	-1.8	0.1
14	Hydroxythiovardenafil	-18.2	10.5	0.3	6.3	-6.9	-7.3	-0.6	5.5	-1.1	-5.3
15	Tadalafil	-18.6	-5.4	-3.6	-2.0	-7.8	-14.7	3.1	-1.1	-1.2	0.1
16	Mirodenafil	-5.9	3.0	-1.8	1.3	-4.9	-7.4	0.0	2.1	-3.1	-5.8
17	Mutaprodenafil	-11.3	7.3	-1.8	5.0	-8.7	-6.9	3.4	4.3	-2.0	-6.4
18	Thiosildenafil	-22.0	6.1	-4.5	4.4	-10.8	-13.3	3.2	2.8	-1.1	-4.3
19	Thiohomosildenafil	-21.8	4.9	-2.3	2.8	-1.0	-5.2	7.5	5.5	0.6	1.0
20	Dithiodesmethylcarbodenafil	-20.3	5.7	-4.7	8.8	-3.5	10.7	7.6	4.2	-0.8	-1.3
21	Thiodimethylsildenafil	-21.4	4.4	-3.7	5.0	-2.5	-13.7	6.2	5.5	-3.1	-0.7
22	Propoxyphenyl-thiohydroxyhomosildenafil	-24.4	8.4	-3.2	2.6	-13.1	-17.1	8.7	2.3	-1.8	-5.9
23	Propoxyphenyl-thiodimethylsildenafil	-20.6	2.2	-3.9	4.2	-7.5	-13.1	5.3	4.4	-2.8	0.5

Table 3.8A: Continued.

No.	Target analytes	ME (%) (n = 9) at 1:2 matrix dilution									
		SPE					QuEChERS				
		PDM	HNY	JLY	HCD	CWG	PDM	HNY	JLY	HCD	CWG
1	Desmethylcarbodenafil	-12.3	0.0	3.8	-2.8	11.1	4.0	15.1	4.3	7.9	-0.7
2	Carbodenafil	-22.4	-0.4	5.4	-1.5	4.6	-3.6	12.3	5.5	-0.5	-4.1
3	N-desethylacetildenafil	-25.3	-1.3	-5.1	-5.8	5.2	-5.9	7.5	5.4	-2.6	-5.1
4	Acetildenafil	-21.6	2.1	12.6	-1.4	10.3	-5.2	6.4	0.5	1.8	-8.2
5	Hydroxyvardenafil	-16.9	3.6	1.3	-0.5	6.4	2.6	18.1	9.0	4.1	3.0
6	Dimethylacetildenafil	-22.7	0.3	-9.4	-2.7	6.6	-3.0	5.6	7.1	1.3	-2.1
7	Vardenafil	-17.8	3.4	6.4	1.9	8.6	0.5	19.6	8.3	4.7	3.3
8	Sildenafil	-24.1	2.4	0.6	-2.5	6.3	0.3	14.2	8.6	3.0	-2.1
9	Homosildenafil	-16.9	1.6	3.0	-1.0	6.5	0.3	13.9	7.1	1.4	0.3
10	Dimethylsildenafil	-12.5	-0.7	4.4	-1.0	7.9	0.0	13.6	7.2	-0.7	-2.4
11	Propoxyphenyl-hydroxyhomosildenafil	-18.3	1.2	2.6	-3.4	2.4	-0.2	16.6	7.5	2.1	-6.2
12	Udenafil	-10.9	4.0	6.3	-0.8	4.7	-2.0	9.6	5.7	3.5	-4.7
13	Propoxyphenyl-sildenafil	-19.8	0.0	2.4	-2.2	4.4	1.0	14.1	7.8	0.3	-3.5
14	Hydroxythiovardenafil	-18.1	5.4	2.3	0.4	-1.1	-4.6	16.5	8.6	4.4	-7.9
15	Tadalafil	41.7	-10.9	1.6	-4.9	1.3	-8.7	3.7	6.0	-3.4	-10.4
16	Mirodenafil	-16.9	3.2	1.1	-2.2	3.4	0.0	11.1	8.4	0.6	-6.6
17	Mutaprodenafil	ND	1.9	3.0	-2.0	0.8	-2.9	13.5	5.9	3.3	-12.6
18	Thiosildenafil	-30.6	-0.2	1.5	-2.5	1.5	-2.9	13.2	9.9	4.9	-13.5
19	Thiohomosildenafil	-27.6	-0.1	3.2	0.0	5.1	-3.4	12.8	7.8	2.4	-4.9
20	Dithiodesmethylcarbodenafil	-33.9	-5.1	2.6	0.1	5.2	-2.3	10.4	9.1	1.8	-5.4
21	Thiodimethylsildenafil	-26.5	-1.4	5.7	0.3	3.4	-3.1	11.4	6.1	3.4	-6.2
22	Propoxyphenyl-thiohydroxyhomosildenafil	-28.8	-2.2	0.5	-1.6	2.8	-8.1	15.6	10.5	-1.1	-15.5
23	Propoxyphenyl-thiodimethylsildenafil	-27.3	-2.8	4.7	1.3	6.9	-6.9	10.5	7.1	2.1	-12.0

(Abbreviations: PDM, powdered drink mix; HNY, honey; JLY, jelly; HCD, hard candy; CWG, chewing gum)

Table 3.8B: Matrix effect (ME) of 23 targeted phosphodiesterase 5 (PDE5) inhibitors using dilute and shoot (D&S), liquid-liquid extraction (LLE), solid-phase extraction (SPE), and modified quick, easy, cheap, effective, rugged, and safe (QuEChERS) technique at 1:10 matrix dilution.

No.	Target analytes	ME (%) (n = 9) at 1:10 matrix dilution									
		D&S					LLE				
		PDM	HNY	JLY	HCD	CWG	PDM	HNY	JLY	HCD	CWG
1	Desmethylcarbodenafil	-7.1	-6.6	-0.5	8.7	3.7	-4.1	-2.6	-1.2	-0.8	7.6
2	Carbodenafil	-14.3	-7.5	-0.1	2.0	4.1	1.5	-2.1	0.9	-1.4	3.0
3	N-desethylacetildenafil	5.5	-9.0	-0.5	3.4	5.1	-8.1	-2.3	-1.3	0.7	3.0
4	Acetildenafil	7.7	-7.1	-4.5	1.4	5.3	-2.8	-3.6	-2.6	2.1	3.0
5	Hydroxyvardenafil	2.4	-4.8	1.5	0.5	5.4	-0.6	-3.8	2.4	-0.6	2.3
6	Dimethylacetildenafil	4.6	-3.5	-3.3	1.0	0.6	-2.4	2.4	-3.7	2.3	2.3
7	Vardenafil	2.0	-3.9	-2.1	2.8	2.8	1.5	1.0	1.3	2.0	3.2
8	Sildenafil	1.6	-3.9	2.4	6.8	4.9	-0.6	-4.7	1.4	1.4	3.4
9	Homosildenafil	1.7	-1.8	1.1	3.3	5.0	-0.5	-3.5	-1.0	2.0	4.7
10	Dimethylsildenafil	1.5	-4.3	2.2	2.3	4.5	-0.7	-2.3	-0.5	0.6	3.4
11	Propoxyphenyl-hydroxyhomosildenafil	0.2	-1.1	3.5	5.8	7.7	-1.2	-3.0	3.4	2.6	4.6
12	Udenafil	1.8	-3.4	-2.1	4.1	1.4	-1.6	-2.8	3.5	1.1	4.6
13	Propoxyphenyl-sildenafil	-0.3	-2.7	2.2	4.4	4.6	1.5	-5.6	0.7	0.7	2.7
14	Hydroxythiovardenafil	3.1	-3.6	-0.3	2.2	0.9	0.1	-1.6	2.3	1.2	2.7
15	Tadalafil	-2.4	-4.9	3.0	5.8	7.2	-2.3	-2.0	5.2	8.2	3.6
16	Mirodenafil	0.8	-5.8	1.8	-0.1	3.1	0.7	-2.3	2.2	2.6	0.6
17	Mutaprodenafil	0.1	-0.2	-1.0	7.4	2.3	-1.3	-1.2	1.3	2.1	3.0
18	Thiosildenafil	7.9	1.4	0.6	5.7	2.7	0.3	-1.8	2.1	2.1	4.8
19	Thiohomosildenafil	6.7	-2.1	-1.1	3.4	3.1	4.8	-2.0	2.1	2.2	4.3
20	Dithiodesmethylcarbodenafil	1.9	-0.4	0.2	5.5	2.3	0.1	-1.6	2.7	2.2	3.7
21	Thiodimethylsildenafil	5.5	-2.3	-1.4	5.0	3.0	-3.0	0.9	1.7	-1.4	3.0
22	Propoxyphenyl-thiohydroxyhomosildenafil	3.1	-0.7	2.7	4.0	3.7	-1.0	-5.1	4.8	3.3	6.5
23	Propoxyphenyl-thiodimethylsildenafil	4.5	-3.8	-1.1	4.0	5.2	-0.2	-1.1	1.3	-1.3	4.2

Table 3.8B: Continued.

No.	Target analytes	ME (%) (n = 9) at 1:10 matrix dilution									
		SPE					QuEChERS				
		PDM	HNY	JLY	HCD	CWG	PDM	HNY	JLY	HCD	CWG
1	Desmethylcarbodenafil	3.4	3.7	3.6	2.3	7.6	6.3	3.7	-4.1	4.4	2.9
2	Carbodenafil	-0.7	0.2	3.5	0.7	3.0	3.8	3.5	-3.1	-1.6	2.3
3	N-desethylacetildenafil	-2.3	1.5	1.7	-6.8	3.0	4.8	4.9	-1.0	0.8	0.3
4	Acetildenafil	-3.8	1.5	2.6	-2.3	3.0	4.4	4.1	-9.2	-1.5	-3.5
5	Hydroxyvardenafil	-1.0	0.3	4.5	0.4	2.3	5.7	1.7	-0.2	-0.4	3.9
6	Dimethylacetildenafil	-4.2	0.0	-6.9	-5.0	2.3	4.4	1.7	4.9	-4.5	-1.8
7	Vardenafil	-2.4	-2.3	3.5	-1.9	3.2	5.2	7.8	-1.0	-1.4	0.2
8	Sildenafil	-2.3	2.5	3.6	3.0	3.4	3.7	3.8	-1.5	0.7	-0.1
9	Homosildenafil	-1.3	0.2	5.4	-2.9	4.7	4.4	4.9	-1.1	-1.3	0.1
10	Dimethylsildenafil	0.6	0.3	2.7	-0.6	3.4	3.1	4.6	-0.5	-2.9	1.0
11	Propoxyphenyl-hydroxyhomosildenafil	-1.2	3.2	6.6	3.4	4.6	5.2	6.3	-0.6	3.0	-0.2
12	Udenafil	-2.8	-0.1	6.0	0.3	4.6	5.8	5.7	-1.4	0.1	-4.8
13	Propoxyphenyl-sildenafil	-1.6	3.1	6.0	0.8	2.7	4.8	2.3	-1.2	0.4	0.4
14	Hydroxythiovardenafil	-2.3	-2.4	2.5	-1.0	2.7	4.2	4.7	-0.4	-2.9	-0.7
15	Tadalafil	-12.6	6.6	7.6	8.1	3.6	2.7	6.8	-1.2	5.2	-2.8
16	Mirodenafil	-5.9	2.0	7.4	1.7	0.6	8.8	-0.1	2.9	2.6	1.8
17	Mutaprodenafil	ND	0.4	2.2	-1.6	3.0	5.1	8.1	-1.4	-0.9	-5.2
18	Thiosildenafil	-4.4	0.2	5.7	-0.3	4.8	4.1	7.2	-0.2	0.8	-4.1
19	Thiohomosildenafil	-4.0	0.4	1.1	-2.1	4.3	4.6	6.1	0.9	0.9	-2.7
20	Dithiodesmethylcarbodenafil	-5.3	-2.6	2.2	0.9	3.7	4.2	4.7	1.6	2.0	0.5
21	Thiodimethylsildenafil	-4.3	5.0	2.5	0.0	3.0	5.1	5.4	-1.0	-1.1	-1.3
22	Propoxyphenyl-thiohydroxyhomosildenafil	-1.4	4.6	6.4	4.4	6.5	1.1	4.9	1.1	2.0	-3.5
23	Propoxyphenyl-thiodimethylsildenafil	-4.2	1.7	2.1	-0.2	4.2	2.2	5.4	0.7	-1.8	-2.6

(Abbreviations: PDM, powdered drink mix; HNY, honey; JLY, jelly; HCD, hard candy; CWG, chewing gum)

Table 3.8C: Matrix effect (ME) of 23 targeted phosphodiesterase 5 (PDE5) inhibitors using dilute and shoot (D&S), liquid-liquid extraction (LLE), solid-phase extraction (SPE), and modified quick, easy, cheap, effective, rugged, and safe (QuEChERS) technique at 1:100 matrix dilution.

No.	Target analytes	ME (%) (n = 9) at 1:100 matrix dilution									
		D&S					LLE				
		PDM	HNY	JLY	HCD	CWG	PDM	HNY	JLY	HCD	CWG
1	Desmethylcarbodenafil	0.2	-6.8	-0.5	2.3	1.5	-5.4	-0.1	1.4	-6.7	3.0
2	Carbodenafil	-1.2	-6.1	0.2	-2.6	4.8	-0.2	-0.6	0.9	-5.6	3.0
3	N-desethylacetildenafil	2.8	-6.3	1.5	2.2	0.5	-2.1	-2.1	0.0	-3.8	2.1
4	Acetildenafil	2.2	-4.7	-2.1	-2.3	2.9	-3.3	-2.6	-0.5	-3.8	1.2
5	Hydroxyvardenafil	0.1	-4.8	0.6	-3.1	0.0	-1.8	0.1	-0.1	-4.6	3.4
6	Dimethylacetildenafil	1.1	-3.6	-1.9	2.9	-1.5	-3.1	3.7	-1.8	-7.1	1.8
7	Vardenafil	-1.0	-4.8	-2.1	-0.5	-0.8	-0.7	1.7	-0.4	-2.8	2.2
8	Sildenafil	-1.1	-3.3	-0.4	3.4	3.3	-0.3	-3.8	0.8	-3.1	3.6
9	Homosildenafil	0.5	-3.6	-0.8	-0.5	0.6	-4.3	-3.1	-1.0	-3.9	2.3
10	Dimethylsildenafil	-0.8	-2.4	0.9	0.1	1.6	-2.5	-2.4	-0.8	-2.5	3.1
11	Propoxyphenyl-hydroxyhomosildenafil	-0.9	-2.4	1.2	-0.1	3.0	-1.8	-2.7	1.1	-2.9	2.6
12	Udenafil	-0.4	-4.7	-3.8	0.7	1.4	-1.0	-0.5	2.2	-4.6	3.7
13	Propoxyphenyl-sildenafil	-1.1	-3.9	-2.9	1.5	0.0	-4.2	-0.4	-0.7	-3.4	4.0
14	Hydroxythiovardenafil	-1.8	-3.4	-0.8	-2.0	-0.8	-2.3	2.0	-0.2	-3.5	2.6
15	Tadalafil	1.3	-3.3	0.5	3.0	3.9	-2.1	-1.5	3.8	2.1	4.0
16	Mirodenafil	-4.0	-2.4	-1.8	-3.3	4.2	-0.5	-1.6	1.6	-1.7	3.6
17	Mutaprodenafil	-0.8	-2.9	-2.5	3.3	0.4	-2.3	0.0	-0.6	-3.7	2.8
18	Thiosildenafil	-2.3	-2.3	-1.3	4.3	1.7	1.0	-0.1	0.6	-2.9	3.8
19	Thiohomosildenafil	-2.2	-2.0	-1.4	-0.1	1.7	2.5	0.9	1.3	-2.6	2.7
20	Dithiodesmethylcarbodenafil	-4.1	-2.3	0.5	1.0	-0.4	-3.0	-1.9	0.3	-2.0	2.6
21	Thiodimethylsildenafil	-1.9	-3.4	-1.5	2.0	1.5	-4.2	3.1	2.3	-4.5	3.7
22	Propoxyphenyl-thiohydroxyhomosildenafil	-2.7	-0.6	0.9	2.1	1.8	-1.1	-5.3	2.3	-1.3	4.0
23	Propoxyphenyl-thiodimethylsildenafil	-0.5	-3.1	-0.6	0.0	3.5	-2.3	1.6	1.2	-4.6	2.8

Table 3.8C: Continued.

No.	Target analytes	ME (%) (n = 9) at 1:100 matrix dilution									
		SPE					QuEChERS				
		PDM	HNY	JLY	HCD	CWG	PDM	HNY	JLY	HCD	CWG
1	Desmethylcarbodenafil	-3.2	0.3	2.8	-1.0	4.5	-4.0	-4.3	-5.9	-9.2	0.3
2	Carbodenafil	-1.9	0.2	4.2	-0.5	2.7	1.9	0.3	0.7	0.4	1.2
3	N-desethylacetildenafil	-5.2	0.9	1.2	-2.9	2.5	1.4	1.3	0.5	-5.5	-0.8
4	Acetildenafil	-6.1	4.0	2.4	0.2	2.1	1.0	0.2	-8.1	-3.2	-2.5
5	Hydroxyvardenafil	-6.4	0.6	1.2	-0.4	2.9	2.7	-0.4	-1.4	-0.8	1.5
6	Dimethylacetildenafil	-9.5	-0.2	1.3	-8.4	5.2	1.1	1.2	1.9	-3.5	0.4
7	Vardenafil	-5.1	-1.3	3.2	-1.0	4.5	2.2	1.0	-2.0	-0.7	-1.8
8	Sildenafil	-3.6	2.3	5.1	0.5	2.8	1.7	-0.1	-1.9	1.0	0.1
9	Homosildenafil	-3.5	0.0	4.5	-0.3	5.5	1.9	0.0	-2.8	-1.8	0.3
10	Dimethylsildenafil	-5.3	1.1	2.5	-1.9	3.8	0.5	1.7	-0.5	-3.6	2.6
11	Propoxyphenyl-hydroxyhomosildenafil	-3.4	-0.2	5.8	-0.4	4.6	2.0	2.1	-2.0	3.1	-0.3
12	Udenafil	-3.8	-0.3	3.7	-1.6	4.8	3.2	1.7	0.0	-0.5	-1.0
13	Propoxyphenyl-sildenafil	-3.5	2.6	4.2	-0.6	4.0	0.4	-1.0	-1.2	-0.8	-0.3
14	Hydroxythiovardenafil	-5.6	0.1	3.0	-1.1	4.3	-0.4	-1.5	-2.9	-1.6	-0.3
15	Tadalafil	-5.1	6.5	5.8	4.4	0.5	3.4	-0.6	-3.7	-1.1	1.4
16	Mirodenafil	-3.9	1.9	6.1	1.2	1.5	4.1	-1.5	-1.1	1.1	2.2
17	Mutaprodenafil	ND	1.2	2.5	-1.5	4.1	2.6	1.0	-0.2	-0.4	-3.6
18	Thiosildenafil	-3.6	0.8	4.5	0.5	5.7	-1.0	1.3	-2.2	-1.2	-1.6
19	Thiohomosildenafil	-5.1	0.5	1.8	-1.3	4.7	-2.0	1.7	-0.1	-1.3	-1.3
20	Dithiodesmethylcarbodenafil	-5.4	-3.0	2.2	-0.2	5.2	-2.1	0.4	0.5	-1.2	-0.9
21	Thiodimethylsildenafil	-6.2	2.5	2.7	-1.9	4.4	0.3	0.6	-2.2	-1.1	-1.3
22	Propoxyphenyl-thiohydroxyhomosildenafil	-2.9	2.2	5.5	1.1	6.5	-4.2	-1.1	-0.5	-1.1	-1.1
23	Propoxyphenyl-thiodimethylsildenafil	-5.8	0.7	2.3	-0.9	4.6	-1.7	0.1	-0.2	-1.8	-0.8

(Abbreviations: PDM, powdered drink mix; HNY, honey; JLY, jelly; HCD, hard candy; CWG, chewing gum)

Table 3.8D: Mean extraction recovery (RE) of 23 targeted phosphodiesterase 5 (PDE5) inhibitors at low (0.1 µg/mL); medium (Med, 0.4 µg/mL); and high (1 µg/mL) quality control levels using liquid-liquid extraction (LLE) technique.

No.	Target analytes	Mean RE (%) (n = 3) LLE								
		Powdered drink mix			Honey			Jelly		
		Low	Med	High	Low	Med	High	Low	Med	High
1	Desmethylcarbodenafil	91.6	103.1	103.7	108.2	123.1	121.9	102.6	95.0	115.6
2	Carbodenafil	102.9	102.6	103.7	110.5	123.6	117.5	111.5	98.5	117.1
3	N-desethylacetildenafil	32.4	21.6	40.3	15.8	42.9	55.2	8.2	28.3	53.5
4	Acetildenafil	67.3	56.0	55.8	14.9	63.3	88.4	6.6	36.4	78.1
5	Hydroxyvardenafil	105.7	101.6	98.1	143.5	137.1	119.3	127.3	106.6	120.4
6	Dimethylacetildenafil	79.5	63.2	63.1	29.5	92.6	101.8	15.8	56.3	95.2
7	Vardenafil	99.5	105.6	105.7	116.6	129.5	120.1	109.7	101.2	116.9
8	Sildenafil	111.5	96.5	108.3	129.9	131.8	117.6	128.2	105.1	116.7
9	Homosildenafil	116.7	99.3	116.0	131.7	132.0	115.4	128.6	107.7	122.0
10	Dimethylsildenafil	114.9	101.6	113.3	138.6	133.3	117.9	135.6	108.1	121.1
11	Propoxyphenyl-hydroxyhomosildenafil	95.5	108.6	105.7	117.9	125.4	113.5	116.4	98.2	115.2
12	Udenafil	98.3	106.4	105.4	113.6	126.9	116.8	111.0	99.7	117.6
13	Propoxyphenyl-sildenafil	96.4	96.1	101.0	106.1	121.5	111.1	105.6	95.4	113.0
14	Hydroxythiovardenafil	31.4	29.3	57.1	37.7	82.0	97.5	35.3	63.5	93.5
15	Tadalafil	96.9	110.4	106.7	110.8	118.8	119.4	108.9	94.5	116.7
16	Mirodenafil	91.7	94.8	102.9	105.8	119.2	111.3	100.7	91.4	111.0
17	Mutaprodenafil	94.4	76.5	82.3	118.6	125.7	115.0	111.4	98.9	116.5
18	Thiosildenafil	27.7	30.0	64.5	28.8	80.3	97.1	25.5	63.4	92.1
19	Thiohomosildenafil	27.0	31.8	66.0	27.5	76.9	98.1	26.1	64.8	94.1
20	Dithiodesmethylcarbodenafil	20.8	34.0	60.2	11.1	63.8	94.1	9.2	53.0	84.7
21	Thiodimethylsildenafil	27.4	31.5	66.3	28.4	82.9	99.2	27.6	66.4	98.0
22	Propoxyphenyl-thiohydroxyhomosildenafil	30.2	33.2	63.6	35.1	83.9	98.4	30.4	66.4	96.6
23	Propoxyphenyl-thiodimethylsildenafil	31.3	34.6	68.2	37.1	84.1	101.0	35.0	71.1	100.6

Table 3.8D: Continued.

No.	Target analytes	Mean RE (%) (n = 3) LLE					
		Hard candy			Chewing gum		
		Low	Med	High	Low	Med	High
1	Desmethylcarbodenafil	121.4	123.0	128.3	93.6	91.4	104.1
2	Carbodenafil	122.6	122.0	126.0	102.5	94.9	105.4
3	N-desethylacetildenafil	9.2	42.1	50.9	3.7	26.3	49.6
4	Acetildenafil	3.8	46.2	83.9	2.2	28.5	62.9
5	Hydroxyvardenafil	128.9	129.7	128.5	114.9	99.4	107.7
6	Dimethylacetildenafil	12.9	70.7	99.9	8.5	39.3	74.8
7	Vardenafil	119.5	124.7	129.9	103.0	95.5	106.7
8	Sildenafil	133.8	129.4	124.7	110.1	97.2	105.2
9	Homosildenafil	138.9	129.9	126.0	119.0	99.6	106.4
10	Dimethylsildenafil	139.3	133.9	130.8	118.7	100.4	108.5
11	Propoxyphenyl-hydroxyhomosildenafil	119.5	120.3	121.7	111.2	96.5	105.9
12	Udenafil	122.0	124.3	127.0	107.1	96.2	108.2
13	Propoxyphenyl-sildenafil	114.2	114.8	120.6	93.9	90.4	100.7
14	Hydroxythiovardenafil	59.0	88.0	103.9	35.8	63.6	88.7
15	Tadalafil	107.0	117.0	124.1	98.9	87.6	99.1
16	Mirodenafil	111.8	116.8	121.4	99.1	92.1	105.7
17	Mutaprodenafil	122.8	124.9	128.3	103.7	94.1	106.9
18	Thiosildenafil	51.9	85.7	102.5	20.3	59.5	84.8
19	Thiohomosildenafil	55.0	87.0	104.5	20.7	56.6	82.5
20	Dithiodesmethylcarbodenafil	29.3	70.6	94.5	4.4	40.6	71.4
21	Thiodimethylsildenafil	58.9	91.3	108.3	23.7	59.4	85.6
22	Propoxyphenyl-thiohydroxyhomosildenafil	60.6	88.8	103.9	29.8	64.1	87.6
23	Propoxyphenyl-thiodimethylsildenafil	63.5	96.6	109.2	31.1	64.1	88.5

Table 3.8E: Mean extraction recovery (RE) of 23 targeted phosphodiesterase 5 (PDE5) inhibitors at low (0.1 µg/mL); medium (Med, 0.4 µg/mL); and high (1 µg/mL) quality control levels using solid-phase extraction (SPE) technique.

No.	Target analytes	Mean RE (%) (n = 3) SPE								
		Powdered drink mix			Honey			Jelly		
		Low	Med	High	Low	Med	High	Low	Med	High
1	Desmethylcarbodenafil	118.6	97.9	92.5	100.7	103.0	113.6	102.5	109.5	106.2
2	Carbodenafil	122.5	100.8	60.5	85.8	98.0	104.7	91.4	106.1	104.9
3	N-desethylacetildenafil	27.0	29.9	53.1	ND	ND	7.4	ND	6.9	9.8
4	Acetildenafil	96.8	85.7	82.8	57.7	73.3	93.5	77.4	98.7	94.6
5	Hydroxyvardenafil	122.6	101.5	96.1	126.7	120.0	116.2	93.2	103.4	102.5
6	Dimethylacetildenafil	96.4	81.5	81.5	57.2	80.6	93.6	52.9	90.1	105.3
7	Vardenafil	112.3	94.0	92.4	111.1	109.7	111.8	88.9	106.3	105.5
8	Sildenafil	128.1	104.6	103.1	101.3	104.3	106.9	97.7	108.5	105.1
9	Homosildenafil	123.4	99.0	92.7	96.6	99.5	104.2	90.7	104.0	102.7
10	Dimethylsildenafil	122.1	98.4	91.8	90.0	96.2	103.5	90.1	107.1	106.5
11	Propoxyphenyl-hydroxyhomosildenafil	100.5	92.6	94.8	76.1	89.0	99.8	68.7	93.4	96.4
12	Udenafil	76.8	75.3	73.2	57.5	63.1	83.4	31.5	74.1	84.8
13	Propoxyphenyl-sildenafil	99.0	91.4	92.6	83.5	96.3	102.4	81.1	101.5	104.0
14	Hydroxythiovardenafil	31.2	47.9	68.9	30.0	73.9	87.8	24.3	67.2	82.2
15	Tadalafil	117.7	94.1	62.7	52.1	69.6	81.3	123.1	109.7	106.8
16	Mirodenafil	57.4	54.9	59.5	53.3	53.5	68.6	35.5	57.1	70.7
17	Mutaprodenafil	ND	ND	ND	50.0	47.4	61.3	22.5	43.9	61.8
18	Thiosildenafil	34.2	53.5	85.4	39.8	67.6	83.4	21.3	73.7	86.0
19	Thiohomosildenafil	32.1	51.3	76.4	37.1	64.5	81.7	24.3	75.2	87.2
20	Dithiodesmethylcarbodenafil	34.1	54.0	73.2	33.7	53.6	74.8	19.8	74.7	83.3
21	Thiodimethylsildenafil	38.1	54.8	78.8	37.8	65.3	83.4	27.0	78.7	87.0
22	Propoxyphenyl-thiohydroxyhomosildenafil	20.8	45.4	79.7	22.4	58.9	80.1	10.7	63.4	78.1
23	Propoxyphenyl-thiodimethylsildenafil	30.0	46.7	71.6	32.9	53.5	74.8	19.5	67.2	80.5

Table 3.8E: Continued.

No.	Target analytes	Mean RE (%) (n = 3) SPE					
		Hard candy			Chewing gum		
		Low	Med	High	Low	Med	High
1	Desmethylcarbodenafil	109.4	117.6	123.5	94.1	92.6	98.4
2	Carbodenafil	102.9	116.6	118.2	101.5	91.9	98.2
3	N-desethylacetildenafil	ND	0.0	7.9	ND	ND	ND
4	Acetildenafil	43.3	71.1	105.1	5.1	5.1	4.2
5	Hydroxyvardenafil	161.3	145.3	126.7	99.6	102.3	106.2
6	Dimethylacetildenafil	47.9	75.0	101.5	ND	ND	ND
7	Vardenafil	139.6	134.6	123.5	102.8	98.9	99.5
8	Sildenafil	128.0	123.5	119.4	91.5	95.6	98.5
9	Homosildenafil	118.9	116.8	118.1	98.2	96.8	96.5
10	Dimethylsildenafil	120.3	123.9	124.5	99.3	88.8	96.7
11	Propoxyphenyl-hydroxyhomosildenafil	95.1	104.8	112.5	76.1	78.0	79.5
12	Udenafil	66.8	78.3	100.4	ND	0.9	0.2
13	Propoxyphenyl-sildenafil	98.0	113.8	115.8	85.9	94.8	103.3
14	Hydroxythiovardenafil	25.4	70.5	102.3	68.3	79.5	94.2
15	Tadalafil	72.2	96.2	104.5	82.1	92.2	102.5
16	Mirodenafil	39.8	50.5	66.3	78.0	73.3	79.3
17	Mutaprodenafil	43.0	50.0	62.7	81.9	72.8	76.0
18	Thiosildenafil	37.0	78.4	102.5	63.7	75.4	90.4
19	Thiohomosildenafil	43.6	78.0	103.8	27.1	73.0	88.2
20	Dithiodesmethylcarbodenafil	46.3	72.8	93.5	92.1	65.5	79.7
21	Thiodimethylsildenafil	44.6	86.3	106.3	83.7	76.7	89.1
22	Propoxyphenyl-thiohydroxyhomosildenafil	21.9	70.2	97.0	56.7	72.2	87.4
23	Propoxyphenyl-thiodimethylsildenafil	43.2	73.5	101.1	59.4	71.5	83.5

Table 3.8F: Mean extraction recovery (RE) of 23 targeted phosphodiesterase 5 (PDE5) inhibitors at low (0.1 µg/mL); medium (Med, 0.4 µg/mL); and high (1 µg/mL) quality control levels using modified quick, easy, cheap, effective, rugged, and safe (QuEChERS) technique.

No.	Target analytes	Mean RE (%) (n = 3) Modified QuEChERS								
		Powdered drink mix			Honey			Jelly		
		Low	Med	High	Low	Med	High	Low	Med	High
1	Desmethylcarbodenafil	118.5	111.8	100.5	106.3	104.2	103.5	113.9	110.3	106.6
2	Carbodenafil	43.5	54.3	49.7	107.0	106.5	102.1	114.6	109.2	106.2
3	N-desethylacetildenafil	93.3	88.6	77.8	104.8	105.0	101.4	97.5	94.5	94.3
4	Acetildenafil	114.2	107.7	98.7	113.0	104.5	104.5	104.5	101.4	99.5
5	Hydroxyvardenafil	121.3	109.4	98.1	108.0	104.2	104.0	111.1	109.7	104.3
6	Dimethylacetildenafil	113.7	107.2	97.5	112.4	106.8	104.7	103.9	101.1	101.4
7	Vardenafil	119.0	108.6	101.3	110.7	107.1	104.1	108.4	107.4	104.6
8	Sildenafil	115.0	109.8	100.1	100.4	103.2	100.0	118.2	112.7	105.6
9	Homosildenafil	113.8	111.5	101.5	106.6	106.4	102.0	112.0	109.8	106.6
10	Dimethylsildenafil	113.6	112.9	100.5	109.2	107.2	102.5	111.8	107.2	106.4
11	Propoxyphenyl-hydroxyhomosildenafil	117.7	113.5	101.1	109.2	106.4	103.5	112.4	111.0	105.6
12	Udenafil	116.6	111.2	99.1	113.4	108.3	103.0	110.8	108.1	104.9
13	Propoxyphenyl-sildenafil	116.8	108.9	102.0	108.4	105.3	103.1	112.4	110.7	105.3
14	Hydroxythiovardenafil	117.4	109.0	99.4	109.2	106.6	103.4	112.0	107.4	104.5
15	Tadalafil	121.6	115.3	103.5	119.3	106.0	101.8	106.9	114.4	107.1
16	Mirodenafil	114.8	111.2	99.8	107.0	105.9	101.3	112.2	110.4	107.1
17	Mutaprodenafil	114.8	107.3	99.9	112.7	108.2	103.5	108.4	107.4	105.1
18	Thiosildenafil	118.6	109.2	100.9	109.4	107.9	103.5	110.1	110.6	104.3
19	Thiohomosildenafil	113.7	109.3	102.4	110.1	108.1	103.9	110.3	110.0	105.7
20	Dithiodesmethylcarbodenafil	118.5	106.9	100.3	112.8	106.6	105.8	108.4	109.7	104.7
21	Thiodimethylsildenafil	116.8	108.4	101.4	111.4	108.1	103.1	109.3	108.8	105.4
22	Propoxyphenyl-thiohydroxyhomosildenafil	121.9	113.2	101.8	110.5	107.3	103.1	109.5	111.6	104.7
23	Propoxyphenyl-thiodimethylsildenafil	116.0	110.0	102.0	112.7	107.7	103.5	110.6	109.4	105.9

Table 3.8F: Continued.

No.	Target analytes	Mean RE (%) (n = 3) Modified QuEChERS					
		Hard candy			Chewing gum		
		Low	Med	High	Low	Med	High
1	Desmethylcarbodenafil	111.5	94.8	97.1	100.9	95.8	98.0
2	Carbodenafil	106.0	95.0	99.2	103.0	97.4	102.9
3	N-desethylacetildenafil	115.2	94.4	94.3	80.5	78.1	82.2
4	Acetildenafil	112.2	102.3	105.0	104.1	103.5	100.6
5	Hydroxyvardenafil	110.3	98.0	98.3	101.2	95.7	101.5
6	Dimethylacetildenafil	111.0	101.9	101.2	101.0	94.8	98.4
7	Vardenafil	118.6	108.6	102.8	103.3	98.5	103.5
8	Sildenafil	95.7	91.2	95.4	107.1	98.2	102.7
9	Homosildenafil	101.6	93.8	97.0	104.6	99.1	104.1
10	Dimethylsildenafil	96.8	94.4	100.5	105.8	97.9	104.0
11	Propoxyphenyl-hydroxyhomosildenafil	101.3	93.0	98.4	101.8	96.8	101.6
12	Udenafil	114.3	101.7	102.2	110.8	100.1	102.5
13	Propoxyphenyl-sildenafil	101.4	89.8	96.3	102.8	97.9	102.3
14	Hydroxythiovardenafil	110.2	101.2	100.7	105.4	100.8	104.7
15	Tadalafil	75.5	79.3	90.8	107.2	100.2	100.4
16	Mirodenafil	108.4	94.6	96.0	103.8	99.1	102.8
17	Mutaprodenafil	123.9	104.7	102.2	104.5	100.1	105.3
18	Thiosildenafil	90.6	90.7	95.9	105.4	105.4	106.0
19	Thiohomosildenafil	92.3	93.0	97.4	107.6	100.2	105.2
20	Dithiodesmethylcarbodenafil	100.3	95.0	100.3	108.6	100.9	105.3
21	Thiodimethylsildenafil	89.5	93.4	98.7	111.3	100.4	105.2
22	Propoxyphenyl-thiohydroxyhomosildenafil	82.9	88.8	95.7	107.6	101.6	105.4
23	Propoxyphenyl-thiodimethylsildenafil	87.5	93.5	100.3	111.7	101.0	106.5

Table 3.8G: Retention time (RT), theoretical accurate mass of protonated molecule ($[M+H]^+$) precursor ion, product ions, coefficient of determination (r^2), and limit of detection (LOD) of 23 targeted phosphodiesterase 5 (PDE5) inhibitors.

No.	Target analytes	RT (min)	Theoretical accurate mass of $[M+H]^+$ (*m/z*)	Product ion 1 (*m/z*)	Product ion 2 (*m/z*)	r^2	LOD (ng/mL)
1	Desmethylcarbodenafil	8.77	439.2452	311.1139	339.1452	0.9989	10
2	Carbodenafil	9.24	453.2609	311.1139	339.1452	0.9994	10
3	N-desethylacetildenafil	9.68	439.2452	325.1295	297.1346	0.9993	40
4	Acetildenafil	10.64	467.2765	297.1346	127.1230	0.9971	10
5	Hydroxyvardenafil	10.81	505.2228	312.1581	151.0866	0.9996	10
6	Dimethylacetildenafil	11.15	467.2765	297.1346	127.1230	0.9966	10
7	Vardenafil	11.48	489.2279	312.1581	151.0866	0.9990	60
8	Sildenafil	13.34	475.2122	283.1190	100.0995	0.9995	10
9	Homosildenafil	13.91	489.2279	283.1190	113.1073	0.9988	40
10	Dimethylsildenafil	14.66	489.2279	283.1190	113.1073	0.9990	20
11	Propoxyphenyl-hydroxyhomosildenafil	15.68	519.2384	129.1022	283.1190	0.9995	10
12	Udenafil	15.90	517.2592	112.1121	283.1190	0.9995	10
13	Propoxyphenyl-sildenafil	16.07	489.2279	100.0995	283.1190	0.9975	10
14	Hydroxythiovardenafil	18.33	521.1999	167.0637	328.1352	0.9995	40
15	Tadalafil	20.86	390.1448	135.0441	169.0760	0.9960	40
16	Mirodenafil	21.45	532.2588	312.1343	296.1394	0.9995	10
17	Mutaprodenafil	21.62	630.2275	113.1073	142.0070	0.9976	10
18	Thiosildenafil	24.74	491.1894	100.0995	299.0961	0.9992	30
19	Thiohomosildenafil	25.74	505.2050	299.0961	113.1073	0.9982	60
20	Dithiodesmethylcarbodenafil	26.08	471.1995	343.0682	371.0995	0.9961	10
21	Thiodimethylsildenafil	26.50	505.2050	113.1073	299.0961	0.9991	70
22	Propoxyphenyl-thiohydroxyhomosildenafil	27.26	535.2156	129.1022	299.0961	0.9991	20
23	Propoxyphenyl-thiodimethylsildenafil	30.09	519.2207	113.1073	299.0961	0.9991	10

Table 3.8H: Accuracy of 23 targeted phosphodiesterase 5 (PDE5) inhibitors at low (0.1 µg/mL); medium (0.4 µg/mL); and high (1 µg/mL) quality control levels.

No.	Target analytes	Accuracy (Mean ± SD, %) (n = 3)					
		Powdered drink mix			Honey		
		Low	Medium	High	Low	Medium	High
1	Desmethylcarbodenafil	119.2 ± 1.7	96.1 ± 1.8	107.1 ± 4.0	101.8 ± 3.7	107.5 ± 0.5	107.0 ± 1.0
2	Carbodenafil	118.1 ± 2.4	97.4 ± 1.6	101.3 ± 1.6	100.3 ± 2.6	107.0 ± 0.9	108.2 ± 1.1
3	N-desethylacetildenafil	110.9 ± 5.9	93.8 ± 0.7	105.9 ± 1.1	94.8 ± 4.7	98.1 ± 1.5	102.2 ± 1.7
4	Acetildenafil	102.0 ± 0.9	111.1 ± 7.9	107.0 ± 1.0	80.1 ± 6.9	94.8 ± 1.2	104.1 ± 2.2
5	Hydroxyvardenafil	99.2 ± 1.1	100.8 ± 1.8	105.2 ± 0.8	104.3 ± 3.3	107.8 ± 1.1	109.4 ± 1.6
6	Dimethylacetildenafil	117.9 ± 2.1	93.7 ± 3.7	108.0 ± 3.2	97.6 ± 2.7	100.5 ± 2.4	103.2 ± 0.8
7	Vardenafil	88.8 ± 2.3	102.8 ± 1.7	105.4 ± 0.6	101.8 ± 4.9	107.3 ± 2.4	111.8 ± 0.9
8	Sildenafil	110.3 ± 0.8	96.9 ± 1.0	105.4 ± 0.4	97.6 ± 5.5	106.7 ± 0.6	107.5 ± 1.6
9	Homosildenafil	103.4 ± 0.1	99.5 ± 1.5	105.0 ± 0.9	89.7 ± 4.5	109.5 ± 1.8	108.2 ± 1.0
10	Dimethylsildenafil	110.9 ± 0.9	96.5 ± 0.8	103.3 ± 1.8	96.4 ± 3.3	107.5 ± 1.5	109.8 ± 2.0
11	Propoxyphenyl-hydroxyhomosildenafil	110.2 ± 4.5	95.6 ± 0.5	103.8 ± 0.8	103.1 ± 5.5	107.1 ± 1.4	109.3 ± 0.8
12	Udenafil	106.1 ± 1.6	98.4 ± 0.4	105.3 ± 0.8	94.1 ± 1.1	107.1 ± 1.0	108.5 ± 1.5
13	Propoxyphenyl-sildenafil	100.7 ± 2.2	99.9 ± 1.9	104.9 ± 0.5	85.4 ± 3.7	112.2 ± 0.8	109.5 ± 0.9
14	Hydroxythiovardenafil	88.0 ± 1.9	100.2 ± 0.9	104.5 ± 0.2	100.3 ± 4.7	106.3 ± 1.0	109.1 ± 2.1
15	Tadalafil	104.4 ± 1.4	95.1 ± 4.5	104.3 ± 0.9	89.9 ± 7.9	116.5 ± 1.9	99.6 ± 0.9
16	Mirodenafil	116.7 ± 6.8	93.5 ± 1.0	104.2 ± 0.6	103.7 ± 2.1	104.3 ± 2.5	109.6 ± 1.5
17	Mutaprodenafil	81.5 ± 0.6	101.6 ± 1.8	104.5 ± 1.3	94.2 ± 2.2	114.1 ± 0.6	106.7 ± 1.8
18	Thiosildenafil	84.3 ± 3.4	99.6 ± 1.7	104.9 ± 0.9	92.3 ± 4.7	109.6 ± 1.3	108.8 ± 0.7
19	Thiohomosildenafil	83.2 ± 1.3	102.6 ± 1.5	104.1 ± 0.7	109.7 ± 2.8	113.7 ± 2.6	108.1 ± 1.0
20	Dithiodesmethylcarbodenafil	77.4 ± 4.5	103.1 ± 2.4	103.9 ± 1.2	93.3 ± 5.5	113.4 ± 1.0	110.9 ± 2.8
21	Thiodimethylsildenafil	97.3 ± 1.4	99.3 ± 2.8	103.7 ± 0.8	96.7 ± 3.5	106.4 ± 2.7	111.3 ± 1.6
22	Propoxyphenyl-thiohydroxyhomosildenafil	94.7 ± 1.3	97.9 ± 1.4	104.4 ± 1.1	111.0 ± 5.5	103.6 ± 1.2	110.7 ± 1.6
23	Propoxyphenyl-thiodimethylsildenafil	98.4 ± 1.6	97.7 ± 1.8	104.8 ± 0.8	100.8 ± 2.9	104.5 ± 0.2	107.1 ± 1.4

Table 3.8H: Continued.

No.	Target analytes	Accuracy (Mean ± SD, %) (n = 3)					
		Jelly			Hard candy		
		Low	Medium	High	Low	Medium	High
1	Desmethylcarbodenafil	90.5 ± 4.1	93.6 ± 3.0	104.0 ± 2.0	114.9 ± 1.1	100.7 ± 0.9	99.7 ± 1.1
2	Carbodenafil	89.7 ± 2.3	97.4 ± 1.6	105.7 ± 1.3	116.9 ± 4.0	99.2 ± 1.0	100.5 ± 0.7
3	N-desethylacetildenafil	99.5 ± 2.4	98.5 ± 2.0	103.6 ± 1.2	115.1 ± 1.0	91.3 ± 1.8	96.1 ± 1.0
4	Acetildenafil	87.1 ± 1.2	98.8 ± 1.3	105.2 ± 0.8	98.6 ± 0.5	87.4 ± 0.8	93.6 ± 0.5
5	Hydroxyvardenafil	97.3 ± 1.0	98.1 ± 2.0	104.8 ± 0.4	115.0 ± 0.7	99.6 ± 0.2	100.5 ± 0.9
6	Dimethylacetildenafil	111.0 ± 4.3	100.2 ± 4.2	104.4 ± 4.8	113.1 ± 3.1	91.4 ± 2.6	94.8 ± 0.0
7	Vardenafil	94.0 ± 2.4	100.1 ± 1.6	105.5 ± 0.6	113.7 ± 2.5	99.7 ± 0.6	101.2 ± 2.7
8	Sildenafil	94.4 ± 1.6	101.5 ± 0.9	102.5 ± 1.5	111.6 ± 3.1	100.9 ± 0.4	101.5 ± 1.8
9	Homosildenafil	87.7 ± 1.7	102.3 ± 1.2	105.8 ± 1.1	103.4 ± 1.2	102.8 ± 1.6	100.1 ± 2.4
10	Dimethylsildenafil	96.5 ± 2.3	101.7 ± 1.3	105.8 ± 1.3	109.1 ± 0.9	99.2 ± 1.8	101.5 ± 1.4
11	Propoxyphenyl-hydroxyhomosildenafil	97.2 ± 7.3	99.4 ± 1.2	104.0 ± 0.8	114.5 ± 1.3	98.9 ± 0.7	101.3 ± 0.9
12	Udenafil	93.8 ± 1.2	98.6 ± 0.3	104.3 ± 0.9	105.5 ± 1.4	100.9 ± 1.3	102.8 ± 1.2
13	Propoxyphenyl-sildenafil	80.9 ± 2.3	103.0 ± 2.4	104.1 ± 1.2	99.1 ± 2.2	105.1 ± 1.0	100.8 ± 1.3
14	Hydroxythiovardenafil	94.9 ± 1.8	97.9 ± 1.5	103.6 ± 0.5	110.6 ± 0.6	99.4 ± 1.4	100.9 ± 1.9
15	Tadalafil	86.5 ± 8.4	100.8 ± 5.1	104.8 ± 2.8	111.6 ± 7.1	110.4 ± 1.6	95.1 ± 1.7
16	Mirodenafil	101.8 ± 0.8	97.9 ± 0.8	104.5 ± 1.3	112.0 ± 1.1	97.0 ± 1.2	102.9 ± 0.4
17	Mutaprodenafil	81.9 ± 3.1	102.1 ± 1.3	105.2 ± 1.0	106.7 ± 0.7	105.4 ± 1.7	101.2 ± 0.8
18	Thiosildenafil	100.0 ± 2.5	102.3 ± 1.1	104.6 ± 1.6	106.0 ± 2.1	102.7 ± 0.6	101.5 ± 1.3
19	Thiohomosildenafil	89.7 ± 0.6	105.9 ± 0.8	106.4 ± 0.2	122.0 ± 1.2	106.2 ± 0.8	101.3 ± 1.1
20	Dithiodesmethylcarbodenafil	84.0 ± 2.3	100.6 ± 1.7	106.1 ± 1.5	103.1 ± 2.2	108.1 ± 1.1	106.5 ± 0.3
21	Thiodimethylsildenafil	102.9 ± 1.3	103.0 ± 0.5	110.3 ± 1.0	108.0 ± 1.0	98.9 ± 0.8	104.4 ± 0.3
22	Propoxyphenyl-thiohydroxyhomosildenafil	102.2 ± 3.8	98.9 ± 0.7	105.5 ± 1.8	118.3 ± 1.1	95.2 ± 1.6	102.0 ± 0.2
23	Propoxyphenyl-thiodimethylsildenafil	103.1 ± 1.0	99.4 ± 1.1	104.0 ± 1.6	109.1 ± 0.7	99.5 ± 1.1	100.3 ± 0.3

Table 3.8H: Continued.

No.	Target analytes	Accuracy (Mean ± SD, %) (n = 3)		
		Chewing gum		
		Low	Medium	High
1	Desmethylcarbodenafil	100.6 ± 0.8	98.9 ± 1.1	102.5 ± 0.9
2	Carbodenafil	94.6 ± 3.8	103.3 ± 1.7	103.1 ± 2.7
3	N-desethylacetildenafil	100.6 ± 0.9	101.8 ± 1.1	102.6 ± 2.9
4	Acetildenafil	93.0 ± 0.8	104.8 ± 1.7	102.5 ± 2.0
5	Hydroxyvardenafil	98.9 ± 2.1	105.1 ± 1.6	104.0 ± 1.6
6	Dimethylacetildenafil	115.4 ± 3.0	101.0 ± 1.9	103.0 ± 4.5
7	Vardenafil	97.2 ± 1.6	105.1 ± 3.0	104.8 ± 0.9
8	Sildenafil	98.3 ± 0.8	104.9 ± 2.5	103.7 ± 1.0
9	Homosildenafil	91.3 ± 2.9	106.4 ± 0.6	101.6 ± 0.8
10	Dimethylsildenafil	96.8 ± 2.5	104.9 ± 2.7	103.7 ± 2.1
11	Propoxyphenyl-hydroxyhomosildenafil	98.0 ± 1.7	102.4 ± 2.2	103.3 ± 1.9
12	Udenafil	97.3 ± 2.4	104.3 ± 1.6	104.4 ± 1.6
13	Propoxyphenyl-sildenafil	86.9 ± 1.7	106.5 ± 1.6	104.2 ± 1.5
14	Hydroxythiovardenafil	95.3 ± 1.9	98.7 ± 1.8	100.4 ± 1.2
15	Tadalafil	86.0 ± 2.9	116.4 ± 1.8	103.4 ± 2.2
16	Mirodenafil	103.4 ± 2.8	101.5 ± 0.3	102.1 ± 0.4
17	Mutaprodenafil	84.5 ± 2.0	105.5 ± 2.8	101.2 ± 1.0
18	Thiosildenafil	100.7 ± 6.0	103.1 ± 1.1	101.7 ± 0.9
19	Thiohomosildenafil	92.0 ± 2.7	108.6 ± 1.9	105.1 ± 0.6
20	Dithiodesmethylcarbodenafil	86.6 ± 2.2	105.8 ± 2.2	105.6 ± 1.1
21	Thiodimethylsildenafil	102.5 ± 2.1	107.2 ± 1.9	109.4 ± 0.7
22	Propoxyphenyl-thiohydroxyhomosildenafil	104.7 ± 4.8	100.1 ± 1.8	101.0 ± 1.4
23	Propoxyphenyl-thiodimethylsildenafil	104.4 ± 2.0	102.7 ± 1.7	102.5 ± 0.5

Table 3.8I: Repeatability of 23 targeted phosphodiesterase 5 (PDE5) inhibitors at low (L, 0.1 µg/mL); medium (M, 0.4 µg/mL); and high (H, 1 µg/mL) quality control levels.

No.	Target analytes	Repeatability (%RSD) (n = 9)														
		PDM			HNY			JLY			HCD			CWG		
		L	M	H	L	M	H	L	M	H	L	M	H	L	M	H
1	Desmethylcarbodenafil	2.1	2.1	3.8	4.6	0.5	0.9	4.1	3.2	1.9	1.2	0.9	1.1	0.8	1.1	0.9
2	Carbodenafil	3.1	1.8	1.6	3.1	0.9	1.0	2.5	1.6	1.2	4.0	1.0	0.7	3.8	1.6	2.6
3	N-desethylacetildenafil	6.0	0.8	1.1	6.4	1.7	1.7	2.6	2.1	1.1	1.1	2.1	1.0	1.0	1.1	2.8
4	Acetildenafil	1.0	7.4	0.9	8.6	1.3	2.1	1.3	1.3	0.8	0.5	1.0	0.5	0.8	1.6	1.9
5	Hydroxyvardenafil	1.3	1.8	0.8	3.7	1.1	1.5	1.3	2.2	0.4	0.7	0.2	0.9	2.5	1.6	1.6
6	Dimethylacetildenafil	2.4	4.3	3.1	3.5	2.6	0.8	4.8	4.4	4.7	3.3	3.0	0.1	3.1	2.0	4.4
7	Vardenafil	2.4	1.6	0.6	5.7	2.3	0.8	2.6	0.6	1.6	2.5	0.6	2.7	1.7	2.9	0.9
8	Sildenafil	1.1	1.2	0.4	5.9	0.6	1.5	1.7	0.9	1.5	2.9	0.4	1.8	0.8	2.4	0.9
9	Homosildenafil	0.1	1.6	0.9	4.4	1.6	1.0	1.7	1.2	1.1	1.0	1.6	2.4	2.8	0.6	0.8
10	Dimethylsildenafil	1.4	0.9	1.8	3.6	1.4	1.9	2.8	1.3	1.2	0.9	1.8	1.4	3.0	2.7	2.1
11	Propoxyphenyl-hydroxyhomosildenafil	6.2	0.6	0.8	6.3	1.4	0.7	9.0	1.2	0.8	1.3	0.8	0.9	2.1	2.2	1.9
12	Udenafil	1.8	0.5	0.8	1.4	0.9	1.4	1.3	0.3	0.9	1.4	1.3	1.2	2.5	1.5	1.5
13	Propoxyphenyl-sildenafil	2.6	2.0	0.5	3.5	0.7	0.9	2.3	2.2	1.1	1.9	0.9	1.3	1.6	1.4	1.4
14	Hydroxythiovardenafil	2.0	0.8	0.2	5.1	1.0	1.9	2.3	1.6	0.5	0.6	1.5	1.9	2.4	1.9	1.2
15	Tadalafil	3.0	5.6	0.9	8.8	1.6	0.9	7.5	4.7	2.6	6.3	1.4	1.8	2.6	1.4	2.1
16	Mirodenafil	9.5	1.3	0.6	2.6	2.6	1.4	0.9	0.9	1.2	1.3	1.3	0.4	3.2	0.3	0.4
17	Mutaprodenafil	0.6	1.7	1.2	2.4	0.6	1.7	3.2	1.2	0.9	0.7	1.6	0.8	2.0	2.5	0.9
18	Thiosildenafil	5.0	1.8	0.8	4.6	1.2	0.7	2.9	1.1	1.6	1.8	0.6	1.2	7.0	1.1	0.9
19	Thiohomosildenafil	1.6	1.5	0.7	2.6	2.3	0.9	0.6	0.7	0.2	1.0	0.7	1.1	2.7	1.7	0.5
20	Dithiodesmethylcarbodenafil	4.9	2.3	1.2	5.4	0.9	2.5	2.4	1.6	1.4	2.0	1.0	0.3	2.2	2.0	1.0
21	Thiodimethylsildenafil	2.3	3.1	0.8	3.8	2.5	1.4	1.6	0.5	1.0	1.0	0.9	0.3	2.5	1.9	0.7
22	Propoxyphenyl-thiohydroxyhomosildenafil	2.2	1.6	1.1	6.2	1.2	1.5	6.1	0.8	1.7	1.1	1.7	0.2	7.4	2.0	1.5
23	Propoxyphenyl-thiodimethylsildenafil	2.6	2.1	0.8	3.3	0.2	1.4	1.4	1.1	1.6	0.8	1.1	0.3	2.6	1.7	0.5

(Abbreviations: PDM, powdered drink mix; HNY, honey; JLY, jelly; HCD, hard candy; CWG, chewing gum)

Table 3.8J: Intermediate precision of 23 targeted phosphodiesterase 5 (PDE5) inhibitors at low (L, 0.1 µg/mL); medium (M, 0.4 µg/mL); and high (H, 1 µg/mL) quality control levels.

No.	Target analytes	Intermediate precision (%RSD) (n = 9)														
		PDM			HNY			JLY			HCD			CWG		
		L	M	H	L	M	H	L	M	H	L	M	H	L	M	H
1	Desmethylcarbodenafil	2.3	4.9	3.4	3.6	2.0	1.0	4.5	3.2	1.4	1.6	2.0	1.0	6.7	5.4	1.0
2	Carbodenafil	4.2	3.8	2.9	2.6	2.0	0.8	3.5	4.2	1.4	4.2	0.4	0.4	8.4	3.3	2.3
3	N-desethylacetildenafil	1.9	3.8	4.1	7.9	2.4	6.5	5.4	3.8	1.6	2.5	0.8	2.3	7.9	6.0	2.0
4	Acetildenafil	4.9	2.0	1.5	8.5	0.7	0.4	1.8	4.2	1.4	4.1	2.4	1.0	2.3	1.9	2.5
5	Hydroxyvardenafil	2.3	2.2	1.5	3.7	2.9	1.9	7.8	5.4	3.2	3.2	1.6	2.0	10.4	5.5	4.3
6	Dimethylacetildenafil	1.1	4.3	2.9	3.7	1.2	1.0	0.6	0.6	4.8	7.0	0.9	2.7	2.1	3.5	1.4
7	Vardenafil	6.5	3.3	3.4	0.5	1.5	1.3	7.7	4.7	2.8	3.3	1.8	3.2	11.4	6.5	2.3
8	Sildenafil	3.7	3.1	1.7	5.7	0.7	0.8	7.7	4.2	3.5	2.3	1.4	1.1	14.6	6.2	4.4
9	Homosildenafil	0.3	2.7	1.7	1.6	2.6	0.7	6.3	3.5	3.3	1.3	2.0	1.3	8.5	4.6	2.1
10	Dimethylsildenafil	0.9	5.9	4.1	2.8	1.5	1.4	4.6	2.5	2.7	2.2	1.1	2.0	7.6	4.4	1.1
11	Propoxyphenyl-hydroxyhomosildenafil	1.9	6.5	5.3	1.5	1.2	1.3	5.3	3.8	2.1	3.1	1.6	1.9	10.4	5.5	3.7
12	Udenafil	4.2	1.3	2.1	1.3	1.7	0.7	2.5	2.6	2.8	1.3	1.9	1.9	2.8	3.3	2.2
13	Propoxyphenyl-sildenafil	0.3	4.0	2.4	3.2	2.1	0.7	7.3	2.8	4.6	2.2	0.3	0.7	10.3	4.7	0.9
14	Hydroxythiovardenafil	5.2	1.3	2.5	3.7	2.4	1.7	7.1	5.2	3.5	1.8	2.7	3.0	9.4	6.2	3.6
15	Tadalafil	7.6	3.4	3.4	7.7	2.9	2.2	6.7	0.9	4.9	5.5	3.0	2.3	9.3	5.7	4.3
16	Mirodenafil	3.5	4.0	3.1	2.4	1.0	0.1	4.6	3.6	1.9	3.9	0.8	1.8	3.8	4.6	2.4
17	Mutaprodenafil	9.8	1.2	1.6	1.4	0.4	0.6	5.8	2.7	1.6	0.9	1.1	1.5	8.9	3.6	2.2
18	Thiosildenafil	4.5	0.8	1.4	4.1	1.3	1.4	11.1	5.1	3.1	0.5	2.1	2.3	16.5	7.2	4.8
19	Thiohomosildenafil	7.3	1.3	0.2	3.4	1.1	0.5	7.0	4.2	3.0	3.3	1.3	2.4	11.1	5.7	4.8
20	Dithiodesmethylcarbodenafil	5.0	1.0	0.7	2.3	1.2	1.4	14.8	7.3	4.7	3.5	0.9	1.7	16.7	7.6	4.5
21	Thiodimethylsildenafil	3.5	1.4	2.2	4.5	1.6	0.4	7.7	5.6	3.8	1.7	1.8	1.7	10.6	5.8	3.9
22	Propoxyphenyl-thiohydroxyhomosildenafil	3.1	3.9	3.8	1.6	1.2	2.3	9.4	4.0	4.1	3.5	3.0	2.8	13.7	7.3	4.8
23	Propoxyphenyl-thiodimethylsildenafil	2.5	2.6	3.0	2.5	1.6	1.4	5.0	5.5	3.4	2.7	2.1	2.0	10.5	6.2	4.6

(Abbreviations: PDM, powdered drink mix; HNY, honey; JLY, jelly; HCD, hard candy; CWG, chewing gum)

CHAPTER 4

Determination of phosphodiesterase 5 (PDE5) inhibitors in instant coffee premixes using liquid chromatography-high-resolution mass spectrometry (LC-HRMS)

4.1 FOREWORD

The following two manuscripts, in Chapter 4, were published in *Talanta* (https://doi.org/10.1016/j.talanta.2019.05.078) with the method development, as well as the optimisation and validation of the analytical procedure published in *Data in Brief* (https://doi.org/10.1016/j.dib.2019.104234). The detection, identification, and eventual quantification of phosphodiesterase 5 (PDE5) inhibitors, particularly their novel analogues, are demanding and time-consuming tasks. Recently, unscrupulous manufacturers have found ways to conceal PDE5 inhibitors within complex matrices, such as instant coffee premixes, which may hinder detection, and thus, evade the consequences of law enforcement. This chapter developed, optimised, and validated a liquid chromatography-high-resolution mass spectrometry-based method for simultaneous screening, identification, and quantification of PDE5 inhibitors and their analogues as adulterants in instant coffee premixes. The full spectral information via suspected-target and non-targeted strategies was utilised to screen these adulterants. The method was then applied to 25 samples of instant coffee premixes that claimed to enhance male sexual performance. The manuscript addressed the growing popularity of PDE5 inhibitors' adulteration in instant coffee premixes and the continuous emergence of their novel analogues, as the number of available literature remain scarce. Mr Ahmad Yusri Mohd Yusop, Dr Linda

Xiao, and Professor Shanlin Fu authored the manuscripts. Mohd Yusop AY performed the experimental work, data analysis, and initial draft preparation including supplementary data with manuscript edits provided by Xiao L and Fu S. The article section, figure, table, equation, and reference numbering was adjusted to align with the chronology of this thesis and may not reflect those published in the printed or online version.

4.2 ABSTRACT

As a widely consumed beverage, coffee tends to be a target for intentional adulteration. This study describes the application of modified quick, easy, cheap, effective, rugged, and safe (QuEChERS) coupled to liquid chromatography-high-resolution mass spectrometry (LC-HRMS) for simultaneous screening, identification, and quantification of undeclared phosphodiesterase 5 (PDE5) inhibitors in instant coffee premixes (ICPs). The mass spectrometer was operated in auto MS/MS acquisition for simultaneous MS and MS/MS experiments. Qualitative establishments from the suspected-target screening and targeted identification processes led to an unambiguous analyte assignment from the protonated molecule ($[M+H]^+$) precursor ion which is subsequently used for quantification of 23 targeted PDE5 inhibitors. The analytical method validation covered specificity, linearity, range, accuracy, limit of detection (LOD), limit of quantification (LOQ), precisions, matrix effect (ME), and extraction recovery (RE). The specificity was established using the optimised chromatographic separation as well as the distinguishable $[M+H]^+$ precursor ion. The linearity of each target analyte was demonstrated with a coefficient of determination (r^2) of >0.9960 over the expected range of sample concentrations. The accuracy ranged from 88.1% to 119.3% with LOD and LOQ of <70 ng/mL and 80 ng/mL, respectively. Excellent precisions were established within 0.4%–9.1% of the relative standard deviation. An insignificant ME within -5.2% to +8.7% was achieved using three different strategies of chromatography, sample extraction, and sample dilution. The RE was good for all target analytes within 84.7%–123.5% except for N-desethylacetildenafil at low (53.8%) and medium (65.1%) quality control levels. The method was successfully applied to 25 samples of ICPs where 17 of them

were found to be adulterated with PDE5 inhibitors and their analogues. Further quantification revealed the total amount of these adulterants ranged from 2.77 to 121.64 mg per sachet.

4.3 INTRODUCTION

Coffee is among the most favoured beverages throughout the world [1], leading to the advent of instant coffee premixes (ICPs) which typically packaged in a single serving sachet. These coffee products often comprise other ingredients such as creamer, sugar and ingredients to enrich flavour and texture [2,3]. Sometimes, they are fortified with vitamins and minerals [4]. Unfortunately, ICPs are also known to be adulterated with synthetic drugs which claim to enhance male sexual performance such as phosphodiesterase 5 (PDE5) inhibitors and their analogues.

An analogue of PDE5 inhibitors is synthesised by minor modifications to the parent structure of the approved drugs which will alter their physical and chemical properties [5]. Additionally, there are no clinical studies performed on these analogues to ensure their efficacy and safety [6]. To date, more than 90 unapproved analogues of PDE5 inhibitors have been discovered and described in the literature as adulterants. Since 2010 up to the end of 2018, the United States Food and Drug Administration has issued seven warnings regarding ICPs tainted with PDE5 inhibitors and their analogues [7], specifically those that were made in Malaysia [8].

Liquid chromatography (LC) coupled with mass spectrometry (MS) has been most popular in the detection and analysis of PDE5 inhibitors and their analogues. Although low-resolution MS was frequently used [9-11], high-resolution MS (HRMS) proves to be superior [12-14] as it delivers full spectral information with excellent mass accuracy on top of isotopic reliability, aiding

suspected-target screening [15] and targeted identification processes [16]. It also enables embedding non-targeted screening into a developed method for retrospective and prospective applications [17]. To date, analysis of PDE5 inhibitors has been primarily targeting health supplements, particularly in pharmaceutical dosage form [18]. Due to the relatively high concentration of analytes in these products and the relatively simple matrix involved, these published methods are not applicable in the analysis of PDE5 inhibitors in ICPs. The low analyte level and the complex matrix nature of ICPs in combination with the growing number of novel PDE5 analogues available for adulteration represent a real analytical challenge for forensic drug testing laboratories.

This study focused on developing an LC-HRMS based analytical method that is capable of accurately detecting and quantifying PDE5 inhibitors and their analogues down to trace levels in ICPs. Method development involved optimisation of chromatographic separation, MS conditions, and sample preparation, described in [19]. Method validation covered specificity, linearity, range, accuracy, limit of detection (LOD), limit of quantification (LOQ), precisions, matrix effect (ME), and extraction recovery (RE). The method was applied to real sample analysis incorporating suspected-target screening, targeted identification, quantification, and non-targeted screening approaches. To the best of our knowledge, this is the first report to comprehensively address the analytical challenge for a reliable determination of PDE5 inhibitors as adulterants in ICPs.

4.4 MATERIALS AND METHODS

4.4.1 Chemicals and reagents

Certified reference materials were purchased from TLC Pharmaceutical Standards Ltd. (Aurora, Ontario, Canada). They are desmethylcarbodenafil (1), carbodenafil (2), N-desethylacetildenafil (3), acetildenafil (4), hydroxyvardenafil (5), dimethylacetildenafil (6), vardenafil (7), sildenafil (8), homosildenafil (9), dimethylsildenafil (10), propoxyphenyl-hydroxyhomosildenafil (11), udenafil (12), propoxyphenyl-sildenafil (13), hydroxythiovardenafil (14), tadalafil (15), mirodenafil (16), mutaprodenafil (17), thiosildenafil (18), thiohomosildenafil (19), dithiodesmethylcarbodenafil (20), thiodimethylsildenafil (21), propoxyphenyl-thiohydroxyhomosildenafil (22), and propoxyphenyl-thiodimethylsildenafil (23). Their chemical structures are presented in Fig. 4.9A (supplementary data).

LC-MS grade methanol and acetonitrile were purchased from Chem-Supply Pty Ltd. (Gillman, SA, Australia). LC-MS grade formic acid and analytical grade ammonium formate were purchased from Sigma Aldrich Pty Ltd. (Castle Hill, NSW, Australia). Ultrapure water (18.2 MΩ-cm) was obtained from a Sartorius arium® pro ultrapure water system (Goettingen, Germany). Restek Q-sep QuEChERS extraction salts (EN 15662) was purchased from LECO Australia Pty Ltd. (Castle Hill, NSW, Australia).

4.4.2 Standard solution preparation

All 23 individual stock solutions of PDE5 inhibitors were prepared separately in LC-MS grade methanol at 1 mg/mL and stored in the dark at 4°C until analysis. A mixture of all standards (working solution) was prepared fresh for each analysis

from the stock solutions by further dilution in methanol to make up to 25 µg/mL concentration.

4.4.3 Sample collection and storage

A total of 25 distinct brands of ICPs were acquired from Malaysia. These samples are highly suspected to be adulterated with PDE5 inhibitors based on the references to male sexual performance in their brand names, label claims, images, botanical ingredients, or advertising materials. Out of the total, 13 samples were kindly donated by the Pharmacy Enforcement Division, Ministry of Health Malaysia, obtained from surveillance activities (7 samples), and by confiscation at the international airport (2 samples) and international seaport (4 samples) during routine inspections by pharmacy enforcement officers. The other 12 samples were purchased through online shopping platforms in Malaysia. All distinct samples were coded and labelled as SPL001 to SPL025. These samples were deposited in a plastic zip-lock bag separately and then stored in an airtight container in the dark. A blank ICP, free from any analyte of interests was sourced from a local supermarket and used for method development and validation.

4.4.4 Sample preparation

First, the whole content of a sachet of an ICP sample was weighed. Then, 100 mg of the sample was dissolved in 5 mL of acetonitrile and methanol (50:50, v/v). The resulting solution was then transferred into a tube prefilled with QuEChERS salts for the extraction procedure. Finally, the upper layer was filtered and diluted with methanol at 1:10 dilution level for analysis. The blank ICP was treated in the

same manner as the steps described for the sample analysis. The full extraction procedures can be found in Section 4.6.2.

4.4.5 LC-HRMS conditions

The chromatographic separation was performed using an Agilent Technologies (Santa Clara, CA, USA) 1290 Infinity II LC system coupled to an Agilent Technologies 6510 quadrupole time of flight-mass spectrometer (QTOF-MS). The LC system was fitted with a reverse-phase high-performance LC column from Merck KGaA (Darmstadt, Germany) Chromolith® High-Resolution RP-18 end-capped (100 × 4.6 mm, 2.0 µm) with solvent A (10 mM ammonium formate in ultrapure water) and solvent B (acetonitrile). Both solvents were acidified with 0.1% v/v formic acid as the binary mobile phase system. The QTOF-MS was operated in positive electrospray ionisation mode with auto MS/MS acquisition. Specific details on the LC-HRMS conditions are described in Section 4.6.2.

4.4.6 Method validation and data analysis

Method validation was performed in accordance with the guideline set by the International Conference on Harmonisation [20] covering specificity, linearity, range, accuracy, LOD, LOQ, and precisions. The ME and RE were also evaluated for each target analyte in the blank ICP matrix following the published procedures [21]. All analyses were done in triplicate.

The specificity was assessed for each target analyte based on their chromatographic resolution and their unique accurate mass of the protonated molecule ($[M+H]^+$) precursor ion from the MS experiment. The presence of two

fragment ions corresponding to each targeted PDE5 inhibitor was established from the MS/MS experiment. To further confirm the identity of each target analyte, the average intensity ratio between the first and the second fragment ion was compared to those obtained from the linearity assessment with an acceptable value of ±30%. The effects of interferences, especially from the blank ICP matrix, were ascertained by the evaluation of three levels of quality control (QC) analytes and analyte-free extracted blank matrix.

Six-point external calibration curves were constructed for each target analyte by diluting the working solution in methanol at concentrations ranged from 0.08 to 1.2 µg/mL. The individual analyte peak areas, from the $[M+H]^+$ precursor ion versus analyte concentrations, were utilised to construct an external calibration curve. A regression analysis was done to determine the linearity based on the coefficient of determination (r^2) and the regression equation was used to calculate the QC analytes and samples concentrations. The linear range was established based on the lower (trace level) and upper (lowest recommended dose) concentrations of analyte expected in adulterated ICPs.

The accuracy was established at low, medium, and high QC levels. All target analytes were spiked into an extracted blank ICP, and the resulting peak area of the $[M+H]^+$ precursor ion was fitted to the regression equation of the external calibration curve to determine its concentration. Comparison of the observed analyte concentration versus the expected concentration at the same QC level was expressed as a percentage of accuracy with an acceptable value of ±25%.

The LOD and LOQ were determined experimentally based on the visual evaluation approach. For LOD, solutions were prepared with an initial 100 ng/ml concentration of target analytes. The solutions were then decreased by 10 ng/ml each down to the final solution of 10 ng/ml. The LOD was set at the lowest concentration of target analyte that can be reliably detected based on the presence and the average intensity ratio of two fragment ions described in the specificity assessment. Meanwhile, the LOQ was defined as the lowest concentration of the calibration curve, where each target analyte can be quantified with an acceptable percentage of accuracy of ±25% and precision based on the percentage of relative standard deviation (%RSD) of less than 20%.

Using the same QC analytes in an extracted blank matrix, precisions were determined based on repeatability and intermediate precision at low, medium, and high QC levels. Repeatability and intermediate precision were established at intra- and inter-day, respectively, and expressed as a %RSD of the peak areas of the $[M+H]^+$ precursor ion with an acceptable value of less than 20%.

The ME was evaluated based on the post-extraction addition method by comparing the slopes of the matrix-matched calibration curve versus those of the external calibration curve in a neat solution as expressed by Eq. 4.4.6. Both calibration curves were constructed using the same concentration as the QC analytes. The percentage of ME was then categorised in accordance with the set criteria of insignificant (0% to ±10%), acceptable (±10% to ±20%), moderate (±20% to ±50%), and severe (-50%<>+50%), where a positive value indicates ionisation enhancement while a negative value indicates ionisation suppression.

$$ME\ (\%) = \left[\frac{Slope_{matrix\ matched\ standards}}{Slope_{standards\ in\ neat\ solution}} - 1\right] \times 100 \qquad \text{(Eq. 4.4.6)}$$

The RE was determined by comparing the peak areas of the $[M+H]^+$ precursor ion of target analytes spiked into the blank ICP matrix before extraction versus those spiked into an extracted blank matrix at the same concentration. The RE was expressed in percentage at low, medium, and high QC levels with an acceptable value of ±25%.

All qualitative and quantitative data were processed using Agilent Technologies Mass Hunter workstation software version B.07.00 and personal compound database and library (PCDL) manager software version B.04.00. All other calculations were done using Microsoft (Redmond, WA, USA) Excel 2016 (Microsoft Office).

4.4.7 Workflow for determination of PDE5 inhibitors in instant coffee premixes

The targeted analysis workflow employed (1) the suspected-target screening, (2) the targeted identification, and (3) the quantification of identified PDE5 inhibitors. The non-targeted screening workflow covered both top-down and bottom-up approaches to identify novel PDE5 inhibitors. Fig. 4.4.7 summarises the LC-HRMS workflow for the targeted analysis and the non-targeted screening employed in this study.

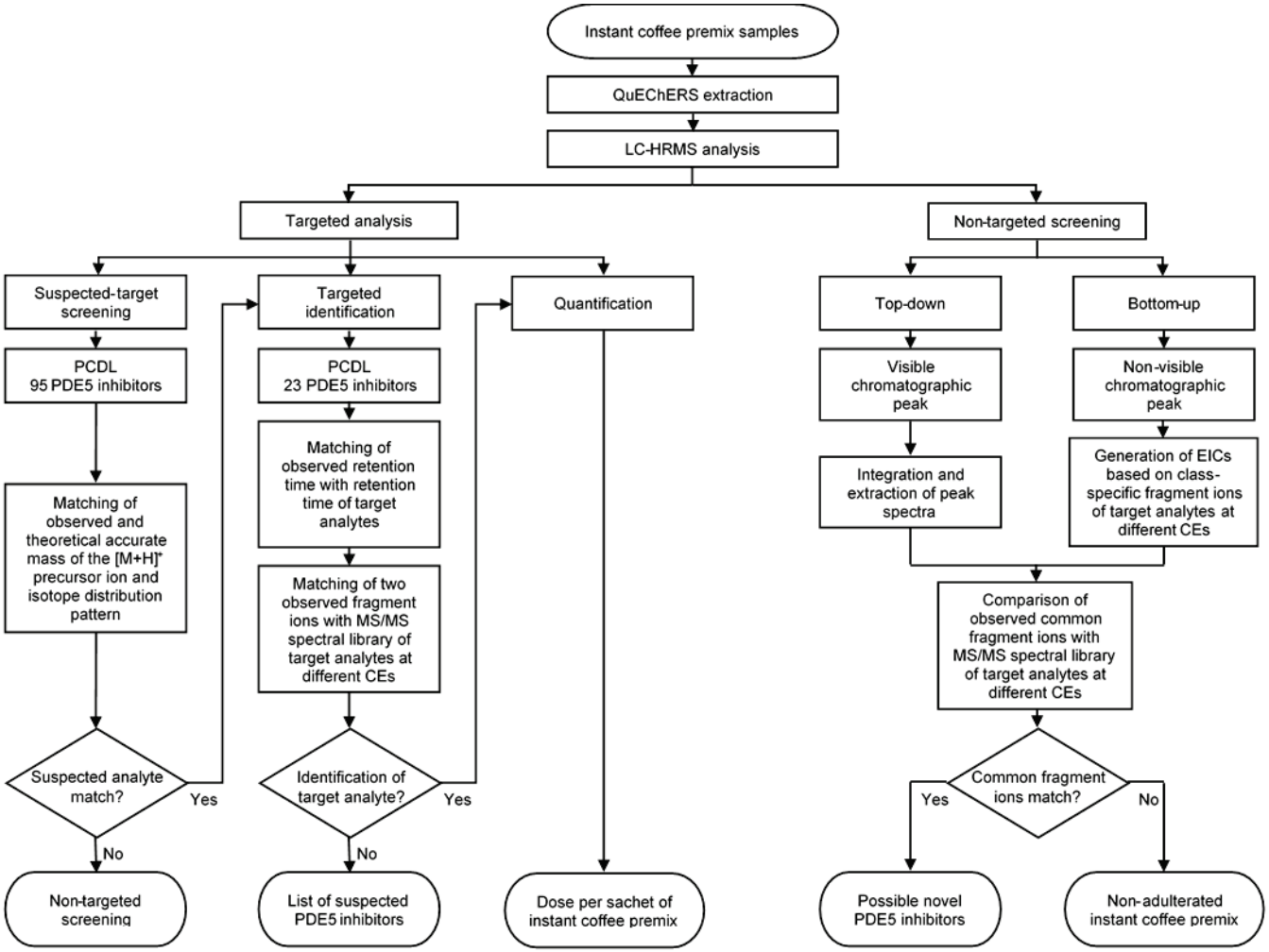


Fig. 4.4.7: Liquid chromatography-high-resolution mass spectrometry (LC-HRMS) workflow for the determination of phosphodiesterase 5 (PDE5) inhibitors in instant coffee premixes. (Abbreviations: QuEChERS, quick, easy, cheap, effective, rugged, and safe; PCDL, personal compound database and library; $[M+H]^+$, protonated molecule; CEs, collision energies)

The initial suspected-target screening workflow was based on a matching algorithm when an observed accurate mass of the $[M+H]^+$ precursor ion was compared to those theoretical ones in the database for a possible match and thus possible presence of a PDE5 inhibitor. Moreover, the isotope distribution pattern was also compared for a match based on its abundance and spacing. For this purpose, a personal MS compound database was created using the PCDL software based on the currently known PDE5 inhibitors found as adulterants in literature. The database contained a total of 95 PDE5 inhibitors with a comprehensive collection of the compound name, molecular formula and structure, and exact mass. The mass accuracy for the MS matching was set at 5 ppm windows with isotope abundance distribution and spacing score of more than 80%. A positive match of the suspected PDE5 inhibitors will be subjected to the targeted identification workflow while a negative match will be further investigated using the non-targeted screening workflow.

The targeted identification workflow relied on the matching of the observed retention time and two observed fragment ions with those of target analytes stored in the same database which included only the 23 PDE5 inhibitors. The same database comprises additional information on the retention time and MS/MS spectral library of target analytes at different collision energies (CEs). The mass accuracy for the MS/MS matching was set at 20 ppm windows with a retention time difference of up to ±0.25 minutes. The rest of the detected PDE5 inhibitors from the suspected-target screening are listed as suspected-target analytes.

The quantification workflow was only applied to samples positive in the targeted identification process. The final dose of the adulterants in each ICP sachet was calculated based on Eq. 4.4.7. The quantification levels were divided into subtherapeutic, therapeutic, and supratherapeutic based on the dose recommended by the approved PDE5 inhibitors. For the comparative purpose of this study, the quantification levels of unapproved PDE5 inhibitors analogues were linked to the therapeutic dosage of their corresponding approved drugs, i.e. 25 to 100 mg for sildenafil and 5 to 20 mg for vardenafil and tadalafil. The determination of trace concentrations was based on a definition set by the International Union of Pure and Applied Chemistry (IUPAC) [22].

$$Final\ dose = \frac{Average\ conc.\ from\ reg.eq.}{(Analysis\ conc. \times Dilution\ level)} \times Wt.per\ sachet \qquad \text{(Eq. 4.4.7)}$$

where:
$Average\ conc.\ from\ reg.\ eq.$ = concentration of target analyte calculated from the regression equation of the external calibration curve (n=3)
$Analysis\ conc.$ = concentration of an ICP used in sample preparation
$Dilution\ level$ = level of dilution from the initial analysis concentration
$Wt.\ per\ sachet$ = total weight of ICP per sachet

The non-targeted screening workflow was employed for further investigation of negative samples from the suspected-target screening. The non-targeted screening approach used in this study was adapted and modified according to the critical review by Pasin et al. [17]. Based on the visual inspection of the chromatographic peak, the top-down and bottom-up approaches were both employed to detect any novel PDE5 inhibitors. A top-down approach was utilised for visible chromatographic peaks. All visible peaks within the base peak chromatogram (BPC) were integrated and extracted to reveal the mass spectra. Each mass spectrum was interrogated with the highest abundance peak selected

as a possible $[M+H]^+$ precursor ion of novel PDE5 inhibitors. The relationship between the selected $[M+H]^+$ precursor ion was established with the fragment ions of target analytes via product ion scan at MS/MS level of the Mass Hunter workstation software to reveal any common fragmentation pattern.

Conversely, a bottom-up approach was utilised for non-visible chromatographic peaks where the extracted ion chromatograms (EICs) were generated based on the fragment ions of target analytes at different CEs. Using this approach, no prior knowledge of the $[M+H]^+$ precursor ion is available. Therefore, all possible $[M+H]^+$ precursor ions generated from the MS experiment were considered as novel PDE5 inhibitors. The presence of class-specific EICs of the product ion scan at MS/MS level may reveal the presence of novel PDE5 inhibitors which can be further interrogated and linked with their distinct $[M+H]^+$ precursor ion. Both of these approaches aimed to reveal any common fragmentation pattern that could be linked to any known PDE5 inhibitors and thus, deduce the potential of identifying novel PDE5 inhibitors.

4.5 RESULTS AND DISCUSSION

4.5.1 Analytical method optimisation and validation

The analytical method optimisation as a whole addressed the issue of MEs from complex matrices such as ICPs. Also, the presence of four different groups of structural isomers was tackled chromatographically, leading to a baseline chromatographic separation, enhancing the specificity of each isomeric analyte. Other chromatographic optimisation discussed in Section 4.6.3 resulted in improved peak shape and resolution, and reproducible retention time for each target analyte. The presence of sodium adducts was addressed during the MS optimisation and thus improved the selectivity and sensitivity of the MS and MS/MS experiments. The modified QuEChERS extraction procedure was successfully developed following poor MEs using the conventional dilute and shoot technique during the sample preparation optimisation. In conclusion, the success of the analytical method optimisations discussed in this study is significant for a definitive screening, identification, and quantification of PDE5 inhibitors and their analogues from ICPs.

The specificity was successfully demonstrated using the developed chromatographic separation as presented in Fig. 4.5.1. Target analytes in extracted blank ICP at all QC levels could be correctly identified using the distinguishable $[M+H]^+$ precursor ion without any interference from the matrix components. Conversely, the analyte-free extracted blank matrix returned insignificant signals corresponding to all target analytes at their retention times. The presence of two fragment ions correspondingly ensured the specificity of the

method and the average intensity ratio confirmed the identity of the target analytes. These data are presented in Table 4.6.6A.

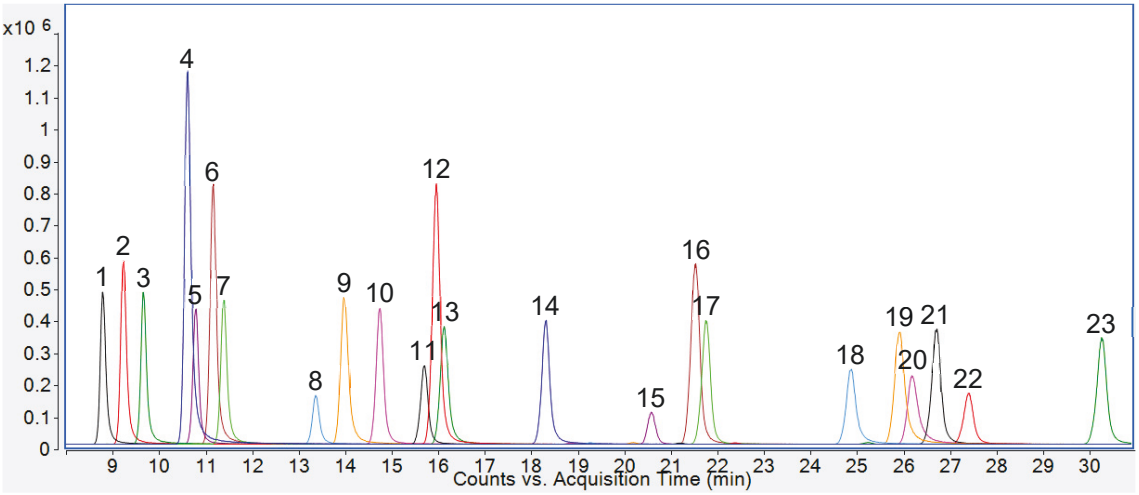


Fig. 4.5.1: Representative extracted ion chromatogram (EIC) of the protonated molecule ($[M+H]^+$) precursor ion of 23 targeted phosphodiesterase 5 (PDE5) inhibitors mixture in neat solution at a concentration level of 1.2 µg/mL.

The linearity of the method was confirmed for each target analyte with a coefficient of determination (r^2) larger than 0.9960. The selected range proved to suffice for quantification of target analytes ranging from trace level up to supratherapeutic concentrations from the ICP matrix. The percentage of accuracy ranged from 88.1% to 119.3% at low; 94.8% to 110.3% at medium; and 100.6% to 109.3% at high QC level. The LOD and LOQ for all target analytes ranged from 10 to 70 ng/mL and 80 ng/mL, respectively. These results are presented in Table 4.6.6B.

Table 4.6.6C shows the results of precisions, ME and RE. The method produced good repeatability at low, medium, and high QC levels with the %RSD ranging from 0.4% to 7.3%; 1.0% to 6.2%; and 0.6% to 3.1%, respectively. In agreement

with the repeatability results, the intermediate precision was calculated to be within 0.6%–7.2% at low; 0.6%–7.7% at medium; and 0.5%–9.1% at high QC level. Insignificant MEs were observed for all target analytes within -5.2% to +8.7% whereas the RE proved to be satisfactory at all QC levels within 84.7%–123.5% except for N-desethylacetildenafil at low (53.8%) and medium (65.1%) QC levels.

4.5.2 Analysis of PDE5 inhibitors in instant coffee premixes

A total of 25 ICP samples were submitted to the LC-HRMS analysis for the determination of PDE5 inhibitors. The initial suspected-target screening resulted in 17 positive samples, of which 15 were further confirmed using the targeted identification process and quantified. The non-targeted screening workflow detected no suspicious compounds, so there were no analytes of novel PDE5 inhibitors flagged from the ICP samples. In summary, 9 samples were adulterated with one PDE5 inhibitor, 2 samples with two inhibitors, and the rest 6 samples with three and four inhibitors for each 3 samples, respectively, as shown in Fig. 4.9B (a) and (b) (supplementary data).

Collectively, eight distinct PDE5 inhibitors were determined using the targeted identification workflow while another two highly suspected adulterants were detected through the suspected-target screening workflow. The most prominent adulterant was sildenafil which was identified in 4 samples as a single adulterant and 5 samples in combinations with other PDE5 inhibitors. Other adulterants of PDE5 inhibitors discovered in this study included dimethylsildenadil, thiodimethylsildenafil, and thiosildenafil (5 samples each), tadalafil (3 samples),

desmethylcarbodenafil (2 samples), and propoxyphenyl-thiodimethylsildenafil and propoxyphenyl-sildenafil (1 sample each) either in combination with each other or as a single adulterant.

Only 15 samples were quantified with these adulterants found at subtherapeutic levels up to supratherapeutic concentrations ranged from 2.77 to 121.64 mg per sachet of the ICP sample. Although distinct PDE5 inhibitors may be quantified at trace, subtherapeutic, and therapeutic levels, a combination of these adulterants in one sachet of ICP may subsequently result in supratherapeutic concentrations as presented in SPL004, SPL015, SPL019, SPL020, and SPL024. The sample dilution approach employed in this study proved to be excellent for the determination of PDE5 inhibitors at trace and subtherapeutic levels. For quantification of adulterants at therapeutic and supratherapeutic concentrations, the dilution level of up to 1:100 was deemed to be sufficient. However, the fact that multiple adulterants may be present in a sample and often at different concentration levels, required at least another further sample dilution for accurate and precise quantification of each target analyte. A detailed content of each sachet of ICP samples is presented in Table 4.5.2 and Fig. 4.9B (c) (supplementary data) summarises the results.

Table 4.5.2: The contents of phosphodiesterase 5 (PDE5) inhibitors in each sachet of instant coffee premix samples.

Label	Weight per sachet (g)	Identified analytes (average dose per sachet in mg - quantification level)				
		Analyte 1	Analyte 2	Analyte 3	Analyte 4	Total analyte
SPL001	20.21	Desmethylcarbodenafil (106.02 - SPR)	ND	ND	ND	106.02 SPR
SPL002	24.81	Thiosildenafil (2.77 - SUB)	Hydroxy thiohomosildenafil*	ND	ND	2.77 SUB
SPL003	23.37	Dimethylsildenafil (0.85 - TRC)	Propoxyphenyl-thiodimethylsildenafil (4.12 - SUB)	Thiodimethylsildenafil (20.39 - SUB)	ND	25.36 THE
SPL004	19.75	Tadalafil (27.03 - SPR)	Sildenafil (41.86 - THE)	ND	ND	68.89 SPR
SPL005	25.50	Compound X*	ND	ND	ND	NA
SPL006	24.40	Hydroxythiohomo-sildenafil*	ND	ND	ND	NA
SPL007	20.88	Sildenafil (84.93 - THE)	ND	ND	ND	84.93 THE
SPL008	20.31	ND	ND	ND	ND	ND
SPL009	25.50	ND	ND	ND	ND	ND
SPL010	30.06	ND	ND	ND	ND	ND
SPL011	8.26	ND	ND	ND	ND	ND
SPL012	21.61	Sildenafil (83.69 - THE)	ND	ND	ND	83.69 THE

SPL013	25.03	Sildenafil (86.56 - THE)	ND	ND	ND	83.56 THE
SPL014	29.67	ND	ND	ND	ND	ND
SPL015	19.23	Dimethylsildenafil (0.60 - TRC)	Sildenafil (0.85 - TRC)	Thiodimethylsildenafil (29.15 - THE)	Thiosildenafil (91.04 - THE)	121.64 SPR
SPL016	17.59	ND	ND	ND	ND	ND
SPL017	19.66	Desmethylcarbodenafil (9.47 - SUB)	ND	ND	ND	9.47 SUB
SPL018	24.13	ND	ND	ND	ND	ND
SPL019	19.18	Dimethylsildenafil (1.32 - TRC)	Thiosildenafil (22.18 - SUB)	Thiodimethylsildenafil (91.55 - THE)	ND	115.05 SPR
SPL020	20.12	Propoxyphenyl-sildenafil (Detected)	Tadalafil (2.33 - SUB)	Sildenafil (97.82 - THE)	ND	100.15 SPR
SPL021	18.08	Tadalafil (36.02 - SPR)	ND	ND	ND	36.02 SPR
SPL022	19.81	Sildenafil (68.90 - THE)	ND	ND	ND	68.90 THE
SPL023	24.39	ND	ND	ND	ND	ND
SPL024	20.06	Dimethylsildenafil (Detected)	Sildenafil (1.11 - TRC)	Thiodimethylsildenafil (31.40 - THE)	Thiosildenafil (84.16 - THE)	117.32 SPR
SPL025	23.47	Dimethylsildenafil (3.08 - SUB)	Sildenafil (4.43 - SUB)	Thiodimethylsildenafil (8.59 - SUB)	Thiosildenafil (40.50 - THE)	56.60 THE

(Abbreviations: ND, not detected; TRC, trace; SUB, subtherapeutic; THE, therapeutic; SPR, supratherapeutic; NA, not applicable)
*suspected-target screening

Qualitative establishments from the suspected-target screening and targeted identification processes had revealed two highly suspected PDE5 inhibitors in three different samples. SPL002 and SPL006 exhibited a prominent peak at 23.63 and 23.65 minutes, respectively, for each of their BPC. Each of these samples was initially matched with two possible structural isomers, i.e. hydroxythiohomosildenafil and hydroxythiovardenafil based on its $[M+H]^+$ precursor ion at *m/z* 521.1999, with mass errors of 0.00 ppm for SPL002 and 0.19 ppm for SPL006. Moreover, the isotope abundance distribution and spacing score of more than 90% correspondingly approved the matched compounds. The suspected compound has a similar fragmentation pattern with thiohomosildenafil at three different CEs and hence, construed its identity as a possible analogue of thiohomosildenafil. Due to the additional 16 Da mass unit of hydroxythiohomosildenafil, which corresponds to an oxygen atom, their fragmentation patterns are expected to be the same [23].

In contrast, the BPC of SPL005 revealed a prominent peak at 27.85 minutes which was initially assigned as an unknown compound X with *m/z* 499.2310 for its $[M+H]^+$ precursor ion as shown in Fig. 4.5.2 (a). The suspected-target screening revealed matching for two possible structural isomers, namely 3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil [24] and dithiopropylcarbodenafil [25] with a mass error of 0.40 ppm for their $[M+H]^+$ precursor ion. Further investigation of the collision-induced dissociation (CID) process of compound X revealed two unique fragment ions at *m/z* 371.0995 and *m/z* 343.0682 which were also present in the CID spectrum of dithiodesmethylcarbodenafil (20) run at 10, 20, and 40 eV CEs, shown in Fig.

4.5.2 (b) as a representative at 20 eV CE. The data suggest strongly that compound X is a structural analogue of dithiodesmethylcarbodenafil with an extra 28 Da mass unit (C_2H_4). Only 2 isomers are shown in Fig. 4.5.2 (c) with varying R groups, although many other possible R group variations may exist for the structure.

Although the ultimate identity of compound X cannot be concluded with the obtained data, the presence of either 3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil, dithiopropylcarbodenafil, or any other possible structural isomers as an adulterant in an ICP has not been reported in the literature before. To unambiguously confirm the structure of compound X, the use of complementary techniques, such as nuclear magnetic resonance (NMR), would be highly valuable following analyte isolation and purification. Alternatively, identification might be achieved if the certified reference materials of various structural isomers are available. Future investigation for full structural elucidation is warranted as compound X might be a novel PDE5 inhibitor.

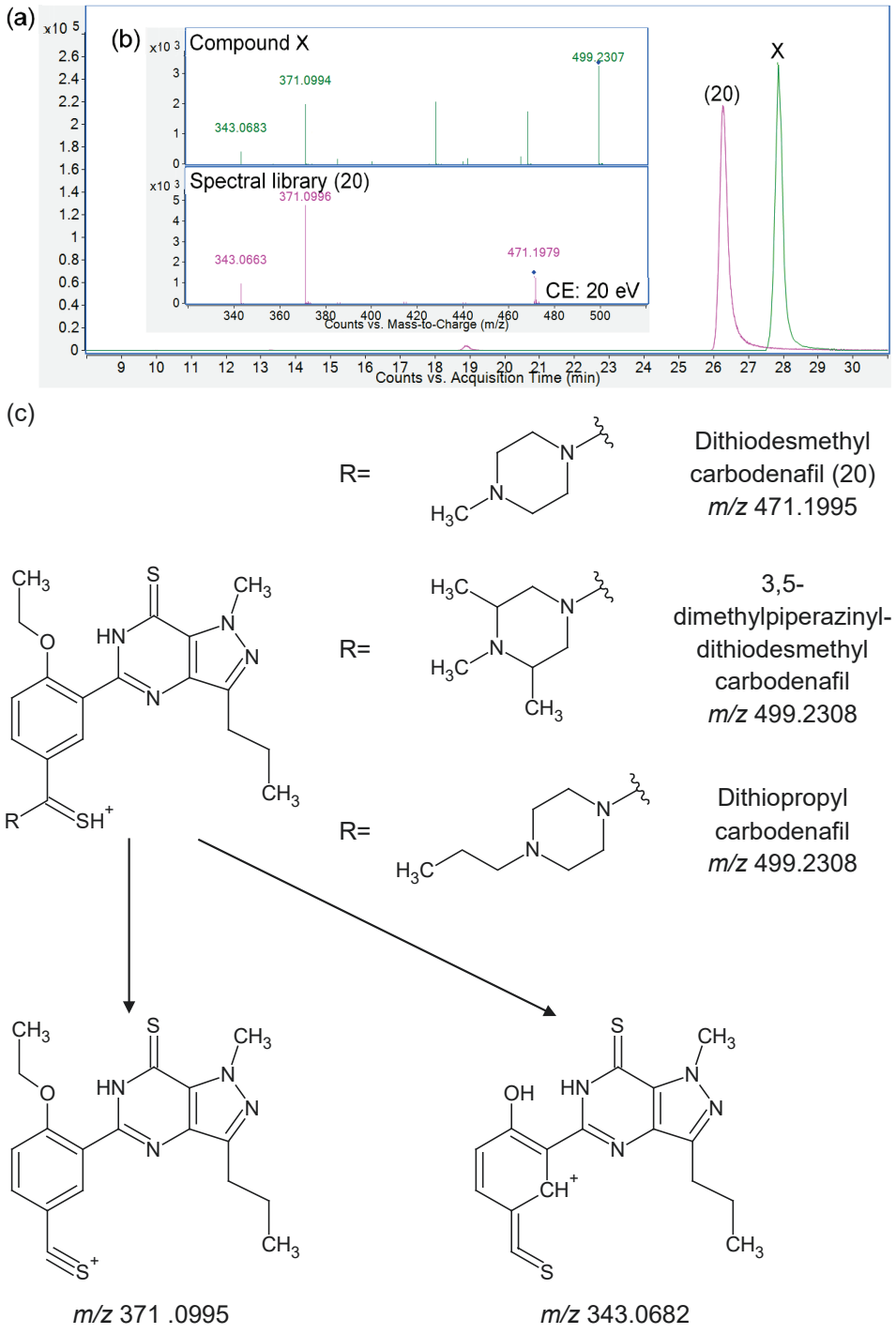


Fig. 4.5.2: Comparison between dithiodesmethylcarbodenafil (20) from the personal compound database and library (PCDL) spectral library with the unknown compound X of sample SPL005 using the suspected-target screening approach with (a) overlaid extracted ion chromatogram (EIC) of the protonated molecule ($[M+H]^+$) precursor ion, (b) comparison of fragment ions based on common fragments at 20 eV collision energy, and (c) proposed common fragmentation pattern shared by dithiodesmethylcarbodenafil (20) and compound X (only showing two possible isomers, 3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil and dithiopropylcarbodenafil, among other isomeric variations).

4.6 DATA ON THE OPTIMISATION AND VALIDATION OF A LIQUID CHROMATOGRAPHY-HIGH-RESOLUTION MASS SPECTROMETRY (LC-HRMS) TO ESTABLISH THE PRESENCE OF PHOSPHODIESTERASE 5 (PDE5) INHIBITORS IN INSTANT COFFEE PREMIXES

4.6.1 Abstract

This paper presents the data on the optimisation and validation of a liquid chromatography-high-resolution mass spectrometry (LC-HRMS) to establish the presence of phosphodiesterase 5 (PDE5) inhibitors and their analogues as adulterants in instant coffee premixes. The method development data covered chromatographic optimisation for better analyte separation and isomeric resolution, mass spectrometry optimisation for high sensitivity and sample preparation optimisation for high extraction recovery (RE) and low matrix effect (ME). The validation data covered specificity, linearity, range, accuracy, limit of detection, limit of quantification, precisions, ME, and RE. The optimisation and validation data presented here is related to the article: “Determination of phosphodiesterase 5 (PDE5) inhibitors in instant coffee premixes using liquid chromatography-high-resolution mass spectrometry (LC-HRMS)” [26].

Specifications table

Subject	Chemistry
Specific subject area	Analytical chemistry
Type of data	Table and extracted ion chromatograms (EICs)
How data was acquired	Liquid chromatography-high-resolution mass spectrometry (LC-HRMS) Agilent Technologies (Santa Clara, CA, USA) 1290 Infinity II LC system coupled to Agilent Technologies 6510 quadrupole time of flight-mass spectrometer (QTOF-MS)
Data format	Extracted and analysed LC-HRMS data
Parameters for data collection	Extraction of analyte-free blank instant coffee premix using dilute and shoot technique with methanol and modified quick, easy, cheap, effective, rugged, and safe (QuEChERS) procedure
Description of data collection	Liquid chromatography-high-resolution mass spectrometry (LC-HRMS) for the determination of phosphodiesterase 5 (PDE5) inhibitors and their analogues as adulterants in instant coffee premixes
Data source location	Institution: University of Technology Sydney City/Town/Region: Ultimo, NSW, 2007 Country: Australia Latitude and longitude for collected data: 33.8832° S, 151.2005° E
Data accessibility	Data are available with this article
Related research article	A.Y. Mohd Yusop, L. Xiao, S. Fu, Determination of phosphodiesterase 5 (PDE5) inhibitors in instant coffee premixes using liquid chromatography-high-resolution mass spectrometry (LC-HRMS), Talanta 204 (2019) 36-43. https://doi.org/10.1016/j.talanta.2019.05.078

Value of the data

- The comprehensive optimisation procedures presented in this paper are valuable for those working on LC-HRMS method development, especially for complex matrices.
- Optimisations of chromatography, sample extraction, and sample dilution are the key to the minimisation of matrix effect.
- The validation data would serve as a reference for forensic drug testing laboratories working on a similar aim of combating adulterated consumable products.

Data

Fig. 4.6.3 shows the chromatographic separation of four different groups of structural isomers of phosphodiesterase 5 (PDE5) inhibitors displaying the extracted ion chromatograms (EICs) of the protonated molecule ($[M+H]^+$) precursor ions. Table 4.6.5 presents the comparison of matrix effect (ME) for instant coffee premix matrix using different extraction technique while Tables 4.6.6A–C summarise the validation data of the analytical method. The optimisation of the analytical method additionally described in this paper.

4.6.2 Experimental design, materials, and methods

The detail on the chemicals and reagents, standard solution and blank matrix preparation, and data analysis are described in [26].

Sample preparation

The whole content of an ICP sachet was weighed into a 50 mL polypropylene tube and recorded. Then, 100 mg of the powder was weighed into a 15 mL polypropylene tube and dissolved in 5 mL of acetonitrile and methanol (50:50, v/v), followed by vortex mixing for 1 minute, sonication for 20 minutes, and centrifugation for 5 minutes at 2500 × *g*, successively. The whole upper solution was then transferred into another 50 mL polypropylene tube prefilled with half of a sachet of the Restek Q-sep QuEChERS salts. Immediately, the sample solution was vortexed for 1 minute followed by centrifugation for 5 minutes at 2500 × *g* to separate the solid extraction salts. The upper layer was filtered using a 0.22 µm PTFE syringe filter and then diluted with methanol at 1:10 dilution level for analysis. The blank ICP was treated in the same manner as the steps described for the sample analysis. For quantification purpose, whenever the analyte concentration was beyond the linear range, the sample solution was further diluted with methanol to fit the resulting concentration within the range of the constructed external calibration curve.

LC-HRMS conditions

The chromatographic separation was performed using an Agilent Technologies (Santa Clara, CA, USA) 1290 Infinity II LC system coupled to an Agilent Technologies 6510 quadrupole time of flight-mass spectrometer (QTOF-MS)

equipped with a dual electrospray ionisation (ESI) nebuliser. The LC system was fitted with a reverse-phase high-performance LC column from Merck KGaA (Darmstadt, Germany) Chromolith® High-Resolution RP-18 end-capped (100 × 4.6 mm, 2.0 µm) with column compartment temperature maintained at 20°C.

The binary mobile phase composition comprised solvent A (10 mM ammonium formate in ultrapure water) and solvent B (acetonitrile). Both solvents were acidified with 0.1% v/v formic acid. The gradient elution was initiated at 5% B and held for 1 minute, followed by a linear boost for 1 minute to 25% B. It was then slowly ramped up to 50% B for 30 minutes followed by a linear boost to 95% B for 1 minute. For the isocratic hold at high organic solvent, 95% B was held for 1 minute before immediately returning the system at 34.01 minutes to the initial gradient of 5% B for 6 minutes. The flow rate was maintained at 0.4 mL/min for the first 34 minutes before immediately ramping it to 1 mL/min between 34.01 to 40 minutes. Post-run equilibration was maintained at 0.4 mL/min for 5 minutes before the next injection. The injection volume was set for 5 µL with the autosampler compartment temperature maintained at 10°C.

The QTOF-MS was operated at a low mass range of *m/z* 1700, calibrated before each chromatographic run to achieve an excellent mass accuracy. ESI in positive ionisation mode was employed with flow-dependent source parameters set at 300°C for gas temperature, 12 L/min for drying gas flow, and 32 psig for nebuliser pressure. The compound-dependent source parameters were set at 3500 V for capillary voltage and 175 V for fragmentor voltage. Other common source parameters were maintained at 65 V for skimmer voltage and 750 V for OCT 1

RF Vpp. An auto MS/MS acquisition was selected for simultaneous MS and MS/MS experiments within a mass range of *m/z* 100 to 1100. The acquisition rates were set at 1 and 3 spectra/sec for the MS and MS/MS experiments, respectively, with a narrow isolation width of *m/z* ~1.3. For the fragmentation of the $[M+H]^+$ precursor ion, the collision energy (CE) was fixed at 10, 20, and 40 eV in a separate scan with nitrogen as the collision gas. The reference mass solution was continuously infused through the reference nebuliser at a steady pressure of 5 psig.

4.6.3 Optimisation of chromatographic separation

The simultaneous separation of a multi-analyte analysis can be a difficult task. Critical attention must be given for each target analyte to achieve an excellent chromatographic separation for reliable screening, identification, and quantification. The presence of four different groups of structural isomers with their $[M+H]^+$ precursor ions, i.e. Group A (*m/z* 439.2452): desmethylcarbodenafil (1) and N-desethylacetildenafil (3); Group B (*m/z* 467.2765): acetildenafil (4) and dimethylacetildenafil (6); Group C (*m/z* 489.2279): vardenafil (7), homosildenafil (9), dimethylsildenafil (10), and propoxyphenyl-sildenafil (13); and Group D (*m/z* 505.2050): thiohomosildenafil (19) and thiodimethylsildenafil (21) among the 23 targeted PDE5 inhibitors need to be addressed to achieve, if possible, an acceptable baseline chromatographic separation. Furthermore, matrix components from the instant coffee premix may cause a significant ME and impair the overall analytical method performance. Thus, the chromatographic separation will be optimised to resolve these issues apart from achieving a good peak shape and resolution, and reproducible retention time.

During the early stage of method development, the suitability and performance of two different columns were assessed using the initial scouting method. Both columns are of reverse phase but with slightly different specifications. The first column tested was Nucleoshell RP 18 (100 × 4.6 mm, 2.7 µm) produced a very broad peak with severe peak tailing for most target analytes. The second column, Chromolith® High Resolution RP-18 end-capped (100 × 4.6 mm, 2.0 µm), displayed an exceptional narrow peak for almost all target analytes with peak asymmetry factor of less than 1.2 based on the symmetrical shape of a Gaussian peak. Although both columns have identical length and internal diameter, the reduction in the particle size of the Chromolith® column led to higher peak efficiencies and thus was chosen for the final methodology.

Organic solvents such as acetonitrile and methanol [27,28] are widely utilised in the analysis of PDE5 inhibitors using LC-MS/MS technique, were initially assessed. Acetonitrile produced a good chromatographic separation for most target analytes compared to methanol and thus selected as the organic mobile phase. Due to the presence of multiple basic amine groups within PDE5 inhibitors, pH-dependent chromatographic problems are expected to be observed as these analytes may exist in both neutral and ionised forms. Therefore, the pK_a values of these target analytes were first evaluated using ChemAxon (Budapest, Hungary) software which revealed some significant variations depending on their chemical structure. Taking all these pK_a values into consideration led to the selection of ammonium formate as a matrix modifier attributable to its pK_a values and useful pH range, and its superior volatility, which is essential for LC-MS/MS analysis. The ammonium formate was assessed at three different concentrations

of 5, 10, and 20 mM. The 10 mM buffer solution proved to be adequate to achieve reproducible retention and improved separation with excellent peak shape and resolution for all target analytes in the presence of 0.1% v/v formic acid in the mobile phases.

The elution profile covered 5% to 95% of the organic solvent to account for the suspected-target and non-targeted screening approaches. Also, the chromatographic gap at the first quarter of the runtime permitted any highly polar and unretained matrix components to be eluted first, minimising the possibilities of co-elution between matrix components and target analytes. The same principle applied in the last quarter of the chromatographic run where strongly retained matrix components eluted. These strategies were adopted to minimise any interference from the instant coffee premix matrix which may lead to a significant ion suppression or ion enhancement of target analytes.

The chromatographic elution profile incorporated an extensive column re-equilibration segment in the developed method. Immediately after conditioning at 34.01 minutes, the re-equilibrium time was maintained at the initial gradient percentage for about 6 minutes by ramping up the flow rate to 1 mL/min. Consequently, the column was flushed with approximately five times of the column volume before the next sample injection. Apart from achieving a good separation and a constant retention time for all target analytes, quantitative variability was minimised, and the carry-over effect was not observed in subsequent analysis. Besides, all four different groups of structural isomers were

separated down to a baseline level, ensuring the specificity of each target analyte as illustrates in Fig. 4.6.3.

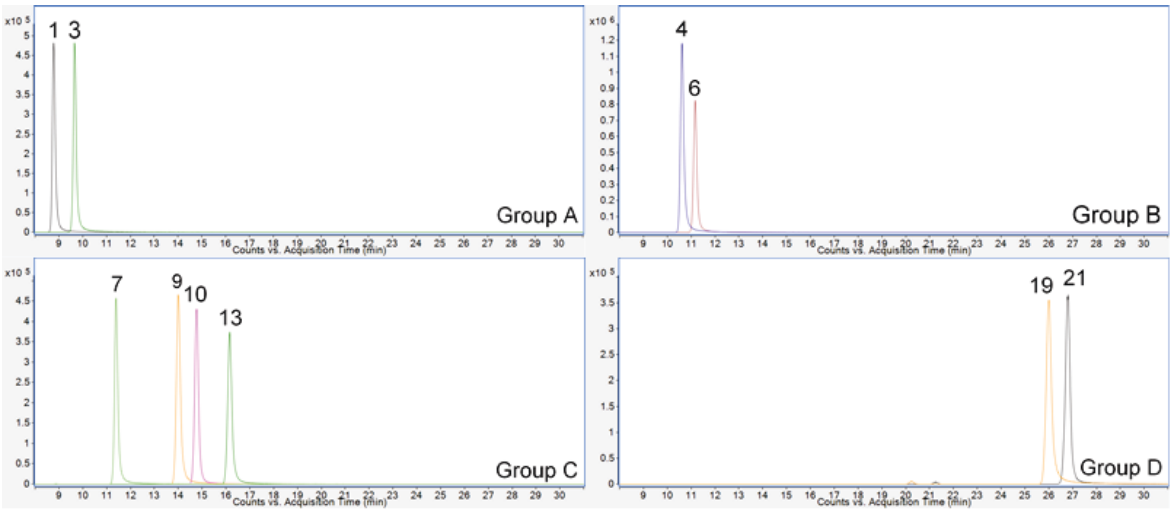


Fig. 4.6.3: Chromatographic separation of structural isomers with extracted ion chromatograms (EICs) of the protonated molecule ($[M+H]^+$) precursor ions of: Group A (*m/z* 439.2452): desmethylcarbodenafil (1) and N desethylacetildenafil (3); Group B (*m/z* 467.2765): acetildenafil (4) and dimethylacetildenafil (6); Group C (*m/z* 489.2279): vardenafil (7), homosildenafil (9), dimethylsildenafil (10), and propoxyphenyl-sildenafil (13); and Group D (*m/z* 505.2050): thiohomosildenafil (19) and thiodimethylsildenafil (21).

4.6.4 Optimisation of MS conditions

The first MS issue encountered during the method development phase was the presence of sodium adducts, especially with analytes containing carbonyl groups. Although this phenomenon often observed in previous studies, it has not been acknowledged and addressed. It is widely known that one of the most common sources of this metal adduct contamination is the laboratory glassware [29]. Plasticware, therefore, was utilised throughout the whole experimental processes. High drying gas temperature may also contribute to the formation of sodium adducts; thus, the temperature was lowered from 350°C to 300°C. These

approaches led to the absence of metal adducts, particularly single charge sodium adducts.

All other MS source parameters were adjusted based on the flow- and compound-dependent parameters. The flow-dependent source parameters, which include nebuliser pressure and drying gas flow, were adjusted based on the flow rate of 0.4 mL/min. The compound-dependent source parameters, i.e. capillary voltage, fragmentor voltage, and CE, were adjusted based on the mass range of *m/z* 100 to 1100. The continuous infusion of two reference masses viz. purine (*m/z* 121.050873) and hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine (*m/z* 922.009798) during each chromatographic run led to the mass accuracy of less than 2 ppm for $[M+H]^+$ precursor ions and less than 5 ppm for fragment ions.

4.6.5 Optimisation of sample preparation

Even though the chromatographic separation and MS conditions have been fully optimised, the widely used dilute and shoot (D&S) technique initially applied on instant coffee premixes with either methanol, acetonitrile, or in combination with ultrapure water often end up in severe and moderate MEs for most target analytes. During the early stage of method development, methanol was chosen as the solvent for the D&S technique on instant coffee premixes as it produced the least number of target analytes exhibiting ME.

The optimisation of sample preparation was mainly evaluated based on the ME and extraction recovery (RE) efficiency. As the D&S technique using methanol is very simple, a dilution approach was further attempted to minimise any possible

ME that may arise from the instant coffee premix matrix. The matrix was assessed at three levels of dilution at 1:2, 1:10, and 1:100 while maintaining the concentration of target analytes at low, medium, and high quality control (QC) levels.

As expected, the D&S technique at the lowest dilution level of 1:2 had caused severe and moderate ionisation suppressions for three and seven analytes, respectively. Even at 1:10 dilution, severe MEs were still prominent for the same three analytes that belong to the acetyl-bonded analogue of sildenafil. The MEs for the D&S technique were found to be reduced in proportion to the matrix dilution and turn out to be insignificant at 1:100 dilution. However, one major concern at this dilution level was the ability of the developed procedure to detect target analytes at trace concentrations.

Consequently, another sample extraction technique was assessed in a review of the first attempted D&S technique. The quick, easy, cheap, effective, rugged, and safe (QuEChERS) extraction technique was selected primarily based on its time-saving advantage of sample preparation. To the best of our knowledge, only two previous studies had employed the AOAC official 2007.01 QuEChERS method to determine PDE5 inhibitors in herbal dietary supplements [30] and Chinese tonic liquors [31]. On the contrary, in this study, the EN 15662 QuEChERS salts were chosen due to its claimed capacity in removing polar interferences, sugars, and fats that might be present in the sample matrix. Unfortunately, the official buffered EN 15662 method using acetonitrile and hydrated instant coffee premix give rise to poor RE for some target analytes. Therefore, several modified

QuEChERS procedures were attempted on a trial-and-error basis with varying solvent mixtures, sample loads and QuEChERS salts quantities. The final methodology is given in detail in Section 4.6.2.

The modified QuEChERS extraction procedure was also evaluated at three dilution levels like the D&S technique discussed above. The instant coffee premix matrix at 1:10 dilution exhibited an insignificant ME for all target analytes with the percentage of within -5.22 to +8.67. Equally, the RE proved to be excellent for all target analytes at low, medium, and high QC levels within ±25% except for N-desethylacetildenafil at low (53.8%) and medium (65.1%) QC levels. The analysis of real samples had evinced that any trace analyte was highly unlikely to produce false-negative identification at this dilution level as opposed to a higher level of dilution. Table 4.6.5 displays the comparison of the ME for all 23 targeted PDE5 inhibitors using the D&S technique with methanol against the modified QuEChERS procedure on the instant coffee premix at different dilution levels.

4.6.6 Method validation

The analytical method validation was performed in accordance with the described procedure in Section 4.4.6. The data on each validation parameter are presented in Tables 4.6.6A–C.

Table 4.6.5: Matrix effect (ME) for instant coffee premix using dilute and shoot (D&S) technique with methanol and modified quick, easy, cheap, effective, rugged, and safe (QuEChERS) procedure at three levels of dilution.

No.	Analytes	ME (%) (n=9)					
		Dilution 1:2		Dilution 1:10		Dilution 1:100	
		D&S	QuEChERS	D&S	QuEChERS	D&S	QuEChERS
1	Desmethylcarbodenafil	-19.7	-9.8	-6.4	+1.2	-0.9	-0.2
2	Carbodenafil	-21.1	-9.7	-5.1	+1.0	+0.3	0.0
3	N-desethylacetildenafil	-95.4	-23.3	-62.3	+0.1	+1.7	+1.5
4	Acetildenafil	-100.0	-36.7	-73.0	-5.2	-1.7	+0.3
5	Hydroxyvardenafil	-3.4	-6.1	+4.8	+5.3	+0.7	+2.7
6	Dimethylacetildenafil	-96.7	-22.7	-63.9	-2.9	-3.3	+1.2
7	Vardenafil	-2.9	-8.9	+1.5	+0.2	-1.1	-0.4
8	Sildenafil	-19.3	-11.8	-4.4	+1.3	0.0	+1.9
9	Homosildenafil	-23.0	-9.5	-8.4	+1.4	-1.9	+0.7
10	Dimethylsildenafil	-18.2	-6.0	-5.6	+5.3	-2.2	+3.1
11	Propoxyphenyl-hydroxyhomosildenafil	-12.9	-6.7	-2.7	+5.6	+0.1	+4.4
12	Udenafil	-15.2	-10.5	-4.3	+2.1	-0.5	+1.0
13	Propoxyphenyl-sildenafil	-20.7	-13.1	-8.3	-0.5	-3.1	+1.1
14	Hydroxythiovardenafil	-14.0	-10.1	-1.9	+5.4	-1.5	+3.4
15	Tadalafil	-23.3	-9.4	-10.2	+3.4	-1.9	+3.2
16	Mirodenafil	-14.6	-9.4	-3.9	+1.0	-2.2	+1.4
17	Mutaprodenafil	-11.8	-10.1	-3.5	+2.1	-1.7	+0.2
18	Thiosildenafil	-15.9	-7.1	-3.2	+5.1	-3.0	+3.6
19	Thiohomosildenafil	-21.4	-12.1	-5.4	+5.6	-2.9	+3.0
20	Dithiodesmethylcarbodenafil	-10.6	-8.9	-2.9	-4.9	-0.2	+0.2
21	Thiodimethylsildenafil	-24.0	-11.3	-4.2	+8.7	-2.6	+3.8
22	Propoxyphenyl-thiohydroxyhomosildenafil	-9.0	-2.8	+3.6	+7.3	-1.4	+5.8
23	Propoxyphenyl-thiodimethylsildenafil	-22.7	-6.9	-4.8	+6.4	-2.9	+4.2

Table 4.6.6A: Retention time (RT), accurate mass of protonated molecule ($[M+H]^+$) precursor ion, mass error, and fragment ions of 23 targeted phosphodiesterase 5 (PDE5) inhibitors.

No.	Analytes	RT (min)	Accurate mass of $[M+H]^+$ precursor ion (*m/z*)		Mass error (ppm)	Fragment ion 1 (*m/z*)	Mass error (ppm)	Fragment ion 2 (*m/z*)	Mass error (ppm)
			Theoretical	Observed					
1	Desmethylcarbodenafil	8.78	439.2452	439.2452	0.00	311.1139	-3.21	339.1452	-2.36
2	Carbodenafil	9.23	453.2609	453.2600	-1.96	311.1139	-1.61	339.1452	-2.36
3	N-desethylacetildenafil	9.65	439.2452	439.2454	0.46	325.1295	-3.08	297.1346	3.37
4	Acetildenafil	10.62	467.2765	467.2764	-0.21	297.1346	-1.35	127.1230	0.00
5	Hydroxyvardenafil	10.80	505.2228	505.2222	-1.19	312.1581	-3.20	151.0866	-5.29
6	Dimethylacetildenafil	11.17	467.2765	467.2763	-0.43	297.1346	-2.02	127.1230	-4.72
7	Vardenafil	11.39	489.2279	489.2276	-0.61	312.1581	-1.60	151.0866	-1.32
8	Sildenafil	13.38	475.2122	475.2119	-0.63	283.1190	-3.53	100.0995	-5.99
9	Homosildenafil	13.99	489.2279	489.2274	-1.02	283.1190	-4.24	113.1073	-3.54
10	Dimethylsildenafil	14.77	489.2279	489.2279	0.00	283.1190	-0.35	113.1073	-1.77
11	Propoxyphenyl-hydroxyhomosildenafil	15.74	519.2384	519.2391	1.35	129.1022	6.20	283.1190	-6.71
12	Udenafil	15.99	517.2592	517.2594	0.39	112.1121	-2.68	283.1190	-3.53
13	Propoxyphenyl-sildenafil	16.16	489.2279	489.2286	1.43	100.0995	-1.00	283.1190	-5.65
14	Hydroxythiovardenafil	18.36	521.1999	521.2000	0.19	167.0637	-2.99	328.1352	-1.83
15	Tadalafil	20.63	390.1448	390.1444	-1.03	135.0441	-5.92	169.0760	-1.77
16	Mirodenafil	21.59	532.2588	532.2588	0.00	312.1343	-2.88	296.1394	-0.34
17	Mutaprodenafil	21.81	630.2275	630.2275	0.00	113.1073	0.88	142.0070	-0.70
18	Thiosildenafil	24.96	491.1894	491.1886	-1.63	100.0995	-1.00	299.0961	-5.02
19	Thiohomosildenafil	25.99	505.2050	505.2049	-0.20	299.0961	-5.35	113.1073	-5.30
20	Dithiodesmethylcarbodenafil	26.26	471.1995	471.1994	-0.21	343.0682	-2.04	371.0995	-1.62
21	Thiodimethylsildenafil	26.81	505.2050	505.2048	-0.40	113.1073	-3.54	299.0961	-3.34
22	Propoxyphenyl-thiohydroxyhomosildenafil	27.48	535.2156	535.2155	-0.19	129.1022	3.10	299.0961	0.33
23	Propoxyphenyl-thiodimethylsildenafil	30.36	519.2207	519.2210	0.58	113.1073	1.77	299.0961	-1.00

Table 4.6.6B: Coefficient of determination (r^2), accuracy, limit of detection (LOD), and limit of quantification (LOQ) of 23 targeted phosphodiesterase 5 (PDE5) inhibitors.

No.	Analytes	r^2	Accuracy (Mean ± SD, %) (n=3)			LOD (ng/mL)	LOQ (ng/mL)
			Low	Medium	High		
1	Desmethylcarbodenafil	0.9979	116.5 ± 3.4	102.9 ± 1.9	108.8 ± 2.2	10	80
2	Carbodenafil	0.9982	119.3 ± 1.1	100.4 ± 2.8	107.8 ± 1.6	10	80
3	N-desethylacetildenafil	0.9980	105.8 ± 2.5	96.6 ± 4.1	104.1 ± 2.8	30	80
4	Acetildenafil	0.9969	106.3 ± 0.4	106.8 ± 6.4	100.6 ± 1.7	20	80
5	Hydroxyvardenafil	0.9985	103.5 ± 2.2	108.8 ± 3.2	108.6 ± 1.5	10	80
6	Dimethylacetildenafil	0.9960	117.5 ± 3.0	94.8 ± 3.8	109.2 ± 1.9	20	80
7	Vardenafil	0.9972	94.8 ± 1.2	110.3 ± 1.4	107.0 ± 1.2	20	80
8	Sildenafil	0.9989	110.9 ± 1.3	101.0 ± 1.6	109.3 ± 1.5	30	80
9	Homosildenafil	0.9993	108.3 ± 1.0	105.8 ± 1.1	107.3 ± 1.2	40	80
10	Dimethylsildenafil	0.9989	116.5 ± 0.8	103.5 ± 1.2	107.7 ± 0.8	30	80
11	Propoxyphenyl-hydroxyhomosildenafil	0.9984	116.8 ± 3.6	103.2 ± 1.4	107.9 ± 2.4	70	80
12	Udenafil	0.9994	109.2 ± 1.9	105.8 ± 1.2	108.0 ± 1.4	10	80
13	Propoxyphenyl-sildenafil	0.9988	106.2 ± 1.0	107.4 ± 2.4	107.3 ± 1.6	10	80
14	Hydroxythiovardenafil	0.9984	98.5 ± 1.6	107.8 ± 1.0	107.5 ± 0.7	20	80
15	Tadalafil	0.9983	119.2 ± 4.4	106.5 ± 1.8	107.0 ± 3.1	60	80
16	Mirodenafil	0.9963	117.3 ± 1.4	101.6 ± 2.4	106.5 ± 0.7	10	80
17	Mutaprodenafil	0.9977	88.1 ± 1.6	108.4 ± 1.6	107.3 ± 1.4	20	80
18	Thiosildenafil	0.9972	97.5 ± 1.9	109.0 ± 1.6	107.6 ± 1.4	30	80
19	Thiohomosildenafil	0.9972	97.5 ± 1.5	109.6 ± 1.7	106.9 ± 1.9	10	80
20	Dithiodesmethylcarbodenafil	0.9960	90.7 ± 2.5	109.9 ± 1.3	106.7 ± 2.0	10	80
21	Thiodimethylsildenafil	0.9992	107.4 ± 2.2	105.2 ± 2.1	106.8 ± 0.8	20	80
22	Propoxyphenyl-thiohydroxyhomosildenafil	0.9986	104.3 ± 2.8	109.2 ± 1.2	108.4 ± 0.6	20	80
23	Propoxyphenyl-thiodimethylsildenafil	0.9989	106.9 ± 4.9	105.1 ± 1.6	108.6 ± 1.2	10	80

Table 4.6.6C: Precisions, matrix effect (ME), and extraction recovery (RE) of 23 targeted phosphodiesterase 5 (PDE5) inhibitors.

No.	Analytes	Precisions (%RSD) (n=9)						ME (%) (n=9)	RE (Mean ± SD, %) (n=3)		
		Repeatability			Intermediate						
		L	M	H	L	M	H		Low	Medium	High
1	Desmethylcarbodenafil	4.4	2.0	2.1	3.1	2.9	3.0	+1.2	117.4 ± 6.1	112.5 ± 1.8	97.6 ± 1.1
2	Carbodenafil	1.4	3.1	1.5	5.3	4.1	1.7	+1.0	115.1 ± 2.2	111.4 ± 0.9	100.3 ± 0.3
3	N-desethylacetildenafil	2.7	4.4	2.7	3.0	1.5	2.0	+0.1	53.8 ± 0.8	65.1 ± 2.0	95.1 ± 0.6
4	Acetildenafil	0.4	6.2	1.7	6.7	7.7	9.1	-5.2	87.3 ± 0.1	84.7 ± 0.4	111.0 ± 4.5
5	Hydroxyvardenafil	2.5	3.1	1.4	6.1	1.5	2.6	+5.3	116.7 ± 2.6	116.6 ± 1.2	106.4 ± 0.4
6	Dimethylacetildenafil	3.4	4.3	1.8	1.4	2.1	1.0	-2.9	85.1 ± 4.6	83.9 ± 4.4	89.1 ± 4.4
7	Vardenafil	1.2	1.2	1.1	2.3	2.6	2.9	+0.2	123.0 ± 8.6	115.5 ± 1.8	108.9 ± 1.1
8	Sildenafil	1.7	1.7	1.4	5.6	3.1	2.6	+1.3	113.7 ± 4.5	109.5 ± 1.2	103.9 ± 1.8
9	Homosildenafil	1.2	1.1	1.2	3.6	1.8	1.4	+1.4	116.0 ± 5.4	110.1 ± 1.8	103.5 ± 0.8
10	Dimethylsildenafil	1.1	1.3	0.8	6.9	2.7	4.1	+5.3	115.3 ± 6.0	109.0 ± 1.6	103.1 ± 0.5
11	Propoxyphenyl-hydroxyhomosildenafil	4.5	1.5	2.3	7.2	7.3	5.5	+5.6	117.3 ± 6.7	114.3 ± 0.6	99.1 ± 11.8
12	Udenafil	2.1	1.2	1.3	0.6	1.2	2.2	+2.1	113.3 ± 2.8	109.4 ± 1.2	102.3 ± 1.6
13	Propoxyphenyl-sildenafil	1.1	2.3	1.5	6.2	3.2	1.6	-0.5	123.5 ± 0.2	120.4 ± 2.2	108.5 ± 0.6
14	Hydroxythiovardenafil	1.6	1.0	0.6	4.0	2.2	2.3	+5.4	111.1 ± 1.4	109.4 ± 1.8	107.7 ± 0.3
15	Tadalafil	7.2	1.9	3.1	6.7	1.4	1.1	+3.4	103.5 ± 8.3	101.5 ± 4.0	99.3 ± 2.2
16	Mirodenafil	2.0	2.6	0.7	2.3	2.7	3.1	+1.0	111.0 ± 3.4	109.7 ± 1.5	103.5 ± 1.3
17	Mutaprodenafil	1.7	1.4	1.3	1.2	1.3	0.5	+2.1	119.6 ± 5.6	112.0 ± 1.5	105.9 ± 0.9
18	Thiosildenafil	2.3	1.5	1.3	3.6	1.9	0.9	+5.1	114.2 ± 4.6	108.8 ± 0.6	107.1 ± 1.4
19	Thiohomosildenafil	1.6	1.6	1.8	1.7	1.6	1.1	+5.6	114.1 ± 3.7	106.7 ± 0.9	110.1 ± 1.9
20	Dithiodesmethylcarbodenafil	2.4	1.1	1.9	6.6	1.3	0.5	-4.9	111.9 ± 2.1	105.6 ± 5.2	107.2 ± 5.2
21	Thiodimethylsildenafil	3.1	2.2	0.8	1.7	1.9	1.7	+8.7	113.4 ± 3.9	107.0 ± 0.4	112.9 ± 0.5
22	Propoxyphenyl-thiohydroxyhomosildenafil	3.9	1.2	0.6	5.7	2.1	4.1	+7.3	111.0 ± 4.0	110.6 ± 1.5	109.4 ± 1.6
23	Propoxyphenyl-thiodimethylsildenafil	7.3	1.7	1.2	1.0	0.6	1.4	+6.4	111.0 ± 4.2	107.1 ± 0.8	104.8 ± 1.2

(Abbreviations: L, low; M, medium; H, high)

4.7 CONCLUSION

A modified QuEChERS extraction procedure coupled to LC-HRMS analysis was fully optimised and validated to determine PDE5 inhibitors and their analogues found as adulterants in ICPs. The process of screening, identification, and quantification were done simultaneously with detailed procedures and examples discussed in this study. These adulterants were comprehensively screened via the suspected-target and non-targeted approaches, utilising the full spectral information of the simultaneous MS and MS/MS experiments. The optimisation of chromatography, sample extraction, and sample dilution led to the minimisation of ME for all 23 targeted PDE5 inhibitors [19]. The applicability of the developed method was then demonstrated using 25 ICP samples. Typically, consumers tend to take extra precaution when taking health supplements, especially in pharmaceutical dosage form compared to consumable products, such as ICPs. Therefore, this kind of adulterated products will put the public at the absolute risk owing to its easy accessibility, either through conventional or online markets. The strategies proposed in this study would be beneficial to tackle the problems of adulterated ICPs, especially with PDE5 inhibitors and their analogues to safeguard the public health.

4.8 REFERENCES

1. Toci AT, Farah A, Pezza HR, Pezza L. Coffee adulteration: more than two decades of research. *Crit Rev Anal Chem* 2016;46(2):83-92. doi:https://doi.org/10.1080/10408347.2014.966185
2. Stipp GK, Inventor; US Patent 5433962, assignee. Rapidly soluble flavored instant coffee product. 1995.
3. Villagran FV, Dria GJ, Bruno Jr DJ, Small LE, Inventors; US Patent 6048567, assignee. Higher density foamable instant coffee products for preparing cappuccino like beverages. 2000.
4. Atkinson JR, Deis DA, Marchio AL, Inventors; US Patent 6207203, assignee. Fortified coffee drink. 2001.
5. Poon WT, Lam YH, Lai CK, Chan AY, Mak TW. Analogues of erectile dysfunction drugs: an under-recognised threat. *Hong Kong Med J* 2007;13(5):359-363.
6. Skalicka-Wozniak K, Georgiev MI, Orhan IE. Adulteration of herbal sexual enhancers and slimmers: the wish for better sexual well-being and perfect body can be risky. *Food Chem Toxicol* 2017;108:355-364. doi:https://doi.org/10.1016/j.fct.2016.06.018
7. United States Food and Drug Administration. Tainted products marketed as dietary supplements 2018. https://www.accessdata.fda.gov/scripts/sda/sdNavigation.cfm?sd=tainted_supplements_cder. Accessed 1 January 2019.

8. FDA warns consumers about health risks of herbal coffee products from Malaysia containing undeclared active pharmaceutical ingredients and milk 2017. https://www.fda.gov/Drugs/DrugSafety/ucm572569.htm. Accessed 1 January 2019.
9. Gratz SR, Flurer CL, Wolnik KA. Analysis of undeclared synthetic phosphodiesterase-5 inhibitors in dietary supplements and herbal matrices by LC-ESI-MS and LC-UV. *J Pharm Biomed Anal* 2004;36(3):525-533. doi:https://doi.org/10.1016/j.jpba.2004.07.004
10. Jeong JH, Lee JH, Kim HJ, et al. LC-ESI-MS/MS analysis of phosphodiesterase-5 inhibitors and their analogues in foods and dietary supplements in Korea. *Food Addit Contam Part B Surveill* 2016;9(1):1-8. doi:https://doi.org/10.1080/19393210.2014.968220
11. Park SR, Lee JG, Roh SH, et al. Determination of PDE-5 inhibitors and appetite suppressants in adulterated dietary supplements using LC/PDA and LC/MS. *Food Addit Contam Part B Surveill* 2012;5(1):29-32. doi:https://doi.org/10.1080/19393210.2012.656706
12. Gratz SR, Gamble BM, Flurer RA. Accurate mass measurement using Fourier transform ion cyclotron resonance mass spectrometry for structure elucidation of designer drug analogs of tadalafil, vardenafil and sildenafil in herbal and pharmaceutical matrices. *Rapid Commun Mass Spectrom* 2006;20(15):2317-2327. doi:https://doi.org/10.1002/rcm.2594
13. Kee C-L, Ge X, Low M-Y, Koh H-L. Structural elucidation of a new sildenafil analogue using high-resolution Orbitrap mass spectrometry. *Rapid Commun Mass Spectrom* 2013;27(12):1380-1384. doi:https://doi.org/10.1002/rcm.6590

14. Savaliya AA, Shah RP, Prasad B, Singh S. Screening of Indian aphrodisiac ayurvedic/herbal healthcare products for adulteration with sildenafil, tadalafil and/or vardenafil using LC/PDA and extracted ion LC-MS/TOF. *J Pharm Biomed Anal* 2010;52(3):406-409. doi:https://doi.org/10.1016/j.jpba.2009.05.021

15. Zhang F, Wang H, Zhang L, et al. Suspected-target pesticide screening using gas chromatography-quadrupole time-of-flight mass spectrometry with high resolution deconvolution and retention index/mass spectrum library. *Talanta* 2014;128:156-163. doi:https://doi.org/10.1016/j.talanta.2014.04.068

16. Xian F, Hendrickson CL, Marshall AG. High Resolution Mass Spectrometry. *Anal Chem* 2012;84(2):708-719. doi:https://doi.org/10.1021/ac203191t

17. Pasin D, Cawley A, Bidny S, Fu S. Current applications of high-resolution mass spectrometry for the analysis of new psychoactive substances: a critical review. *Anal Bioanal Chem* 2017;409(25):5821-5836. doi:https://doi.org/10.1007/s00216-017-0441-4

18. Rocha T, Amaral JS, Oliveira MBPP. Adulteration of dietary supplements by the illegal addition of synthetic drugs: a review. *Compr Rev Food Sci Food Saf* 2016;15(1):43-62. doi:https://doi.org/10.1111/1541-4337.12173

19. Mohd Yusop AY, Xiao L, Fu S. Data on the optimisation and validation of a liquid chromatography-high-resolution mass spectrometry (LC-HRMS) to establish the presence of phosphodiesterase 5 (PDE5) inhibitors in instant coffee premixes. *Data Brief* 2019;25:104234. doi:https://doi.org/10.1016/j.dib.2019.104234

20. ICH. ICH harmonized tripartite guideline: validation of analytical procedures: text and methodology Q2(R1). In: International Conference on Harmonization of technical requirements for registration of pharmaceuticals for human use; 2005.

21. Furey A, Moriarty M, Bane V, Kinsella B, Lehane M. Ion suppression; a critical review on causes, evaluation, prevention and applications. *Talanta* 2013;115:104-122. doi:https://doi.org/10.1016/j.talanta.2013.03.048

22. Namieśnik J. Trace Analysis - Challenges and Problems. *Crit Rev Anal Chem* 2002;32(4):271-300. doi:https://doi.org/10.1080/10408340290765579

23. Li L, Low MY, Aliwarga F, et al. Isolation and identification of hydroxythiohomosildenafil in herbal dietary supplements sold as sexual performance enhancement products. *Food Addit Contam Part A* 2009;26(2):145-151. doi:https://doi.org/10.1080/02652030802368757

24. Kee CL, Low MY, Ge X. Isolation and characterization of a novel dithio-carbodenafil analogue from a health supplement. *J Pharm Biomed Anal* 2017;137:132-138. doi:https://doi.org/10.1016/j.jpba.2017.01.010

25. Yun J, Shin KJ, Choi J, Kwon K, Jo C-H. Identification and structural elucidation of a new sildenafil analogue, dithiopropylcarbodenafil, from a premixed powder intended as a dietary supplement. *J Chromatogr B* 2018;1072:273-281. doi:https://doi.org/10.1016/j.jchromb.2017.11.029

26. Mohd Yusop AY, Xiao L, Fu S. Determination of phosphodiesterase 5 (PDE5) inhibitors in instant coffee premixes using liquid chromatography-high-resolution mass spectrometry (LC-HRMS). *Talanta* 2019;204:36-43. doi:https://doi.org/10.1016/j.talanta.2019.05.078

27. Shi F, Guo C, Gong L, et al. Application of a high resolution benchtop quadrupole-Orbitrap mass spectrometry for the rapid screening, confirmation and quantification of illegal adulterated phosphodiesterase-5 inhibitors in herbal medicines and dietary supplements. *J Chromatogr A* 2014;1344:91-98. doi:https://doi.org/10.1016/j.chroma.2013.12.030

28. Strano-Rossi S, Odoardi S, Castrignano E, Serpelloni G, Chiarotti M. Liquid chromatography-high resolution mass spectrometry (LC-HRMS) determination of stimulants, anorectic drugs and phosphodiesterase 5 inhibitors (PDE5I) in food supplements. *J Pharm Biomed Anal* 2015;106:144-152. doi:https://doi.org/10.1016/j.jpba.2014.06.011

29. Cech NB, Enke CG. Practical implications of some recent studies in electrospray ionization fundamentals. *Mass Spectrom Rev* 2001;20(6):362-387. doi:https://doi.org/10.1002/mas.10008

30. Vaclavik L, Krynitsky AJ, Rader JI. Targeted analysis of multiple pharmaceuticals, plant toxins and other secondary metabolites in herbal dietary supplements by ultra-high performance liquid chromatography-quadrupole-orbital ion trap mass spectrometry. *Anal Chim Acta* 2014;810:45-60. doi:https://doi.org/10.1016/j.aca.2013.12.006

31. Ding B, Wang Z, Xie J, Zeng G, Chen W. Phosphodiesterase-5 inhibitors in Chinese tonic liquors by liquid chromatography coupled with quadrupole time of flight mass spectrometry. *Food Addit Contam Part B Surveill* 2018;11(3):214-222. doi:https://doi.org/10.1080/19393210.2018.1488769

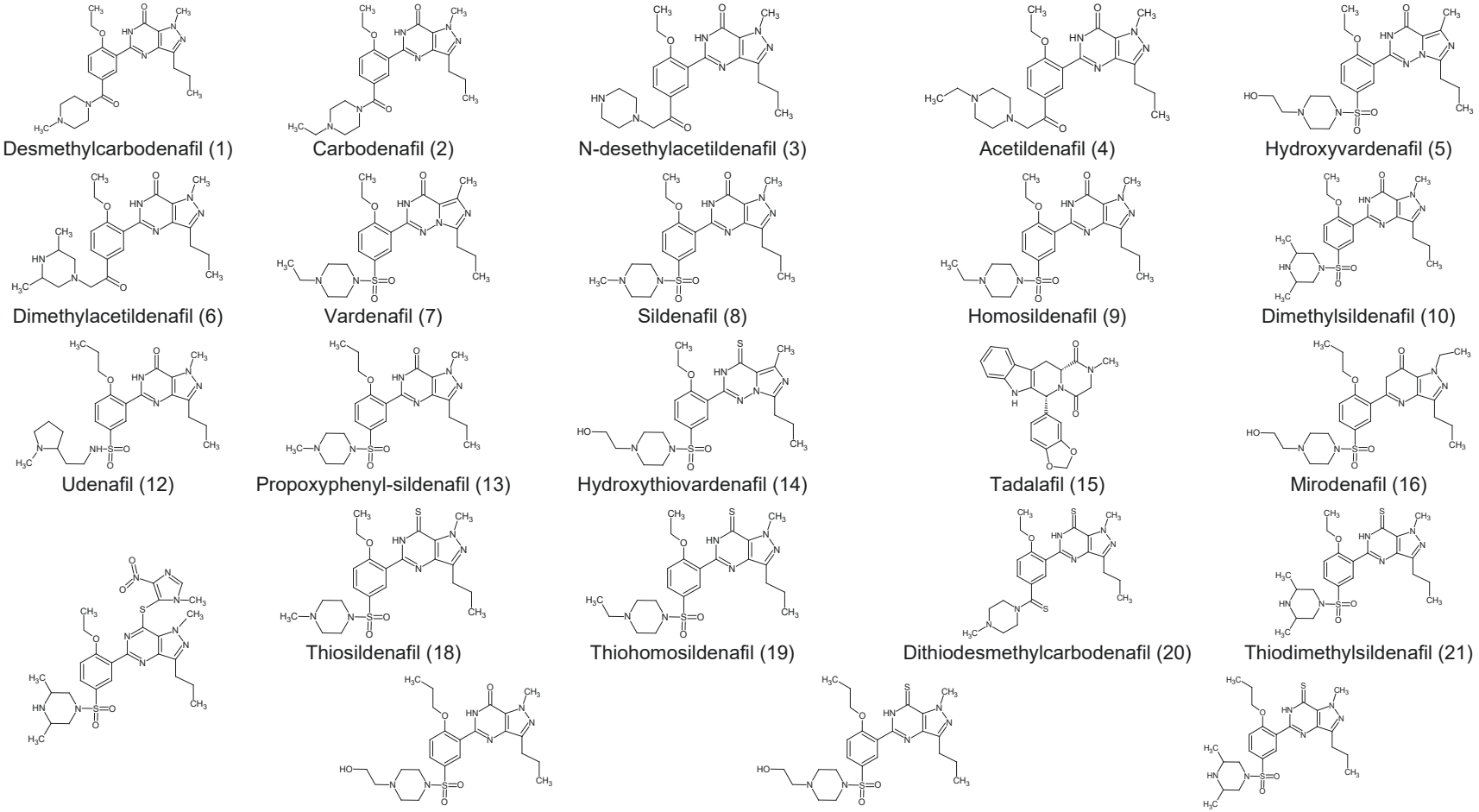
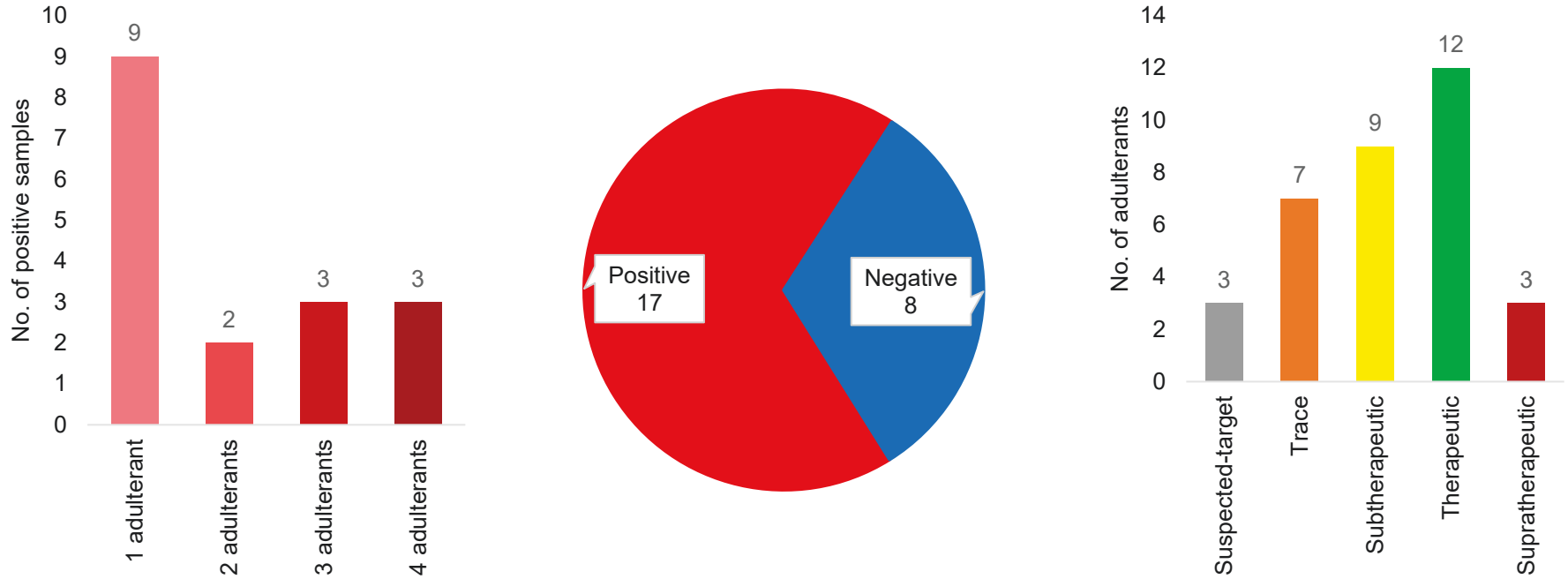


Fig. 4.9A: Chemical structures of 23 targeted phosphodiesterase 5 (PDE5) inhibitors.



(a) Number of adulterants per sample (n=17 positive samples)

(b) LC-HRMS analysis results (n=25 samples)

(c) Quantification level of adulterants (n=34 PDE5 inhibitors)

Fig. 4.9B: Results summary of phosphodiesterase 5 (PDE5) inhibitors in instant coffee premix samples.

CHAPTER 5

Isolation and identification of an isomeric sildenafil analogue as an adulterant in an instant coffee premix

5.1 FOREWORD

The following manuscript, in Chapter 5, has been submitted for publication. The continuous proliferation of novel phosphodiesterase 5 (PDE5) inhibitors analogues already poses a challenge to forensic drug testing laboratories. Worse, their structural isomers may not be readily distinguished, even with high-resolution mass spectrometry (HRMS) data. Consequently, the adulterated products may be distributed in the market undetected, putting consumers at grave risk, due to the unknown safety and toxicology profiles. An instant coffee premix sample, labelled as SPL005, was purchased from an established online retailer based in Malaysia. SPL005 is promoted to improve erectile function, prolong an erection, and prevent premature ejaculation, to name a few. This chapter aims to elucidate the structure of compound X, initially suspected as a novel sildenafil analogue from SPL005 using suspected-target and non-targeted screenings of a liquid chromatography-HRMS in Chapter 4, and flagged as potentially adulterated sample using PDE5 inhibition assay in Chapter 7. Despite the advantages of an HRMS technique, structural isomers belonging to the same group of PDE5 inhibitors such as compound X, could not be distinguished as they shared the same fragmentation patterns. Compound X was, therefore, isolated from SPL005 using liquid chromatography-diode array detection, and its structure was subsequently elucidated with liquid chromatography-ultraviolet spectroscopy and nuclear magnetic resonance spectroscopy. The manuscript emphasised the

equally important application of complementary analytical techniques to unambiguously identified an isomeric PDE5 inhibitor. Mr Ahmad Yusri Mohd Yusop, Dr Linda Xiao, and Professor Shanlin Fu authored the manuscript. Mohd Yusop AY performed the experimental work, data analysis, and initial draft preparation including supplementary data with manuscript edits provided by Xiao L and Fu S.

5.2 ABSTRACT

The proliferation of adulterated health foods and beverages in the market demands a comprehensive analytical strategy to identify the adulterants, particularly those of isomeric phosphodiesterase 5 (PDE5) inhibitors. An instant coffee premix (ICP) purchased from an online retailer was flagged for suspected adulteration through PDE5 inhibition assay. The ICP was then analysed using suspected-target and non-targeted screenings of a liquid chromatography-quadrupole time-of-flight mass spectrometry. Based on these findings, a PDE5 inhibitor initially assigned as compound X was isolated from the ICP by employing a liquid chromatography-diode array detection before its structural elucidation with liquid chromatography-ultraviolet spectroscopy (LC-UV) and nuclear magnetic resonance (NMR) spectroscopy. The suspected-target screening matched the protonated molecule ($[M+H]^+$) precursor ion of compound X at *m/z* 499.2310 with two suspected analytes that are structural isomers of one another. The fragmentation patterns of compound X were comparable to those analogues in the dithiocarbodenafil group through the non-targeted screening. These findings, complemented by the LC-UV and NMR spectroscopy data, together with the chromatographic separation of related structural isomers, conclude the identity of compound X. To our best knowledge, this is the first study to report the presence of 3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil in an ICP sample.

5.3 INTRODUCTION

Herbal-based consumable products are widely perceived as healthy and safe compared to modern medicines [1]. Catchphrases such as all-natural, certified organic, and chemical-free are usually associated with these products to attract consumers. Moreover, the dispersal of misleading information, prominently through social networking media, along with aggressive Internet marketing strategies, has frequently deceived consumers [2]. Among the most prevalent are health foods and beverages that advertise to enhance male sexual performance [3]. These products often stated on their labels to supposedly made up of herbal aphrodisiacs such as *Panax ginseng*, *Eurycoma longifolia*, and *Lepidium meyenii*, to name a few [4].

Regrettably, this lucrative market entices a widespread adulteration, particularly with synthetic erectile dysfunction drugs, namely phosphodiesterase 5 (PDE5) inhibitors [5]. Worse, these products usually contain analogues of the approved drugs viz. sildenafil, vardenafil, and tadalafil, which frequently passed through undetected as it is not included in the routine targeted screening procedure applied by forensic drug testing laboratories [6]. An analogue of PDE5 inhibitors is often synthesised by minor modifications to the parent structure of the approved drugs; thus, altering their physical and chemical properties [7]. Furthermore, some of these analogues are structural isomers of one another, making their identification a challenging task [8].

Clinical studies have shown that the approved PDE5 inhibitors may produce common side effects such as headache, flushing, dyspepsia, and abnormal vision [9]. Besides, they may also cause severe drug-drug interactions in patients on nitrates or α-blockers [10]. Contrarily, structural modifications on the unapproved analogues may impact their absorption, distribution, metabolism, and excretion, which could result in unpredictable potency and side-effects [11]. For example, a sildenafil analogue, namely propoxyphenyl-thiohydroxyhomosildenafil, is ten-fold more potent in inhibiting PDE5 enzyme compared to sildenafil [12]. Therefore, at the same dose, the analogue is more likely to cause severe side effects compared to sildenafil. Another analogue, acetildenafil, has been reported to trigger ataxia, a side effect that was never documented for PDE5 inhibitors before [7]. This adulteration trend raises serious concerns about food safety and public health, as consumers are often unaware of the risks associated with consuming such products [13]. A fatality case associated with a sildenafil analogue, i.e. desmethylcarbodenafil [14], highlights the need for a comprehensive analytical strategy that may reveal the presence of PDE5 inhibitors, particularly those of the unapproved analogues.

In this study, an instant coffee premix (ICP) was submitted to a comprehensive analytical procedure with a pre-screening using PDE5 inhibition assay, where it was flagged for suspected adulteration. The ICP was then analysed to detect specific PDE5 inhibitors through suspected-target and non-targeted screenings. A suspected PDE5 inhibitor, initially assigned as compound X, was isolated from the ICP to determine its identity.

5.4 MATERIALS AND METHODS

5.4.1 Chemicals and reagents

An ICP (SPL005) promoted as a male sexual performance product was purchased from an established online retailer based in Malaysia. Certified reference materials (CRMs) of dithiodesmethylcarbodenafil, sildenafil impurity 12 (3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil), and sildenafil impurity 18 (dithiopropylcarbodenafil) were purchased from TLC Pharmaceutical Standards Ltd. (Aurora, Ontario, Canada). LC-MS grade methanol and acetonitrile were purchased from Chem-Supply Pty Ltd. (Gillman, SA, Australia) while Sigma Aldrich Pty Ltd. (Castle Hill, NSW, Australia) supplied the LC-MS grade formic acid, analytical grade ammonium formate, and deuterated chloroform ($CDCl_3$). Ultrapure water (18.2 MΩ-cm) was collected from a Sartorius arium® pro ultrapure water system (Goettingen, Germany) while LECO Australia Pty Ltd. (Castle Hill, NSW, Australia) provided the QuEChERS extraction salt (EN 15662).

5.4.2 Screening of food products

Health foods and beverages marketed with claims to enhance male sexual performance were pre-screened using PDE5 inhibition assay [15]. In brief, the bioactivity-based assay utilised a fluorescence polarisation technique to screen PDE5 inhibitors in foods and beverages, by competing with fluorescein-labelled cyclic-3',5'-guanosine monophosphate (PDE5 substrate) to bind to PDE5 enzyme. Suspected products were flagged by their average PDE5 inhibition above the threshold value of the blank instant coffee premix matrix.

The suspected products were then analysed to detect specific PDE5 inhibitors, through suspected-target and non-targeted screenings, with an Agilent Technologies (Santa Clara, CA, USA) 1290 Infinity II liquid chromatography system coupled to an Agilent Technologies 6510 quadrupole time-of-flight mass spectrometer (LC-QTOF-MS) as described previously [16]. Briefly, the chromatographic separation was carried out using a reverse-phase high-performance LC column from Merck KGaA (Darmstadt, Germany) Chromolith® High-Resolution RP-18 end-capped (100 × 4.6 mm, 2.0 µm) with 10 mM ammonium formate in ultrapure water (solvent A) and acetonitrile (solvent B) as the binary mobile phase system. Both solvents were acidified with 0.1% v/v formic acid. The gradient elution was set as follows: 5% B for 0–1 min, 5%–25% B for 1–2 min, 25%–50% B for 2–32 min, 50%–95% B for 32–33 min, and 95% B for 33–34 min at 0.4 mL/min. The elution was immediately returned to the initial gradient at 34.01 min for 6 min at 1 mL/min with post-run equilibration maintained at 0.4 mL/min, 5 min before the next injection. The QTOF-MS was operated in positive electrospray ionisation mode using a data-dependent acquisition.

SPL005, in the form of an ICP, is promoted to improve erectile function, prolong an erection, and prevent premature ejaculation, among others. The online advertisement also included a certificate of analysis stating that the ICP is free from adulterants such as sildenafil, vardenafil, and tadalafil. The ingredients listed on the ICP sachet were as follows: *Eurycoma longifolia*, *Lepidium meyenii*, arabica coffee, goat's milk, creamer, and brown sugar.

5.4.3 Standard solution preparation

Each CRM was prepared into a stock solution of 1 mg/mL in methanol and stored at 4°C in the dark. A working solution was freshly prepared for each analysis from the stock solution by further dilution in methanol.

5.4.4 Sample preparation

One-third of SPL005 contents (25.5 g per ICP sachet) were extracted using a modified QuEChERS procedure described previously [17]. In short, 100 mg of the sample was dissolved in 5 mL of acetonitrile and methanol (1:1, v/v), sequentially via 1-min vortexing, 20-min sonication, and 5-min centrifugation at 2500 × *g*. The resulting mixture was then transferred into a tube prefilled with QuEChERS salt for extraction, by vortexing for 1 min, followed by centrifuging for 5 min at 2500 × *g*. The solutions were filtered and combined into a round bottom flask. The volume of the filtrate was subsequently reduced to 5 mL using a rotary evaporator.

5.4.5 Isolation of compound X

Compound X was isolated from SPL005 through an Agilent Technologies 1290 Infinity LC system fitted with an end-capped high-performance LC column: Nucleoshell RP 18 (100 × 4.6 mm, 2.7 µm) from Macherey-Nagel GmbH & Co. KG (Duren, Germany). The column compartment temperature was maintained at 20°C with an injection volume of 20 µL. The mobile phases, consisting of solvent A (10 mM ammonium formate in ultrapure water) and solvent B (acetonitrile), were acidified with 0.1% v/v of formic acid. A shorter gradient elution program was devised specifically for the isolation of compound X at 0.4 mL/min as follows:

5% B for 0–1 min, 5%–45% B for 1–2 min, 45%–65% B for 2–8 min, 65%–95% B for 8–9 min, and 95% B for 9–10 min. The system was immediately returned to the initial gradient with post-run equilibration maintained for 3 min before the next injection.

The fraction of compound X was collected following a diode array detection (DAD) at 356 nm using an Agilent Technologies 1290 Infinity DAD. The procedure was repeated a number of times to obtain enough compound X for the liquid chromatography-ultraviolet spectroscopy (LC-UV) and nuclear magnetic resonance (NMR) spectroscopy analysis. The collected fractions were then combined and placed under a gentle stream of nitrogen gas to remove the residual solvents.

5.4.6 LC-UV analysis

The UV spectra were recorded on-line during the chromatographic run from 200–400 nm with an Agilent Technologies 1290 Infinity LC coupled to an Agilent Technologies 1290 Infinity DAD using the same chromatographic conditions as the screening of food products, with the UV signal monitored at 356 nm.

5.4.7 NMR Spectroscopy

The isolated compound X was dissolved in $CDCl_3$ with ^{1}H and ^{13}C NMR spectra recorded using an Agilent Technologies 500 MHz NMR spectrometer coupled to an Agilent Technologies 7510-AS automated NMR sample changer at room temperature. The acquisition was performed at 499.86 MHz within 1024 scans for ^{1}H NMR and 125.70 MHz within 10000 scans for ^{13}C NMR. All the chemical

shifts were reported in δ (ppm); measured relative to $CDCl_3$ (^1H δ= 7.26, ^{13}C δ= 77.0). The coupling constants (*J* values) were expressed in Hertz (Hz).

5.4.8 Data analysis

The qualitative and quantitative data of the LC-QTOF-MS, LC-DAD, and LC-UV analyses were processed through an Agilent Technologies Mass Hunter workstation software version B.07.00, Mass Hunter qualitative analysis software version B.07.00, and personal compound database and library (PCDL) manager software version B.04.00. A Bruker (Billerica, MA, USA) TopSpin software version 4.0.6 was applied to analyse the NMR data.

5.5 RESULTS AND DISCUSSION

5.5.1 Screening of SPL005

SPL005 was initially flagged for suspected adulteration through PDE5 inhibition assay, where it showed to inhibit the PDE5 enzyme [15]. An LC-QTOF-MS analysis [16] later unveiled the presence of one unidentified peak, initially assigned as compound X at 27.85 min of the base peak chromatogram (BPC) (Fig. 5.5.1 (a)). The full-scan MS in Fig. 5.5.1 (b) shows a protonated molecule ($[M+H]^+$) at *m/z* 499.2310, suggesting a chemical formula of $C_{25}H_{34}N_6OS_2$ with a mass error of 0.40 ppm. The matching scores of the observed mass, isotopic abundance distribution, and isotopic spacing for compound X were also ascertained to be >80%. The suspected-target screening [16] matched the $[M+H]^+$ precursor ion with two suspected analytes, i.e. 3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil and dithiopropylcarbodenafil, via the PCDL library.

In addition to this, the product ion scan employing the non-targeted screening [16] revealed the presence of two product ions corresponding to the common fragmentation patterns shared by the dithiocarbodenafil group of analogues, presented in Table 5.5.1. Fig. 5.5.1 (c) further displays the product ions' signals at *m/z* 343.0680 and *m/z* 371.0996, which aligned with the peak of compound X within ±20 ppm mass error. Dithiodesmethylcarbodenafil CRM, which represents the dithiocarbodenafil group of analogues, shared the same common fragmentation patterns as compound X at averaged collision energies, shown in Fig. 5.5.1 (d).

These findings indicated that compound X belongs to the dithiocarbodenafil group of analogues. However, both of the suspected analytes are structural isomers of one another. Besides, four other possible structural isomers could be generated based on these findings. Complementary technique such as LC-UV and NMR spectroscopy would, therefore, be highly valuable following analyte isolation and purification.

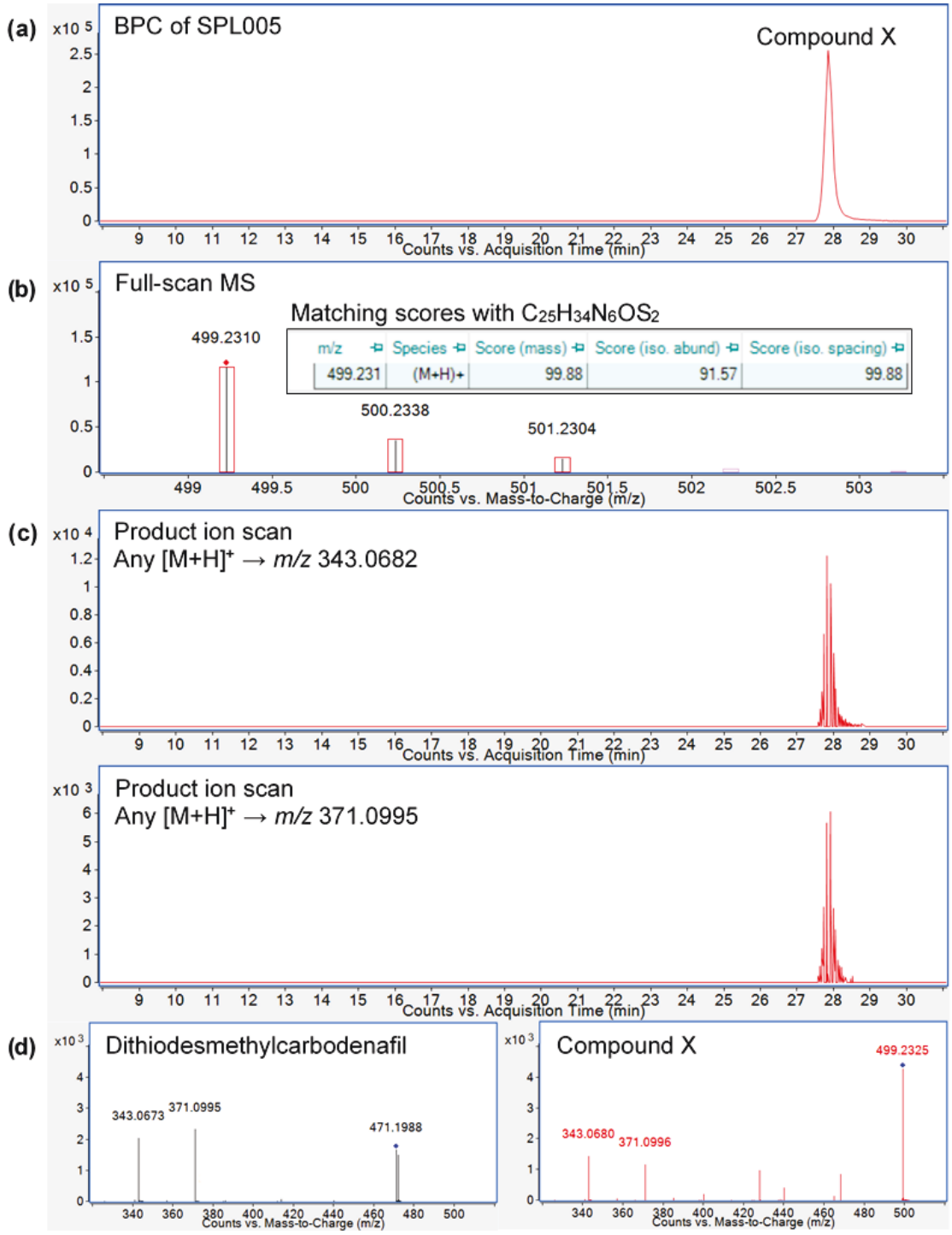
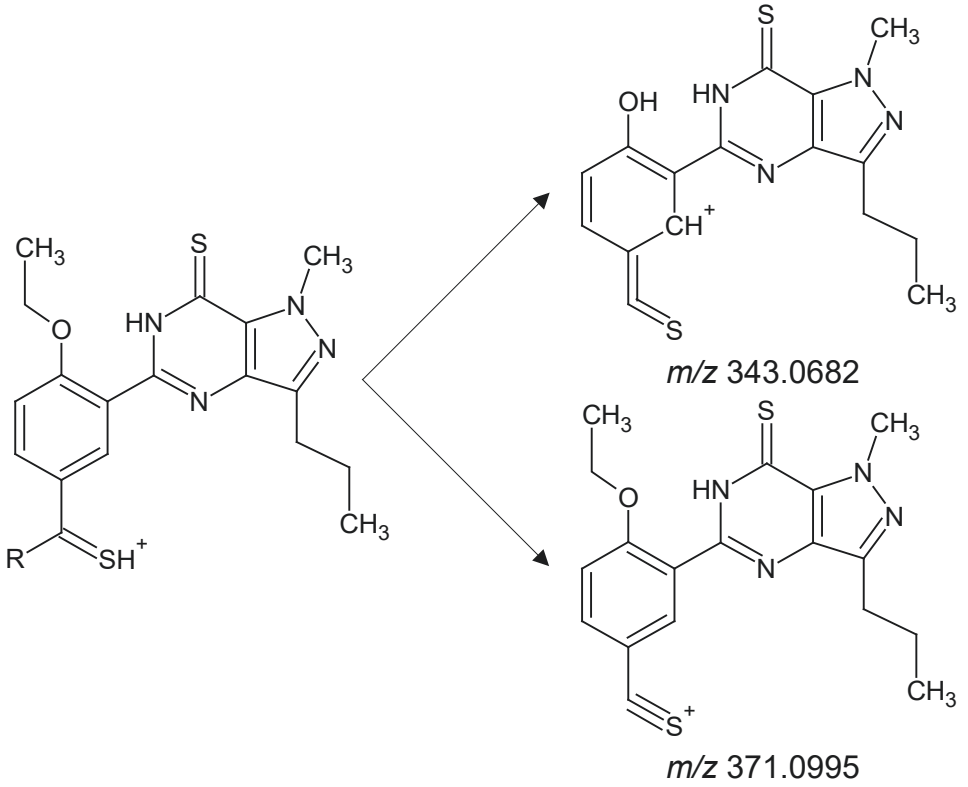
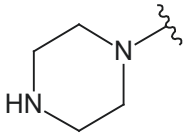
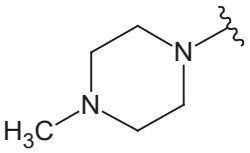
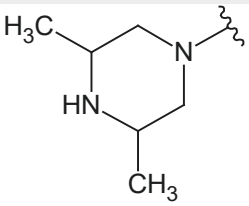
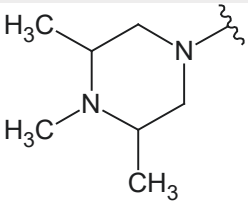
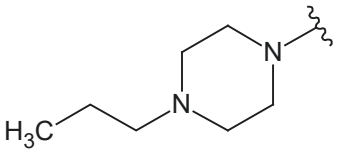


Fig. 5.5.1: (a) Base peak chromatogram (BPC) of SPL005 with one unidentified peak, initially assigned as compound X; (b) full-scan MS with a protonated molecule ($[M+H]^+$) precursor ion at *m/z* 499.2310 (also showing the matching scores of the observed mass, isotopic abundance distribution, and isotopic spacing of compound X with $C_{25}H_{34}N_6OS_2$); (c) product ion scan employing the non-targeted screening at *m/z* 343.0682 and *m/z* 371.0995; (d) common fragmentation patterns shared by compound X and dithiodesmethylcarbodenafil at averaged collision energies.

Table 5.5.1: Proposed common fragmentation patterns shared by dithiocarbodenafil group of analogues.

Dithiocarbodenafil group of analogues		
 *m/z* 343.0682 *m/z* 371.0995		
R = 		
Dithiodesethyl carbodenafil *m/z* 457.1839	Dithiodesmethyl carbodenafil *m/z* 471.1995	Dimethyldithiodenafil *m/z* 485.2152
R = 		
3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil *m/z* 499.2308		Dithiopropylcarbodenafil *m/z* 499.2308

5.5.2 LC-DAD and LC-UV of compound X

Compound X (2 mg) in the form of pale-yellow solid was isolated from the LC-DAD and then analysed by employing LC-UV and NMR spectroscopy. Fig. 5.5.2 displays the UV spectrum of compound X with maximum absorbance at 249, 284, and 357 nm, similar to that of dithiodesmethylcarbodenafil. The 3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil and dithiopropylcarbodenafil also exhibited similar UV spectrum patterns, overlaid as a comparison.

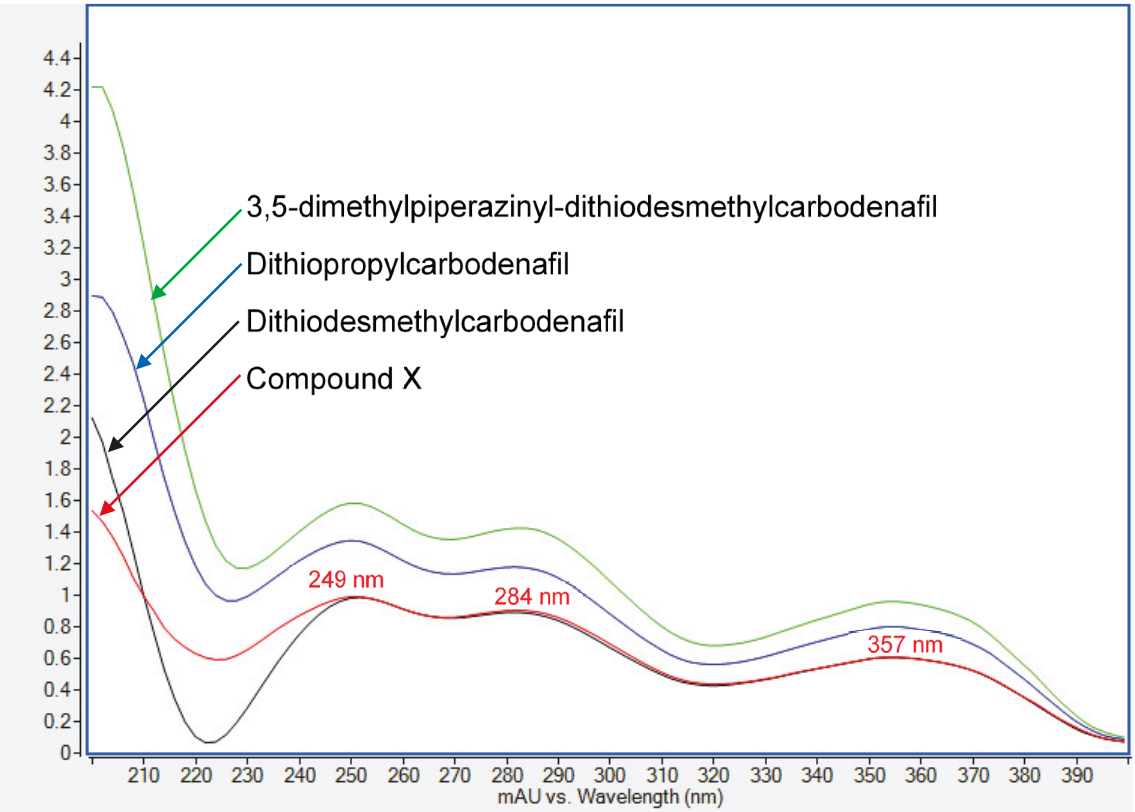
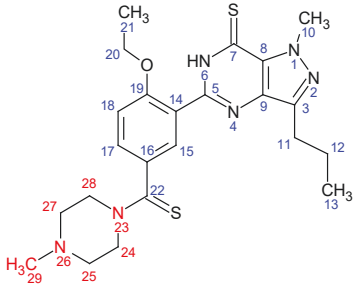
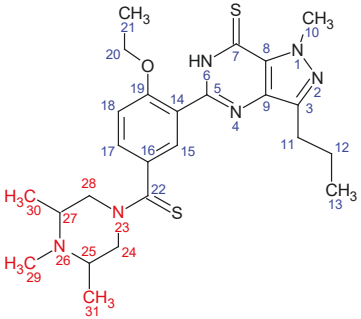
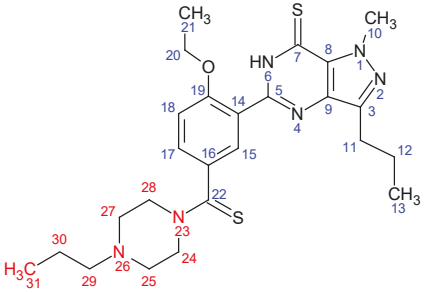
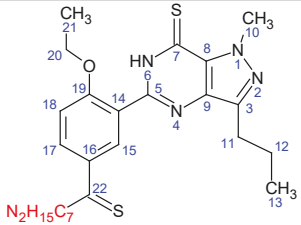


Fig. 5.5.2: Overlaid ultraviolet (UV) spectra of three structurally related phosphodiesterase 5 (PDE5) inhibitor standards at 1 µg/mL and compound X isolated from SPL005.

5.5.3 NMR spectroscopy of compound X

Table 5.5.3 compiles the ^{1}H and ^{13}C NMR data of compound X in comparison with the structurally related PDE5 inhibitors i.e. dithiodesmethylcarbodenafil [18], 3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil [19], and dithiopropylcarbodenafil [20]. The ^{1}H and ^{13}C NMR signal assignments of compound X, as well as 3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil and dithiopropylcarbodenafil, were comparable to that of dithiodesmethylcarbodenafil, except for the piperazine ring environment at positions 24–31. Indeed, all PDE5 inhibitors within the dithiocarbodenafil group of analogues possess similar skeletal configurations at positions 1–23, except for the different substitutes on the piperazine ring. Compound X was, therefore, characterised based on this skeletal structure.

Table 5.5.3: ^{1}H and ^{13}C nuclear magnetic resonance (NMR) data (δ in ppm, *J* in Hz) of compound X and structurally related phosphodiesterase 5 (PDE5) inhibitors.

	Dithiodesmethylcarbodenafil [18]		**3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil [19]**		**Dithiopropylcarbodenafil [20]**		**Compound X**	
								
	^{1}H (δH)	^{13}C (δC)	^{1}H (δH)	^{13}C (δC)	^{1}H (δH)	^{13}C (δC)	^{1}H (δH)*	^{13}C (δC)
1								
2								
3		146.2		144.7		146.2		144.6
4								
5		147.0		148.2		147.0		146.7
6	12.59 (1H, s)		13.30 (1H, s)		12.61 (1H, brs)		12.18 (s)	
7		171.8		171.6		171.7		170.2
8		132.3		131.8		132.3		132.2
9		134.1		133.6		134.1		133.7
10	4.52 (3H, s)	39.2	4.43 (3H, s)	39.5	4.53 (3H, s)	39.4	4.02 (s)	39.4

11	2.93 (2H, t, 7.5)	27.6	2.82 (2H, t, 7.0)	26.9	2.93 (2H, t, 7.5)	27.6	3.02 (t, 7.5)	26.2
12	1.87 (2H, sextet, 7.5)	22.3	1.75 (2H, sextet, 7.5)	22.0	1.86 (2H, sextet, 7.5)	22.3	1.89 (sextet, 6.9)	22.6
13	1.01 (3H, t, 7.5)	14.0	0.93 (3H, t, 7.5)	13.8	1.01 (3H, t, 7.4)	14.1	0.88 (t, 7.0)	14.1
14		136.3		134.6		136.3		137.7
15	8.41 (1H, d, 2.5)	128.1	7.70 (1H, d, 2.5)	128.2	8.42 (1H, d, 2.3)	128.1	8.51 (d, 2.5)	128.8
16		118.5		120.6		118.4		121.6
17	7.55 (1H, dd, 2.5, 8.0)	131.7	7.48 (1H, dd, 2.5, 8.0)	130.6	7.57 (1H, dd, 2.3, 8.6)	131.7	7.58 (dd, 1.2, 8.7)	131.8
18	7.06 (1H, d, 8.0)	113.0	7.19 (1H, d, 8.5)	112.6	7.07 (1H, d, 8.6)	113.0	7.09 (d, 8.7)	113.1
19		156.9		157.0		156.9		155.9
20	4.34 (2H, q, 7.0)	66.0	4.21 (2H, q, 7.0)	64.7	4.35 (2H, q, 7.0)	66.0	4.22 (q, 7.2)	65.1
21	1.69 (3H, t, 7.0)	14.8	1.38 (3H, t, 7.0)	14.4	1.70 (3H, t, 7.0)	14.8	1.42 (t, 6.9)	14.5
22		199.3		196.6		198.8		198.1
23								

24	3.73 (2H, brs)	52.0	H_a 2.97 (1H, t, 12), H_e 5.20 (1H, d, 13)	55.1	3.71 (2H, brs)	52.3	3.60 (dd), 3.70 (dd)	51.7
25	2.50 (2H, brs)	55.2	2.31 (1H, m)	56.8	2.52 (2H, t, 4.7)	53.5	2.47 (brs)	53.7
26								
27	2.68 (2H, brs)	54.3	2.21 (1H, m)	57.9	2.69 (2H, brs)	52.6	2.47 (brs)	53.4
28	4.48 (2H, brs)	49.5	H_a 3.15 (1H, t, 11.5), H_e 3.78 (1H, d, 15)	57.5	4.49 (2H, brs)	49.8	3.60 (dd), 3.70 (dd)	50.4
29	2.38 (3H, s)	45.5	2.20 (3H, s)	37.0	2.38 (2H, t, 7.5)	60.1	2.47 (brs)	36.8
30	NA	NA	1.12 (3H, d, 6)	17.7	1.54 (2H, sextet, 7.5)	20.0	1.05 (d, 7.3)	17.4
31	NA	NA	0.92 (3H, d, 6)	17.3	0.93 (3H, t, 7.4)	11.8	1.02 (d, 7.3)	17.4

Note:

a) Positions 1–31 indicate either a hydrogen or carbon signal.

b) Abbreviations: s, singlet; brs, broad singlet; d, doublet; dd, doublet of doublet; t, triplet; q, quartet; m, multiplet.

c) *the number of protons for each ^{1}H NMR signals cannot be established due to the presence of unknown impurities in the isolated compound X.

Fig. 5.8 (supplementary data) shows the ^{1}H NMR spectrum of the isolated compound X. A broad singlet peak at 2.47 ppm was assigned to a methyl group attached to a nitrogen atom at position H-29, based on the ^{1}H NMR signal of dithiodesmethylcarbodenafil [18]. The chemically equivalent protons of the methine groups attached to the same nitrogen atom at positions H-25 and H-27 are predicted to have similar chemical shifts within the range of 2 to 3 ppm. Therefore, they were assigned at 2.47 ppm within the same broad singlet peak. Another chemically equivalent protons of the methylene groups at positions H-24 and H-28 are expected to produce higher chemical shifts due to the diamagnetic anisotropy effect from a nearby thiocarbonyl group compared to those of H-25 and H-27 [18]. Therefore, they were assigned at 3.60 and 3.70 ppm, taking into account the axial and equatorial protons [21]. Finally, the two methyl groups at positions H-30 and H-31 were assigned to two doublet peaks at 1.05 and 1.02 ppm, respectively. As well, the ^{13}C NMR chemical shifts of compound X at positions C-29, C-30, and C-31 were similar to those of 3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil, indicating the presence of three methyl groups attached to the piperazine ring. These results ruled out the possibility of having an N-propylated linear chain group connected to the piperazine ring of compound X.

Isolation and structural elucidation of compound X through the LC-DAD and NMR spectroscopy are rather challenging due to the complexity of the ICP matrix, which typically contains multiple ingredients. Furthermore, the low quantity of the adulterant, often at trace levels relative to the matrix components, demands a larger sample size to isolate sufficient amounts of PDE5 inhibitor for different

types of NMR experiments. Nevertheless, based on the obtained ^{1}H and ^{13}C NMR data, the identity of compound X could still be inferred as the signal assignments were comparable to that of 3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil, particularly for the three methyl groups at positions 29–31 of the piperazine ring.

5.5.4 Confirmation of compound X

The chemical structure of compound X was primarily elucidated from the LC-QTOF-MS, LC-UV, and NMR spectroscopy data. However, to confirm these findings, three CRMs of structurally related PDE5 inhibitors i.e. dithiodesmethylcarbodenafil, 3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil, and dithiopropylcarbodenafil were acquired to unambiguously conclude the identity of compound X, if possible, based on the chromatographic separation. Fig. 5.5.4 shows an overlaid BPCs of the three structurally related PDE5 inhibitor standards at 1 µg/mL and compound X isolated from SPL005. Dithiodesmethylcarbodenafil eluted at 26.26 min, followed by 3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil and dithiopropylcarbodenafil at 27.78 min and 29.46 min, respectively. These findings indicated that the structural isomers were separated down to a baseline level, ensuring the specificity of each analyte.

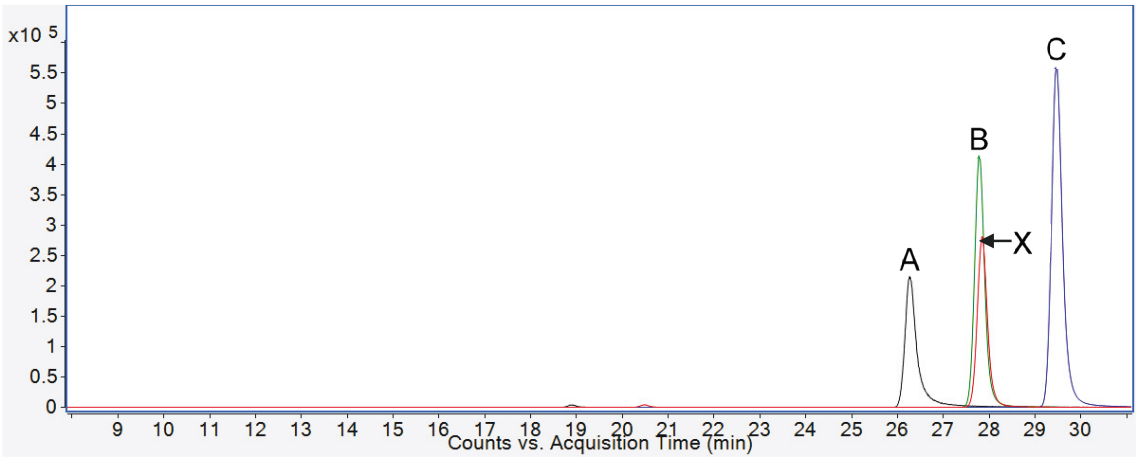


Fig. 5.5.4: Overlaid base peak chromatograms (BPCs) of three structurally related phosphodiesterase 5 (PDE5) inhibitor standards at 1 µg/mL with (A) dithiodesmethylcarbodenafil, (B) 3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil, and (C) dithiopropylcarbodenafil; and (X) compound X isolated from SPL005.

Compound X, isolated from SPL005 eluted at 27.84 min, within ±0.25 min of the retention time of 3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil. Based on this finding, the 1 µg/mL standard solution of 3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil was spiked into the ICP sample solution at 1:10 v/v to substantiate the identity of compound X. The BPC of the spiked ICP showed only one peak at 27.80 min, similarly within ±0.25 min of the retention time of the CRM. These results, complemented by the previous data, concluded the identity of compound X as 3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil. The content of the adulterant was subsequently quantified at 8.4 mg per sachet of the ICP sample.

5.6 CONCLUSION

This study's comprehensive analytical procedure has identified an isomeric sildenafil analogue from an ICP marketed to enhance male sexual performance. Pre-screening with PDE5 inhibition assay and the following LC-QTOF-MS analysis revealed the presence of a suspected compound X. However, the suspected-target screening with an LC-QTOF-MS matched compound X with two suspected analytes that are structural isomers of one another. Compound X was, therefore, isolated from the ICP using an LC-DAD and then submitted to LC-UV and NMR spectroscopy analysis. The UV spectrum, as well as the NMR signals of compound X, closely matched to that of the 3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil. The identity of compound X was finally concluded by comparing its chromatographic separation with the structurally related PDE5 inhibitors. For the identification of structural isomers, baseline separation by way of chromatography is superior as their full spectral information are often indistinguishable, as demonstrated by 3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil and dithiopropylcarbodenafil. To our best knowledge, this is the first study to report an adulterated ICP containing 3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil.

5.7 REFERENCES

1. Žuntar I, Krivohlavek A, Kosić-Vukšić J, Granato D, Bursać Kovačević D, Putnik P. Pharmacological and toxicological health risk of food (herbal) supplements adulterated with erectile dysfunction medications. *Curr Opin Food Sci* 2018;24:9-15. doi:https://doi.org/10.1016/j.cofs.2018.10.012
2. Ismail SF, Abd Ghani Azmi IM, Daud M, Abd Jalil J, Safuan S. Regulatory control of herbal and traditional medicines in Malaysia: issues and concerns. *Int J Bus Soc* 2020;21(S1):192-204.
3. Calahan J, Howard D, Almalki AJ, Gupta MP, Calderón AI. Chemical adulterants in herbal medicinal products: a review. *Planta Med* 2016;82(6):505-515. doi:https://doi.org/10.1055/s-0042-103495
4. Srivatsav A, Balasubramanian A, Pathak UI, et al. Efficacy and safety of common ingredients in aphrodisiacs used for erectile dysfunction: a review. *Sex Med Rev* 2020. doi:https://doi.org/10.1016/j.sxmr.2020.01.001
5. Kee CL, Ge X, Gilard V, Malet-Martino M, Low MY. A review of synthetic phosphodiesterase type 5 inhibitors (PDE-5i) found as adulterants in dietary supplements. *J Pharm Biomed Anal* 2018;147:250-277. doi:https://doi.org/10.1016/j.jpba.2017.07.031
6. Gerssen A, Bovee THF, van Ginkel LA, van Iersel MLPS, Hoogenboom RLAP. Food and feed safety: cases and approaches to identify the responsible toxins and toxicants. *Food Control* 2019;98:9-18. doi:https://doi.org/10.1016/j.foodcont.2018.10.028

7. Poon WT, Lam YH, Lai CK, Chan AY, Mak TW. Analogues of erectile dysfunction drugs: an under-recognised threat. *Hong Kong Med J* 2007;13(5):359-363.

8. Kee C-L, Ge X, Low M-Y. Application of Orbitrap-mass spectrometry to differentiate isomeric sildenafil- and thiosildenafil-like analogues used for the adulteration of dietary supplements. *Food Addit Contam Part A* 2015;32(10):1737-1748. doi:https://doi.org/10.1080/19440049.2015.1062923

9. Baillie GS, Tejeda GS, Kelly MP. Therapeutic targeting of 3′,5′-cyclic nucleotide phosphodiesterases: inhibition and beyond. *Nat Rev Drug Discov* 2019;18(10):770-796. doi:https://doi.org/10.1038/s41573-019-0033-4

10. Ventimiglia E, Capogrosso P, Montorsi F, Salonia A. The safety of phosphodiesterase type 5 inhibitors for erectile dysfunction. *Expert Opin Drug Saf* 2016;15(2):141-152. doi:https://doi.org/10.1517/14740338.2016.1131818

11. Skalicka-Wozniak K, Georgiev MI, Orhan IE. Adulteration of herbal sexual enhancers and slimmers: the wish for better sexual well-being and perfect body can be risky. *Food Chem Toxicol* 2017;108:355-364. doi:https://doi.org/10.1016/j.fct.2016.06.018

12. Balayssac S, Gilard V, Zedde C, Martino R, Malet-Martino M. Analysis of herbal dietary supplements for sexual performance enhancement: first characterization of propoxyphenyl-thiohydroxyhomosildenafil and identification of sildenafil, thiosildenafil, phentolamine and tetrahydropalmatine as adulterants. *J Pharm Biomed Anal* 2012;63:135-150. doi:https://doi.org/10.1016/j.jpba.2012.01.035

13. Rocha T, Amaral JS, Oliveira MBPP. Adulteration of dietary supplements by the illegal addition of synthetic drugs: a review. *Compr Rev Food Sci Food Saf* 2016;15(1):43-62. doi:https://doi.org/10.1111/1541-4337.12173

14. Bakota EL, Kelly AT, Walterscheid JP, Phatak DR. A case report of fatal desmethyl carbodenafil toxicity. *J Anal Toxicol* 2017;41(3):250-255. doi:https://doi.org/10.1093/jat/bkw128

15. Mohd Yusop AY, Xiao L, Fu S. Fluorescence polarisation for high-throughput screening of adulterated food products via phosphodiesterase 5 (PDE5) inhibition assay. *Manuscript submitted for publication* 2020.

16. Mohd Yusop AY, Xiao L, Fu S. Determination of phosphodiesterase 5 (PDE5) inhibitors in instant coffee premixes using liquid chromatography-high-resolution mass spectrometry (LC-HRMS). *Talanta* 2019;204:36-43. doi:https://doi.org/10.1016/j.talanta.2019.05.078

17. Mohd Yusop AY, Xiao L, Fu S. Data on the optimisation and validation of a liquid chromatography-high-resolution mass spectrometry (LC-HRMS) to establish the presence of phosphodiesterase 5 (PDE5) inhibitors in instant coffee premixes. *Data Brief* 2019;25:104234. doi:https://doi.org/10.1016/j.dib.2019.104234

18. Ge X, Li L, Koh H-L, Low M-Y. Identification of a new sildenafil analogue in a health supplement. *J Pharm Biomed Anal* 2011;56(3):491-496. doi:https://doi.org/10.1016/j.jpba.2011.06.004

19. Kee CL, Low MY, Ge X. Isolation and characterization of a novel dithio-carbodenafil analogue from a health supplement. *J Pharm Biomed Anal* 2017;137:132-138. doi:https://doi.org/10.1016/j.jpba.2017.01.010

20. Yun J, Shin KJ, Choi J, Kwon K, Jo C-H. Identification and structural elucidation of a new sildenafil analogue, dithiopropylcarbodenafil, from a premixed powder intended as a dietary supplement. *J Chromatogr B* 2018;1072:273-281. doi:https://doi.org/10.1016/j.jchromb.2017.11.029

21. Reepmeyer JC, Woodruff JT, d'Avignon DA. Structure elucidation of a novel analogue of sildenafil detected as an adulterant in an herbal dietary supplement. *J Pharm Biomed Anal* 2007;43(5):1615-1621. doi:https://doi.org/10.1016/j.jpba.2006.11.037

5.8 SUPPLEMENTARY DATA

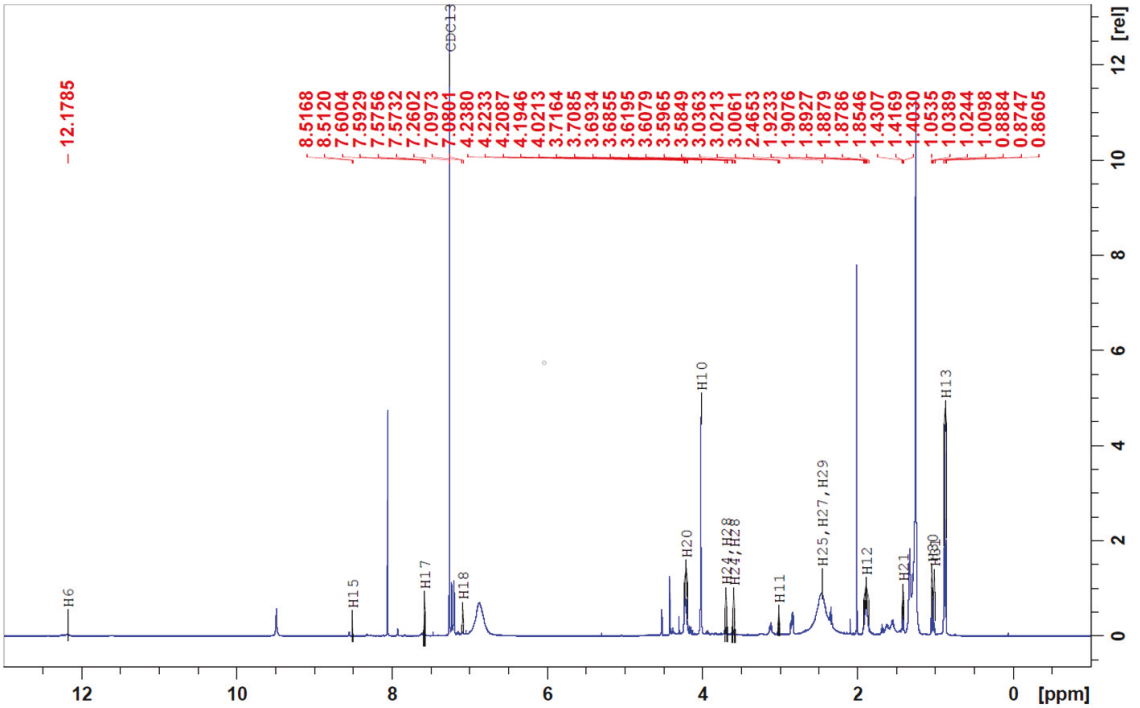


Fig. 5.8: ^{1}H nuclear magnetic resonance (NMR) spectrum of the isolated compound X.

CHAPTER 6

Suspected-target and non-targeted screenings of phosphodiesterase 5 inhibitors in herbal remedies by liquid chromatography-quadrupole time-of-flight mass spectrometry

6.1 FOREWORD

The following manuscript, in Chapter 6, was accepted for publication in a special issue of "Non-targeted screening of drugs" in *Drug Testing and Analysis* (https://doi.org/10.1002/dta.2861). The article is currently available as an "Early View" (Online Version of Record before inclusion in an issue). Liquid chromatography-high-resolution mass spectrometry, in recent years, has become an essential tool in analytical chemistry due to its superior specificity, sensitivity, and the ability to separate multiple analytes from complex matrices. The full-spectral information provided by this technique, particularly in tandem mode, enables researchers to develop targeted, suspected-target, and non-targeted analysis. This chapter describes the application of data-dependent acquisition (DDA) of a liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) to comprehensively screen phosphodiesterase 5 (PDE5) inhibitors in herbal remedies via suspected-target and non-targeted strategies. The suspected-target screening employed a library, developed to comprise 95 PDE5 inhibitors and their analogues, including 23 selected target analytes. The non-targeted screening utilised top-down and bottom-up approaches to flag novel PDE5 inhibitors analogues based on common fragmentation patterns of target analytes. The method was optimised, validated, and applied to screen 52 samples of herbal remedies that claimed to enhance

male sexual performance in capsule and tablet dosage forms. The manuscript explored the applicability of DDA of an LC-QTOF-MS to curb the spread of adulterated herbal remedies. Mr Ahmad Yusri Mohd Yusop, Dr Linda Xiao, and Professor Shanlin Fu authored the manuscript. Mohd Yusop AY performed the experimental work, data analysis, and initial draft preparation including supplementary data with manuscript edits provided by Xiao L and Fu S. The article section, figure, and table numbering was adjusted to align with the chronology of this thesis and may not reflect those published in the online version.

6.2 ABSTRACT

The lucrative market of herbal remedies spurs rampant adulteration, particularly with pharmaceutical drugs and their unapproved analogues. A comprehensive screening strategy is, therefore, warranted to detect these adulterants, and accordingly, to safeguard public health. This study utilises the data-dependent acquisition of a liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) to screen phosphodiesterase 5 (PDE5) inhibitors in herbal remedies using suspected-target and non-targeted strategies. The suspected-target screening employed a library comprising 95 PDE5 inhibitors. The non-targeted screening adopted top-down and bottom-up approaches to flag novel PDE5 inhibitors analogues based on common fragmentation patterns. The LC-QTOF-MS was optimised and validated for capsule and tablet dosage forms using 23 target analytes, selected to represent different groups of PDE5 inhibitors. The method exhibited excellent specificity and linearity with limit of detection and limit of quantification of <40 ng/mL and 80 ng/mL, respectively. The accuracy ranged from 79.0% to 124.7% with precision of <14.9%RSD. The modified QuEChERS extraction provided insignificant matrix effect within -9.1%–8.0% and satisfactory extraction recovery of 71.5%–105.8%. These strategies were utilised to screen 52 herbal remedy samples that claimed to enhance male sexual performance. The suspected-target screening resulted in 33 positive samples, revealing ten target analytes and two suspected analytes. Systematic MS and tandem MS interrogations using the non-targeted screening returned insignificant signals, indicating the absence of potentially novel analogues. The target analytes were quantified from 0.03 to 121.31 mg per dose of each sample. The proposed strategies ensure all PDE5 inhibitors are comprehensively

screened, providing a useful tool to curb the widespread adulteration of herbal remedies.

6.3 INTRODUCTION

Globally, people consume an array of health products to treat minor ailments, prevent illnesses, and boost their health and well-being [1]. Herbal remedies have recently surged to be a substantial part of this market due to the many side effects associated with modern medicines. At present, the herbal industry is one of the most rapidly growing sectors with annual sales over several billion dollars worldwide [2]. Herbal remedies are typically marketed in pharmaceutical dosage forms and apportioned into specific doses.

Herbal remedies commonly claim to be of natural origin, giving the perception of being effective and safe [3]. However, this lucrative market often tempts intentional adulteration with pharmaceutical drugs and their unapproved analogues, aimed to provide the desired efficacy which may pose severe health and life-threatening risks to consumers [2,4]. Among the most prevalent include products adulterated with phosphodiesterase 5 (PDE5) inhibitors and their analogues, frequently marketed to enhance male sexual performance [5].

Novel PDE5 inhibitors analogues used as adulterants pose a challenge to forensic drug testing laboratories, as they may evade detection during routine screening [6]. As a result, the adulterated herbal remedies may be distributed in the market undetected, putting the consumers at absolute risk, owing to the unknown safety and toxicology profiles [7]. Thus far, the literature has identified more than 90 unapproved PDE5 inhibitors analogues as adulterants [8]. From 2015 to 2019, the United States Food and Drug Administration (USFDA) had

reported that 260 out of 390 adulterated products contain PDE5 inhibitors and their analogues [9].

The literature described several analytical methods to determine PDE5 inhibitors in various matrices, for instance, thin-layer chromatography (TLC) [10], gas chromatography-mass spectrometry (GC-MS) [11], Raman spectroscopy [12], and nuclear magnetic resonance (NMR) spectroscopy [13]. More frequently, liquid chromatography (LC) coupled with mass spectrometry (MS) detection in tandem mode has demonstrated to be an indispensable tool in the analysis of PDE5 inhibitors. The analyses used both low-resolution MS [14] and high-resolution MS (HRMS) [8]. However, HRMS has proven to be superior as it delivers full-spectral information for both MS and tandem MS modes simultaneously [15], which provides an unrivalled specificity.

In recent years, researchers are getting more interested in HRMS techniques such as quadrupole time-of-flight MS (QTOF-MS), as they can use the full-spectral information to develop targeted, suspected-target, and non-targeted analysis [15,16]. However, the widely used targeted analysis is limited, depending on the availability of certified reference materials (CRMs) [17]. In the case of PDE5 inhibitors identification, it would not be financially viable for forensic drug testing laboratories to acquire all the available CRMs. Therefore, suspected-target screening provides extended coverage of known analytes without the need for CRMs. Additionally, non-targeted screening can address the growing concerns of the novel PDE5 inhibitors analogues found as adulterants. This

strategy plays a pivotal role to discover those novel analogues based on the common fragmentation patterns of the known PDE5 inhibitors.

This study utilised the data-dependent acquisition (DDA) of an LC-QTOF-MS for comprehensive screening of PDE5 inhibitors and their analogues in herbal remedies. The screening procedure was carefully developed using suspected-target and non-targeted strategies. The analytical method was optimised and validated using 23 target analytes, ensuring robust and reliable performance to determine PDE5 inhibitors in different herbal remedies' matrices. These strategies were then employed to screen 52 distinct samples of herbal remedies that claimed to enhance male sexual performance. The highlighted significant results showcased the applicability of the developed screening strategies.

6.4 MATERIALS AND METHODS

6.4.1 Chemicals and reagents

In total, the 23 CRMs of PDE5 inhibitors purchased from TLC Pharmaceutical Standards Ltd (Aurora, Ontario, Canada) were as follows: (1) desmethylcarbodenafil, (2) carbodenafil, (3) N-desethylacetildenafil, (4) acetildenafil, (5) hydroxyvardenafil, (6) dimethylacetildenafil, (7) vardenafil, (8) sildenafil, (9) homosildenafil, (10) dimethylsildenafil, (11) propoxyphenyl-hydroxyhomosildenafil, (12) udenafil, (13) propoxyphenyl-sildenafil, (14) hydroxythiovardenafil, (15) tadalafil, (16) mirodenafil, (17) mutaprodenafil, (18) thiosildenafil, (19) thiohomosildenafil, (20) dithiodesmethylcarbodenafil, (21) thiodimethylsildenafil, (22) propoxyphenyl-thiohydroxyhomosildenafil, and (23) propoxyphenyl-thiodimethylsildenafil. Each of the CRM was carefully selected as target analytes to represent different groups of PDE5 inhibitors based on the structural similarities, as presented in Tables 6.8A–F (supplementary data).

The vendor for methanol and acetonitrile of LC-MS grade was Chem-Supply Pty Ltd (Gillman, SA, Australia); while Sigma Aldrich Pty Ltd (Castle Hill, NSW, Australia) supplied the formic acid of LC-MS grade and ammonium formate of analytical grade. Ultrapure water (18.2 MΩ-cm) was collected from a Sartorius arium® pro ultrapure water system (Goettingen, Germany); and LECO Australia Pty Ltd (Castle Hill, NSW, Australia) supplied the quick, easy, cheap, effective, rugged, and safe (QuEChERS) extraction salt (EN 15662). Each sachet of the QuEChERS extraction salt is composed of 4 g magnesium sulphate, 1 g sodium chloride, 1 g trisodium citrate dihydrate, and 0.5 g disodium hydrogen citrate sesquihydrate.

6.4.2 Standard solution preparation

The stock solution of each CRM was prepared in methanol at 1 mg/mL and stored in the dark at 4°C. A mixture of all CRMs (working solution) was freshly prepared for each analysis from the stock solutions by further dilution in methanol to make up to 25 µg/mL concentration.

6.4.3 Sample collection and storage

Altogether, the 52 distinct herbal remedy samples in capsule and tablet dosage forms were obtained from Malaysia (44 samples) and Australia (8 samples). These suspected samples were selected based on brand names, label claims, images, herbal ingredients, or advertising materials related to male sexual performance. The Pharmacy Enforcement Division, Ministry of Health Malaysia, kindly donated most of these samples which were confiscated at the international airport (10 samples) and international seaport (16 samples), including those from routine market surveillance activities (17 samples). The remainder of the samples were purchased from various online shopping platforms based in Malaysia (1 sample) and Australia (8 samples). Each sample was labelled as SPC001 to SPC032 for capsule samples and SPT001 to SPT020 for tablet samples. These samples were deposited in a plastic zip-lock bag individually and then stored in an airtight container in the dark.

A representative blank matrix of capsule or tablet, free from any analyte of interests, was sourced from a local pharmacy in Australia and used for method optimisation and validation. The compositions of the capsule utilised as a blank matrix were as follows: *Epimedium sagittatum*, *Eleutherococcus senticosus*,

Tribulus terrestris, *Dulacia inopiflora*, zinc oxide, and encapsulating aids. Meanwhile, the tablet employed as a blank matrix was composed of *Morinda officinalis*, *Epimedium sagittatum*, *Panax ginseng*, *Schisandra chinensis*, *Serenoa repens*, lycopene, zinc amino acid chelates, calcium hydrogen phosphate, carnauba wax, microcrystalline cellulose, chlorophyllin-copper complex, croscarmellose sodium, magnesium stearate, silica, and tablet coating ingredients. The constituents of these two blank matrices were stated on the products' labels.

6.4.4 Sample preparation

The initial weight of each sample was recorded according to the recommended dose on its label. Then, using an electric grinder for capsules or a mortar and pestle for tablets, the entire recommended dosage was homogenised. For instrumental analysis, 100 mg of the homogenised sample was weighed in a polypropylene tube and then extracted with 5 mL of acetonitrile and methanol (1:1, v/v) by 1-min vortex mixing, 20-min sonication, and 5-min centrifugation at 2500 × *g*, successively. The resulting mixture was then transferred into another polypropylene tube prefilled with half of a sachet of the QuEChERS extraction salt (2 g magnesium sulphate, 0.5 g sodium chloride, 0.5 g trisodium citrate dihydrate, and 0.25 g disodium hydrogen citrate sesquihydrate); and vortexed for 1 min, followed by centrifugation for 5 min at 2500 × *g* to separate the solid residues. The upper layer was filtered using a 0.22 μm PTFE syringe filter and diluted with methanol at 1:10 dilution level for analysis. The blank matrices were treated in the same manner as the sample analysis. For quantification purpose,

the sample solution was further diluted with methanol whenever the target analyte concentration was beyond the linear range of the external calibration curve.

6.4.5 LC-QTOF-MS conditions and data analysis

This study employed an Agilent Technologies (Santa Clara, CA, USA) 1290 Infinity II LC system coupled to an Agilent Technologies 6510 QTOF-MS using our previously developed methodology [8,18]. The chromatographic separation was carried out using a reverse-phase high-performance LC column from Merck KGaA (Darmstadt, Germany) Chromolith® High-Resolution RP-18 end-capped (100 × 4.6 mm, 2.0 µm) with column compartment temperature maintained at 20°C.

The injection volume was set at 5 µL with the autosampler compartment temperature maintained at 10°C. The mobile phases were acidified with 0.1% v/v formic acid and consisted of solvent A (10 mM ammonium formate in ultrapure water) and solvent B (acetonitrile). The settings of the gradient elution were as follows: 5% B for 0–1 min, 5%–25% B for 1–2 min, 25%–50% B for 2–32 min, 50%–95% B for 32–33 min, and 95% B for 33–34 min at 0.4 mL/min. The elution was immediately returned to the initial gradient at 34.01 min for 6 min at 1 mL/min. Post-run equilibration was set for 5 min at 0.4 mL/min before the next injection.

The QTOF-MS, equipped with a dual electrospray ionisation (ESI) nebuliser was calibrated at a low mass range of *m/z* 1700 before each chromatographic run to achieve a typically attainable mass accuracy within ±5 ppm for precursor ion and ±20 ppm for product ion. ESI in positive ionisation mode was employed using the

following experimental parameters: 300°C for gas temperature, 12 L/min for drying gas flow, 32 psig for nebuliser pressure, 3500 V for capillary voltage, 175 V for fragmentor voltage, 65 V for skimmer voltage, and 750 V for OCT 1 RF Vpp.

A DDA (auto MS/MS) mode was selected for simultaneous MS and tandem MS experiments within a mass-to-charge range of *m/z* 100 to 1100. The acquisition rates' settings were 1 and 3 spectra/sec for the MS and tandem MS experiments, respectively, within a narrow isolation width of *m/z* ~1.3. The collision-induced dissociation experiments were performed at fixed collision energies (CEs) of 10, 20, and 40 eV in a separate scan with nitrogen as the collision gas. The reference mass solution containing purine (*m/z* 121.050873) and hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine (*m/z* 922.009798) were continuously infused throughout the chromatographic run at a steady pressure of 5 psig.

Agilent Technologies Mass Hunter workstation software version B.07.00, Mass Hunter qualitative analysis software version B.07.00, and personal compound database and library (PCDL) manager software version B.04.00 were used to process all qualitative and quantitative data. All other calculations were done using Microsoft (Redmond, WA, USA) Excel 2016 (Microsoft Office).

6.4.6 Analytical method validation

The analytical method validation was performed for specificity, linearity, limit of detection (LOD), and limit of quantification (LOQ) [19]. The accuracy, precision, matrix effect (ME), and extraction recovery (RE) were also evaluated for each target analyte in each of the blank matrices of capsule and tablet at low (0.1

μg/mL), medium (0.4 μg/mL), and high (1 μg/mL) quality control (QC) levels following the recommended procedures [20]. All validation parameters were analysed in triplicate.

Each target analyte was evaluated for specificity based on (1) the chromatographic separation and (2) the high-resolution mass of the protonated molecule ($[M+H]^+$). The tandem MS experiment was then used to establish the presence of two product ions corresponding to each target analyte. The average intensity ratio between the first and the second product ion at average CEs was compared to those obtained from the matrix-matched QC analytes within ±30% and thus, confirming the target analytes' identity. The extent of interfering components from the extracted blank matrices was also ascertained at the retention time of each target analytes.

An external calibration curve was constructed using the peak areas of each target analyte from the $[M+H]^+$ precursor ion versus their concentrations. The linearity was then determined based on the coefficient of determination (r^2), and the regression equation was used to calculate the QC analytes and samples concentrations. The lowest and highest concentrations of target analytes expected in adulterated herbal remedies were applied for the linear range.

The LOD was determined experimentally by tapering down each point of 10 ng/mL of the working solution concentration starting from 100 to 10 ng/mL. The LOD was then selected based on the lowest concentration of target analyte that can be reliably identified as defined in the specificity assessment. The LOQ was

established at the lowest concentration of the external calibration curve with acceptable accuracy and precision. Whenever a background noise is present, the signal-to-noise ratio was ascertained at >3 for LOD and >10 for LOQ.

The accuracy and precision were established at three QC levels. The extracted blank matrices were spiked with the working solution and submitted to analysis. The observed target analyte concentration versus the expected concentration at the same QC level was expressed as a percentage of accuracy with an acceptable value of ±25%. Precision was determined using the same QC analytes at intra-day for repeatability and inter-day for intermediate precision. The results were then expressed as a percentage of the relative standard deviation (%RSD) of the peak areas of the $[M+H]^+$ precursor ion with an acceptable value of <20%.

Using the same QC levels, the ME was evaluated based on the post-extraction addition method [8]. The slopes of the matrix-matched calibration curve versus those of the external calibration curve were compared to determine the percentage of ionisation suppression (negative value) or ionisation enhancement (positive value). The ME categories for each target analyte in both matrices were as follows: insignificant (0% to ±10%), acceptable (±10% to ±20%), moderate (±20% to ±50%), and severe (less than -50% or more than +50%). In contrast, the comparison of the peak areas of the $[M+H]^+$ precursor ion of target analytes spiked into the blank matrices before extraction versus those spiked into an extracted blank matrix at the same QC level, generated the percentage of RE with an acceptable value of ±25%.

6.4.7 Suspected-target and non-targeted screenings of herbal remedies

The screening of PDE5 inhibitors and their analogues as adulterants in herbal remedies was performed using suspected-target and non-targeted strategies. For the suspected-target screening, a library was constructed using the PCDL manager software. The library comprised of 95 PDE5 inhibitors and their analogues that are presently known as adulterants (listed in Table 6.8G of the supplementary data). Specific details such as compound name, molecular formula and structure, and exact mass were manually stored in this library. Also, extended details from the LC-QTOF-MS analysis of the 23 target analytes were imported from the Mass Hunter qualitative analysis software into the same library, which includes the retention time and tandem MS spectra at different CEs.

The suspected-target screening was employed to match the observed accurate mass of the $[M+H]^+$ precursor ion of the sample to those theoretical ones in the library within ±5 ppm mass tolerance. The matching scores of the observed mass, isotopic abundance distribution, and isotopic spacing for each analyte were also ascertained to be >80%. Based on these findings, a list of matched analytes was generated. Next, the observed tandem MS spectra of the sample were compared to those of target analytes within ±20 ppm mass tolerance. Subsequently, the identity of each target analyte was confirmed by comparing the observed retention time to those of the CRMs within ±0.25 min tolerance.

At this stage, there are two possible outcomes for positive samples, where: (1) sample matched with target analytes and (2) sample matched with suspected analytes. Only samples in outcome number (1) were submitted to quantification.

Whereas, a list of suspected analytes was generated for outcome number (2). The negative samples were tentatively categorised as possible non-adulterated samples and were submitted to the non-targeted screening.

The non-targeted screening was performed using comprehensive top-down and bottom-up approaches to screen visible and non-visible chromatographic peak, respectively. The screening was adapted and modified following a critical review by Pasin et al. [15] to flag novel PDE5 inhibitors analogues based on common fragmentation patterns of target analytes. The visible peaks within a base peak chromatogram (BPC) were integrated and extracted using the top-down approach to reveal the mass spectra. Each of this spectrum was then interrogated for the $[M+H]^+$ precursor ions. Using the product ion scan of the Mass Hunter qualitative analysis software, the link between the pre-determined $[M+H]^+$ precursor ions and the product ions of target analytes was established at the specific retention time of each chromatographic peak.

In contrast, with the bottom-up approach, all generated $[M+H]^+$ precursor ions during the chromatographic run were considered to establish if there is any link to the product ions of target analytes. Therefore, additional investigations are required to ultimately establish the correct $[M+H]^+$ precursor-product ion pair. In both approaches, any two tandem MS signals observed belonging to the same group of PDE5 inhibitors within ±20 ppm mass tolerance would reveal the presence of a novel analogue.

6.5 RESULTS AND DISCUSSION

6.5.1 Method optimisation

The simultaneous separation of multiple PDE5 inhibitors with structural similarities is critical for a reliable determination of these adulterants, particularly from complex matrices such as herbal remedies. The mobile phases, matrix modifier, and chromatographic column were initially selected based on the physical and chemical properties of target analytes to obtain optimum chromatographic resolution. The chromatographic separation was then optimised by varying the LC parameters such as injection volume, flow rate, column temperature, elution gradient, and elution time. The MS conditions were tuned according to the flow- and compound-dependent parameters to improve the method sensitivity. The chromatographic separation and MS conditions were optimised following the previous literature [18].

Although the LC-QTOF-MS via ESI is superior in detecting analytes from complex matrices, its performance is often hindered by the presence of ME, which often lead to errors in quantification [21]. Therefore, the presence of either ionisation suppression or ionisation enhancement needs to be addressed to minimise the possibilities of false-negative and false-positive results. Consequently, two extraction techniques were compared and assessed based on the ME and RE efficiency to resolve this issue. The ME was also evaluated at three levels of matrix dilution while maintaining the target analytes concentration at three QC levels.

The widely used dilute-and-shoot (D&S) technique was initially performed to analyse the PDE5 inhibitors in the blank matrices. Methanol was chosen as the solvent for this technique based on previous literature [22,23]. The capsule matrix produced moderate ME for two and acceptable ME for eight target analytes at 1:2 matrix dilution. The remaining 13 target analytes showed insignificant ME. However, non-detection of several target analytes at the same matrix dilution was observed for the tablet matrix at low and medium QC levels. Therefore, the ME cannot be determined for seven target analytes, particularly those with pyrazolopyrimidine-7-thione and imidazotriazine-4-thione moiety. The same problem persisted for the tablet matrix at a higher 1:10 matrix dilution. In general, the ME was minimised to insignificant percentages with increasing matrix dilution from 1:2 to 1:100 for all target analytes in both matrices.

From the D&S ME assessment, the presence of the tablet matrix had resulted in a complete loss of MS signals which subsequently led to false-negative results of the seven target analytes. Consequently, another sample extraction technique was assessed to overcome this problem. After several trial-and-error [18], a modified QuEChERS procedure was developed specifically to resolve the ME issue. The application of the modified QuEChERS extraction in combination with appropriate matrix dilution had resulted in insignificant ME percentages for both matrices at 1:10 and 1:100 matrix dilution. Finally, the 1:10 matrix dilution was selected and submitted to RE assessment. Table 6.8H (supplementary data) presents the full ME assessment results of the blank matrices using D&S technique and modified QuEChERS extraction at three levels of matrix dilution.

6.5.2 Analytical method validation

Table 6.8I (supplementary data) shows the specificity, linearity, and sensitivity results of the analytical method. The presence of each target analyte was ascertained using the optimised chromatographic separation and the full-scan MS data of the $[M+H]^+$ precursor ion. Isomeric analytes were chromatographically resolved, qualifying their specificity. Furthermore, the presence of two product ions from the tandem MS experiment confirmed the target analytes' identities. The effects of interferences from the extracted blank matrices were established to be trivial. Moreover, the carry-over effect was not observed in the subsequent analysis using the optimised chromatographic separation as the reverse-phase high-performance LC column was flushed with approximately five times of the column volume starting from 34.01 to 40 min at 1 mL/min before the next sample injection. The linear relationship between the peak areas of target analytes and their concentrations was verified by r^2 of >0.9870 within the selected range of 0.08 to 1.2 µg/mL. The LOD was determined between 10 and 40 ng/mL, while the LOQ was fixed at 80 ng/mL for all target analytes.

Supplementary Table 6.8J and K respectively, present the accuracy and precision data. Excellent accuracy was obtained for both capsule and tablet matrices. The capsule matrix produced the percentage of accuracy ranged from 90.8% to 123.1% at low; 94.4% to 104.9% at medium; and 95.6% to 103.4% at high QC level. The percentage of accuracy for tablet matrix at low, medium, and high QC levels were within 79.0%–124.7%; 93.8%–109.9%; and 90.8%–103.9%, respectively. The precision was also satisfactory with the %RSD of <14.9%. The

repeatability and intermediate precision for both matrices were calculated within 0.3%–8.6% and 0.1%–14.9% of RSD, respectively, at all QC levels.

As mentioned in Section 6.5.1, the ME was within insignificant percentages for all target analytes. The MEs for capsule and tablet matrices were within -9.1%–1.7% and -3.7%–8.0%, respectively. Table 6.8L (supplementary data) shows the RE results of the modified QuEChERS extraction. The RE was satisfactory within 72.6%–105.8% for capsule matrix and 71.5%–102.3% for tablet matrix.

6.5.3 Screenings of herbal remedies for PDE5 inhibitors

A total of 52 distinct herbal remedy samples obtained from Malaysia and Australia were comprehensively screened using suspected-target and non-targeted strategies. The top three countries of origin based on the products' label are Malaysia, China (Hong Kong), and Indonesia. Most of these samples claimed to contain *Eurycoma longifolia*, *Tribulus terrestris*, and *Panax ginseng*, which are usually regarded as herbal aphrodisiacs. Table 6.5.3A compiles the analysis results of the adulterated herbal remedy samples.

Table 6.5.3A: Identification of target analytes and detection of suspected analytes in adulterated herbal remedy samples.

Sample	Target analytes identified (average weight per dose in mg - quantification level)	Total average weight per dose in mg - quantification level	Herbs claimed on the label (top 3 herbs)	Product origin claimed on the label
SPC001	1. Sildenafil (0.11 - SUB)	0.11 - SUB	*Panax ginseng, Tribulus terrestris, Ginkgo biloba*	Canada
SPC003	1. Sildenafil (33.50 - THE)	33.50 - THE	*Panax ginseng, Cordyceps sinensis, Epimedium*	Not stated
SPC005	1. Thiodimethyl-sildenafil (19.96 - SUB) 2. Thiosildenafil (0.06 - SUB) 3. Dimethylsildenafil (0.03 - TRC) 4. Hydroxythiohomo-sildenafil*	20.05 - SUB	*Mulberry leaves, yam roots, Rhodiola rosea*	Norway
SPC008	1. Sildenafil (116.96 - SPR)	116.96 - SPR	*Myristica fragrans, Pausinystalia yohimbe, Eurycoma longifolia*	Indonesia
SPC010	1. Sildenafil (35.73 - THE)	35.73 - THE	Unspecified herbs	Malaysia
SPC011	1. Tadalafil (53.96 - SPR)	53.96 - SPR	*Tribulus terrestris, Lepidium meyenii, Eurycoma longifolia*	Malaysia

SPC012	1. Sildenafil (73.18 - THE)	73.18 - THE	*Epimedium grandiflorum, Eurycoma longifolia, Serenoa repens*	United States
SPC015	1. Sildenafil (74.98 - THE) 2. Propoxyphenyl-sildenafil (<LOQ)	74.98 - THE	*Eurycoma longifolia, Ginkgo biloba, Tribulus terrestris*	Malaysia
SPC017	1. Propoxyphenyl-thiohydroxyhomo-sildenafil (3.99 - SUB) 2. Propoxyphenyl-hydroxyhomo-sildenafil (0.08 - SUB)	4.07 - SUB	*Eurycoma longifolia, Panax ginseng, Cordyceps sinensis*	Malaysia
SPC019	1. Sildenafil (111.27 - SPR)	111.27 - SPR	*Tribulus terrestris, Paullinia cupana, Citrus aurantium*	Not stated
SPC021	1. Sildenafil (116.31 - SPR) 2. Propoxyphenyl-sildenafil (<LOQ)	116.31 - SPR	*Curcuma longa, cactus extract*	Thailand
SPC022	1. Sildenafil (71.02 - THE) 2. Propoxyphenyl-sildenafil (<LOQ)	71.02 - THE	*Crocus sativus, Cordyceps sinensis, snow lotus flower*	Hong Kong
SPC023	1. Tadalafil (34.77 - SPR)	34.77 - SPR	*Eurycoma longifolia, Lepidium meyenii, Lycium barbarum*	Malaysia

SPC027	1. Thiodimethyl-sildenafil (120.15 - SPR) 2. Dimethylsildenafil (0.99 - SUB) 3. Thiosildenafil (0.09 - TRC) 4. Propoxyphenyl-thiodimethylsildenafil (0.08 - TRC)	121.31 - SPR	*Eucommia ulmoides, Cynomorium songaricum, Ganoderma lucidum*	Malaysia
SPC028	1. Thiodimethyl-sildenafil (23.78 - SUB) 2. Dimethylsildenafil (0.06 - SUB) 3. Thiosildenafil (<LOQ)	23.84 - SUB	*Avena sativa, Okra mucilage, Desert cistanche*	Not stated
SPC029	1. Sildenafil (24.66 - SUB) 2. Thiodimethyl-sildenafil (0.02 - TRC)	24.68 - SUB	*Eurycoma longifolia, Ginkgo biloba, Tribulus terrestris*	Not stated
SPC030	1. Sildenafil (99.24 - THE)	99.24 - THE	*Pausinystalia yohimbe, Eurycoma longifolia, Tribulus terrestris*	Indonesia
SPC031	1. Sildenafil (24.75 - SUB)	24.75 - SUB	*Cornus officinalis, Turnera diffusa, Ptychopeta-lum olacoides*	Indonesia
SPC032	1. Sildenafil (0.18 - SUB)	0.18 - SUB	*Eurycoma longifolia, Rehmanniae preparata, Eucommia ulmoides*	Not stated

SPT001	1. Sildenafil (0.03 - TRC)	0.03 - TRC	*Panax quinquefolius, Epimedium, Rhodiola rosea*	Hong Kong
SPT002	1. Sildenafil (0.18 - SUB)	0.18 - SUB	*Pausinystalia yohimbe, Tribulus terrestris, Panax ginseng*	Canada
SPT003	1. Vardenafil (5.48 - THE) 2. Tadalafil (1.41 - SUB) 3. Aminotadalafil* 4. Hydroxythiohomo-sildenafil*	6.89 - THE	*Rhodiola rosea, dodder seed, Angelica*	China
SPT005	1. Sildenafil (67.93 - THE)	67.93 - THE	Unspecified herbs	Not stated
SPT007	1. Sildenafil (107.17 - SPR) 2. Propoxyphenyl-sildenafil (<LOQ)	107.17 - SPR	Unspecified herbs	Hong Kong
SPT008	1. Sildenafil (66.22 - THE) 2. Tadalafil (0.11 - SUB)	66.33 - THE	Unspecified herbs	Hong Kong
SPT012	1. Sildenafil (112.42 - SPR)	112.42 - SPR	*Boschniakia rossica, ginseng, medlar*	Hong Kong
SPT013	1. Sildenafil (100.20 - SPR)	100.20 - SPR	*Cordyceps, Tianshan snow lotus, Cistanche*	Not stated
SPT015	1. Sildenafil (88.43 - THE)	88.43 - THE	Unspecified herbs	Hong Kong
SPT016	1. Sildenafil (113.94 - SPR)	113.94 - SPR	*Boschniakia rossica, ginseng, medlar*	Hong Kong

SPT017	1. Aminotadalafil*	-	*Cynomorium songaricum, kudzu roots, Cordyceps*	United States
SPT018	1. Sildenafil (91.90 - THE)	91.90 - THE	*Boschniakia rossica, ginseng, medlar*	Hong Kong
SPT019	1. Sildenafil (59.49 - THE)	59.49 - THE	*Cordyceps sinensis, ginseng*	Hong Kong
SPT020	1. Sildenafil (73.35 - THE)	73.35 - THE	Unspecified herbs	Hong Kong

(Abbreviations: TRC, trace; SUB, subtherapeutic; THE, therapeutic; SPR, supratherapeutic; LOQ, limit of quantification)
*suspected analyte

The suspected-target screening generated a list of 12 matched analytes from 33 samples. The tandem MS and retention time matching subsequently confirmed the identity of each analyte, particularly distinguishing those with isomeric configurations. Based on these findings, ten target analytes were identified from 32 samples and quantified. Just two suspected analytes, i.e. aminotadalafil and hydroxythiohomosildenafil, were detected from 3 samples (2 samples contained a combination of target analytes and suspected analytes while another 1 sample contained only suspected analyte). The remaining 19 of possibly non-adulterated samples were submitted to the non-targeted screening. Systematic MS and tandem MS interrogations using top-down and bottom-up approaches returned insignificant signals, and no novel PDE5 inhibitors analogues were detected; thereby, confirming the negative results.

Fig. 6.5.3A summarises the identification of target analytes and the detection of suspected analytes in adulterated herbal remedy samples. The active ingredient of Viagra®: sildenafil, was identified in nearly half of the samples. It was found in 19 samples as a sole adulterant and 6 samples in combinations with other PDE5 inhibitors. Other target analytes identified in this study were as follows: propoxyphenyl-sildenafil, tadalafil, and thiodimethylsildenafil (4 samples each); dimethylsildenafil and thiosildenafil (3 samples each); and propoxyphenyl-hydroxyhomosildenafil, propoxyphenyl-thiodimethylsildenafil, propoxyphenyl-thiohydroxyhomosildenafil, and vardenafil (1 sample each). These target analytes can either be present as a sole adulterant or in combinations of up to four different adulterants in any one sample.

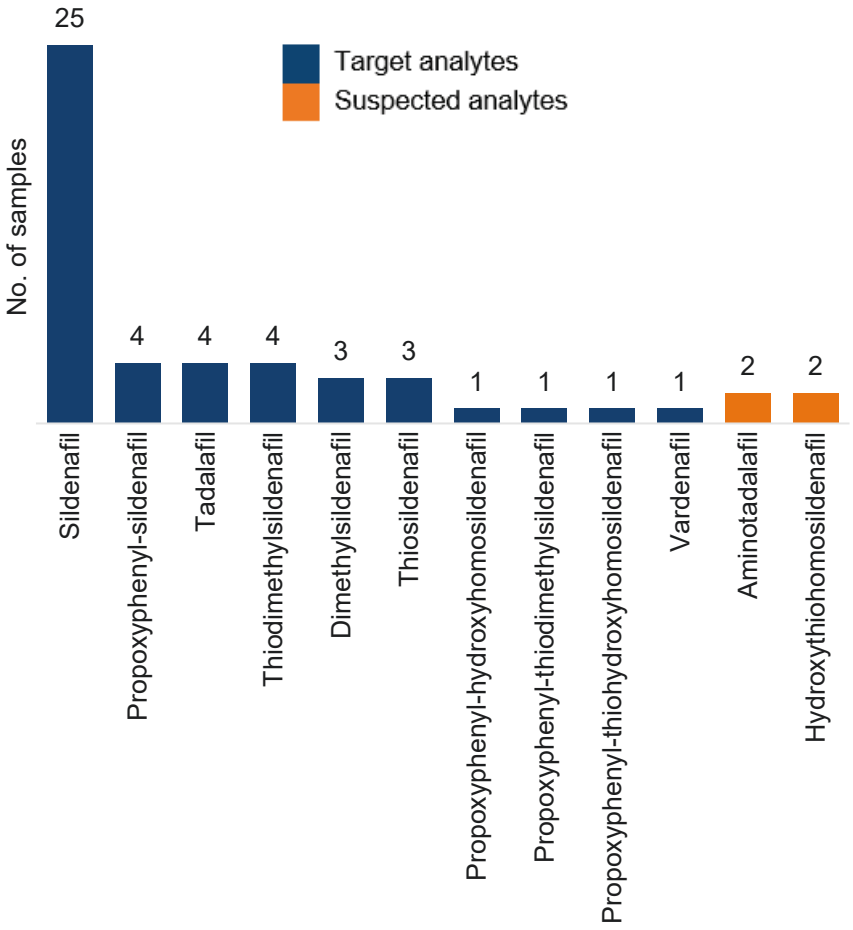


Fig. 6.5.3A: Identification of target analytes and detection of suspected analytes in adulterated herbal remedy samples.

These findings indicated that sildenafil is the most prevalent adulterant detected among the 33 adulterated samples. Similarly, analogues of sildenafil are frequently detected compared to those of tadalafil and vardenafil. The trends may be attributed to the easily accessible and inexpensive cost of raw materials to obtain or synthesise the adulterants [24]. Furthermore, the synthesis steps are readily available from the patent literature, which could yield hundreds of active sildenafil analogues [25].

The total target analytes quantified for each recommended dose ranged from 0.03 to 121.31 mg per sample. These findings were then categorised based on the recommended dose of the approved PDE5 inhibitors (i.e. 25–100 mg for sildenafil and 5–20 mg for vardenafil and tadalafil) [26], summarised in Fig. 6.5.3B. The quantification of target analytes was indicative of supratherapeutic level for 10 samples. Sildenafil (7 samples) and tadalafil (2 samples), in particular, were quantified exceeding their maximum therapeutic dose of 100 mg and 20 mg, respectively. Sample SPC027 notably contains four distinct analogues of sildenafil, combined to produce a supratherapeutic level of PDE5 inhibitors. The high dose of these adulterants generally increases the incidence of side effects which could easily jeopardise consumers' health and well-being. In some cases, concurrent consumption with nitrates or α-blockers may lead to life-threatening hypotension [27]. The remainder of the samples were categorised as follows: therapeutic level (13 samples), subtherapeutic level (8 samples), and trace level (1 sample).

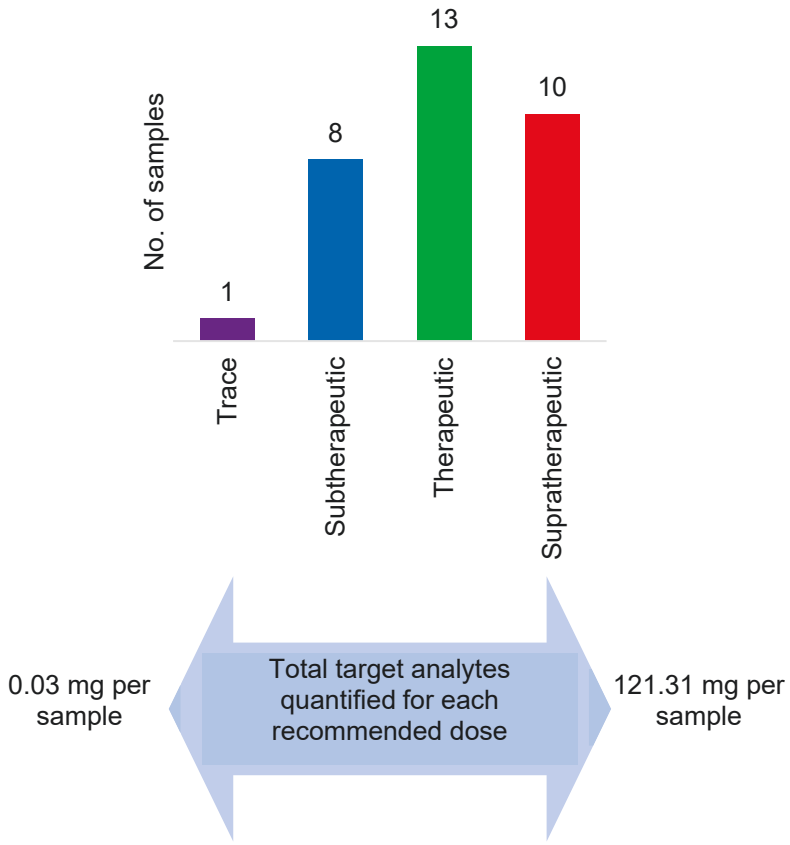


Fig. 6.5.3B: Quantification level of target analytes in adulterated herbal remedy samples.

Table 6.5.3B compiles the suspected analytes detected using the developed screening strategies. Samples SPC005 and SPT003 comprised of target analytes and suspected analytes with a total of four analytes in each sample. In contrast, sample SPT017 contained only one suspected analyte. For example, with the suspected-target screening, an unidentified BPC peak at the retention time of 23.92 min from sample SPC005 was initially matched with two analytes, namely hydroxythiohomosildenafil and hydroxythiovardenafil based on the theoretical $[M+H]^+$ precursor ion of *m/z* 521.1999. As these two isomeric analytes belong to different groups of PDE5 inhibitors analogues, it can be clearly distinguished using the tandem MS spectra. Furthermore, the differences in the retention time of both analytes confirmed the presence of

hydroxythiohomosildenafil, which was similarly detected from sample SPT003. The observed retention time can be tentatively assigned to hydroxythiohomosildenafil to match it with the specific CRM when available, and thus, identify the same analyte in the future.

Table 6.5.3B: Suspected analytes detected from herbal remedy samples.

Sample	Suspected analytes	RT (min)	Theoretical accurate mass of $[M+H]^+$ (*m/z*) (mass error)	Product ion 1 (*m/z*) (mass error)	Product ion 2 (*m/z*) (mass error)
SPC005	1. Hydroxy-thiohomo-sildenafil	23.92	521.1999 (1.0 ppm)	129.1022 (-3.9 ppm)	299.0961 (-1.7 ppm)
SPT003	1. Amino-tadalafil	17.75	391.1401 (-2.6 ppm)	135.0441 (-17.0 ppm)	169.0760 (-3.0 ppm)
	2. Hydroxy-thiohomo-sildenafil	24.20	521.1999 (2.3 ppm)	129.1022 (-7.7 ppm)	299.0961 (-6.0 ppm)
SPT017	1. Amino-tadalafil	17.57	391.1401 (0.5 ppm)	135.0441 (-2.2 ppm)	169.0760 (-1.8 ppm)

(Abbreviations: RT, retention time, $[M+H]^+$, protonated molecule precursor ion)

The non-targeted screening did not detect any novel PDE5 inhibitors analogues among the study samples. Its effectiveness and validity, however, can be demonstrated by the detection of aminotadalafil (a known analyte) in sample SPT003 (Fig. 6.5.3C). By using the top-down approach, an unidentified BPC peak at 17.75 min was integrated and extracted, revealing the observed $[M+H]^+$ precursor ion of *m/z* 391.1391. The link between the pre-determined $[M+H]^+$ precursor ion and the product ions of target analytes was established using the product ion scan of the Mass Hunter qualitative analysis software. As a result, the tandem MS signals at the same retention time were specific to the common

fragmentation pattern of tadalafil within ±20 ppm mass tolerance at *m/z* 135.0441 and 169.0760. Based on these findings, the “novel” analogue (aminotadalafil) can be flagged and narrowed down into the tadalafil group of analogues.

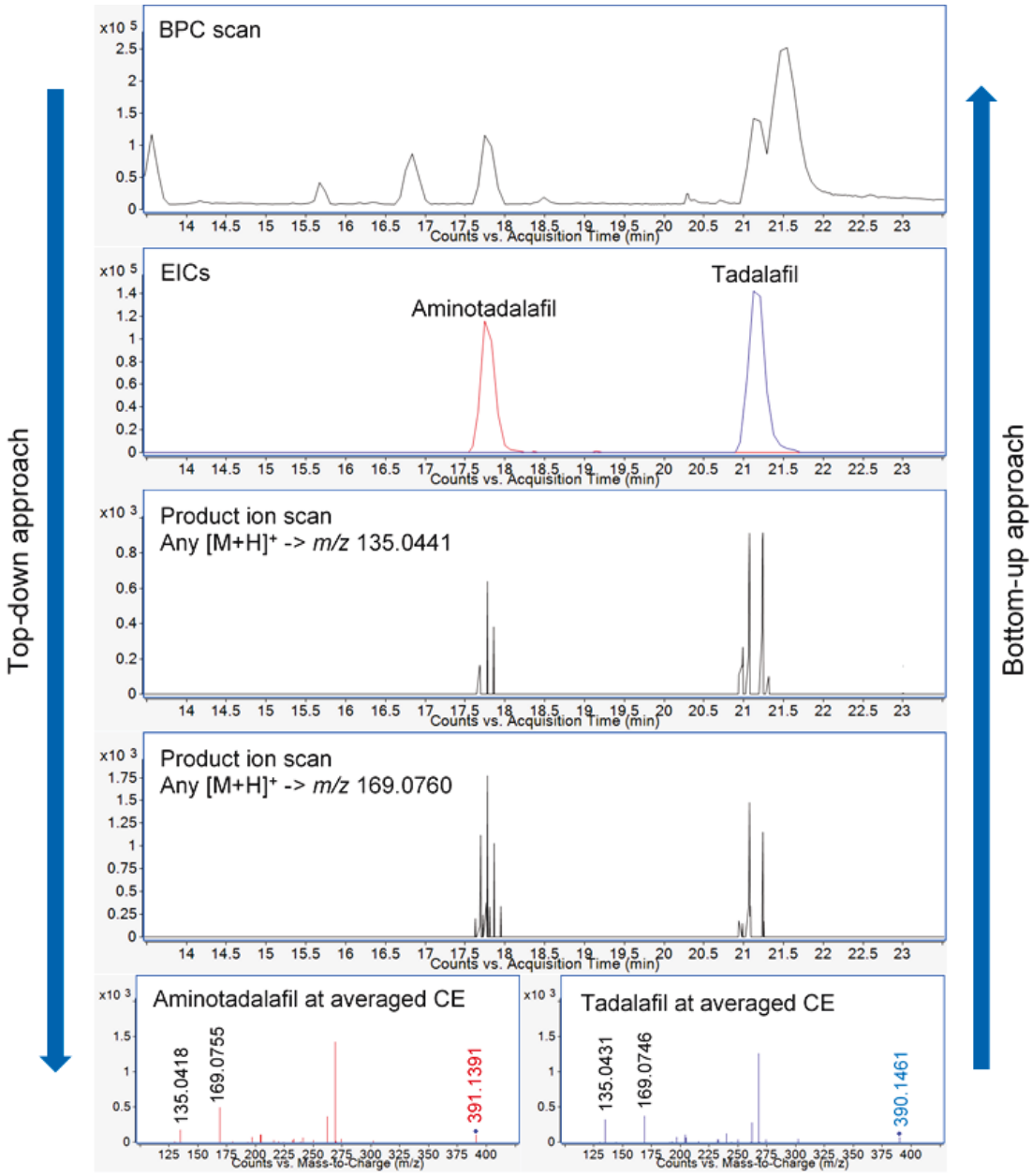


Fig. 6.5.3C: Representative base peak chromatogram (BPC); overlaid extracted ion chromatograms (EICs) of aminotadalafil and tadalafil; and tandem MS spectra of sample SPT003 demonstrating the non-targeted screening based on top-down and bottom-up approaches.

Contrarily, the bottom-up approach utilised all generated $[M+H]^+$ precursor ions during the chromatographic run to establish if there is any link to the product ions of target analytes. For sample SPT003, two tandem MS signals were detected using the product ion scan at different retention times for both product ions of tadalafil. These findings indicated the presence of two different analytes belonging to the tadalafil group of analogues. Indeed, one of the analytes was tadalafil based on the matching of the retention time at 21.12 min. Thorough investigations established the link between the observed $[M+H]^+$ precursor ion of the “novel” analogue (aminotadalafil) at *m/z* 391.1391 and the product ions of tadalafil. From these findings, the chemical formula or structure of the “novel” analogue (aminotadalafil) can be predicted based on tadalafil and tentatively assigned before further structural elucidation. Fig. 6.5.3D presents the proposed common fragmentation pattern shared by the tadalafil group of analogues. Although sample SPT003 contained other analytes such as vardenafil and hydroxythiohomosildenafil, no interferences of the tandem MS signals were observed at the *m/z* of tadalafil product ions.

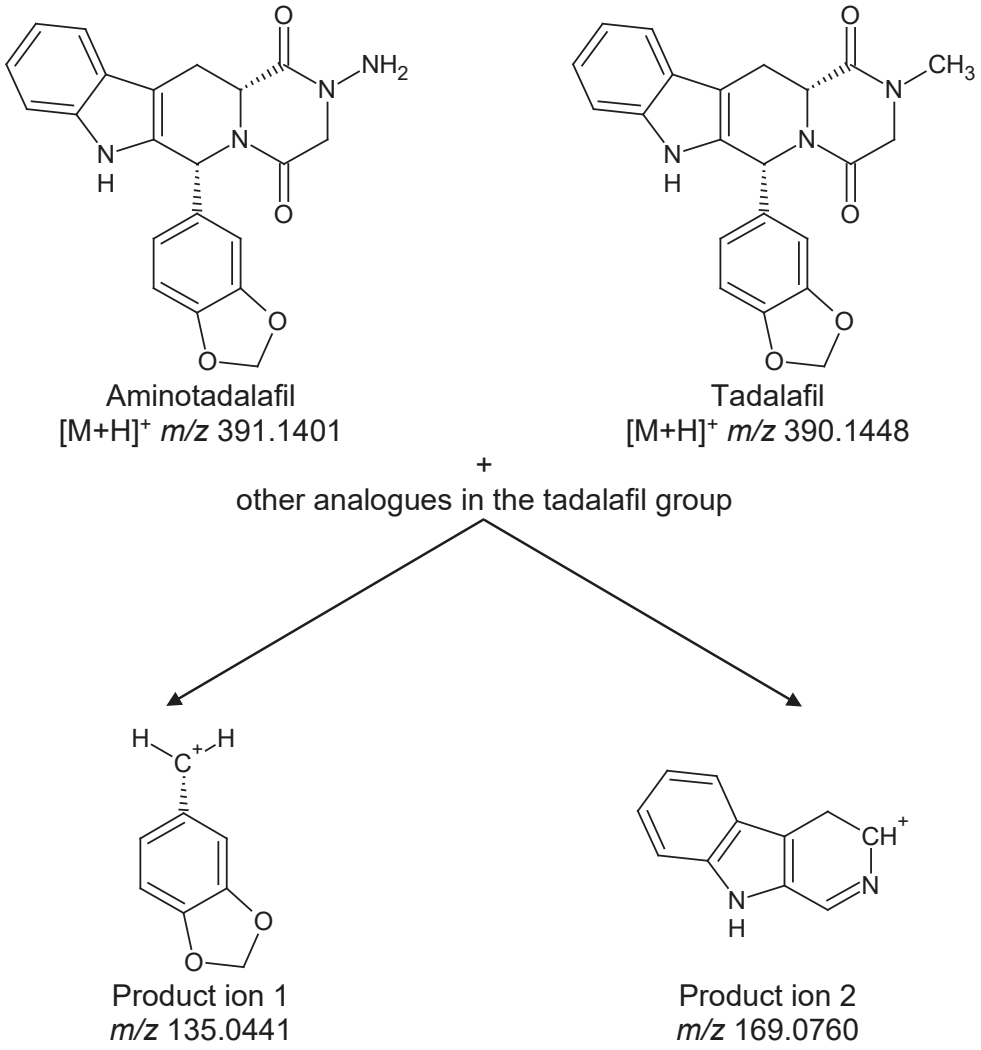


Fig. 6.5.3D: The proposed common fragmentation pattern shared by the tadalafil group of analogues.

The suspected analytes detected in this study, i.e. aminotadalafil and hydroxythiohomosildenafil, had been initially reported as adulterants in herbal remedy capsules [28-30]. The USFDA had also warned consumers on the dangers of these unapproved PDE5 inhibitors analogues, detected in 26 products marketed to enhance male sexual performance [31,32]. As exhibited by sample SPT003, these two analytes had previously detected in pairs either with [33] or without [34] other PDE5 inhibitors.

6.6 CONCLUSION

This study explored the applicability of an LC-QTOF-MS for comprehensive screening of PDE5 inhibitors and their analogues in herbal remedies using suspected-target and non-targeted strategies. The method was fully optimised and validated for 23 target analytes to screen 52 herbal remedy samples in capsule and tablet dosage forms. The screening strategies revealed 33 positive samples, identifying ten target analytes and detecting two suspected analytes. The target analytes were quantified from 0.03 to 121.31 mg for each of the recommended dose of the samples. The DDA provides cleaner spectra where the observed product ions could be easily linked to their $[M+H]^+$ precursor ion. The screening strategies discussed in this study would be beneficial to curb the widespread of adulterated herbal remedies, particularly those with PDE5 inhibitors and their analogues. It is vital to ensure that herbal remedies do not pose any health risks to consumers and thus, protecting their safety.

6.7 REFERENCES

1. Boon H, Bozinovski N. A systematic narrative review of the evidence for labeling of natural health products and dietary supplements. *J Altern Complement Med* 2019;25(8):777-788. doi:https://doi.org/10.1089/acm.2018.0533
2. Skalicka-Wozniak K, Georgiev MI, Orhan IE. Adulteration of herbal sexual enhancers and slimmers: the wish for better sexual well-being and perfect body can be risky. *Food Chem Toxicol* 2017;108:355-364. doi:https://doi.org/10.1016/j.fct.2016.06.018
3. Haneef J, Shaharyar M, Husain A, et al. Analytical methods for the detection of undeclared synthetic drugs in traditional herbal medicines as adulterants. *Drug Test Anal* 2013;5(8):607-613. doi:https://doi.org/10.1002/dta.1482
4. Primpray V, Chailapakul O, Tokeshi M, Rojanarata T, Laiwattanapaisal W. A paper-based analytical device coupled with electrochemical detection for the determination of dexamethasone and prednisolone in adulterated traditional medicines. *Anal Chim Acta* 2019;1078:16-23. doi:https://doi.org/10.1016/j.aca.2019.05.072
5. Fibigr J, Šatínský D, Solich P. Current trends in the analysis and quality control of food supplements based on plant extracts. *Anal Chim Acta* 2018;1036:1-15. doi:https://doi.org/10.1016/j.aca.2018.08.017
6. Marcus DM. Dietary supplements: What's in a name? What's in the bottle? *Drug Test Anal* 2016;8(3-4):410-412. doi:https://doi.org/10.1002/dta.1855

7. Rocha T, Amaral JS, Oliveira MBPP. Adulteration of dietary supplements by the illegal addition of synthetic drugs: a review. *Compr Rev Food Sci Food Saf* 2016;15(1):43-62. doi:https://doi.org/10.1111/1541-4337.12173

8. Mohd Yusop AY, Xiao L, Fu S. Determination of phosphodiesterase 5 (PDE5) inhibitors in instant coffee premixes using liquid chromatography-high-resolution mass spectrometry (LC-HRMS). *Talanta* 2019;204:36-43. doi:https://doi.org/10.1016/j.talanta.2019.05.078

9. United States Food and Drug Administration. Tainted products marketed as dietary supplements 2019. https://www.accessdata.fda.gov/scripts/sda/sdNavigation.cfm?filter=&sortColumn=1d&sd=tainted_supplements_cder&page=1. Accessed 21 March 2020.

10. Do TT, Theocharis G, Reich E. Simultaneous detection of three phosphodiesterase type 5 inhibitors and eight of their analogs in lifestyle products and screening for adulterants by high-performance thin-layer chromatography. *J AOAC Int* 2015;98(5):1226-1233. doi:https://doi.org/10.5740/jaoacint.14-285

11. Mokhtar SU, Chin ST, Kee CL, Low MY, Drummer OH, Marriott PJ. Rapid determination of sildenafil and its analogues in dietary supplements using gas chromatography-triple quadrupole mass spectrometry. *J Pharm Biomed Anal* 2016;121:188-196. doi:https://doi.org/10.1016/j.jpba.2016.01.034

12. Minh DTC, Thi LA, Huyen NTT, Van Vu L, Anh NTK, Ha PTT. Detection of sildenafil adulterated in herbal products using thin layer chromatography combined with surface enhanced Raman spectroscopy: “double coffee-ring effect” based enhancement. *J Pharm Biomed Anal* 2019;174:340-347. doi:https://doi.org/10.1016/j.jpba.2019.05.043

13. Gilard V, Balayssac S, Tinaugus A, Martins N, Martino R, Malet-Martino M. Detection, identification and quantification by ^{1}H NMR of adulterants in 150 herbal dietary supplements marketed for improving sexual performance. *J Pharm Biomed Anal* 2015;102:476-493. doi:https://doi.org/10.1016/j.jpba.2014.10.011

14. Lee JH, Park HN, Park OR, Kim NS, Park S-K, Kang H. Screening of illegal sexual enhancement supplements and counterfeit drugs sold in the online and offline markets between 2014 and 2017. *Forensic Sci Int* 2019;298:10-19. doi:https://doi.org/10.1016/j.forsciint.2019.02.014

15. Pasin D, Cawley A, Bidny S, Fu S. Current applications of high-resolution mass spectrometry for the analysis of new psychoactive substances: a critical review. *Anal Bioanal Chem* 2017;409(25):5821-5836. doi:https://doi.org/10.1007/s00216-017-0441-4

16. Álvarez-Ruiz R, Picó Y. Sequential window acquisition of all theoretical fragments versus information dependent acquisition for suspected-screening of pharmaceuticals in sediments and mussels by ultra-high pressure liquid chromatography-quadrupole time-of-flight-mass spectrometry. *J Chromatogr A* 2019;1595:81-90. doi:https://doi.org/10.1016/j.chroma.2019.02.041

17. von Eyken A, Ramachandran S, Bayen S. Suspected-target screening for the assessment of plastic-related chemicals in honey. *Food Control* 2020;109:106941. doi:https://doi.org/10.1016/j.foodcont.2019.106941

18. Mohd Yusop AY, Xiao L, Fu S. Data on the optimisation and validation of a liquid chromatography-high-resolution mass spectrometry (LC-HRMS) to establish the presence of phosphodiesterase 5 (PDE5) inhibitors in instant coffee premixes. *Data Brief* 2019;25:104234. doi:https://doi.org/10.1016/j.dib.2019.104234

19. ICH. ICH harmonized tripartite guideline: validation of analytical procedures: text and methodology Q2(R1). In: International Conference on Harmonization of technical requirements for registration of pharmaceuticals for human use; 2005.

20. Kruve A, Rebane R, Kipper K, et al. Tutorial review on validation of liquid chromatography-mass spectrometry methods: part II. *Anal Chim Acta* 2015;870:8-28. doi:https://doi.org/10.1016/j.aca.2015.02.016

21. Kruve A, Rebane R, Kipper K, et al. Tutorial review on validation of liquid chromatography-mass spectrometry methods: part I. *Anal Chim Acta* 2015;870:29-44. doi:https://doi.org/10.1016/j.aca.2015.02.017

22. Lee S, Ji D, Park M, Chung KH. Development of a comprehensive spectral library of sildenafil and related active analogues using LC-QTOF-MS and its application for screening counterfeit pharmaceuticals. *Forensic Sci Int* 2015;257:182-188. doi:https://doi.org/10.1016/j.forsciint.2015.08.016

23. Strano-Rossi S, Odoardi S, Castrignano E, Serpelloni G, Chiarotti M. Liquid chromatography-high resolution mass spectrometry (LC-HRMS) determination of stimulants, anorectic drugs and phosphodiesterase 5 inhibitors (PDE5I) in food supplements. *J Pharm Biomed Anal* 2015;106:144-152. doi:https://doi.org/10.1016/j.jpba.2014.06.011

24. Patel DN, Li L, Kee C-L, Ge X, Low M-Y, Koh H-L. Screening of synthetic PDE-5 inhibitors and their analogues as adulterants: analytical techniques and challenges. *J Pharm Biomed Anal* 2014;87:176-190. doi:https://doi.org/10.1016/j.jpba.2013.04.037

25. Bell AS, Brown D, Terrett NK. Pyrazolopyrimidinone antianginal agents. In. United States: Google Patents; 1993.

26. Bruzziches R, Francomano D, Gareri P, Lenzi A, Aversa A. An update on pharmacological treatment of erectile dysfunction with phosphodiesterase type 5 inhibitors. *Expert Opin Pharmacother* 2013;14(10):1333-1344. doi:https://doi.org/10.1517/14656566.2013.799665

27. Ventimiglia E, Capogrosso P, Montorsi F, Salonia A. The safety of phosphodiesterase type 5 inhibitors for erectile dysfunction. *Expert Opin Drug Saf* 2016;15(2):141-152. doi:https://doi.org/10.1517/14740338.2016.1131818

28. Zou P, Hou P, Oh SS-Y, Low M-Y, Koh H-L. Electrospray tandem mass spectrometric investigations of tadalafil and its analogue. *Rapid Commun Mass Spectrom* 2006;20(22):3488-3490. doi:https://doi.org/10.1002/rcm.2752

29. Zou P, Hou P, Low M-Y, Koh H-L. Structural elucidation of a tadalafil analogue found as an adulterant of a herbal product. *Food Addit Contam* 2006;23(5):446-451. doi:https://doi.org/10.1080/02652030500479690

30. Li L, Low MY, Aliwarga F, et al. Isolation and identification of hydroxythiohomosildenafil in herbal dietary supplements sold as sexual performance enhancement products. *Food Addit Contam Part A* 2009;26(2):145-151. doi:https://doi.org/10.1080/02652030802368757

31. United States Food and Drug Administration. Tainted products marketed as dietary supplements 2019. https://www.accessdata.fda.gov/scripts/sda/sdNavigation.cfm?filter=aminotadalafil&sortColumn=1d&sd=tainted_supplements_cder&page=1. Accessed 11 January 2020.

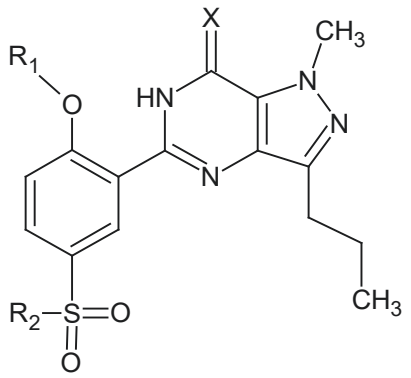
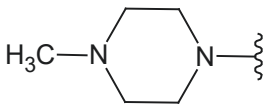
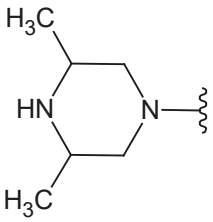
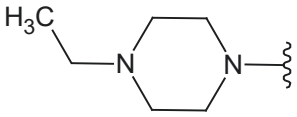
32. United States Food and Drug Administration. Tainted products marketed as dietary supplements 2019. https://www.accessdata.fda.gov/scripts/sda/sdNavigation.cfm?filter=hydroxythiohomosildenafil&sortColumn=1d&sd=tainted_supplements_cder&page=1. Accessed 11 January 2020.

33. United States Food and Drug Administration. Public notification: Alpha Male contains hidden drug ingredient 2013. https://www.fda.gov/drugs/medication-health-fraud/public-notification-alpha-male-contains-hidden-drug-ingredient. Accessed 11 January 2020.

34. United States Food and Drug Administration. Tainted products marketed as dietary supplements 2013. https://www.accessdata.fda.gov/scripts/sda/sdNavigation.cfm?filter=rezzrx&sortColumn=1d&sd=tainted_supplements_cder&page=1. Accessed 11 January 2020.

6.8 SUPPLEMENTARY DATA

Table 6.8A: Sildenafil group of analogues.

Sildenafil group			
			
	Sildenafil analogues X = O $R_1 = CH_3CH_2$	**Propoxyphenyl-sildenafil analogues** X = O $R_1 = CH_3CH_2CH_2$	**Thiosildenafil analogues** X = S $R_1 = CH_3CH_2$	**Propoxyphenyl-thiosildenafil analogues** X = S $R_1 = CH_3CH_2CH_2$
R_2 =				
	(8) Sildenafil	(13) Propoxyphenyl-sildenafil	(18) Thiosildenafil	-
R_2 =				
	(10) Dimethyl-sildenafil	-	(21) Thiodimethyl-sildenafil	(23) Propoxyphenyl-thiodimethyl-sildenafil
R_2 =				
	(9) Homosildenafil	-	(19) Thiohomo-sildenafil	-

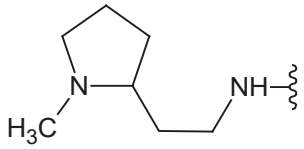
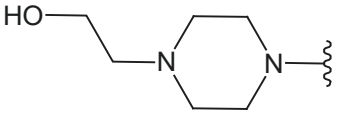
R_2 = 				
	-	(12) Udenafil	-	-
R_2 = 				
	-	(11) Propoxyphenyl-hydroxyhomo-sildenafil	-	(22) Propoxyphenyl-thiohydroxyhomo-sildenafil

Table 6.8B: Acetildenafil group of analogues.

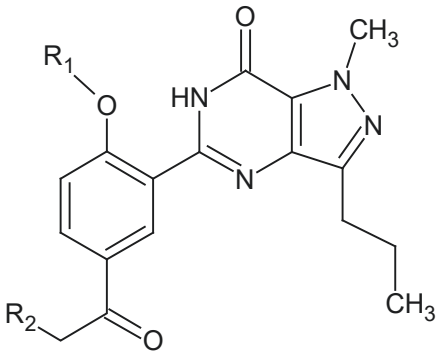
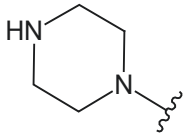
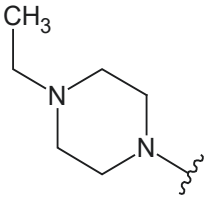
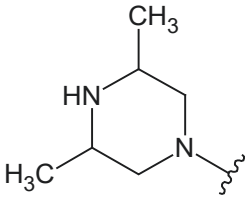
Acetildenafil group			
			
Acetildenafil analogues $R_1 = CH_3CH_2$			
R_2 =			
	(3) N-desethylacetildenafil	(4) Acetildenafil	(6) Dimethylacetildenafil

Table 6.8C: Carbodenafil group of analogues.

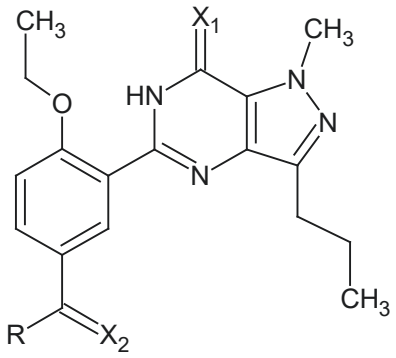
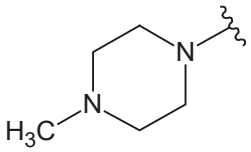
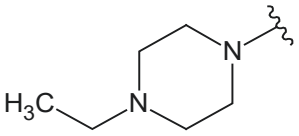
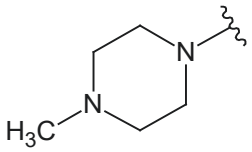
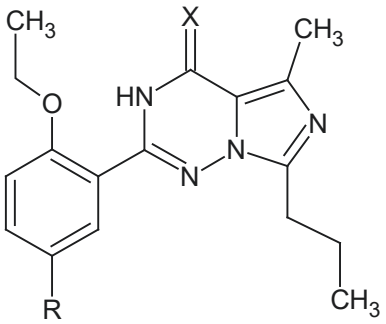
Carbodenafil group		
		
	Carbodenafil analogues X_1 = O X_2 = O	**Dithiocarbodenafil analogue** X_1 = S X_2 = S
R =		 
	(1) Desmethyl-carbodenafil	(2) Carbodenafil (20) Dithiodesmethyl-carbodenafil

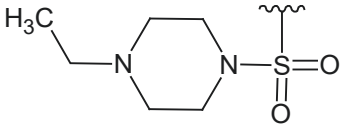
Table 6.8D: Vardenafil group of analogues.

Vardenafil group

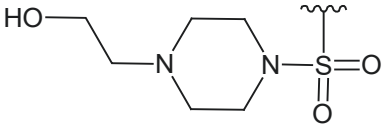


Vardenafil analogues

X = O

R = 

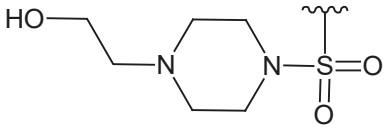
(7)
Vardenafil



(5)
Hydroxyvardenafil

Thiovardenafil analogue

X = S

R = 

(14)
Hydroxythiovardenafil

Table 6.8E: Tadalafil group of analogues.

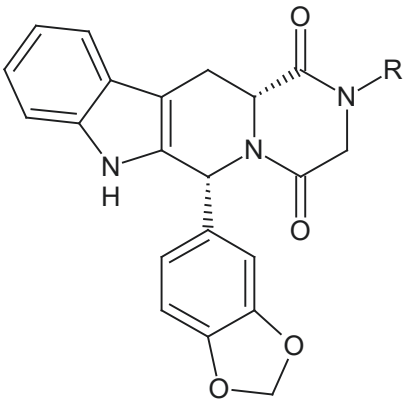
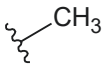
Tadalafil group

Tadalafil analogue
R = 
(15) Tadalafil

Table 6.8F: Miscellaneous phosphodiesterase 5 (PDE5) inhibitors.

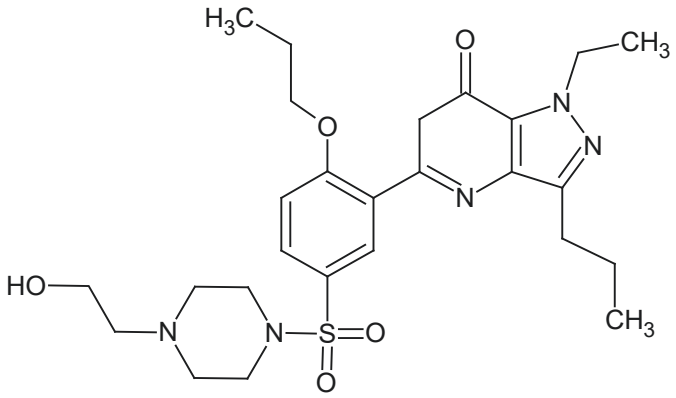
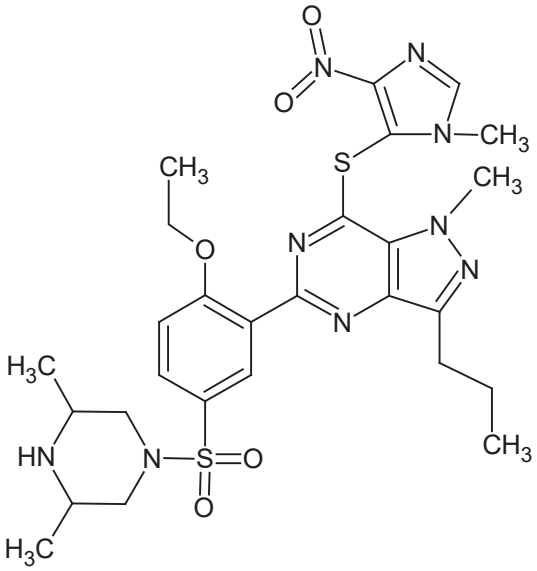
Miscellaneous PDE5 inhibitors

(16) Mirodenafil

(17) Mutaprodenafil

Table 6.8G: Phosphodiesterase 5 (PDE5) inhibitors and their analogues included in the personal compound database and library (PCDL).

No.	Compound name	Molecular formula	Exact mass
1	Desmethylcarbodenafil	$C_{23}H_{30}N_6O_3$	438.2379
2	Carbodenafil	$C_{24}H_{32}N_6O_3$	452.2536
3	N-desethylacetildenafil	$C_{23}H_{30}N_6O_3$	438.2379
4	Acetildenafil	$C_{25}H_{34}N_6O_3$	466.2692
5	Hydroxyvardenafil	$C_{23}H_{32}N_6O_5S$	504.2155
6	Dimethylacetildenafil	$C_{25}H_{34}N_6O_3$	466.2692
7	Vardenafil	$C_{23}H_{32}N_6O_4S$	488.2206
8	Sildenafil	$C_{22}H_{30}N_6O_4S$	474.2049
9	Homosildenafil	$C_{23}H_{32}N_6O_4S$	488.2206
10	Dimethylsildenafil	$C_{23}H_{32}N_6O_4S$	488.2206
11	Propoxyphenyl-hydroxyhomosildenafil	$C_{24}H_{34}N_6O_5S$	518.2311
12	Udenafil	$C_{25}H_{36}N_6O_4S$	516.2519
13	Propoxyphenyl-sildenafil	$C_{23}H_{32}N_6O_4S$	488.2206
14	Hydroxythiovardenafil	$C_{23}H_{32}N_6O_4S_2$	520.1926
15	Tadalafil	$C_{22}H_{19}N_3O_4$	389.1376
16	Mutaprodenafil	$C_{27}H_{35}N_9O_5S_2$	629.2203
17	Mirodenafil	$C_{26}H_{37}N_5O_5S$	531.2515
18	Thiosildenafil	$C_{22}H_{30}N_6O_3S_2$	490.1821
19	Thiohomosildenafil	$C_{23}H_{32}N_6O_3S_2$	504.1977
20	Dithiodesmethylcarbodenafil	$C_{23}H_{30}N_6OS_2$	470.1923
21	Thiodimethylsildenafil	$C_{23}H_{32}N_6O_3S_2$	504.1977
22	Propoxyphenyl-thiohydroxyhomosildenafil	$C_{24}H_{34}N_6O_4S_2$	534.2083
23	Propoxyphenyl-thiodimethylsildenafil	$C_{24}H_{34}N_6O_3S_2$	518.2134
24	Desulfovardenafil	$C_{17}H_{20}N_4O_2$	312.1586
25	Gendenafil	$C_{19}H_{22}N_4O_3$	354.1692
26	Acetil acid	$C_{18}H_{20}N_4O_4$	356.1485
27	Norneovardenafil	$C_{18}H_{20}N_4O_4$	356.1485
28	Nitrodenafil	$C_{17}H_{19}N_5O_4$	357.1437
29	Dihydroxydenafil	$C_{19}H_{24}N_4O_4$	372.1798
30	Nortadalafil	$C_{21}H_{17}N_3O_4$	375.1219
31	Chlorodenafil	$C_{19}H_{21}ClN_4O_3$	388.1302
32	Trans-tadalafil	$C_{22}H_{19}N_3O_4$	389.1376
33	Benzamidenafil	$C_{19}H_{23}N_3O_6$	389.1587
34	Trans-aminotadalafil	$C_{21}H_{18}N_4O_4$	390.1328
35	Aminotadalafil	$C_{21}H_{18}N_4O_4$	390.1328
36	Hydroxychlorodenafil	$C_{19}H_{23}ClN_4O_3$	390.1459
37	Homotadalafil	$C_{23}H_{21}N_3O_4$	403.1532
38	Aminosildenafil	$C_{18}H_{23}N_5O_4S$	405.1471
39	E-dichlorodenafil	$C_{19}H_{20}Cl_2N_4O_2$	406.0963
40	Z-dichlorodenafil	$C_{19}H_{20}Cl_2N_4O_2$	406.0963
41	Desmethylpiperazinyl propoxysildenafil	$C_{18}H_{22}N_4O_5S$	406.1311
42	Depiperazinothiosildenafil	$C_{17}H_{20}N_4O_4S_2$	408.0926
43	Isopropylnortadalafil	$C_{24}H_{23}N_3O_4$	417.1689
44	2-Hydroxyethylnortadalafil	$C_{23}H_{21}N_3O_5$	419.1481
45	Desethylcarbodenafil	$C_{22}H_{28}N_6O_3$	424.2223

46	Chloropretadalafil	$C_{22}H_{19}ClN_2O_5$	426.0982
47	N-butylnortadalafil	$C_{25}H_{25}N_3O_4$	431.1845
48	Acetaminotadalafil	$C_{23}H_{20}N_4O_5$	432.1434
49	N-3-hydroxypropylnortadalafil	$C_{24}H_{23}N_3O_5$	433.1638
50	2-Hydroxypropylnortadalafil	$C_{24}H_{23}N_3O_5$	433.1638
51	Piperidino acetildenafil	$C_{24}H_{31}N_5O_3$	437.2427
52	Chloropropanoylpretadalafil	$C_{23}H_{21}ClN_2O_5$	440.1139
53	Trans-cyclopentyltadalafil	$C_{26}H_{25}N_3O_4$	443.1845
54	Cyclopentyltadalafil	$C_{26}H_{25}N_3O_4$	443.1845
55	Thioquinapiperifil	$C_{24}H_{28}N_6OS$	448.2045
56	Noracetildenafil	$C_{24}H_{32}N_6O_3$	452.2536
57	Dithiodesethylcarbodenafil	$C_{22}H_{28}N_6OS_2$	456.1766
58	Norneosildenafil	$C_{22}H_{29}N_5O_4S$	459.1940
59	Pseudovardenafil	$C_{22}H_{29}N_5O_4S$	459.1940
60	Tadalafil YJ-05	$C_{27}H_{29}N_3O_4$	459.2158
61	N-desethylvardenafil	$C_{21}H_{28}N_6O_4S$	460.1893
62	N-desmethylsildenafil	$C_{21}H_{28}N_6O_4S$	460.1893
63	Descarbonsildenafil	$C_{21}H_{30}N_6O_4S$	462.2049
64	Diethylaminopretadalafil	$C_{26}H_{29}N_3O_5$	463.2107
65	Acetilvardenafil	$C_{25}H_{34}N_6O_3$	466.2692
66	Propoxyphenyl-noracetildenafil	$C_{25}H_{34}N_6O_3$	466.2692
67	Dimethylthiocarbodenafil	$C_{24}H_{32}N_6O_2S$	468.2307
68	Hydroxycarbodenafil	$C_{24}H_{32}N_6O_4$	468.2485
69	Oxoacetildenafil	$C_{25}H_{32}N_6O_4$	480.2485
70	Hydroxyacetildenafil	$C_{25}H_{34}N_6O_4$	482.2642
71	Isopiperazinonafil	$C_{25}H_{34}N_6O_4$	482.2642
72	Piperazinonafil	$C_{25}H_{34}N_6O_4$	482.2642
73	Avanafil	$C_{23}H_{26}ClN_7O_3$	483.1786
74	Dimethyldithiodenafil	$C_{24}H_{32}N_6OS_2$	484.2079
75	N-octylnortadalafil	$C_{29}H_{33}N_3O_4$	487.2471
76	Dipropylaminopretadalafil	$C_{28}H_{33}N_3O_5$	491.2420
77	Dioxoacetildenafil	$C_{25}H_{30}N_6O_5$	494.2278
78	Interaction prod. of aminotadalafil & 5-hydroxymethylfurfural	$C_{27}H_{22}N_4O_6$	498.1539
79	Dithiopropylcarbodenafil	$C_{25}H_{34}N_6OS_2$	498.2236
80	3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil	$C_{25}H_{34}N_6OS_2$	498.2236
81	Propoxyphenyl-dimethylsildenafil	$C_{24}H_{34}N_6O_4S$	502.2362
82	N-phenylpropenyltadalafil	$C_{30}H_{24}N_4O_4$	504.1798
83	Propoxyphenyl-thiosildenafil	$C_{23}H_{32}N_6O_3S_2$	504.1977
84	Hydroxyhomosildenafil	$C_{23}H_{32}N_6O_5S$	504.2155
85	Propoxyphenyl-isobutyldimethylsildenafil	$C_{25}H_{36}N_6O_4S$	516.2519
86	Propoxyphenyl-thiohomosildenafil	$C_{24}H_{34}N_6O_3S_2$	518.2134
87	Hydroxythiohomosildenafil	$C_{23}H_{32}N_6O_4S_2$	520.1926
88	Cyclopentynafil	$C_{26}H_{36}N_6O_4S$	528.2519
89	Benzylsildenafil	$C_{28}H_{34}N_6O_4S$	550.2362
90	Cinnamyldenafil	$C_{32}H_{38}N_6O_3$	554.3005
91	Isonitrosoprodenafil	$C_{27}H_{35}N_9O_5S_2$	629.2203
92	Bisprenortadalafil	$C_{43}H_{35}N_5O_9$	765.2435

93	Trans-bisprehomotadalafil	$C_{46}H_{43}N_5O_{10}$	825.3010
94	Trans-bisprecyclopentyl tadalafil	$C_{49}H_{47}N_5O_{10}$	865.3323
95	Lodenafil carbonate	$C_{47}H_{62}N_{12}O_{11}S_2$	1034.4102

Table 6.8H: Matrix effect (ME) for capsule and tablet blank matrices using dilute and shoot (D) technique and modified quick, easy, cheap, effective, rugged, and safe (Q) extraction at three levels of matrix dilution.

No.	Analytes	ME (%) (n=9)											
		Dilution 1:2				Dilution 1:10				Dilution 1:100			
		Capsule		Tablet		Capsule		Tablet		Capsule		Tablet	
		D	Q	D	Q	D	Q	D	Q	D	Q	D	Q
1	Desmethylcarbodenafil	4.4	-1.8	3.2	-16.5	1.9	-6.7	-0.3	-0.2	2.9	-5.3	5.6	3.1
2	Carbodenafil	-2.0	-6.0	4.8	-1.8	0.8	-6.5	0.7	1.7	1.5	-5.8	4.0	3.2
3	N-desethylacetildenafil	-11.4	-11.2	1.2	0.4	-0.5	-1.7	0.9	6.1	0.6	2.9	2.0	6.4
4	Acetildenafil	-2.3	-2.0	4.4	-2.3	-2.8	-6.1	3.1	0.0	0.9	-6.0	6.2	2.6
5	Hydroxyvardenafil	-13.7	-9.0	3.7	-0.4	-1.5	-5.2	8.4	8.0	-1.0	-3.6	5.1	2.1
6	Dimethylacetildenafil	-2.6	-3.2	8.2	-2.1	1.2	1.2	-5.6	-0.8	3.6	1.0	3.3	3.1
7	Vardenafil	-4.9	-0.5	6.6	3.1	-1.3	-5.3	2.5	4.8	1.3	-3.7	2.0	4.4
8	Sildenafil	0.4	0.8	3.1	-1.1	-0.3	-1.0	7.9	4.0	1.4	-2.2	4.4	4.1
9	Homosildenafil	-3.3	-1.5	0.7	-1.2	-5.9	-3.3	8.2	0.9	-1.9	-4.0	5.0	2.9
10	Dimethylsildenafil	-10.0	-2.5	4.4	-1.5	2.7	1.3	6.7	2.2	4.1	0.2	2.5	4.3
11	Propoxyphenyl-hydroxyhomosildenafil	-11.1	-8.3	2.8	-0.4	0.7	-0.9	6.4	6.4	1.6	-2.4	4.2	5.3
12	Udenafil	-5.8	-5.0	2.9	-4.1	-3.7	-2.7	3.2	3.1	-2.4	-3.8	3.9	4.3
13	Propoxyphenyl-sildenafil	2.6	2.7	-2.6	-1.3	-0.3	-4.1	0.7	0.5	-1.2	-3.4	4.9	1.6
14	Hydroxythiovardenafil	-12.2	-5.7	-	-6.1	-1.6	-5.1	-	4.8	0.7	-3.5	5.0	1.8
15	Tadalafil	-25.7	-16.5	9.5	-15.8	0.1	-9.1	0.2	-2.4	-1.8	-1.2	3.8	2.5
16	Mirodenafil	-9.0	-10.0	-10.1	-13.5	1.9	-6.4	-1.9	1.3	2.9	-4.8	3.7	3.7
17	Mutaprodenafil	-10.5	-6.6	-4.2	-12.6	-2.7	-0.4	-1.1	-0.1	-2.1	-2.3	1.5	1.8
18	Thiosildenafil	-3.1	-0.7	-	-7.3	-1.5	-2.7	-	0.9	-2.0	-1.5	3.9	1.6
19	Thiohomosildenafil	-2.3	-0.3	-	-17.4	-2.1	-2.5	-	-1.1	-1.3	-2.2	8.3	0.3
20	Dithiodesmethylcarbodenafil	-2.7	-0.2	-	-2.7	-1.4	-4.2	-	-3.7	-2.1	-2.9	3.4	-1.9
21	Thiodimethylsildenafil	-13.0	-6.3	-	-17.1	0.7	-1.1	-	-1.4	-0.5	-1.8	8.3	1.3
22	Propoxyphenyl-thiohydroxyhomosildenafil	-21.5	-14.2	-	-13.2	0.2	1.7	-	0.8	-0.9	-1.1	6.1	0.8
23	Propoxyphenyl-thiodimethylsildenafil	-14.2	-8.1	-	-18.8	0.1	-1.6	-	-3.5	1.1	-1.9	5.9	-0.1

Table 6.8I: Retention time (RT), theoretical accurate mass of protonated molecule ($[M+H]^+$) precursor ion, mass error, product ions, coefficient of determination (r^2), and limit of detection (LOD) of 23 targeted phosphodiesterase 5 (PDE5) inhibitors.

No.	Analytes	RT (min)	Theoretical accurate mass of $[M+H]^+$ (*m/z*)	Mass error (ppm)		Product ion 1 (*m/z*)	Product ion 2 (*m/z*)	r^2	LOD (ng/mL)
				Cap	Tab				
1	Desmethylcarbodenafil	8.94	439.2452	-1.1	0.4	311.1139	339.1452	0.9992	10
2	Carbodenafil	9.41	453.2609	-0.3	-0.1	311.1139	339.1452	0.9992	10
3	N-desethylacetildenafil	9.85	439.2452	-0.8	0.3	325.1295	297.1346	0.9961	40
4	Acetildenafil	10.84	467.2765	0.8	-0.7	297.1346	127.1230	0.9991	10
5	Hydroxyvardenafil	11.01	505.2228	-0.5	0.1	312.1581	151.0866	0.9951	10
6	Dimethylacetildenafil	11.35	467.2765	-2.2	-1.1	297.1346	127.1230	0.9985	30
7	Vardenafil	11.69	489.2279	-0.8	1.4	312.1581	151.0866	0.9986	20
8	Sildenafil	13.66	475.2122	-1.0	-1.1	283.1190	100.0995	0.9995	30
9	Homosildenafil	14.22	489.2279	2.0	-3.0	283.1190	113.1073	0.9997	10
10	Dimethylsildenafil	14.99	489.2279	-0.4	1.2	283.1190	113.1073	0.9966	20
11	Propoxyphenyl-hydroxyhomosildenafil	16.04	519.2384	-2.4	-0.4	129.1022	283.1190	0.9954	10
12	Udenafil	16.26	517.2592	-2.2	0.2	112.1121	283.1190	0.9991	10
13	Propoxyphenyl-sildenafil	16.43	489.2279	0.3	-0.5	100.0995	283.1190	0.9985	10
14	Hydroxythiovardenafil	18.72	521.1999	1.3	-1.7	167.0637	328.1352	0.9948	10
15	Tadalafil	21.14	390.1448	-1.6	-3.6	135.0441	169.0760	0.9870	40
16	Mirodenafil	21.91	532.2588	-0.7	-0.2	312.1343	296.1394	0.9976	10
17	Mutaprodenafil	22.08	630.2275	-0.6	-2.2	113.1073	142.0070	0.9984	10
18	Thiosildenafil	25.24	491.1894	-1.2	-1.9	100.0995	299.0961	0.9994	20
19	Thiohomosildenafil	26.29	505.2050	-1.0	-2.8	299.0961	113.1073	0.9989	20
20	Dithiodesmethylcarbodenafil	26.54	471.1995	-3.1	-0.9	343.0682	371.0995	0.9993	10
21	Thiodimethylsildenafil	27.05	505.2050	0.0	-2.0	113.1073	299.0961	0.9955	10
22	Propoxyphenyl-thiohydroxyhomosildenafil	27.81	535.2156	-2.0	0.8	129.1022	299.0961	0.9887	10
23	Propoxyphenyl-thiodimethylsildenafil	30.59	519.2207	-1.1	-0.9	113.1073	299.0961	0.9929	20

Table 6.8J: Accuracy of 23 targeted phosphodiesterase 5 (PDE5) inhibitors.

No.	Analytes	Accuracy (Mean ± SD, %) (n=3)					
		Capsule			Tablet		
		Low	Medium	High	Low	Medium	High
1	Desmethylcarbodenafil	101.2 ± 2.8	99.4 ± 2.5	101.5 ± 1.4	122.0 ± 2.0	96.7 ± 2.0	97.2 ± 1.2
2	Carbodenafil	104.5 ± 2.9	100.7 ± 1.5	100.9 ± 1.7	117.1 ± 1.7	104.0 ± 0.7	100.8 ± 1.1
3	N-desethylacetildenafil	116.2 ± 2.2	94.4 ± 0.8	99.7 ± 1.2	123.6 ± 2.3	99.9 ± 1.8	101.1 ± 0.5
4	Acetildenafil	96.4 ± 2.7	101.1 ± 0.9	103.4 ± 0.3	101.6 ± 1.3	104.1 ± 2.3	102.0 ± 0.6
5	Hydroxyvardenafil	116.0 ± 1.3	99.7 ± 1.6	98.2 ± 0.5	97.5 ± 2.4	106.0 ± 1.6	102.5 ± 0.7
6	Dimethylacetildenafil	112.7 ± 1.0	97.3 ± 0.9	99.8 ± 0.8	106.1 ± 5.0	101.6 ± 3.2	103.9 ± 0.8
7	Vardenafil	107.0 ± 0.9	100.3 ± 1.0	99.6 ± 1.3	115.9 ± 7.8	104.3 ± 2.4	101.8 ± 2.4
8	Sildenafil	100.1 ± 3.3	100.7 ± 2.4	101.4 ± 0.6	111.7 ± 3.2	106.2 ± 0.8	101.1 ± 1.2
9	Homosildenafil	100.9 ± 1.5	102.7 ± 1.5	100.5 ± 1.2	103.7 ± 1.7	107.2 ± 1.6	101.1 ± 2.7
10	Dimethylsildenafil	111.9 ± 0.5	98.3 ± 0.7	98.8 ± 1.6	121.5 ± 2.9	104.2 ± 0.8	100.1 ± 0.3
11	Propoxyphenyl-hydroxyhomosildenafil	118.2 ± 1.4	97.3 ± 1.9	98.5 ± 1.0	88.3 ± 3.1	103.6 ± 0.5	100.6 ± 0.9
12	Udenafil	111.4 ± 1.0	99.5 ± 2.3	100.6 ± 0.5	124.7 ± 1.7	102.6 ± 1.2	100.0 ± 1.1
13	Propoxyphenyl-sildenafil	90.8 ± 3.8	104.9 ± 0.8	101.7 ± 1.4	87.9 ± 4.7	106.0 ± 1.4	98.3 ± 0.6
14	Hydroxythiovardenafil	115.3 ± 0.6	97.4 ± 2.0	97.3 ± 0.7	102.8 ± 2.3	102.0 ± 0.7	98.6 ± 1.4
15	Tadalafil	120.8 ± 5.0	95.6 ± 2.9	96.0 ± 3.6	93.9 ± 11.4	109.9 ± 1.4	99.6 ± 2.3
16	Mirodenafil	112.8 ± 1.5	97.5 ± 0.5	100.1 ± 1.1	87.2 ± 4.0	96.3 ± 0.8	96.6 ± 1.2
17	Mutaprodenafil	106.1 ± 1.4	101.5 ± 0.5	98.6 ± 0.4	116.8 ± 1.4	107.2 ± 2.3	99.9 ± 0.8
18	Thiosildenafil	97.3 ± 2.4	102.5 ± 0.5	101.3 ± 2.2	82.6 ± 1.0	96.7 ± 1.3	92.8 ± 1.8
19	Thiohomosildenafil	94.0 ± 0.8	104.6 ± 0.9	100.9 ± 1.4	92.5 ± 1.1	93.8 ± 5.1	90.8 ± 0.6
20	Dithiodesmethylcarbodenafil	101.1 ± 3.0	102.9 ± 2.4	100.6 ± 1.3	88.1 ± 3.5	101.3 ± 0.9	94.2 ± 1.1
21	Thiodimethylsildenafil	114.2 ± 1.5	98.1 ± 1.8	97.8 ± 1.3	95.1 ± 2.0	95.0 ± 5.0	94.6 ± 1.2
22	Propoxyphenyl-thiohydroxyhomosildenafil	123.1 ± 5.6	97.0 ± 2.1	95.6 ± 0.7	79.0 ± 3.8	97.1 ± 1.4	92.7 ± 1.2
23	Propoxyphenyl-thiodimethylsildenafil	120.3 ± 2.2	98.2 ± 2.5	97.3 ± 1.1	105.2 ± 3.6	98.6 ± 1.2	96.8 ± 0.8

Table 6.8K: Precision of 23 targeted phosphodiesterase 5 (PDE5) inhibitors.

No.	Analytes	Precisions (%RSD) (n=9)											
		Repeatability						Intermediate precision					
		Capsule			Tablet			Capsule			Tablet		
		Low	Med	High	Low	Med	High	Low	Med	High	Low	Med	High
1	Desmethylcarbodenafil	3.0	2.5	1.4	1.5	2.0	1.2	2.9	2.2	1.6	1.8	1.2	3.7
2	Carbodenafil	3.0	1.5	1.7	1.2	0.6	1.0	3.5	6.7	1.4	3.6	2.1	4.0
3	N-desethylacetildenafil	2.5	0.9	1.2	1.8	1.8	0.5	3.2	2.6	1.1	1.5	0.1	1.7
4	Acetildenafil	2.9	0.9	0.3	1.0	2.1	0.6	3.3	3.3	1.8	1.4	3.3	3.7
5	Hydroxyvardenafil	1.3	1.7	0.6	1.7	1.4	0.7	9.8	6.7	1.2	2.5	8.6	5.6
6	Dimethylacetildenafil	1.0	0.9	0.8	4.2	3.1	0.7	5.1	5.2	2.8	0.8	4.3	5.5
7	Vardenafil	0.8	1.0	1.3	5.6	2.2	2.3	10.1	5.4	1.1	4.9	10.3	10.9
8	Sildenafil	3.2	2.3	0.6	2.3	0.7	1.2	4.3	6.1	1.3	1.0	2.4	2.8
9	Homosildenafil	1.4	1.5	1.2	1.0	1.3	2.5	6.3	5.8	3.0	2.0	3.1	6.4
10	Dimethylsildenafil	0.5	0.8	1.6	2.0	0.7	0.3	4.7	4.3	2.3	1.7	2.3	5.4
11	Propoxyphenyl-hydroxyhomosildenafil	1.7	2.1	1.0	2.8	0.5	0.9	1.5	3.6	1.5	0.7	12.2	2.5
12	Udenafil	1.0	2.4	0.5	1.2	1.1	1.0	2.6	1.2	3.8	2.2	2.8	4.8
13	Propoxyphenyl-sildenafil	3.2	0.7	1.4	2.9	1.1	0.6	3.3	5.6	0.9	1.1	2.2	3.5
14	Hydroxythiovardenafil	0.6	2.2	0.7	1.6	0.6	1.4	11.3	6.0	0.7	1.9	14.0	10.5
15	Tadalafil	5.9	3.3	3.8	8.6	1.2	2.2	10.3	8.1	4.8	6.2	1.2	2.1
16	Mirodenafil	1.7	0.6	1.1	3.9	0.8	1.2	1.9	2.3	1.5	0.6	0.6	2.0
17	Mutaprodenafil	1.4	0.5	0.4	1.0	2.0	0.7	6.7	2.0	2.7	2.5	4.9	4.8
18	Thiosildenafil	2.2	0.4	2.1	0.7	1.2	1.8	8.3	4.7	0.7	1.8	12.6	7.2
19	Thiohomosildenafil	0.6	0.8	1.3	0.7	4.6	0.6	5.9	4.5	1.8	3.5	14.9	8.2
20	Dithiodesmethylcarbodenafil	2.8	2.3	1.3	2.6	0.8	1.1	10.6	4.2	2.7	2.1	5.3	5.6
21	Thiodimethylsildenafil	1.7	1.9	1.3	1.5	4.8	1.3	3.5	3.8	0.5	1.9	9.0	5.9
22	Propoxyphenyl-thiohydroxyhomosildenafil	6.4	2.4	0.8	3.4	1.3	1.3	10.8	6.7	2.0	3.9	6.5	2.4
23	Propoxyphenyl-thiodimethylsildenafil	2.4	2.7	1.1	2.7	1.1	0.8	5.1	3.7	2.0	0.8	8.0	7.3

(Abbreviation: Med, medium)

Table 6.8L: Extraction recovery (RE) of 23 targeted phosphodiesterase 5 (PDE5) inhibitors.

No.	Analytes	RE (Mean ± SD, %) (n=3)					
		Capsule			Tablet		
		Low	Medium	High	Low	Medium	High
1	Desmethylcarbodenafil	97.5 ± 1.3	93.2 ± 3.1	93.9 ± 1.2	76.0 ± 2.1	81.3 ± 0.8	77.4 ± 0.8
2	Carbodenafil	102.5 ± 3.2	97.5 ± 3.9	94.7 ± 0.4	94.2 ± 4.1	98.0 ± 1.5	97.3 ± 1.6
3	N-desethylacetildenafil	73.2 ± 3.5	74.5 ± 3.3	72.6 ± 1.1	88.8 ± 6.0	89.0 ± 3.1	87.7 ± 0.8
4	Acetildenafil	85.6 ± 2.7	90.9 ± 5.0	90.9 ± 1.7	96.0 ± 2.5	98.9 ± 2.2	98.0 ± 1.3
5	Hydroxyvardenafil	91.7 ± 0.6	94.6 ± 5.0	88.6 ± 5.6	100.6 ± 6.0	98.6 ± 2.9	96.1 ± 2.7
6	Dimethylacetildenafil	95.7 ± 8.0	99.0 ± 4.5	91.0 ± 4.4	102.3 ± 5.4	100.2 ± 6.4	99.3 ± 1.0
7	Vardenafil	99.9 ± 1.4	100.9 ± 3.2	95.7 ± 1.7	98.9 ± 8.3	99.0 ± 2.3	97.6 ± 1.6
8	Sildenafil	100.9 ± 3.8	98.6 ± 3.7	95.9 ± 0.9	96.1 ± 2.0	95.2 ± 0.9	94.2 ± 0.6
9	Homosildenafil	100.9 ± 0.6	100.1 ± 4.6	95.5 ± 2.6	97.8 ± 2.3	96.4 ± 2.3	95.4 ± 1.1
10	Dimethylsildenafil	90.0 ± 4.8	93.2 ± 3.9	92.0 ± 3.5	99.4 ± 1.4	96.0 ± 1.7	96.4 ±1.5
11	Propoxyphenyl-hydroxyhomosildenafil	97.9 ± 2.7	97.3 ± 3.3	92.1 ± 2.8	95.0 ± 4.3	95.8 ± 2.7	95.8 ± 0.7
12	Udenafil	88.2 ± 1.4	93.2 ± 6.0	92.1 ± 0.3	98.1 ± 3.0	98.2 ± 3.5	97.5 ± 0.3
13	Propoxyphenyl-sildenafil	99.7 ± 0.7	98.4 ± 5.7	96.1 ± 0.9	95.6 ± 2.1	93.9 ± 1.6	94.9 ± 1.0
14	Hydroxythiovardenafil	95.8 ± 1.7	97.9 ± 5.4	90.4 ± 4.9	86.5 ± 1.4	94.9 ± 3.6	95.2 ± 1.9
15	Tadalafil	105.8 ± 12.1	102.4 ± 5.7	95.1 ± 6.4	89.4 ± 4.3	96.9 ± 4.4	86.3 ± 1.7
16	Mirodenafil	100.0 ± 1.8	99.0 ± 5.0	94.7 ± 4.6	97.1 ± 2.3	97.1 ± 2.2	92.5 ± 1.1
17	Mutaprodenafil	90.3 ± 4.3	94.6 ± 4.0	92.8 ± 2.3	92.1 ± 5.8	96.5 ± 3.1	95.7 ± 1.4
18	Thiosildenafil	89.9 ± 0.8	95.4 ± 4.7	94.4 ± 1.2	83.5 ± 0.8	92.7 ± 2.3	95.2 ± 1.1
19	Thiohomosildenafil	90.1 ± 0.8	95.8 ± 4.4	95.0 ± 0.8	74.7 ± 2.1	90.2 ± 3.6	94.6 ± 0.4
20	Dithiodesmethylcarbodenafil	97.2 ± 2.4	99.7 ± 3.6	96.2 ± 1.7	96.9 ± 2.6	97.2 ± 1.4	96.0 ± 0.6
21	Thiodimethylsildenafil	85.0 ± 1.9	91.8 ± 6.0	91.5 ± 3.2	71.5 ± 1.8	90.5 ± 1.8	93.2 ± 0.5
22	Propoxyphenyl-thiohydroxyhomosildenafil	95.7 ± 5.7	98.0 ± 4.3	88.6 ± 6.4	79.8 ± 9.1	91.4 ± 4.0	92.8 ± 2.0
23	Propoxyphenyl-thiodimethylsildenafil	82.8 ± 4.4	92.0 ± 5.8	90.5 ± 4.2	75.3 ± 1.8	90.0 ± 1.9	95.5 ± 2.5

CHAPTER 7

Fluorescence polarisation for high-throughput screening of adulterated food products via phosphodiesterase 5 (PDE5) inhibition assay

7.1 FOREWORD

The following manuscript, in Chapter 7, was accepted for publication in a special issue of "Non-targeted screening of drugs" in Drug Testing and Analysis (https://doi.org/10.1002/dta.2926). The article is currently available as an "Accepted Articles" (Accepted, unedited articles published online and citable). A liquid chromatography-high-resolution mass spectrometry, though accurate, precise, and reliable; demands higher operational and maintenance costs with limited sample throughput. It also requires trained and qualified operators to handle the analytical instrument and interpret the mass spectrometry data. Therefore, the quest for a simple, rapid, cheap, and preferably, a non-targeted screening test to detect phosphodiesterase 5 (PDE5) inhibitors and their analogues as adulterants, is highly coveted for routine casework. This chapter established an enzyme inhibition assay via a simple mix-incubate-read format to rapidly screen PDE5 inhibitors and their analogues found as adulterants in selected food products. The bioactivity-based screening assay utilised fluorescein-labelled cyclic-3',5'-guanosine monophosphate as substrates for the human recombinant PDE5A1 enzyme, aided by the presence of nanoparticle phosphate-binding beads on their fluorescence polarisation. The method was optimised, validated, and applied to screen 50 food samples that claimed to enhance male sexual performance. The results were then verified using

confirmatory liquid chromatography-high-resolution mass spectrometry analysis. The manuscript showcased the high-throughput potential of the PDE5 inhibition assay, which could be utilised into an automated screening procedure in the future. Mr Ahmad Yusri Mohd Yusop, Dr Linda Xiao, and Professor Shanlin Fu authored the manuscript. Mohd Yusop AY performed the experimental work, data analysis, and initial draft preparation including supplementary data with manuscript edits provided by Xiao L and Fu S. The article section, figure, table, and equation numbering was adjusted to align with the chronology of this thesis and may not reflect those published in the online version.

7.2 ABSTRACT

The surge in the consumption of food products containing herbal aphrodisiacs has driven their widespread adulteration. A rapid screening strategy is, therefore, warranted to curb this problem. This study established an enzyme inhibition assay to screen phosphodiesterase 5 (PDE5) inhibitors found as adulterants in selected food products. Fluorescein-labelled cyclic-3',5'-guanosine monophosphate was utilised as substrates for the human recombinant PDE5A1 enzyme, aided by the presence of nanoparticle phosphate-binding beads on their fluorescence polarisation. The sample preparation was optimised to improve the enzyme inhibition efficiency, and subsequently applied to calculate the threshold values of six blank food matrices to discriminate the adulterated food products. The assay was validated using sildenafil, producing an IC_{50} of 4.2 nM. The applicability of the assay procedure was demonstrated by screening 50 food samples that claimed to enhance male sexual performance. The results were subsequently verified using confirmatory liquid chromatography-high-resolution mass spectrometry (LC-HRMS) analysis. Altogether, 49 samples inhibited the PDE5 enzyme with percentage inhibition within 75.7%–105.5% and were registered as possibly adulterated samples, while one powdered drink mix sample was marked as non-adulterated. The LC-HRMS analysis agreed with the assay results for all food products except for the instant coffee premix (ICP) samples. False-positive results were obtained for the ICP samples (8/25 or 32%), due to possible PDE5 inhibition by caffeine in the sample matrix. The broad-based assay, established via a simple mix-incubate-read format, exhibited promising potential for high-throughput screening of PDE5 inhibitors in various

food products, except those with naturally-occurring phosphodiesterase inhibitors such as caffeine.

7.3 INTRODUCTION

The immense success of sildenafil, vardenafil, and tadalafil has since led to the massive influx of adulterated herbal remedies into the market, typically labelled to contain herbal aphrodisiacs with claims to enhance male sexual performance. These adulterated products are frequently marketed as herbal medicines and dietary supplements; and advertised as all-natural, without any side-effects [1,2]. However, in recent years, the trend has shifted towards food products as they are not heavily regulated compared to those in pharmaceutical dosage forms [3]. These food products can be easily purchased through drugstores, supermarkets, convenience stores, herbal shops, restaurants, electronic commerce platforms, and black markets [4]. Most of them, unfortunately, were found to be adulterated with phosphodiesterase 5 (PDE5) inhibitors and their analogues [5,6]. The widespread adulteration has sparked an elevated food safety and public health concerns, as consumers are often unaware of the risks associated with the consumption of such products [7].

PDE5 inhibitors are generally synthesised to mimic the structure of the purine ring of cyclic-3',5'-guanosine monophosphate (cGMP) [8]. Due to the structural similarities, these drugs competitively bind to the catalytic domain of PDE5 enzyme, subsequently inhibiting the cGMP degradation; thus, enhancing the effects of nitric oxide. The series of events sustain cGMP levels and prolong penile erection [9]. PDE5 enzyme, on the contrary, acts through a negative feedback control mechanism in the corpus cavernosum. It degrades cGMP to the inactive 5'-guanosine monophosphate (GMP), resulting in penile detumescence [10,11]. Based on the penile erection mechanism, the differences between cGMP

(substrate) and GMP (product) levels may indicate the presence or absence of PDE5 inhibitors.

At present, several analytical methods have been utilised to determine PDE5 inhibitors in various matrices [12-15]. More commonly, liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) has proven to be invaluable in the analysis of PDE5 inhibitors [16,17]. However, LC-MS/MS, in general, demands higher operational and maintenance costs with limited sample throughput. It also requires experienced users to operate the analytical instrument and interpret the MS data [18]. Therefore, a rapid, simple, and cheap screening test is warranted to discriminate adulterated from the non-adulterated food products, particularly those with PDE5 inhibitors and their analogues.

Only a few rapid screening tests have been proposed to discriminate PDE5 inhibitors in adulterated or counterfeit products based on colour changes [19] and immunochromatographic assay [20-22]. However, both of these techniques are targeted for a distinct PDE5 inhibitor or a group of PDE5 inhibitors, making them limited to broad-based screening. A study has recently proposed a broad-based screening of PDE5 inhibitors in herbal dietary supplements via the PDE5 inhibition assay [23]. The assay utilised fluorescence intensity measurements of tetramethyl rhodamine-labelled cGMP in the presence of zirconyl chloride octahydrate as a quenching agent. This study, however, was not validated using real samples and the need to measure the fluorescence intensity at seven points over a length of time may not be well-suited for high-throughput screening.

Herein, a broad-based enzyme inhibition assay was established via a simple mix-incubate-read format to rapidly screen PDE5 inhibitors, found as adulterants in selected food products. A PDE5-specific cGMP fused with a fluorescein fluorophore via a 9-atom spacer moiety, known as FAM-cGMP, was utilised as substrates for the human recombinant PDE5A1 enzyme. The substrate depletion and the product formation from the PDE5 enzyme activity were measured using their molecular movements and rotations, aided by the presence of nanoparticle phosphate-binding beads on their fluorescence polarisation (FP). The assay was optimised and validated to improve the enzyme inhibition efficiency and to certify the robustness of the assay performance, respectively. Altogether, 50 distinct food samples that claimed to enhance male sexual performance were submitted to the PDE5 inhibition assay, and the results were subsequently verified using confirmatory liquid chromatography-high-resolution mass spectrometry (LC-HRMS) analysis. To the best of our knowledge, this is the first study utilising the FP technique to rapidly screen PDE5 inhibitors as adulterants in food products via PDE5 inhibition assay. This paper also highlighted the advantages as well as the shortcomings encountered in detecting the adulterated food products.

7.4 MATERIALS AND METHODS

7.4.1 Chemicals and reagents

The PDE5A1 assay kit (Catalogue No. 60351) was purchased from BPS Bioscience Inc. (San Diego, CA, United States). It comprised the following: human recombinant PDE5A1 enzyme (PDE5 enzyme) 0.36 mg/mL (Catalogue No. 60050), fluorescein-labelled cGMP substrate (FAM-cGMP) 20 µM (Catalogue No. 60201), phosphodiesterase (PDE) assay buffer (Catalogue No. 60393), PDE binding agent (Catalogue No. 60390), PDE binding agent diluent for cGMP (Catalogue No. 60392), and Greiner 384-well microtiter plate (black, low binding, flat bottom) with a clear lid. The vendor for sildenafil certified reference material was TLC Pharmaceutical Standards Ltd. (Aurora, Ontario, Canada); while Sigma Aldrich Pty Ltd. (Castle Hill, NSW, Australia) supplied the dimethyl sulfoxide (DMSO) of analytical grade.

7.4.2 Standard solution preparation

The stock solution of sildenafil was prepared at 1 mM in DMSO and stored at 4°C in the dark. To validate the PDE5 inhibition assay, different concentration solutions of sildenafil ranging from 0.01 to 100 µM were prepared from the stock solution, serially diluted in DMSO. Each of these solutions was further diluted at 10-fold in PDE assay buffer before being submitted to the assay, producing a final 100-fold dilution of sildenafil in each microtiter plate well.

7.4.3 Sample collection and storage

A total of 50 distinct food samples were obtained from Malaysia (48 samples) and Australia (2 samples), in the form of instant coffee premix (ICP, 25 samples), powdered drink mix (PDM, 16 samples), honey (HNY, 4 samples), jelly (JLY, 2 samples), hard candy (HCD, 2 samples), and chewing gum (CWG, 1 sample). These suspected adulterated food products were selected based on the brand names, label claims, images, herbal ingredients, or advertising materials with connotations of male sexual performance. The Pharmacy Enforcement Division, Ministry of Health Malaysia, kindly donated two-thirds of these samples, which were confiscated by the pharmacy enforcement officers at the international airport (5 samples) and international seaport (10 samples), as well as from routine market surveillance activities (19 samples). The rest of the samples were purchased from various electronic commerce platforms established in Malaysia (14 samples) and Australia (2 samples). The samples were kept in separate plastic zip-lock bags and stored in an airtight container in the dark. Blank matrices of each food products, free from any analyte of interests, were sourced from a local supermarket and used to establish the threshold value of PDE5 inhibition for adulterated food products.

7.4.4 Sample preparation

The initial weight of each sample was recorded based on the recommended intake on its label. These samples were divided into group A (ICP, PDM, and HNY; with average recommended intake of >5 mg) and group B (JLY, HCD, and CWG; with average recommended intake of <5 mg). The samples in group A were taken directly from their sachets, while samples from group B were initially

homogenised with mortar and pestle. For PDE5 inhibition assay, 50 mg of group A samples or 10 mg of group B samples were weighed in a polypropylene tube and then extracted with 5 mL of DMSO via 1-min vortex mixing, 20-min sonication and 5-min centrifugation at 2500 × *g*, successively. Using a 0.22 mm PTFE syringe filter, the upper layer was filtered and diluted for enzyme inhibition assay with the PDE assay buffer at 10-fold dilution, yielding a final 100-fold dilution of samples in each microtiter plate well. The blank matrices were given the same treatment as the steps described above.

7.4.5 PDE5 inhibition assay protocol

Table 7.4.5 outlines the schematic three-step protocol of the PDE5 inhibition assay. The PDE5 inhibition assay established in this study was adapted and modified according to the manufacturer's instruction [24]. Initially, the stock solutions of the PDE5 enzyme and FAM-cGMP substrate were respectively diluted with PDE assay buffer to produce 10 pg/μL and 200 nM working solutions. The reagents, PDE5 inhibitors, and samples solutions were pipetted into each well of the microtiter plate according to step 1. Subsequently, each assay; comprised of blank, substrate control, positive control, and sample analysis; was covered with the microtiter plate's lid and incubated at room temperature for an hour. Step 2 involved the addition of 50 μL PDE binding agent into each well, initially diluted 100-fold with PDE binding agent diluent. The mixtures were covered with the microtiter plate's lid and incubated at room temperature for 20 min with slow shaking before submitting it to FP measurements via Tecan Infinite M1000 Pro plate reader (Tecan Group Limited, Switzerland) in step 3. The wavelength of the FP was set within 5 nm bandwidth for excitation at 470 nm,

and within 20 nm bandwidth for emission at 528 nm. The gain and Z-position values were automatically calculated from the positive control well. The calibration was performed from the substrate control well by correcting the G-factor to achieve a fixed value of 22 mP. The readings were captured at 10 flashes with a settling time of 500 ms. All measurements were done in triplicates, and the results obtained were automatically subtracted with the blank well readings.

Table 7.4.5: Schematic three-step protocol of the phosphodiesterase 5 (PDE5) inhibition assay.

Reagents	Blank	Substrate control	Positive control	Sample analysis
FAM-cGMP (200 nM)	-	12.5 µL	12.5 µL	12.5 µL
PDE assay buffer	22.5 µL	10.0 µL	-	-
PDE5 inhibitors/ samples	-	-	-	2.5 µL
10% DMSO in PDE assay buffer	2.5 µL	2.5 µL	2.5 µL	-
PDE5 enzyme (10 pg/ µL)	-	-	10.0 µL	10.0 µL
Step 1	Pipette into each microtiter plate well ↓ ↓ ↓ ↓ Total reagents in each microtiter plate well (25 µL) ↓ Incubate at room temperature for an hour			
Step 2	↓ Pipette 50.0 µL diluted binding agent into each microtiter plate well ↓ Incubate at room temperature for 20 min with slow shaking			
Step 3	↓ Measure the fluorescent polarisation (excitation at 470 ± 5 nm and emission 528 ± 20 nm) ↓ Calculate the percentage of inhibition			

Adapted and modified from BPS Bioscience Inc. [24]. (Abbreviations: FAM-cGMP, fluorescein-labelled cyclic-3',5'-guanosine monophosphate; PDE, phosphodiesterase; DMSO, dimethyl sulfoxide)

7.4.6 LC-HRMS analysis

The confirmatory LC-HRMS analysis was employed to verify the findings of the PDE5 inhibition assay using Agilent Technologies (Santa Clara, CA, USA) 1290 Infinity II LC system coupled to an Agilent Technologies 6510 QTOF-MS according to the previous literature [25,26].

7.4.7 Data analysis

The Tecan i-control software version 1.11.1.0 automatically calculated all the FP values. The differences between the parallel and the perpendicular emission light intensities, normalised by the total fluorescence emission intensity of the excitation light plane, generated the absolute FP value based on Eq. 7.4.7A and represented in millipolarisation (mP) unit [27].

$$FP\ (mP) = \frac{\mathrm{I}_{\parallel} - \mathrm{I}_{\perp}}{\mathrm{I}_{\parallel} + \mathrm{I}_{\perp}} \times 1000 \qquad \text{(Eq. 7.4.7A)}$$

where:
$\mathrm{I}_{\parallel}$ = parallel emission light intensities
$\mathrm{I}_{\perp}$ = perpendicular emission light intensities

The substrate control and the positive control theoretically produced 0% and 100% enzyme-substrate activity, respectively, and designated as FP_{SUB} and FP_{POS} each. Therefore, the percentage of PDE5 enzyme activity of a given sample or PDE5 inhibitor (FP_{SPL}) can be determined using Eq. 7.4.7B, while the percentage of PDE5 inhibition was calculated based on Eq. 7.4.7C. The threshold value of PDE5 inhibition ($T_{inhibition}$) was calculated via the 99.7% normal distribution rule for each food products using Eq. 7.4.7D. All of these values were calculated using Microsoft (Redmond, WA, USA) Excel 2016 (Microsoft Office).

$$\%\ of\ PDE5\ activity = \frac{FP_{SPL} - FP_{SUB}}{FP_{POS} - FP_{SUB}} \times 100\% \quad \text{(Eq. 7.4.7B)}$$

$$\%\ of\ PDE5\ inhibition = 100 - \%\ of\ PDE5\ activity \quad \text{(Eq. 7.4.7C)}$$

$$T_{inhibition} = \mu + 3\sigma \quad \text{(Eq. 7.4.7D)}$$

where:
μ = average % of PDE5 inhibition
σ = standard deviation

The calculated percentages of PDE5 activity of sildenafil versus their concentrations were plotted into a concentration-response inhibition curve using Prism GraphPad software version 8.0.1 by GraphPad Software Inc. (San Diego, CA, United States), and then fitted into a non-linear regression model of $\log_{10}$ (inhibitor) versus response (variable slope, four parameters) in Eq. 7.4.7E. The non-linear regression data transformation automatically generated the half-maximal inhibitory concentration (IC_{50}) of sildenafil via the symmetrical sigmoidal curve model.

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{(Log\ IC_{50} - X) \times Hill\ Slope}} \quad \text{(Eq. 7.4.7E)}$$

where:
X = $\log_{10}$ [inhibitor] concentration
Y = % of PDE5 activity
Top = maximum % of PDE5 activity
Bottom = minimum % of PDE5 activity

7.5 RESULTS AND DISCUSSION

7.5.1 PDE5 inhibition assay scheme

The high-throughput screening of PDE5 inhibitors in food products was established via the PDE5 inhibition assay. This bioactivity-based assay utilises an FP technique to screen PDE5 inhibitors such as sildenafil (Fig. 7.5.1A (a)), by competing with FAM-cGMP to bind to the catalytic domain of PDE5 enzyme (Fig. 7.5.1A (b)) [8,10]. Therefore, the assay provides a broad-based screening for multiple PDE5 inhibitors that is non-targeted for a distinct inhibitor or a group of inhibitors, which is helpful to tackle the proliferation of novel analogues, deliberately added into various food products. The assay utilises a PDE5-specific cGMP substrate fused with a fluorescein fluorophore via a 9-atom spacer moiety, known as FAM-cGMP (Fig. 7.5.1A (c)). The cGMP plays a pivotal role in the mechanism of penile erection [28].

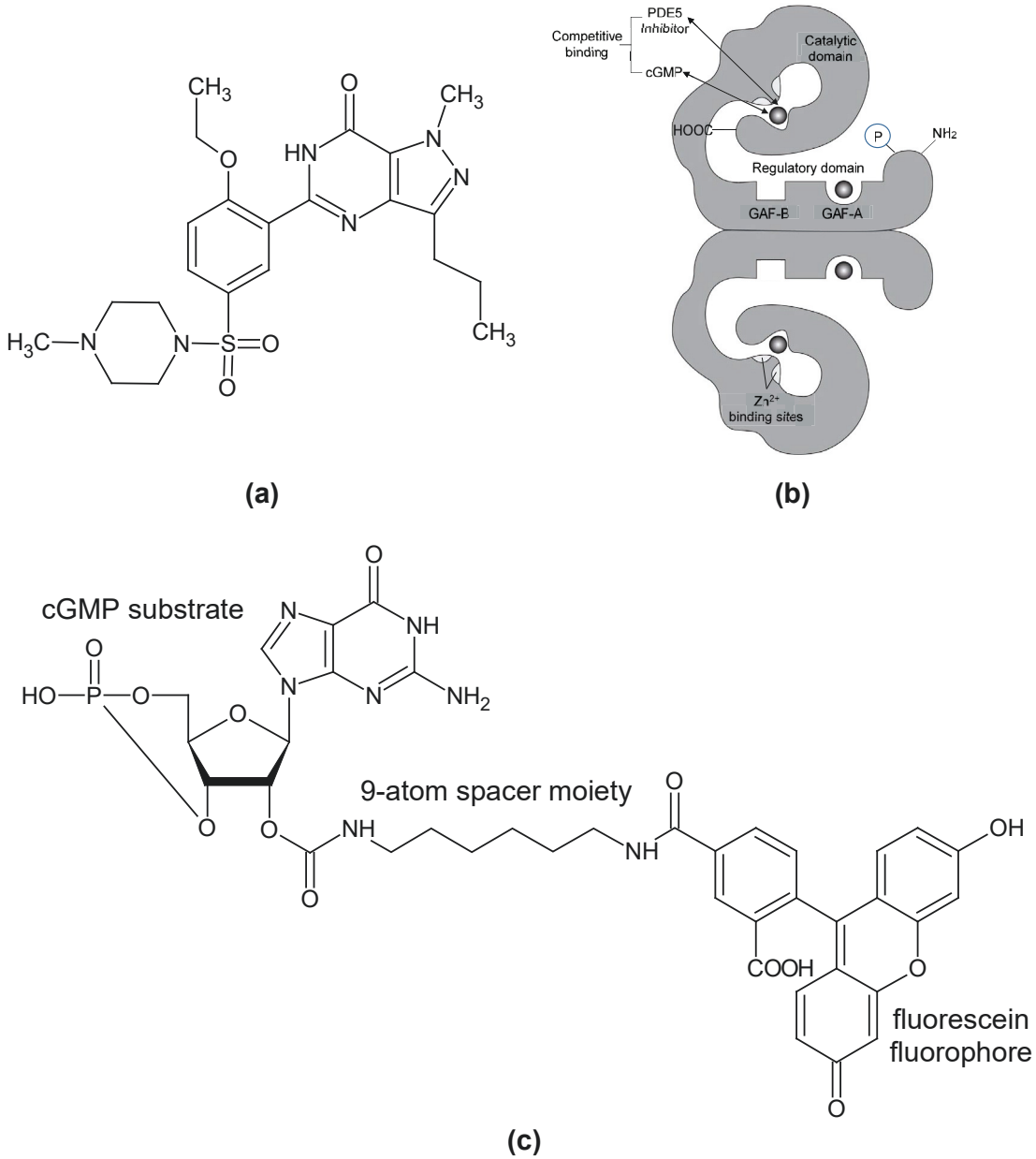


Fig. 7.5.1A: The structure of (a) sildenafil; (b) phosphodiesterase 5 (PDE5) enzyme; and (c) fluorescein-labelled cyclic-3',5'-guanosine monophosphate (FAM-cGMP) substrate.

Fig. 7.5.1B presents the schematic illustration of the PDE5 inhibition assay using FP technique. The enzymatic reaction initiation by the PDE5 enzyme, hydrolysed the phosphodiester bond of FAM-cGMP (substrate) to produce the inactive fluorescein-labelled 5'-guanosine monophosphate (FAM-GMP) (product) over a length of time. Once the incubation period ended, a PDE binding agent composed of nanoparticle beads is added to the assay to selectively bind the phosphate group of the FAM-GMP, consequently increasing its size. As a result, the FAM-cGMP and the FAM-GMP are distinguishable using FP based on the differences in their molecular weight. The low molecular weight FAM-cGMP (small, unbound molecule) produces a rapid rotational movement when excited with polarised light, generating low FP readings via depolarised light emission. Contrarily, the high molecular weight FAM-GMP-bead complex (large, bound molecule) rotates slowly during excitation with polarised light, continuing its polarisation with high FP readings.

The adulteration of food products with PDE5 inhibitors can initially be suspected with low FP readings as their presence blocks the hydrolysis of FAM-cGMP to FAM-GMP. However, to undoubtedly discriminate adulterated from the non-adulterated food products, these FP readings are transformed into the percentage of PDE5 inhibition and then compared with the threshold values obtained for each blank food matrix. The established PDE5 inhibition assay via FP is based on a simple and automation-friendly [29] mix-incubate-read format to screen PDE5 inhibitors in food products. Therefore, the demand for multiple readings over a length of time to monitor the enzymatic reaction progress was eliminated, and thus fitted the assay for high-throughput screening.

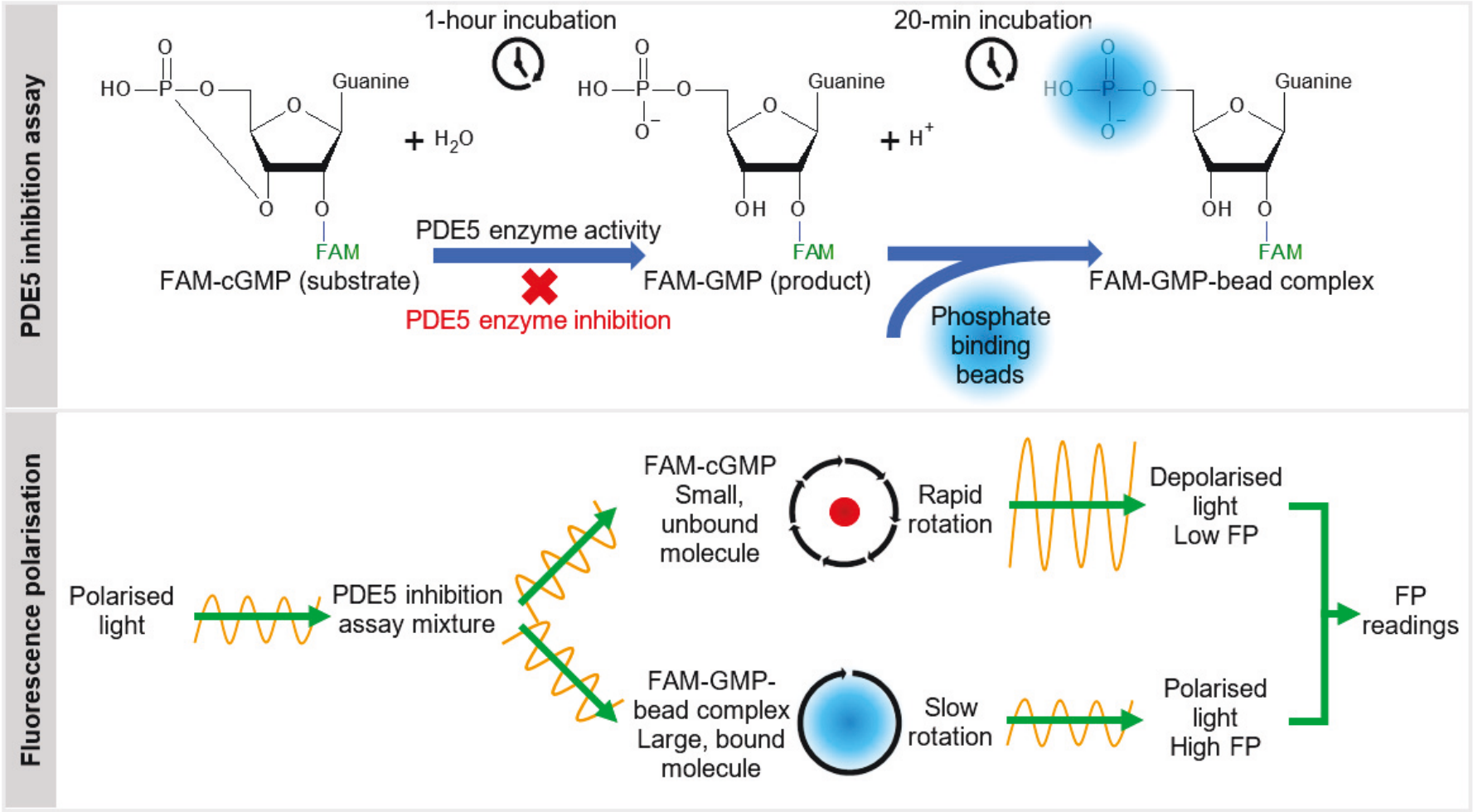


Fig. 7.5.1B: Phosphodiesterase 5 (PDE5) inhibition assay scheme using fluorescence polarisation (FP) technique. Adapted and modified from BPS Bioscience Inc. [24]. (Abbreviations: FAM-cGMP, fluorescein-labelled cyclic-3',5'-guanosine monophosphate; FAM-GMP, fluorescein-labelled 5'-guanosine monophosphate)

7.5.2 Optimisation of sample preparation

The quality of sample preparation is one of the pivotal factors in determining the success of an enzyme inhibition assay. Therefore, it is crucial to prepare the samples into a form that is compatible with the assay procedure. Food products that claimed to enhance male sexual performance may contain an array of PDE5 inhibitors with diverse chemical structures, often exhibiting different inhibitory effects depending on their concentrations. Besides, the information on the potency of almost all unapproved PDE5 inhibitor analogues remains scarce in the literature. Due to these ambiguities, the sample preparation procedure in this study was established based on sildenafil as a representative adulterant, within its recommended dosage of 25–100 mg.

The weight of different types of food products was initially assessed to determine the appropriate ratio of the sample and the adulterant (sildenafil) to produce optimal PDE5 inhibition. The sample weight was then fixed based on these findings for a specific group of food products as detailed in Section 7.4.4. Ideally, the selected sample weight should produce an acceptable sensitivity via inhibitory potency at the lowest level of adulterant, avoiding false-negative results, while, at the same time, preventing oversaturation of the enzyme at the highest adulterant's level. The effect of interferences, particularly from the matrix components of the food products, should also be ascertained to ensure reliable assay performance; thus, avoiding false-positive results.

A threshold value of PDE5 inhibition was established for each blank matrices of the food products to discriminate adulterated from the non-adulterated samples. The threshold value used in this study represents the percentage of PDE5 inhibition at which the likelihood of obtaining a false-positive result from a blank, non-adulterated food product is <0.3% using the 99.7% normal distribution rule. Adulterated food samples are qualified by their average PDE5 inhibition above the threshold value, while those below the threshold are categorised as non-adulterated samples. Each of the obtained threshold values was respectively assigned for a specific food product, as displayed in Fig. 7.5.2.

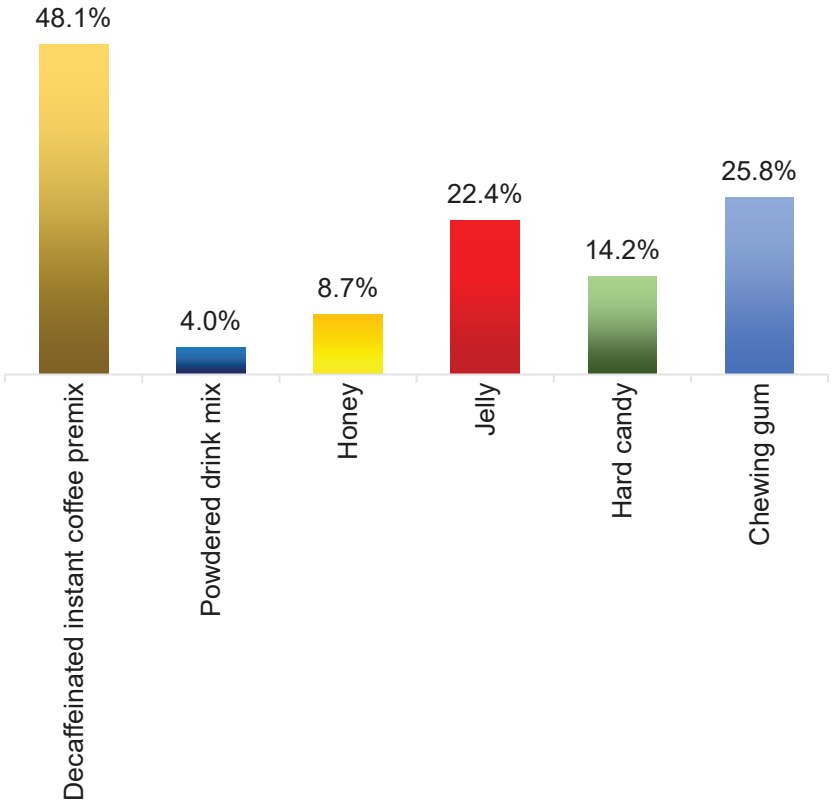


Fig. 7.5.2: Threshold values for each blank matrices of the food product.

The threshold values revealed low PDE5 inhibition within 4.0%–25.8% for all blank matrices of the food products, as expected from any non-adulterated sample, except for the blank ICP. The blank ICP produced an average of 91.0% PDE5 inhibition with a calculated threshold value of 94.3%. The selected blank matrix of the ICP was pre-determined by LC-HRMS analysis to be free from any PDE5 inhibitors and was expected to produce a low PDE5 inhibition. Here, the matrix components may play a significant role in the observed outcome. Given the ambiguities of the ICP sample matrix, which could comprise of either caffeinated or decaffeinated instant coffee, a decaffeinated blank ICP was also tested, yielding a calculated threshold value of 48.1%, to be used to qualify adulterated ICP samples.

7.5.3 Assay validation

The concentrations of enzyme and substrate are fixed at 4 pg/μL and 100 nM, respectively, for each reaction, based on the manufacturer's recommendation. The specific activity of the human recombinant PDE5A1 enzyme is 3100 U/μg, where 1 U represents the amount of enzyme that converts a picomole of cGMP to GMP per min. The specific activity assay exhibited a linear relationship between the PDE5 enzyme concentration and its activity based on the detection of GMP using a malachite green reagent. The molecular weight and purity of the PDE5 enzyme were determined using 4%–20% SDS-PAGE, visualised using Coomassie staining [30].

Apart from monitoring its biological and pharmacological relevance, the PDE5 inhibition assay is validated to certify the robustness of the assay performance. The validation also served to ensure that all the reagents supplied are working as described by its manufacturer. Therefore, an established PDE5 inhibitor, i.e. sildenafil, was chosen, serving as a reference for the enzyme inhibition. Furthermore, sildenafil is the most frequently detected adulterant, reported in many countries worldwide [25,31-33].

Fig. 7.5.3 displays the concentration-response plot of sildenafil using the PDE5 inhibition assay. The sigmoidal curve shows that sildenafil inhibits the PDE5 activity down to a minimum level where the response remained unchanged. The curve conformed to a classic symmetrical sigmoidal shape, as typically observed from any concentration-response plot of an enzyme inhibitor [34,35]. The obtained data fitted well to the regression model in Eq. 7.4.7E with a coefficient of determination (R^2) of 0.9915. Sildenafil inhibits the PDE5 enzyme with an IC_{50} of 4.2 nM. The IC_{50} value is the concentration of an inhibitor required to reduce the enzyme activity by 50%, typically attributed to the potency of an inhibitor [36]. The IC_{50} value of sildenafil obtained from the PDE5 inhibition assay is comparable to those reported from previous studies ranging from 3.5 to 6.6 nM [37-41].

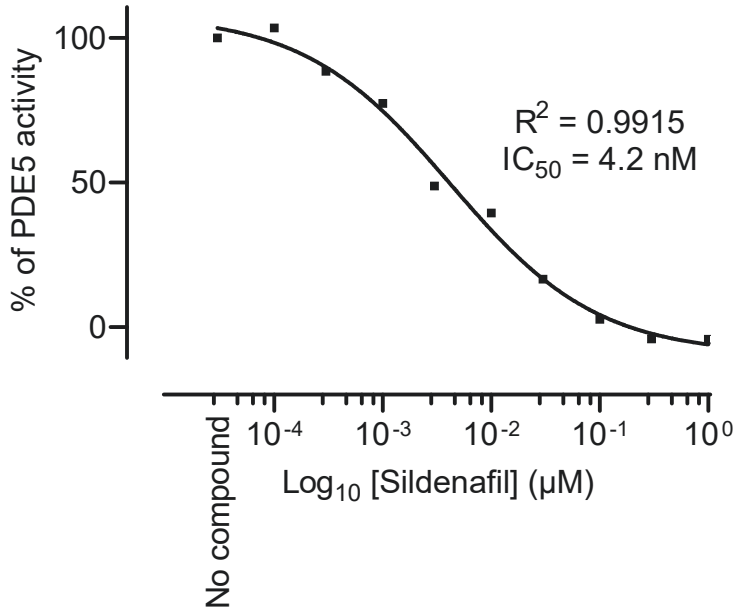


Fig. 7.5.3: The concentration-response plot of sildenafil. (Abbreviations: PDE5, phosphodiesterase 5; R^2, coefficient of determination; IC_{50}, half-maximal inhibitory concentration; Log_{10}, logarithm with base 10)

Nevertheless, the published IC_{50} values of sildenafil may vary significantly, depending on the source and purity of the PDE5 enzyme; the type and concentration of substrate; as well as the selected assay procedure [36,42]. These findings served to validate the overall assay performance, including the recommended concentrations of the PDE5 enzyme and its substrate. The enzyme reaction scheme thus complies with the Michaelis-Menten kinetic model [43].

7.5.4 Analysis of PDE5 inhibitors in food products

Altogether, the 50 distinct food samples were screened using the PDE5 inhibition assay and subsequently verified via the confirmatory LC-HRMS analysis. The primary goal of the enzyme inhibition assay is to rapidly discriminate adulterated from the non-adulterated food products through the presence of PDE5 inhibitors.

Table 7.5.4 summarises the results of the PDE5 inhibition assay, including the confirmatory LC-HRMS analysis. Collectively, 49 samples inhibited the PDE5 enzyme with percentage inhibition within 75.7%–105.5%, significantly above the threshold value, and were registered as possibly adulterated samples. In contrast, only one PDM sample was marked as non-adulterated due to the negative PDE5 inhibition at -3.3%, notably below the threshold value. Altogether, 17 samples produced average PDE5 inhibitions exceeding 100%, which may have been contributed by the high concentrations of PDE5 inhibitors in the sample solutions. In these circumstances, the enzyme may have reached a saturation point within the one hour incubation period where no more inhibitors could bind to the catalytic domain of the PDE5 enzyme [43]. As the PDE5 inhibition assay is based on a simple mix-incubate-read format, the need to monitor the enzymatic reaction progress over a length of time was eliminated, making the saturation point insignificant to the assay outcomes.

Table 7.5.4: Phosphodiesterase 5 (PDE5) inhibition assay results and confirmatory liquid chromatography-high-resolution mass spectrometry (LC-HRMS) analysis of suspected adulterated food samples.

Sample (food product)	Average percentage of PDE5 inhibition - outcome	PDE5 inhibitors detected from the confirmatory LC-HRMS analysis	Conclusion
SPL001 (ICP)[a]	99.7% - adulterated	Desmethylcarbodenafil	True-positive
SPL002 (ICP)[a]	94.2% - adulterated	Thiosildenafil, hydroxythiohomosildenafil*	True-positive
SPL003 (ICP)[a]	91.6% - adulterated	Dimethylsildenafil, propoxyphenyl-thiodimethylsildenafil, thiodimethylsildenafil	True-positive
SPL004 (ICP)[a]	102.6% - adulterated	Sildenafil, tadalafil	True-positive
SPL005 (ICP)[a]	92.9% - adulterated	3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil*	True-positive
SPL006 (ICP)[a]	91.9% - adulterated	Hydroxythiohomosildenafil*	True-positive
SPL007 (ICP)[a]	100.0% - adulterated	Sildenafil	True-positive
SPL008 (ICP)[a]	88.1% - adulterated	Not detected	False-positive
SPL009 (ICP)[a]	90.3% - adulterated	Not detected	False-positive
SPL010 (ICP)[a]	87.7% - adulterated	Not detected	False-positive
SPL011 (ICP)[a]	92.6% - adulterated	Not detected	False-positive
SPL012 (ICP)[a]	100.6% - adulterated	Sildenafil	True-positive
SPL013 (ICP)[a]	96.8% - adulterated	Sildenafil	True-positive
SPL014 (ICP)[a]	91.0% - adulterated	Not detected	False-positive
SPL015 (ICP)[a]	101.0% - adulterated	Sildenafil, dimethylsildenafil, thiosildenafil, thiodimethylsildenafil	True-positive
SPL016 (ICP)[a]	85.5% - adulterated	Not detected	False-positive
SPL017 (ICP)[a]	93.9% - adulterated	Desmethylcarbodenafil	True-positive

SPL018 (ICP)[a]	92.6% - adulterated	Not detected	False-positive
SPL019 (ICP)[a]	101.3% - adulterated	Dimethylsildenafil, thiosildenafil, thiodimethylsildenafil	True-positive
SPL020 (ICP)[a]	101.3% - adulterated	Sildenafil, tadalafil, propoxyphenyl-sildenafil	True-positive
SPL021 (ICP)[a]	98.4% - adulterated	Tadalafil	True-positive
SPL022 (ICP)[a]	99.4% - adulterated	Sildenafil	True-positive
SPL023 (ICP)[a]	83.9% - adulterated	Not detected	False-positive
SPL024 (ICP)[a]	98.7% - adulterated	Sildenafil, dimethylsildenafil, thiosildenafil, thiodimethylsildenafil	True-positive
SPL025 (ICP)[a]	101.3% - adulterated	Sildenafil, dimethylsildenafil, thiosildenafil, thiodimethylsildenafil	True-positive
SPL026 (PDM)	105.5% - adulterated	Sildenafil, tadalafil	True-positive
SPL027 (PDM)	82.9% - adulterated	Propoxyphenyl-thiohydroxyhomosildenafil	True-positive
SPL028 (PDM)	102.8% - adulterated	Tadalafil, thiosildenafil, thiodimethylsildenafil	True-positive
SPL029 (PDM)	102.8% - adulterated	Tadalafil	True-positive
SPL030 (PDM)	103.9% - adulterated	Tadalafil, thiosildenafil	True-positive
SPL031 (PDM)	89.5% - adulterated	Thiosildenafil, thiodimethylsildenafil, propoxyphenyl-thiohydroxyhomosildenafil	True-positive
SPL032 (PDM)	98.9% - adulterated	Sildenafil, dimethylsildenafil, thiosildenafil, thiodimethylsildenafil	True-positive
SPL033 (PDM)	96.1% - adulterated	Tadalafil	True-positive
SPL034 (PDM)	-3.3% - non-adulterated	Not detected	True-negative
SPL035 (PDM)	101.7% - adulterated	Sildenafil, tadalafil, dimethylsildenafil, thiosildenafil, thiodimethylsildenafil	True-positive

SPL036 (PDM)	94.5% - adulterated	Tadalafil, thiosildenafil, thiodimethylsildenafil	True-positive
SPL037 (PDM)	102.2% - adulterated	Sildenafil, tadalafil, dimethylsildenafil, thiosildenafil, thiodimethylsildenafil	True-positive
SPL038 (PDM)	102.2% - adulterated	Sildenafil, tadalafil	True-positive
SPL039 (PDM)	103.3% - adulterated	Sildenafil, tadalafil	True-positive
SPL040 (PDM)	100.6% - adulterated	Dimethylsildenafil, thiodimethylsildenafil	True-positive
SPL041 (PDM)	103.3% - adulterated	Tadalafil	True-positive
SPL042 (HNY)	91.2% - adulterated	Sildenafil, thiosildenafil	True-positive
SPL043 (HNY)	95.6% - adulterated	Sildenafil, thiosildenafil	True-positive
SPL044 (HNY)	75.7% - adulterated	Tadalafil	True-positive
SPL045 (HNY)	92.3% - adulterated	Dimethylsildenafil, thiodimethylsildenafil, propoxyphenyl-thiodimethylsildenafil, propoxyphenyl-dimethylsildenafil*	True-positive
SPL046 (JLY)	91.7% - adulterated	Vardenafil	True-positive
SPL047 (JLY)	100.6% - adulterated	Sildenafil	True-positive
SPL048 (HCD)	99.4% - adulterated	Tadalafil	True-positive
SPL049 (HCD)	85.1% - adulterated	Nortadalafil*	True-positive
SPL050 (CWG)	77.3% - adulterated	Sildenafil, thiosildenafil	True-positive

(Abbreviations: ICP, instant coffee premix; PDM, powdered drink mix; HNY, honey; JLY, jelly; HCD, hard candy; CWG, chewing gum)

*suspected-target analytes

[a]LC-HRMS data published in Mohd Yusop et al. [25]

The PDE5 inhibition assay results, however, were not in full agreement with the confirmatory LC-HRMS analysis, particularly those of the ICP samples which were reported previously [25]. The LC-HRMS analysis resulted in 41 positive samples, with nine distinct PDE5 inhibitors identified via targeted analysis, while another four inhibitors detected by suspected-target screening. Sildenafil again dominated the top list of PDE5 inhibitors found as adulterants in male sexual performance enhancement products, as previously mentioned in Section 7.5.3. It was identified as a sole adulterant in 5 samples and also found in combination with other PDE5 inhibitors in 14 samples. Its popularity is often linked to the accessibility and low cost of raw materials to obtain or synthesise sildenafil [44].

Other PDE5 inhibitors identified via the LC-HRMS targeted analysis included: tadalafil (16 samples); thiodimethylsildenafil and thiosildenafil (13 samples each); dimethylsildenafil (10 samples); desmethylcarbodenafil, propoxyphenyl-thiodimethylsildenafil, and propoxyphenyl-thiohydroxyhomosildenafil (2 samples each); and vardenafil (1 sample). The LC-HRMS analysis additionally detected hydroxythiohomosildenafil (2 samples); and 3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil, nortadalafil, and propoxyphenyl-dimethylsildenafil (1 sample each) via the suspected-target screening. In addition to the one adulterant per sample composition, these adulterants were also found in combination with each other, where each adulterated sample contains as many as five distinct PDE5 inhibitors.

The confirmatory LC-HRMS analysis verified the findings of the PDE5 inhibition assay for all food products, concluded as true-positive and true-negative, except for the ICP samples. The blank ICP had previously produced significant PDE5 inhibition during the establishment of the threshold value in Section 7.5.2. Coincidently, the decaffeinated blank ICP also moderately inhibits the PDE5 enzyme. Both of these blank ICPs were pre-determined by LC-HRMS analysis to be free from any PDE5 inhibitors. Matrix components of the ICPs may, therefore, play a significant role in inhibiting the PDE5 enzyme, which resulted in false-positive outcomes of 8 ICP samples. Zero false-negative results indicated an acceptable sensitivity of the established assay procedure for non-targeted screening.

Coffee is well known to contain caffeine, a central nervous system stimulant, making it the most widely consumed psychoactive substance worldwide [45,46]. Caffeine possesses a chemical structure similar to those of the purine ring of cGMP and cyclic-3',5'-adenosine monophosphate (cAMP). The heterocyclic ring structure of caffeine is also comparable to those of the pyrazolopyrimidine-7-one ring of sildenafil and imidazotriazine-4-one ring of vardenafil. Due to the structural similarities, caffeine may be expected to exhibit the same inhibitory effects as the PDE5 inhibitors. In fact, caffeine is one of the earliest PDE inhibitors, discovered through the bronchodilating effects of coffee [47]. These initial findings suggest that caffeine may act as a non-selective PDE inhibitor, demonstrated by its inhibitory effects on diverse PDE families [48].

The literature about the role of caffeine on selective inhibition of the PDE5 enzyme in human, however, remained scarce. Nonetheless, a couple of studies have demonstrated the up-regulation of cGMP by caffeine, through relaxation of the penile erectile tissue of rabbits [49] and rats [50]. A recent study via computational approach had also predicted the PDE5 inhibition potential of caffeine [51]. Furthermore, data from the National Health and Nutrition Examination Survey from the United States' male respondents revealed a lower incidence of erectile dysfunction with increased caffeine consumptions [52,53]. All of these findings, although via limited evidence, suggest the existence of PDE5 inhibition by caffeine.

Coffee is, therefore, not a suitable matrix for the PDE5 inhibition assay. Other naturally-occurring non-selective PDE inhibitors such as theophylline from tea and theobromine from cocoa [8] may exhibit similar findings. Thus, these kinds of food products should be analysed with caution or excluded altogether from the PDE5 inhibition assay. It is also worth noting that the adulteration of food products with caffeine is currently on the rise [32]. Accordingly, producing definite evidence of food adulteration would be a challenging task for samples with naturally-occurring PDE inhibitors.

7.6 CONCLUSION

A PDE5 inhibition assay was optimised and validated for high-throughput screening of PDE5 inhibitors, found as adulterants in food products. The three-step assay protocol, recorded via FP measurements, relied on a simple mix-incubate-read format, that is automation-friendly. Data interpretation is straightforward, discriminating adulterated food samples based on their PDE5 inhibition above the pre-determined threshold value. Altogether, the 50 distinct food samples, preliminarily screened via the PDE5 inhibition assay, registered 49 possibly adulterated samples, while the remaining 1 sample was marked as non-adulterated. The assay results were then verified using the LC-HRMS via targeted analysis, as well as suspected-target and non-targeted screenings. The confirmatory LC-HRMS analysis was in agreement with the PDE5 inhibition assay results for all food products except for the ICP samples. These findings indicated false-positive results from 8 ICP samples (out of 25 ICP samples in total, or 32%), possibly due to the PDE5 inhibition activity of caffeine present in the sample matrix. The established assay procedure is, therefore, not suitable for certain types of food products such as ICP and those with the presence of naturally-occurring PDE inhibitors. The PDE5 inhibition assay nevertheless has shown promising potential to rapidly screen PDE5 inhibitors as adulterants in other types of food products. A two-tier screening strategy via rapid and confirmatory tests would enhance performance and productivity, where the adulterated samples from the PDE5 inhibition assay can be credibly marked as priority for confirmatory analysis.

7.7 REFERENCES

1. Marcus DM. Dietary supplements: What's in a name? What's in the bottle? *Drug Test Anal* 2016;8(3-4):410-412. doi:https://doi.org/10.1002/dta.1855
2. Skalicka-Wozniak K, Georgiev MI, Orhan IE. Adulteration of herbal sexual enhancers and slimmers: the wish for better sexual well-being and perfect body can be risky. *Food Chem Toxicol* 2017;108:355-364. doi:https://doi.org/10.1016/j.fct.2016.06.018
3. da Justa Neves DB, Caldas ED. Dietary supplements: international legal framework and adulteration profiles, and characteristics of products on the Brazilian clandestine market. *Regul Toxicol Pharmacol* 2015;73(1):93-104. doi:https://doi.org/10.1016/j.yrtph.2015.06.013
4. Gilard V, Balayssac S, Tinaugus A, Martins N, Martino R, Malet-Martino M. Detection, identification and quantification by ^{1}H NMR of adulterants in 150 herbal dietary supplements marketed for improving sexual performance. *J Pharm Biomed Anal* 2015;102:476-493. doi:https://doi.org/10.1016/j.jpba.2014.10.011
5. Haneef J, Shaharyar M, Husain A, et al. Analytical methods for the detection of undeclared synthetic drugs in traditional herbal medicines as adulterants. *Drug Test Anal* 2013;5(8):607-613. doi:https://doi.org/10.1002/dta.1482
6. Fibigr J, Šatínský D, Solich P. Current trends in the analysis and quality control of food supplements based on plant extracts. *Anal Chim Acta* 2018;1036:1-15. doi:https://doi.org/10.1016/j.aca.2018.08.017

7. Rocha T, Amaral JS, Oliveira MBPP. Adulteration of dietary supplements by the illegal addition of synthetic drugs: a review. *Compr Rev Food Sci Food Saf* 2016;15(1):43-62. doi:https://doi.org/10.1111/1541-4337.12173

8. Francis SH, Sekhar KR, Ke H, Corbin JD. Inhibition of cyclic nucleotide phosphodiesterases by methylxanthines and related compounds. In: *Methylxanthines.* Berlin: Springer; 2011:93-133.

9. Huang SA, Lie JD. Phosphodiesterase-5 (PDE_5) inhibitors in the management of erectile dysfunction. *Pharm Ther* 2013;38(7):407-419.

10. Francis SH, Blount MA, Corbin JD. Mammalian cyclic nucleotide phosphodiesterases: molecular mechanisms and physiological functions. *Physiol Rev* 2011;91(2):651-690. doi:https://doi.org/10.1152/physrev.00030.2010

11. Peak TC, Yafi FA, Sangkum P, Hellstrom WJ. Emerging drugs for the treatment of erectile dysfunction. *Expert Opin Emerg Drugs* 2015;20(2):263-275. doi:https://doi.org/10.1517/14728214.2015.1021682

12. Cai Y, Cai T-G, Shi Y, et al. Simultaneous determination of eight PDE5-Is potentially adulterated in herbal dietary supplements with TLC and HPLC-PDA-MS methods. *J Liq Chromatogr Relat Technol* 2010;33(13):1287-1306. doi:https://doi.org/10.1080/10826076.2010.488979

13. Man CN, Noor NM, Lajis R. Identification of thioketone analogues of sildenafil using gas chromatography-mass spectrometry. *J Chromatogr A* 2011;1218(39):7055-7060. doi:https://doi.org/10.1016/j.chroma.2011.08.037

14. Mustazza C, Borioni A, Rodomonte AL, et al. Characterization of sildenafil analogs by MS/MS and NMR: a guidance for detection and structure elucidation of phosphodiesterase-5 inhibitors. *J Pharm Biomed Anal* 2014;96:170-186. doi:https://doi.org/10.1016/j.jpba.2014.03.038

15. Hu X, Fang G, Han A, Fu Y, Tong R, Wang S. Rapid detection of six phosphodiesterase type 5 enzyme inhibitors in healthcare products using thin-layer chromatography and surface enhanced Raman spectroscopy combined with BP neural network. *J Sep Sci* 2017;40(11):2506-2514. doi:https://doi.org/10.1002/jssc.201700024

16. Kim EH, Seo HS, Ki NY, et al. Reliable screening and confirmation of 156 multi-class illegal adulterants in dietary supplements based on extracted common ion chromatograms by ultra-high-performance liquid chromatography-quadrupole/time of flight-mass spectrometry. *J Chromatogr A* 2017;1491:43-56. doi:https://doi.org/10.1016/j.chroma.2017.02.032

17. Jang I, Lee J-u, Lee J-m, et al. LC-MS/MS software for screening unknown erectile dysfunction drugs and analogues: artificial neural network classification, peak-count scoring, simple similarity search, and hybrid similarity search algorithms. *Anal Chem* 2019;91(14):9119-9128. doi:https://doi.org/10.1021/acs.analchem.9b01643

18. Corenblum B, Boyd J. Chapter 8 - Endocrinology and disorders of the reproductive system. In: Sadrzadeh H, Kline G, eds. *Endocrine Biomarkers.* Amsterdam: Elsevier; 2017:351-397.

19. Awofisayo OS, Awofisayo OA, Eyen N. Development of basic tests for sildenafil citrate and sildenafil citrate tablet. *J Clin Med Res* 2010;2(11):175-179.

20. Guo J-B, Xu Y, Huang Z-B, He Q-H, Liu S-W. Development of an immunoassay for rapid screening of vardenafil and its potential analogues in herbal products based on a group specific monoclonal antibody. *Anal Chim Acta* 2010;658(2):197-203. doi:https://doi.org/10.1016/j.aca.2009.11.021

21. Guo J, Liu W, Lan X, Chen H, Xiao Z. Development and evaluation of an immunochromatographic strip for rapid screening of sildenafil-type compounds as illegal additives in functional foods. *Food Addit Contam Part A* 2016;33(7):1095-1104. doi:https://doi.org/10.1080/19440049.2016.1203072

22. He F, Zou T, Yang J, et al. Development of a skeleton-specific antibody and Au nanoparticle-based immunochromatographic sensor for simultaneous detection of various tadalafil adulterants in health food. *Food Agr Immunol* 2019;30(1):349-368. doi:https://doi.org/10.1080/09540105.2019.1585417

23. Santillo MF, Mapa MST. Phosphodiesterase (PDE5) inhibition assay for rapid detection of erectile dysfunction drugs and analogs in sexual enhancement products. *Drug Test Anal* 2018;10(8):1315-1322. doi:https://doi.org/10.1002/dta.2375

24. BPS Bioscience Inc. Data sheet: PDE5A1 assay kit (Catalogue No. 60351) 2020. https://bpsbioscience.com/pub/media/wysiwyg/PDEs/ASSAY_KIT_UPDATE/60351.pdf. Accessed 26 February 2020.

25. Mohd Yusop AY, Xiao L, Fu S. Determination of phosphodiesterase 5 (PDE5) inhibitors in instant coffee premixes using liquid chromatography-high-resolution mass spectrometry (LC-HRMS). *Talanta* 2019;204:36-43. doi:https://doi.org/10.1016/j.talanta.2019.05.078

26. Mohd Yusop AY, Xiao L, Fu S. Data on the optimisation and validation of a liquid chromatography-high-resolution mass spectrometry (LC-HRMS) to establish the presence of phosphodiesterase 5 (PDE5) inhibitors in instant coffee premixes. *Data Brief* 2019;25:104234. doi:https://doi.org/10.1016/j.dib.2019.104234

27. Hall MD, Yasgar A, Peryea T, et al. Fluorescence polarization assays in high-throughput screening and drug discovery: a review. *Methods Appl Fluoresc* 2016;4(2):022001. doi:https://doi.org/10.1088/2050-6120/4/2/022001

28. Corbin JD. Mechanisms of action of PDE5 inhibition in erectile dysfunction. *Int J Impot Res* 2004;16(S1):S4-S7. doi:https://doi.org/10.1038/sj.ijir.3901205

29. Ma H, Deacon S, Horiuchi K. The challenge of selecting protein kinase assays for lead discovery optimization. *Expert Opin Drug Discov* 2008;3(6):607-621. doi:https://doi.org/10.1517/17460441.3.6.607

30. BPS Bioscience Inc. PDE5A1, GST-tag 2020. https://bpsbioscience.com/pde5a-60050. Accessed 4 January 2020.

31. Wang X-B, Zheng J, Li J-J, et al. Simultaneous analysis of 23 illegal adulterated aphrodisiac chemical ingredients in health foods and Chinese traditional patent medicines by ultrahigh performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry. *J Food Drug Anal* 2018;26(3):1138-1153. doi:https://doi.org/10.1016/j.jfda.2018.02.003

32. Biesterbos JWH, Sijm DTHM, van Dam R, Mol HGJ. A health risk for consumers: the presence of adulterated food supplements in the Netherlands. *Food Addit Contam Part A* 2019;36(9):1273-1288. doi:https://doi.org/10.1080/19440049.2019.1633020

33. Lee JH, Park HN, Park S, Lee Y-M, Kang H. Development of a specific fragment pattern-based quadrupole-Orbitrap mass spectrometry method to screen adulterated products of phosphodiesterase-5 inhibitors and their analogues. *Sci Justice* 2019;59(4):433-441. doi:https://doi.org/10.1016/j.scijus.2019.02.006

34. Prinz H. Hill coefficients, dose-response curves and allosteric mechanisms. *J Chem Biol* 2010;3(1):37-44. doi:https://doi.org/10.1007/s12154-009-0029-3

35. Di Veroli GY, Fornari C, Goldlust I, et al. An automated fitting procedure and software for dose-response curves with multiphasic features. *Sci Rep* 2015;5(1):14701. doi:https://doi.org/10.1038/srep14701

36. Bischoff E. Potency, selectivity, and consequences of nonselectivity of PDE inhibition. *Int J Impot Res* 2004;16(S1):S11-S14. doi:https://doi.org/10.1038/sj.ijir.3901208

37. Boolell M, Allen MJ, Ballard SA, et al. Sildenafil: an orally active type 5 cyclic GMP-specific phosphodiesterase inhibitor for the treatment of penile erectile dysfunction. *Int J Impot Res* 1996;8(2):47-52.

38. Ballard SA, Gingell CJ, Tang K, Turner LA, Price ME, Naylor AM. Effects of sildenafil on the relaxation of human corpus cavernosum tissue in vitro and on the activities of cyclic nucleotide phosphodiesterase isozymes. *J Urol* 1998;159(6):2164-2171. doi:https://doi.org/10.1016/S0022-5347(01)63299-3

39. Turko IV, Ballard SA, Francis SH, Corbin JD. Inhibition of cyclic GMP-binding cyclic GMP-specific phosphodiesterase (type 5) by sildenafil and related compounds. *Mol Pharmacol* 1999;56(1):124-130. doi:https://doi.org/10.1124/mol.56.1.124

40. Saenz de Tejada I, Angulo J, Cuevas P, et al. The phosphodiesterase inhibitory selectivity and the in vitro and in vivo potency of the new PDE5 inhibitor vardenafil. *Int J Impot Res* 2001;13(5):282-290. doi:https://doi.org/10.1038/sj.ijir.3900726

41. Blount MA, Beasley A, Zoraghi R, et al. Binding of tritiated sildenafil, tadalafil, or vardenafil to the phosphodiesterase-5 catalytic site displays potency, specificity, heterogeneity, and cGMP stimulation. *Mol Pharmacol* 2004;66(1):144-152. doi:https://doi.org/10.1124/mol.66.1.144

42. Corbin JD, Francis SH, Webb DJ. Phosphodiesterase type 5 as a pharmacologic target in erectile dysfunction. *Urology* 2002;60(2):4-11. doi:https://doi.org/10.1016/S0090-4295(02)01686-2

43. Brooks HB, Geeganage S, Kahl SD, et al. Basics of Enzymatic Assays for HTS. In: Sittampalam GS, Grossman A, Brimacombe K, et al., eds. *Assay Guidance Manual.* Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2012.

44. Patel DN, Li L, Kee C-L, Ge X, Low M-Y, Koh H-L. Screening of synthetic PDE-5 inhibitors and their analogues as adulterants: analytical techniques and challenges. *J Pharm Biomed Anal* 2014;87:176-190. doi:https://doi.org/10.1016/j.jpba.2013.04.037

45. Kole J, Barnhill A. Caffeine content labeling: a missed opportunity for promoting personal and public health. *J Caffeine Res* 2013;3(3):108-113. doi:https://doi.org/10.1089/jcr.2013.0017

46. Jantos R, Stein KM, Flechtenmacher C, Skopp G. A fatal case involving a caffeine-containing fat burner. *Drug Test Anal* 2013;5(9-10):773-776. doi:https://doi.org/10.1002/dta.1485

47. Puzzo D, Sapienza S, Arancio O, Palmeri A. Role of phosphodiesterase 5 in synaptic plasticity and memory. *Neuropsychiatr Dis Treat* 2008;4(2):371-387. doi:https://doi.org/10.2147/ndt.s2447

48. Boswell-Smith V, Spina D, Page CP. Phosphodiesterase inhibitors. *Br J Pharmacol* 2006;147(S1):S252-S257. doi:https://doi.org/10.1038/sj.bjp.0706495

49. Adebiyi A, Adaikan PG. Effect of caffeine on response of rabbit isolated corpus cavernosum to high K+ solution, noradrenaline and transmural electrical stimulation. *Clin Exp Pharmacol Physiol* 2004;31(1-2):82-85. doi:https://doi.org/10.1111/j.1440-1681.2004.03955.x

50. Yang R, Wang J, Chen Y, Sun Z, Wang R, Dai Y. Effect of caffeine on erectile function via up-regulating cavernous cyclic guanosine monophosphate in diabetic rats. *J Androl* 2008;29(5):586-591. doi:https://doi.org/10.2164/jandrol.107.004721

51. Ongaro A, Zagotto G, Memo M, Gianoncelli A, Ribaudo G. Natural phosphodiesterase 5 (PDE5) inhibitors: a computational approach. *Nat Prod Res* 2019:1-6. doi:https://doi.org/10.1080/14786419.2019.1619726

52. Lopez DS, Wang R, Canfield S. MP32-06 Role of caffeine and sugar consumption on erectile dysfunction in US men. *J Urol* 2014;191(4S):e332-e333. doi:https://doi.org/doi:10.1016/j.juro.2014.02.936

53. Lopez DS, Wang R, Tsilidis KK, et al. Role of caffeine intake on erectile dysfunction in US men: results from NHANES 2001-2004. *PloS one* 2015;10(4):e0123547. doi:https://doi.org/10.1371/journal.pone.0123547

CHAPTER 8

Conclusions and recommendations for future work

Erectile dysfunction drugs are highly demanded since the immense success of sildenafil, vardenafil, and tadalafil. These approved phosphodiesterase 5 (PDE5) inhibitors, however, are often deliberately added into herbal remedies, presumably to deliver immediate pharmacological effects, despite their significant health risks to consumers. At present, the advent of their unapproved analogues remains a challenge to forensic drug testing laboratories, as these adulterants may evade detection using conventional targeted screening, and subsequently, pass into the market undetected. Furthermore, the emerging trends of herbal-based food products are alarming, since they are not as heavily regulated compared to those in pharmaceutical dosage forms.

In this study, a two-tier screening strategy, comprising rapid qualitative assay and confirmatory analytical analysis, was established to detect PDE5 inhibitors and their analogues as adulterants in herbal remedies. These comprehensive strategies were applied to 102 herbal remedy samples that claimed to enhance male sexual performance, in pharmaceutical dosage forms (capsule and tablet) and food products (instant coffee premix, powdered drink mix, honey, jelly, hard candy, and chewing gum). The significant results were highlighted to showcase the applicability of the established strategies for routine casework, particularly in a forensic drug testing laboratory.

A rapid screening strategy was established using PDE5 inhibition assay. This bioactivity-based assay utilises a fluorescence polarisation technique to screen PDE5 inhibitors by competing with fluorescein-labelled cyclic-3',5'-guanosine monophosphate to bind to PDE5 enzyme. The assay was optimised and validated to screen 50 herbal-based food samples that claimed to enhance male sexual performance. The PDE5 inhibitions were in agreement with the confirmatory liquid chromatography-high-resolution mass spectrometry (LC-HRMS) analysis for all food products, except for the instant coffee premix samples. Caffeine, a non-selective phosphodiesterase (PDE) inhibitor, is postulated to inhibit the PDE5 enzyme; thus, generating false-positive results for the coffee samples. These findings infer that the assay may not be suitable for certain types of food products, notably those with naturally-occurring PDE inhibitors, such as coffee, tea, and chocolate. The broad-based, non-targeted assay, nevertheless, demonstrated a promising potential to screen PDE5 inhibitors in other types of food products.

A confirmatory LC-HRMS-based method that is universally applicable for various types of matrices, covering an extensive range of known and potentially novel PDE5 inhibitors, is ideal and highly coveted for routine casework. Unfortunately, the LC-HRMS technique, specifically in positive electrospray ionisation mode, is susceptible to matrix effect (ME), leading to various errors, primarily in quantification. Furthermore, the simultaneous determination of multiple PDE5 inhibitors, each with their distinct physical and chemical properties, together with the complexity of herbal remedies, presents an additional challenge for accurate and precise analysis.

Many studies have acknowledged that the probability of having an ionisation suppression or enhancement is proportional to the complexity of the sample matrix. The ME was, therefore, initially tackled for each target analyte in different pharmaceutical and food matrices by optimising the chromatographic separation, sample extraction, and sample dilution. The insignificant ME percentages, within -9.2%–8.8%, were signified with good accuracy of all target analytes within 77.4%–124.7%, when the matrix-matched standards were fitted to the external calibration curve at three quality control levels. Additional validation parameters, i.e. specificity, linearity, limit of detection, limit of quantification, precision, and extraction recovery (RE), were also established within an acceptable range. The development, optimisation, and validation of the targeted LC-HRMS analysis provided a solid foundation for suspected-target and non-targeted screenings.

The suspected-target screening utilised a library comprising 95 PDE5 inhibitors and their analogues, providing extended coverage of known analytes without the need for certified reference materials. A suspected analyte is detected by comparing the accurate mass of the precursor ion to the theoretical ones in the library. The observed product ions are then compared to the common fragmentation patterns of target analytes, confirming the suspected analyte as well as discriminating those with isomeric configurations. Isomeric analytes belonging to the same group of PDE5 inhibitors, however, could not be distinguished as they shared the same common fragmentation patterns.

The non-targeted screening is employed to flag potentially novel PDE5 inhibitors analogues based on the common fragmentation patterns of target analytes. The top-down and bottom-up approaches were established to screen visible and non-visible chromatographic peak, respectively. As demonstrated in this study, both approaches should be engaged concomitantly via systematic MS and tandem MS interrogations to increase confidence in the detection of novel analogues. The non-targeted screening, per se, is informative compared to the conventional ultraviolet (UV) spectra matching, which is typically adopted to flag novel analogues. The full spectral information permits tentative chemical formula to be generated with the capability to predict the potential chemical structure.

In total, 74 out of 102 of the samples, i.e. 73% were found to be adulterated with PDE5 inhibitors and their analogues. The high incidence of adulteration due to the illegal addition of pharmaceutical drugs is manifested by the efficacy of these products, making them highly sought-after by consumers. Some of these products dangerously contained up to five different inhibitors per sample, making them unsafe for consumption; thus, detrimental to consumers' health and well-being. Herbal-based food products exhibited the highest adulteration rate at 82% compared to those in pharmaceutical dosage forms at 63%. This adulteration trend sparks grave concern considering herbal-based food products are readily available and easily accessible through conventional and online markets. Comparable incidences of adulteration were observed for samples obtained from Malaysia (73%) and Australia (70%), suggesting similar adulteration problems in both countries.

At present, the comprehensive two-tier screening strategy should suffice to overcome the challenges faced by forensic drug testing laboratories, discussed throughout this study. The rapid qualitative assay enables potentially adulterated samples to be credibly marked as priority for confirmatory analysis. It also provides preliminary information on the presence of novel analogues, as the PDE5 inhibition assay is non-targeted for a specific inhibitor or a group of inhibitors. The confirmatory LC-HRMS-based method was comprehensively developed, optimised, and validated for targeted analysis as well as suspected-target and non-targeted screenings. Therefore, further analytical improvements are superfluous for the selected matrices. Nevertheless, any new matrices should be critically assessed, particularly for their ME and RE, before they can be fitted into the existing analytical strategies.

The comprehensive screening strategies established in this study may serve as a foundation for the advancement in future methodology. Fluorescence polarisation, for instance, is a versatile and superior solution-based technique, whereby modifications in the molecular weight of a fluorophore are reflected by changes in the polarisation of light, emitted by the sample. Therefore, the PDE5 inhibition assay could be expanded into an automated screening procedure, considering its high-throughput potential via a simple mix-incubate-read format. Furthermore, the data interpretation is straightforward, distinguishing potentially adulterated samples based on their PDE5 inhibition above the pre-determined threshold value.

A streamlined workflow of the suspected-target and non-targeted screenings by LC-HRMS is highly recommended. The development of innovative data processing tools may allow the full spectral information from MS and tandem MS experiments to be automatically analysed and tentatively classified before the final analyte identification and quantification. These would, in turn, enhance the performance and productivity for routine casework in any forensic drug testing laboratories, to keep up with the ever-increasing adulteration cases. However, despite the advantages of an LC-HRMS analysis, the use of other complementary techniques, such as UV spectroscopy and nuclear magnetic resonance spectroscopy, is still crucial to provide orthogonal information, primarily for unambiguous identification of structural isomers and novel PDE5 inhibitors analogues.

The developed screening strategies discussed in this study, regardless, should be extended for different classes of herbal remedies currently prone to be adulterated such as those claimed for slimming, muscle-building, cholesterol-lowering, anti-diabetic, and joint health. Each of these categories of adulterated products should, therefore, be developed, optimised, and validated based on the specific group of analytes. Particular attention should be given to those group of adulterants that are prone to be modified into novel analogues. The findings recorded in this study may serve as a guidance and reference for forensic drug testing laboratories working on a similar aim of combating adulterated herbal remedies.

It may be difficult to predict the future adulteration trend. The extent of these problems, discussed herein, is just the tip of the iceberg. As the drug control authority scrambles to keep up with the ever-changing adulteration patterns, the cunning manufacturers may as well be one step ahead, finding new means to evade detection and circumvent the laws. Therefore, collective and competent measures by relevant governing bodies such as drug control authority, customs and border control, and police are crucial to curb the widespread adulteration of herbal remedies. Perhaps, the mandatory regulations and legislation of all herbal-based products, concerning their quality, safety, and efficacy should be tightened and updated as a move forward to safeguard the public health.