INVESTIGATION ON THE POTENTIAL OF DRIED BLOOD STAIN FOR THE DETECTION OF AMPHETAMINE TYPE STIMULANTS BY ENHANCED METHODS OF LIQUID CHROMATOGRAPHY TANDEM-MASS SPECTROMETRY

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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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INVESTIGATION ON THE POTENTIAL OF DRIED BLOOD STAIN FOR THE DETECTION OF AMPHETAMINE TYPE STIMULANTS BY ENHANCED METHODS OF LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

ABSTRACT

The investigation on the potential of dried blood stain for Amphetamine Type Stimulants (ATS) has been divided into four major parts, from which independent conclusions are drawn. The first part is the development and validation of a novel method for the simultaneous determination of the drugs using liquid chromatography with electrospray ionization tandem mass spectrometer (LC-ESI-MS/MS). The method is enhanced from normal Multi-Reaction Mode (MRM) to Multi-Reaction Mode-Enhanced Product Ion Mode- Multi-Reaction Mode 3 (MRM-EPI-MRM^3) and tested in whole blood specimens (WBS), dried blood stain (DBS) and urine specimens as well as applied to real forensic cases in Malaysia. The ATS are confirmed by their retention times, as well as the respective ions in MRM mode, complemented with higher level of detection using EPI (library matching) and unique identification by combinations of ions in MRM^3 mode. The second part covers another novel technique of Flow Injection Analysis (FIA)-MRM^3 spectrometry for the differentiation of prime critical isomeric compound related to ATS; methamphetamine and phentermine. The technique is designed to shorten the analysis time to 2 min by eliminating the chromatography/separation part and focusing on the strength of MRM^3 mode in spectrometry part. From the validation and real samples testing, the both of isomeric compounds are successfully discriminated and gives a new dimension on high throughput analysis in the forensic field. While the third task evaluates the reliability of DBS versus WBS technique using Mean Concentration Ratio and Bland-Altman statistical analysis. he results show no significant difference for the ATS in the

both techniques proving that the DBS method can be applied interchangeably in the future. The evaluation of DBS has been extended to testing the stability and efficiency of the medium during storage period in part four. The variants studied include the time and temperature in Malaysia for 6-month period of DBS storage. The study proves good stability and efficiency of ATS drugs and establishes the performance of DBS as an alternative and innovative method to be implemented in forensic analytical purposes.

Keywords: MRM-EPI-MRM^3, FIA-MRM^3, Bland-Altman, Mean Concentration Ratio, stability, WBS, Urine, DBS, Forensic Toxicology.

KAJIAN BERKENAAN POTENSI MEDIUM DARAH KERING BAGI PENGESANAN DADAH *AMPHETAMINE TYPE STIMULANTS* MENGGUNAKAN TEKNIK LEBIH KE HADAPAN DENGAN PERALATAN KROMATOGRAFI CECAIR SPEKTROMETRI JISIM

ABSTRAK

Kajian berkenaan dengan menguji potensi medium darah kering bagi pengesanan dadah *Amphetamine Type Stimulants* (ATS) telah dibahagikan kepada empat bidang utama, di mana kesimpulan berdasarkan setiap kajian turut disediakan. Bahagian pertama dalam kajian ini telah memfokuskan kepada pembangunan dan pengesahan teknik analisis yang berbentuk penambahbaikan daripada teknik lazim *Multi-Reaction Mode* (MRM) kepada teknik yang lebih kehadapan iaitu teknik *Multi-Reaction Mode-Enhanced Product Ion Mode- Multi-Reaction Mode 3* (MRM-EPI-MRM^3) dan diuji keatas sampel darah kering, darah, air kencing serta sampel-sampel daripada kes forensik sebenar di Malaysia. ATS telah dapat ditentukan melalui masa tahanan masing-masing, beserta ion-ion berkaitan di mod MRM, dan dilengkapkan dengan tahap keyakinan pengesanan lebih tinggi menggunakan mod EPI (penentuan kesamaan data *library*) serta pengesanan dengan cara yang unik melalui kombinasi ion di mod MRM^3. Bahagian kedua kajian inimenyentuh tentang kaedah bernilai tinggi lain iaitu *Flow Injection Analysis (FIA)-MRM^3 spectrometry* bagi pemisahan dan penentuan dadah isomer kritikal berkaitan ATS; *methamphetamine* dan *phentermine*. Teknik ini telah direka untuk memendekkan masa analisis kepada 2 minit dengan tidak mengambil kira bahagian pemisahan kromatografi serta fokus kepada kekuatan mod MRM^3. Daripada beberapa ujian di peringkat pengesahan serta kes-kes sebenar, kedua-dua dadah isomer ini mampu dipisahkan melalui kaedah ini seterusnya memberi dimensi baru untuk kerja-kerja di bidang forensik yang mempunyai beban analisis tinggi. Sementara bahagian ketiga pula memfokuskan melalui

ujian penilaian terhadap medium DBS berbanding WBS menggunakan kaedah *Mean Concentration Ratio* dan kaedah statistik *Bland- Altman.* Hasil daripada kedua-dua kaedah statistik ini, didapati tiada perbezaan yang signifikan bagi kedua-dua DBS dan WBS dan boleh diaplikasi kelak. Penilaian lanjut melibatkan kestabilan teknik DBS ini turut dijalankan di bahagian keempat sepanjang tempoh penyimpanan sampel. Kajian telah melibatkan varian termasuk masa dan suhu di Malaysia sepanjang sampel disimpan dalam tempoh 6 bulan dalam medium DBS dan hasil kajian telah menunjukkan dadah-dadah ini adalah stabil sepanjang tempoh penyimpanan serta menjadikan DBS layak sebagai medium alternatif untuk diaplikasi dalam tujuan analisis forensik pada masa hadapan.

Kata kunci: *MRM-EPI-MRM^3, FIA-MRM^3, Bland-Altman, Mean Concentration Ratio,* ujian kestabilan, *WBS*, Urin, *DBS*, Toksikologi Forensik.

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LIST OF SYMBOLS AND ABBREVIATIONS

2C-B	:	4-bromo-2,5-dimethoxyphenethylamine
4-MTA	:	4-methylthioamphetamine
6-MAM	:	6-mono-acetyl-morphine
AADK	:	National Anti-Drugs Agency
AF2	:	Excitation energy
ATS	:	AMPHETAMINE TYPE Stimulants
BDB	:	1-(1′,3′-benzodioxol-5′-yl)-2-butanamine
BMI	:	Body mass index
BZE	:	Benzoylecgonine
CAP	:	College of American Pathologists
CDBS	:	Capillary dried blood spots
CE	:	Collision energy
CNS	:	Central Nervous System
CPC	:	Criminal Procedure Code
Cpm	:	counts per minute
CRM	:	Certified reference materials
CuSO4	:	Copper Sulphate
CV	:	Coefficient of variation
CXP	:	Collision exit potential
Da	:	Dalton
DART	:	Direct analysis in real time
DBS	:	Dried blood stain/spot
DBS	:	Dynamic Background Subtraction
DDA	:	Dangerous Drugs Act

DESI	:	Desorption electrospray ionisation
DFT	:	Dynamic Fill Time
DIE	:	Diethylpropione
DOA	:	Drug of abuse
DP	:	Declustering potential
DUS	:	Dried Urine Spot
EDTA	:	Ethylenediaminetetraacetic acid
ELISA	:	Enzyme-Linked Immunosorbent Assay
EMIT	:	Enzyme Multiplied Immunoassay Technique (EMIT)
EP	:	Ephedrine
EP	:	Entrance potential
EPI	:	Enhanced Product Ion
ESI-MS	:	Electro spray ionisation mass spectrometry
EU	:	European Union
EURL	:	European Union Reference Laboratories
EXB	:	Entrance exit barrier
Fe	:	Ferum
FIA	:	Flow Injection Analysis
FPIA	:	Fluorescence polarisation immunoassays
FWHH	:	Full Width Half Height
GC-MS	:	Gas Chromatography Mass Spectrometer
GHB	:	Gamma-Hydro Butyric Acid
HCD	:	Higher Collision Dissociation
HFBA	:	Heptafluorobutyric Anhydride
HRMS	:	High Resolution Mass Spectrometry
IB	:	Ion booster

ICP-MS	:	Inductively Coupled Plasma Mass Spectrometry
IDA	:	Information Dependent Acquisition
ISTD	:	Internal standard
IT-MS	:	Ion-Trap Mass Analyzer
LC-MS/MS	:	Liquid Chromatography Tandem Mass Spectrometer
LCS	:	LeadCare® System
LIT	:	Linear ion trap
LLE	:	Liquid-liquid extraction
LOD	:	Limit of detection
LOQ	:	Limit of quantitation
LSD	:	Lysergic acid diethylamide
m/z	:	Mass-to-charge ratio
MBDB	:	N-methyl-1-(1′,3′-benzodioxol-5′-yl)-2-butanamine
MBTFA	:	*N*-methyl bis(trifluoroacetamide)
MDA	:	3,4-Methylenedioxyamphetamine
MDEA	:	Methylenedioxy-N-ethylamphetamine
MDMA	:	3,4-methylenedioxy-*N*-methylamphetamine
MPH	:	Methylphenidate
MRM	:	Multi-Reaction Mode
MRM3	:	Multistage fragmentation
NIDA	:	National Institute on Drugs Abuse
NPS	:	New psychoactive substances
NTBC	:	2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione
PA	:	Poisons Act
Pb	:	Lead
PEP	:	Pseudoephedrine

PFC	:	Perfluorinated compounds
PFOA	:	Perfluorooctanoic acid
PFOS	:	Perfluorooctanesulfonic acid
PFP	:	Pentafluorophenyl
PMA	:	para-methoxyamphetamine
PPG	:	Polypropylene glycol
PPP	:	Fenproporex
PSA	:	Prostate Specific Antigen
PT	:	Proficiency Testing
PTM	:	Phentermine
QC	:	Quality Control
QQQ	:	Triple quadrupole detectors
RIA	:	Radioimmunoassay
RP	:	Reversed phase
RSD	:	Relative standard deviation
S/N	:	signal-to-noise ratio
SRM	:	Selected Reaction Monitoring
STA	:	Systematic Toxicological Analysis
SWGTOX	:	Scientific Working Group for Forensic Toxicology
TCAs	:	Tricyclic antidepressants
TDM	:	Therapeutic drug monitoring
THC	:	Tetrahydrocannabinol
TIAFT	:	The International Association of Forensic Toxicologists
ToF	:	Time-of-flight
TQMS	:	Triple quadrupole mass spectrometer
UHPLC	:	Ultra-High-Performance Liquid Chromatography

UNODC	:	United Nations on Drugs and Crime
USFDA	:	United States Food and Drug Administration
VAD	:	Vitamin A deficiency
VS	:	Venous serum
WBS	:	Whole blood specimen
WHO	:	World Health Organization
Zn	:	Zinc

CHAPTER 1: INTRODUCTION

1.1 Definition of Drugs of Abuse (DOA)

Drugs of abuse (DOA) in general is the use of a drug with such frequency that the user has physical or mental harm or it impairs social abilities. The National Institute on Drugs Abuse (NIDA) classifies that most DOAs alter a person's thinking and judgment thereby leading to health risks including addiction, drugged driving and infectious disease (NIDA, 2017). DOA as according to United Nations on Drugs and Crime (UNODC, 2016) is any substance that, because of some desirable effect, is used for some purpose other than intended. The intended use could be for a therapeutic effect such as benzodiazepines and opiates analgesics. Another definition is any substance the possession or supply of which is restricted by law because of its potential harmful effect on the user and known as controlled or scheduled substances.

According to the World Health Organization (WHO), scheduled drugs are 'abused' rather than 'misused' drugs. DOA may or may not lead to physical or psychological dependence, a term used by WHO in preference to 'addiction' (WHO, 2016). In most circumstances, controlled substances are also known as illicit drugs or narcotics which frequently encompass heroin, cannabis, cocaine, amphetamine, ecstasy pills and others. In drug control, these substances are specifically listed in Schedule I and II of the 1961 Convention as reported by UNODC (2016).

In Malaysia, the term 'dangerous drugs' instead of 'controlled substances' is used to describe drugs that are not permitted for production and consumption altogether. The two major regulations related to DOA are Dangerous Drugs Act (DDA 1952) and Poisons Act (PA 1952). The documentations under Criminal Procedure Code (CPC) in both legislations set a standard of severity by the punishments it attaches to various crimes (Adler et al., 1998). Hence, the sentences imposed by the acts can reflect the severity of

the drug-related crime. Additional Armed Forces Act 1972 which applied to Royal Malaysia Armed Forces also imposes mandatorily charges related to the armed personnel with DOA.

1.1.1 Definition of Amphetamine Type Stimulants (ATS)

The word "stimulant" refers to agents that stimulate the central nervous system (CNS) and have marked effects on mental function and behavior (Middleberg and Hoan, 2012). The term Amphetamine Type Stimulants (ATS) refers to a group of substances comprising of the illicit street drugs include amphetamine, methamphetamine, methcathinone, the ecstasy-group substances (3,4-methylenedioxy-*N*-methylamphetamine (MDMA) and its analogues), and also those usually employed in therapeutic approaches, such as methylphenidate (MPH), amphepramone or diethylpropione (DIE), mazindol, and fenproporex (PPP). The ATS as described by WHO refers to amphetamine and other similar drugs which acts as stimulant to make people awake, felt energized or alert. ATS have been used during war times to keep soldiers on high alert and it was sometimes used in diet pills to keep people from feeling hungry, hence aiding in weight loss.

Routes of administration are normally through intranasal sniffing when in powder form; pulmonary inhalation and injection when in crystal or liquid forms; and oral ingestion when in capsule or tablet forms (Middleberg & Hoan, 2012). ATS, as pharmacological and toxicological agents have a common phenethylamine structural backbone; and typically impart effects that include but not limited to central nervous system stimulation, hallucinations, anorexia and others. Besides addiction, further abuse of these substances has led to hypertension, arrhythmia, excitability, aggressiveness, psychoses, coma, and death (Deventer *et al.*, 2011; Karila *et al.*, 2010).

From toxicological aspect, ATS in the body is normally metabolized in the liver and produces by-products called metabolites. The metabolism rate depends on many factors such as body size, age and health, as well as water and food intake. Prime example is the metabolism process of methamphetamine produces amphetamine. While MDMA is normally metabolized to 3,4-Methylenedioxyamphetamine (MDA), however, both compounds can be detected in the blood and urine specimen after MDMA use. ATS can remain from two hours to four days. These drugs are quite stable and their elimination rate from the body depends on many factors such as the amount ingested, frequency of use, tolerance levels, metabolism rate, body mass, age, and health levels (MyHEALTH, Malaysia).

ATS drugs in this study consist of the common Amphetamine Type stimulants; methamphetamine and MDMA together with the precursors of pseudoephedrine and ephedrine, the metabolites (amphetamine, MDA, Methylenedioxy-N-ethylamphetamine (MDEA)) and isomeric phentermine. Those targeted compounds are analyzed and discussed in the study.

1.2 The Prevalence of Amphetamine Type Stimulants (ATS)

1.2.1 Global Scenario of ATS

The consumption of ATS is stable and consistent in North America and the European Union but is on the rise in China, the Middle East region, and East, South East and Middle East Asia. The 2016 World Drug Report stated that ATS are still among the on-demand DOA besides opiates, cocaine, cannabis, and new psychoactive substances (NPS) (United Nations, 2016). The trend of global seized drugs shown that ATS is still dominating from 1998-2014 (see Figure 1.1).

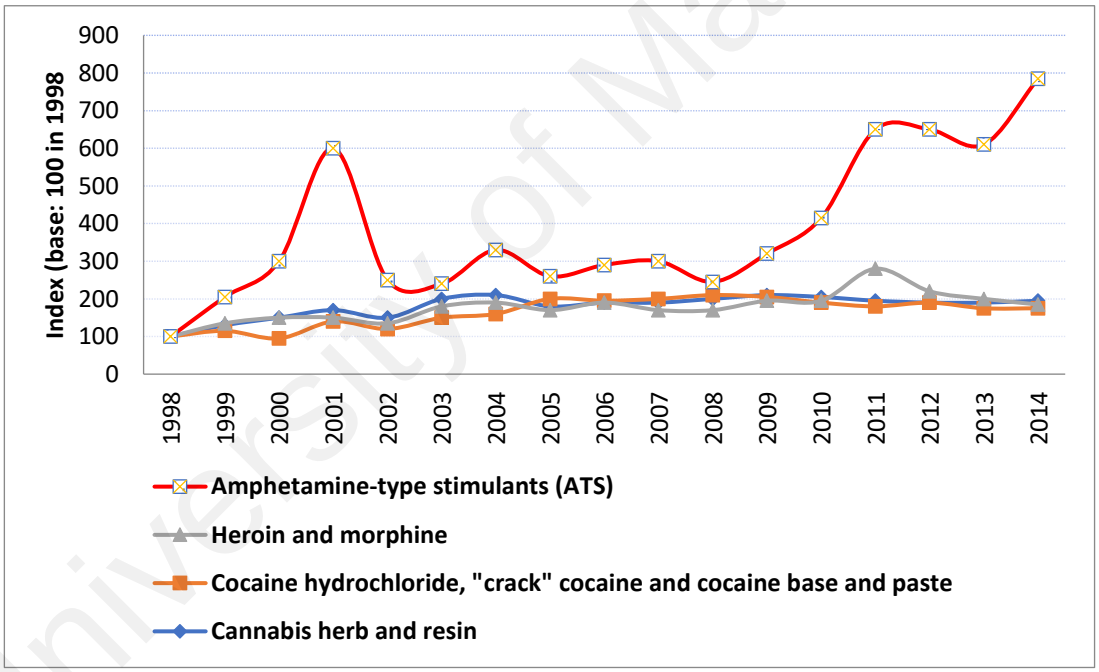


Figure 1.1: Trends in the quantities of drugs seized worldwide; 1998-2014
(Source: UNODC. World Drug Report 2016)

The report also emphasized the trend of poly-drug use, which shown that ATS is of interest to be consumed together with other drugs. ATS have been used with some NPS, and sometimes with other conventional drugs. Among the ATS, methamphetamine plays an important role in the ATS worldwide markets where disorders related to the use of this drug also account for a considerable share on global health problems (Sommers

and Baskin, 2006). Dependence on ATS is a serious worldwide public health problem associated with major medical, psychiatric, cognitive, socioeconomic and legal consequences. The findings on ATS also suggested that the misuse and abuse heightened the risks for violence (Karila *et al.*, 2010).

1.2.2 Local / Malaysia Scenario of ATS

The information from National Anti-Drugs Agency (AADK) is normally referred to illustrate the drugs scenario in Malaysia (AADK, 2016). This agency is responsible for the prevention, treatment, rehabilitation of addicts, and enforcement operations to monitor and combat the drugs occurrences in this country. Table 1.1 summarizes the statistic of type of drugs that are being abused as reported in AADK Annual report (2016).

Table 1.1: Statistic of Type of Drugs of Abuse (DOA) in Malaysia 2016

Category		Cases Status					
		New		Repeated		Total	
		No.	%	No.	%	No.	%
Type of DOA consumed	Opiates*	11,469	48.34	5,516	68.61	16,985	53.47
	Methamphetamine	8,384	35.34	1,723	21.43	10,107	31.82
	Cannabis	1,098	4.63	138	1.72	1,236	3.89
	ATS**	2,737	11.54	658	8.18	3,395	10.69
	Psychotropic Pills***	17	0.07	1	0.01	18	0.06
	Others****	19	0.08	4	0.05	23	0.07
	Total	**23,724**	**100**	**8,040**	**100**	**31,764**	**100**

*Heroine & morphine; **ecstasy, amphetamine & 'Pil Kuda'; ***including benzodiazepines, psychotropic pills & erimin-5; ****including kratom (mitragynine), depressants, hallucinogens, inhalants, etc. Note: The data is not necessarily representing the total number of offenders as these also include poly-drugs users.
(Source: AADK Annual Report 2016)

Table 1.1 indicates that methamphetamine including other ATS of ecstasy, amphetamine, and 'pil kuda' are classified as second most abused in Malaysia drugs after opiates. "Pil Kuda" for example, is categorized as cheap illegal drugs or 'working-class drugs' which is easily accessed. The drugs are often used by labourers and drivers who have to work in tough conditions for very long hours. In some countries, they are known

as the “poor men’s drugs” because they are cheaper than other sophisticated drugs. Meanwhile, methamphetamine, MDMA or ecstasy and amphetamine remained popular in this country. NIDA (2016) reported that those drugs are popular in the nightclub scene and at all-night dance parties ("raves"), affecting a broader range of people. The trend of ATS (combination of methamphetamine and other ATS) abuse is also increasing in this country (see Table 1.2 and Figure 1.2). The dataset has shown that there is a gradual increase in the abuse of nearly all kinds of ATS during 2012 to 2016 and the highest figure recorded on 2016 (about 13,502 cases).

Table 1.2: Type of DOA consumed in Malaysia, 2012-2016

Statistic of Type of Drugs of Abuse (DOA) in Malaysia; 2012-2016							
Year	**Opiates***	**Cannabis**	**Meth**	**ATS****	**Psychotropic Pills*****	**Others******	**Total**
2012	8,472	1,427	4,761	286	66	80	15,101
	56.10%	9.45%	31.53%	1.89%	0.44%	0.53%	100%
2013	16,035	1,885	2,901	476	18	46	21,361
	75.07%	8.82%	13.58%	2.23%	0.08%	0.22%	100%
2014	14,496	1,919	4,117	1,774	6	43	22,355
	64.84%	8.58%	18.42%	7.94%	0.03%	0.09%	100%
2015	16,616	1,389	8,133	1,309	7	25	27,479
	60.48%	5.05%	29.60%	4.76%	0.03%	0.09%	100%
2016	16,985	1,236	10,107	3,395	18	23	31,764
	53.48%	3.89%	31.82%	10.69%	0.06%	0.07%	100%

*Heroine & morphine; **ecstasy, amphetamine & ‘Pil Kuda’; ***including benzodiazepines, psychotropic pills & erimin-5; ****including kratom (mitragynine), depressants, hallucinogens, inhalants, etc. Note: The data is not necessarily representing the total number of offenders as these also include poly-drugs users.
(Source: AADK Annual Report 2016)

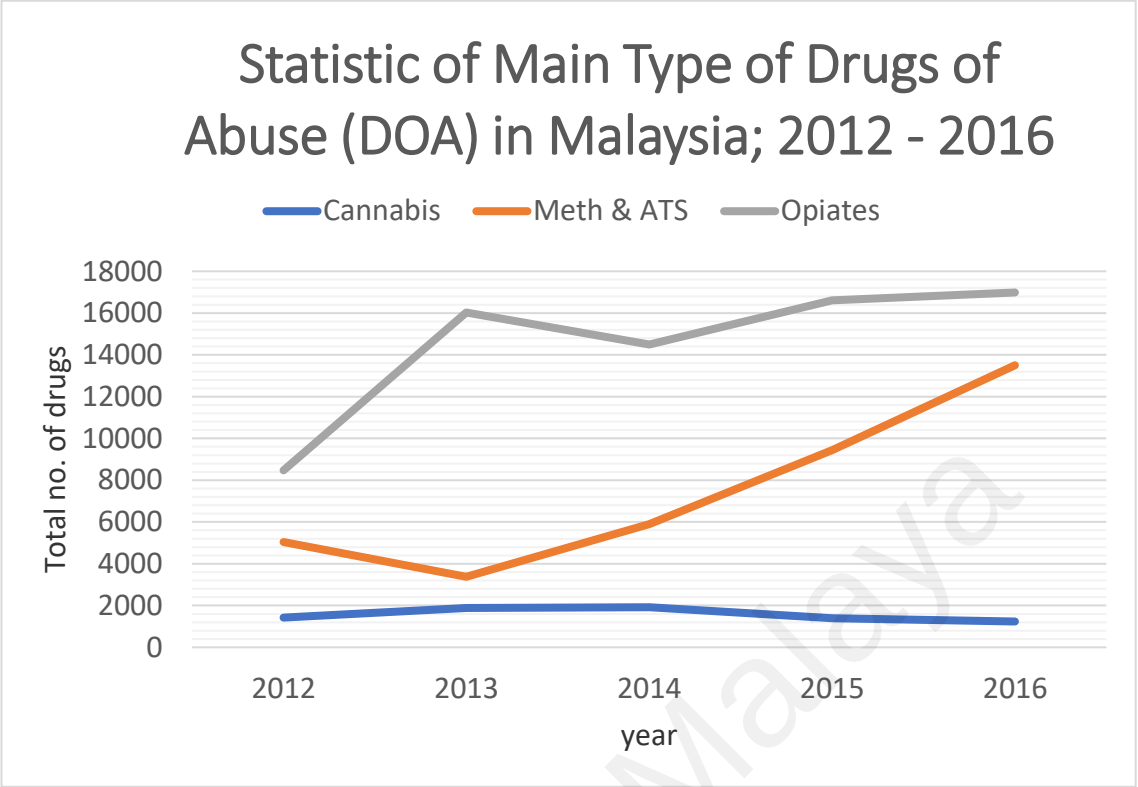


Figure 1.2: Trends of DOA in Malaysia from 2012-2016.

UNODC (2013) reported that the increasing demand for ATS originating from Myanmar, and Malaysia becomes an important transit country, as well as the final destination, for the trafficking. Large quantities of ATS especially methamphetamine was smuggled from the Islamic Republic of Iran, in addition to locally manufactured ones. While information obtained from the Malaysian drug enforcement agencies reveals that most of the methamphetamine available in Malaysia market was smuggled from China, Thailand, and Philippines (Vong, 2004).

1.3 Law and Legislation

In general, law is a system of rules and guidelines which are enforced through social or governmental institutions to govern behavior and regulate conduct. The law is normally enacted by a legislature or other governing body to form a 'statutory law' that also known as legislation. This is one of the most important methods of government to determine the rights and responsibilities of individuals and authorities to whom the legislation applies and as a key in organizing and protecting society and citizens. Malaysian law and legislation that related to the drugs of abuse is associated with several codes such as 399 Criminal Procedure Code, Dangerous Drugs Act 1952, Poison Act 1952 as well as Armed Forces Act 1972 and each of the codes will be discussed in the following section.

1.3.1 399 Criminal Procedure Code

In the statutory document of Act 593 Criminal Procedure Code (CPC), the allocation of Section 399: Reports of certain persons, allows the authorized personnel to perform the examination and analysis and issue a report as evidence in any inquiry, trial or other proceeding under this code in the court. The provisions of this section which apply to the government enforcement agencies, enables the authorized person/agencies to assist in the legal enforcement and combat the crimes including the abuse of dangerous drugs in this country. The following are the persons to whom the provisions of this section apply: (Source: Laws of Malaysia: Act 593. CPC (Revised-2012)

a) *Officers of the Institute for Medical Research;*
b) *Government Medical Officers;*
c) *Chemists in the employment of any Government in Malaysia or of the Government of Singapore;*

d) any person appointed by the Minister by notification in the Gazette, to be a Document Examiner;

e) Inspector of Weights and Measures appointed as such under any written law relating to weights and measures in force in Malaysia; and

f) any person or class of persons to whom the Minister by notification in the Gazette declares that the provisions of this section shall apply.

1.3.2 Dangerous Drugs Act (DDA) 1952

The Dangerous Drugs Acts (DDA 1952) is also a statutory document that described the penalties on drug prosecution in Malaysia. The "dangerous drug" stated in this act refers to any drug or substance, which is for the time being comprised in the First Schedule and for the drugs that been administered to the body in the forensic toxicology field, the allocations of the act are as follows:

Section 15. Self-administration.

(a) a person who consumes, administers to himself or suffers any other person, any dangerous drug specified in the First Schedule. For the purpose of this section, "consumes" includes eat, chew, smoke, swallow, drink, inhale or introduce into the body in any manner or by any means whatsoever.

(b) a person who is found in any premises kept or used for any of the purposes in order that any such dangerous drug may be administered to or smoked or otherwise consumed by him, shall be guilty and shall be liable on conviction to a fine not exceeding five thousand ringgit (RM) or to imprisonment for a term not exceeding two years.

<u>Section 38B</u>. Powers of the Court in respect of persons found guilty under sect. 15 (Source: Laws of Malaysia: Act 593. CPC (Revised-2012)

- Where a person is found guilty of an offence the punishment imposed:

(a) undergo supervision of the Drug Dependents (Treatment and Rehabilitation) Act 1983 for a period of not less than two and not more than three years as may be determined by the court.

1.3.3 Poisons Act (PA) 1952

The Poisons Act 1952 is another statutory document which control the use and abuse of drugs in Malaysia. The act regulates the importation, possession, manufacture, compounding, storage, transport, sale and use of poisons. This act restricts the use of controlled substances/poisons except under the prescription of authorized personnel such as medical practitioners and registered pharmacists as stated in Section 7 and Section 12 of the act, and any offence against this act will be penalized or punished with full jurisdiction by Sessions or Magistrate's Court as stated in Section 33 of the act. For example, as stated in Section 12:

<u>Section 12</u>. Control of compounding of poisons for use in medical treatment.

(1) No person shall dispense, compound or mix any poison with any other substance, whether a poison or not, for the purpose of its being used for medical treatment unless he is-

(a) a registered pharmacist or a person working under the immediate personal supervision of a registered pharmacist;

(b) a person acting in the course of his duties who is employed in a hospital or dispensary maintained by the Government of Malaysia or any State Government or out of public funds or by a charity approved by an order whether general or special of the Director General of Health or in an estate hospital and who is authorized in writing by the registered medical practitioner for the time being in charge of such hospital or dispensary to dispense, compound and mix poison; or

(c) a registered medical practitioner or a person working under the immediate personal supervision of such a practitioner who dispenses, compounds or mixes poisons for the use of such practitioner or of his patients.

1.3.4 Armed Forces Act 1972

Detection of drugs of abuse as listed in the First Schedule of DDA1952 in the Royal Malaysia Armed Forces personnel shall be mandatorily charged as mentioned in Act 77 of Sec 60:

.....unfit to be entrusted with his duty or with any duty which he might be called upon to perform, or behaves in a disorderly manner or in any manner likely to bring discredit on His Majesty's Service.

(Source: Laws of Malaysia: Armed Forces Act 1972)

1.4 ATS Drugs Selected for this Study

In forensic toxicology, it is necessary to determine the drugs as well as their metabolites as the drugs after being ingested will undergo biotransformation via metabolism processes in the body and form other compounds (metabolites).

The information of the drugs and metabolites obtained from quantitative analysis is important for the interpretation of forensic toxicology cases as it describes the level of drugs effect in the body prior to an incidence or death. The level of drugs and metabolites in human body can be categorized as follows (Winek *et al.*, 2001):

a) *Therapeutic Level:* concentration of drugs and/or metabolites present in the blood specimen following therapeutically effective dosage in humans (remedy effect).

b) *Toxic Level:* concentration of drugs and/or, metabolites or chemicals present in the blood specimen that is associated with serious toxic symptom in humans.

c) *Lethal Level:* concentration of drugs and/or, metabolites or chemicals present in the blood specimen that has been reported to cause death or is so far above reported therapeutic or toxic concentrations, that one can judge that it might cause death in humans.

d) *Normal Level:* values under normal represent normal body constituents and others represent values related to normal environmental exposure.

As further explained by Winek *et al.*, (2001) the values are to be applied as a guide in case evaluation and not necessarily absolute. Several factors such as dose, route of

administration, absorption differences, age and sex, tolerance, method of analysis, pathological or disease state, post-mortem redistribution and others may affect the values.

Common ATS selected in this study together with the significant information of the compounds in forensic toxicology is summarized in Table 1.3:

Table 1.3: Common ATS drugs; chemical structure (Chemspider, Royal Society of Chemistry), use/abuse/effect (Baselt, 2000; NIDA, 2016), disposition in body (Baselt, 2000), toxicology level (Winek *et al*. 2001) and legislation/act (Laws of Malaysia, 2012; 1980; 1989; 1972)

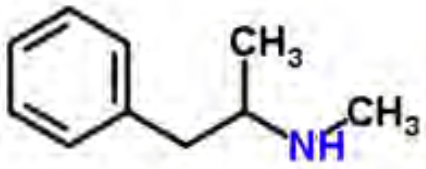
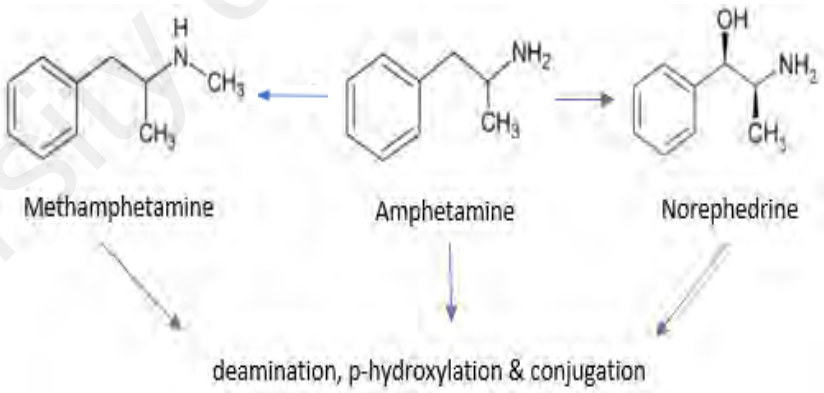
Analyte (1)	**Methamphetamine**
Chemical Structure	
Use/Abuse/Effect	Use/Abuse: a strong central nervous system stimulant as a recreational drug and less commonly as a second-line treatment for attention deficit hyperactivity disorder and obesity. Effect/Toxicity: restlessness, confusion, anxiety, hallucinations, cardiac arrhythmias, hypertension, hyperthermia, paranoid psychosis, circulatory collapse, convulsions, coma.
Disposition in Body	 Methamphetamine undergoes some N-demethylation to amphetamine, its major active metabolite. During normal conditions, up to 43% of a dose is eliminated unchanged in the 24h urine, with 4-7% amphetamine. About 15% is excreted as p-hydroxy methamphetamine and p-hydroxy norephedrine.
Toxicology Level (µg/mL)	-Normal Level: 0.01 – 0.05 -Toxic Level: 0.6 – 5.0 -Lethal Level: >10
Legislation / Act	-listed under Schedule 1 Dangerous Drugs Act 1952 -listed under Schedule 1 Poisons Act 1952

Table 1.3: Common ATS drugs; chemical structure (Chemspider, Royal Society of Chemistry), use/abuse/effect (Baselt, 2000; NIDA, 2016), disposition in body (Baselt, 2000), toxicology level (Winek *et al*. 2001) and legislation/act (Laws of Malaysia, 2012; 1980; 1989; 1972) cont;

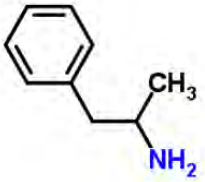
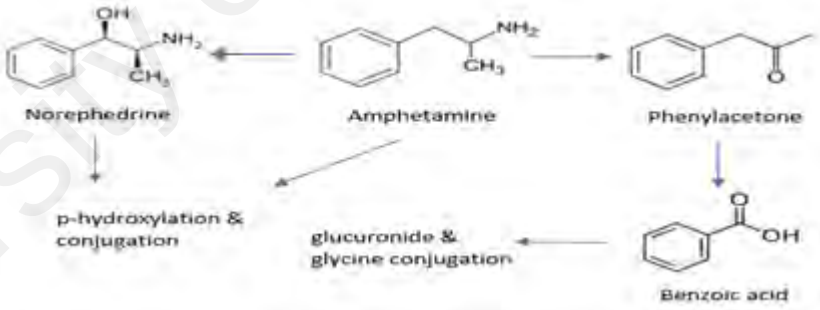
Analyte (2)	**Amphetamine**
Chemical Structure	
Use/Abuse/Effect	Use/Abuse: a potent central nervous system stimulant and is used in the treatment of attention deficit hyperactivity disorder, narcolepsy, and obesity and occasionally as a recreational drug. Effect/Toxicity: euphoria, change in desire for sex, increase wakefulness, improve cognitive control, increase heart rate and blood pressure, weight loss, hallucinations, paranoid psychosis, agitation, hyperthermia, convulsions, unconsciousness and respiratory/cardiac failure.
Disposition in Body	 Amphetamine is largely inactivated during metabolism, deaminated to phenylacetone, oxidized to benzoic acid and excreted as conjugates. The small amount of amphetamine is converted to norephedrine by oxidation, and are subsequently p-hydroxylated. The entire dose of amphetamine probably is eliminated in urine over several days (30% is excreted unchanged, 0.9% as phenylacetone and 2% as norephedrine).
Toxicology Level (µg/mL)	-Normal Level: 0.03 – 0.11 -Toxic Level: >0.5 -Lethal Level: >1
Legislation / Act	-listed under Schedule 1 Dangerous Drugs Act 1952 -listed under Schedule 1 Poisons Act 1952

Table 1.3: Common ATS drugs; chemical structure (Chemspider, Royal Society of Chemistry), use/abuse/effect (Baselt, 2000; NIDA, 2016), disposition in body (Baselt, 2000), toxicology level (Winek *et al*. 2001) and legislation/act (Laws of Malaysia, 2012; 1980; 1989; 1972) cont;

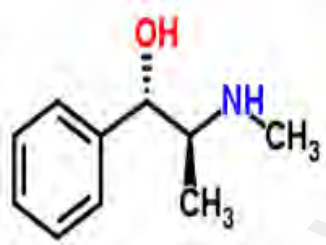
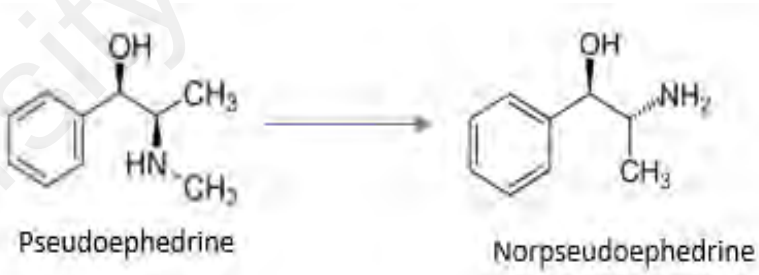
Analyte (3)	**Pseudoephedrine**
Chemical Structure	
Use/Abuse/Effect	Use/Abuse: a sympathomimetic amine and is used as nasal decongestant as well as bronchodilator. Also, a sought-after chemical precursor in the illicit manufacture of methamphetamine and methcathinone. Effect/Toxicity: headache, dizziness, palpitations, tachycardia, restlessness, tremor, anxiety, insomnia, hallucinations, weakness, convulsions, arrhythmias, hypotension and cardiovascular collapse.
Disposition in Body	 Pseudoephedrine is metabolized to norpseudoephedrine through N-demethylation. Up to 88% of a dose is excreted unchanged in urine after 36 hours with less than 1% of norpseudoephedrine present.
Toxicology Level (µg/mL)	-Normal Level: 0.50 – 0.77 -Lethal Level: 19
Legislation / Act	-listed under Schedule 1 Poisons Act 1952

Table 1.3: Common ATS drugs; chemical structure (Chemspider, Royal Society of Chemistry), use/abuse/effect (Baselt, 2000; NIDA, 2016), disposition in body (Baselt, 2000), toxicology level (Winek *et al*. 2001) and legislation/act (Laws of Malaysia, 2012; 1980; 1989; 1972) cont;

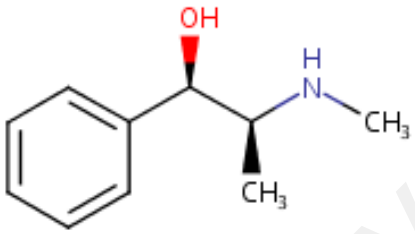
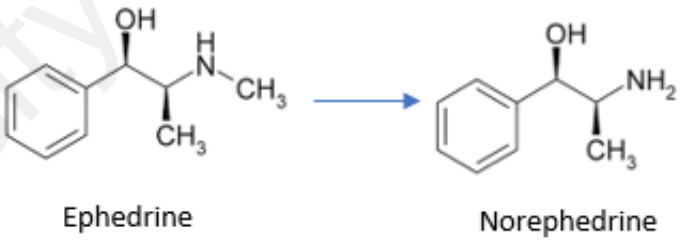
Analyte (4)	**Ephedrine**
Chemical Structure	
Use/Abuse/Effect	<u>Use/Abuse:</u> a naturally occurring sympathomimetic amine and is used for asthma as nasal decongestant, narcolepsy, and obesity. Also, a precursor in the illicit manufacture of methamphetamine. <u>Effect/Toxicity:</u> trouble sleeping, anxiety, headache, hallucinations, high blood pressure, fast heart rate, loss of appetite, inability to urinate, stroke and heart attack.
Disposition in Body	 Ephedrine Norephedrine Ephedrine is metabolized primarily by N-demethylation to norephedrine and to a minor extent by p-hydroxylation and conjugation. 70-80% of ephedrine is eliminated unchanged in urine after 48 hours and about 4% is present as norephedrine.
Toxicology Level (µg/mL)	-Normal Level: 0.068 – 0.10 -Lethal Level: 3.50 -21
Legislation / Act	-listed under Schedule 1 Poisons Act 1952

Table 1.3: Common ATS drugs; chemical structure (Chemspider, Royal Society of Chemistry), use/abuse/effect (Baselt, 2000; NIDA, 2016), disposition in body (Baselt, 2000), toxicology level (Winek *et al*. 2001) and legislation/act (Laws of Malaysia, 2012; 1980; 1989; 1972) cont;

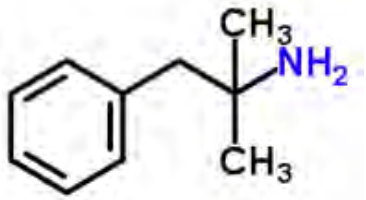
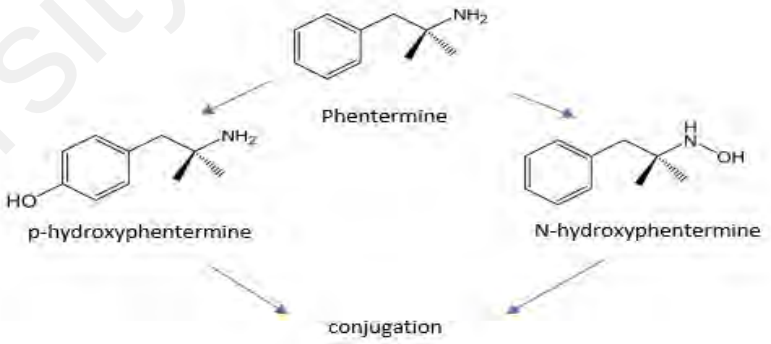
Analyte (5)	**Phentermine**
Chemical Structure	
Use/Abuse/Effect	Use/Abuse: a stimulant that is similar to an amphetamine and is used as an appetite suppressant that can affect the central nervous system. Also as an anorexiant used for short-term (few weeks) weight loss treatment in obese patients. Effect/Toxicity: alter taste, constipation, diarrhoea, insomnia, dizziness, dry mouth, headache, nervousness, hallucinations, psychotic episodes, rapid respiration, cardiac arrhythmias, hyperthermia, convulsions, coma and circulatory collapse.
Disposition in Body	 Phentermine is much less susceptible to metabolism and the primary pathways for biotransformation are p-hydroxylation and N-oxidation. 48% is excreted unchanged in the urine, less than 0.7% is excreted as free and conjugated p-hydroxyphentermine while up to 5% is present as N-hydroxyphentermine and other products of N-oxidation.
Toxicology Level (µg/mL)	-Therapeutic Level: 0.09 – 0.51 -Lethal Level: 1.5 – 7.6
Legislation / Act	-listed under Schedule 1 & Schedule III (Psychotropic Substances) in Poisons Act 1952.

Table 1.3: Common ATS drugs; chemical structure (Chemspider, Royal Society of Chemistry), use/abuse/effect (Baselt, 2000; NIDA, 2016), disposition in body (Baselt, 2000), toxicology level (Winek *et al*. 2001) and legislation/act (Laws of Malaysia, 2012; 1980; 1989; 1972) cont;

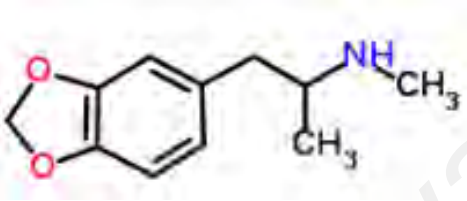
Analyte (6)	**3,4-Methylenedioxymethamphetamine (MDMA)**
Chemical Structure	
Use/Abuse/Effect	Use/Abuse: a recreational drug/club drug/psychoactive drug that has both stimulant and hallucinogenic characteristics and often taken for the feelings of well-being, stimulation, and distortions in time and sensory perceptions that it produces. Effect/Toxicity: visual hallucinations, confusion, agitation, hypotension, seizures, hyperpyrexia, hypoglycaemia, panic disorder and coma.
Disposition in Body	 MDMA MDA MDMA is metabolized to MDA through N-demethylation. Urinary excretion accounts for 65% of the drug and 7% as MDA within 3 days. Other urinary metabolites include mono-and di-hydroxy derivatives of MDMA and MDA in conjugated forms.
Toxicology Level (µg/mL)	-Normal Level: 0.02 – 0.03 -Toxic Level: 0.1 – 1.0
Legislation / Act	-listed under Schedule 1 Dangerous Drugs Act 1952 -listed under Schedule 1 Poisons Act 1952

Table 1.3: Common ATS drugs; chemical structure (Chemspider, Royal Society of Chemistry), use/abuse/effect (Baselt, 2000; NIDA, 2016), disposition in body (Baselt, 2000), toxicology level (Winek *et al*. 2001) and legislation/act (Laws of Malaysia, 2012; 1980; 1989; 1972) cont;

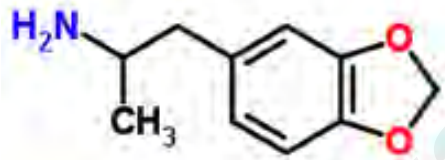
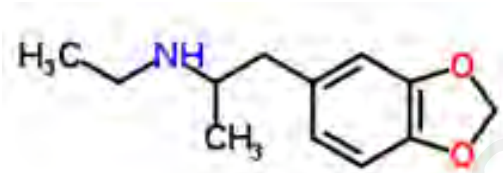
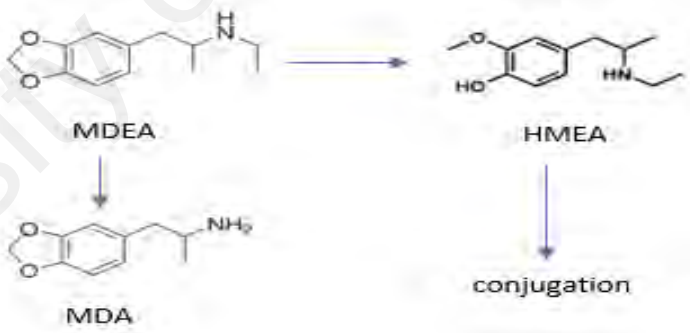
Analyte (7)	**3,4-Methylenedioxyamphetamine (MDA)**
Chemical Structure	
Use/Abuse/Effect	Use/Abuse: a psychotropic amphetamine derivative and is primarily a central stimulant and hallucinogenic in high doses. Effect/Toxicity: agitation, tremor, tachycardia, rapid breathing, pupillary dilation, hyperthermia, muscular rigidity, convulsions and coma.
Disposition in Body	* The human metabolism of MDA has not been studied; urine concentrations in fatal cases of up to 160 mg/L have been recorded and are indication of excretion of substantial portions of unchanged drug. In animals, MDA is metabolized by O-dealkylation, deamination and conjugation.
Toxicology Level (µg/mL)	- Lethal Level: 1.8 – 26
Legislation / Act	-listed under Schedule 1 Dangerous Drugs Act 1952 -listed under Schedule 1 Poisons Act 1952

Table 1.3: Common ATS drugs; chemical structure (Chemspider, Royal Society of Chemistry), use/abuse/effect (Baselt, 2000; NIDA, 2016), disposition in body (Baselt, 2000), toxicology level (Winek *et al*. 2001) and legislation/act (Laws of Malaysia, 2012; 1980; 1989; 1972) cont;

Analyte (8)	**3,4-Methylenedioxy-N-ethylamphetamine (MDEA)**
Chemical Structure	
Use/Abuse/Effect	Use/Abuse: a close chemical analogue of MDMA and is a member of group of methoxylated amphetamine derivatives that has seen widespread abuse for their hallucinogenic properties. Effect/Toxicity: euphoria, perceptual distortions, anxiety, panic, paranoia, depression, bizarre behaviour, agitation, seizures and coma.
Disposition in Body	 MDEA is known to undergo biotransformation via oxidative cleavage of the methylenedioxy ring, conjugation of the resulting phenolic compounds, N-deethylation and deamination. 19% of the drug is excreted in urine after 32 hours with 28% MDA, 32% HMEA and trace amounts of other metabolites.
Toxicology Level (µg/mL)	**Blood or plasma concentrations of MDEA in controlled studies have not been reported, but they are likely to be similar to those observed with MDMA.* (R.C.Baselt)
Legislation / Act	-listed under Schedule 1 Dangerous Drugs Act 1952 -listed under Schedule 1 Poisons Act 1952

CHAPTER 2: REVIEW OF AMPHETAMINE TYPE STIMULANTS (ATS) IN FORENSIC TOXICOLOGY

2.1 Analytical Methods for ATS drugs in Forensic Toxicology

2.1.1 Introduction

The term 'Forensic Toxicology' cover applications of the science and study of poisons to elucidate of questions that arise in judicial proceedings. All analyses performed under toxicology are to detect, identify, quantify, and determine the toxicity of the poison. As mentioned by Paracelsus "*all substances are poisons: there is none which is not a poison. The right dose differentiates a poison from a remedy*" (Amdur *et al.*, 1991).

Drugs classes including DOA is a subgroup of poisons in Forensic Toxicology that demands an overall analytical system to determine their presence in biological specimens. Figure 2.1 below shows the seven major groups of poisons:

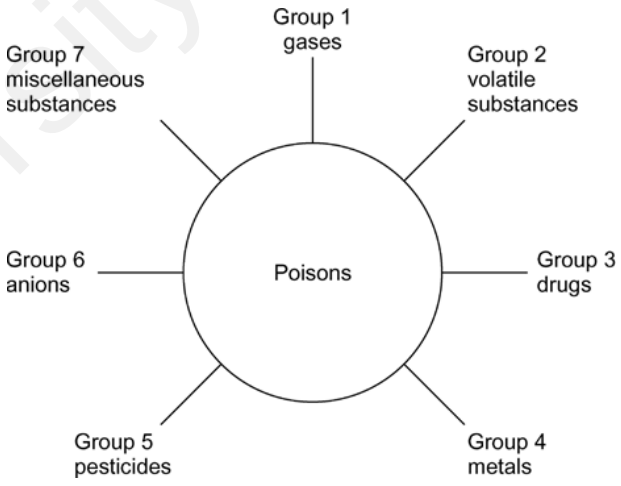


Figure 2.1: Group of Poisons in Forensic Toxicology
(Source: Clarke's Analytical Forensic Toxicology. (2013)).

2.1.2 Specimen for Analysis

Forensic specimens are collected in variable compositions that include ante- and post-mortem specimens (Polettini, 2006). Blood and urine are commonly used; while alternative matrices include stomach content, vitreous humor, bile, hair, nails and other relevant biological specimens are also used in poisons or drugs detection (Polettini, 2006; Drummer, 2001).

Previously, the detection of illicit drugs in clinical and forensic field are performed primarily on urine specimens (Braithwaite *et al.*, 1995). However, it was found the quantitative determination using blood samples are more effective. During 1990s, the importance of drugs analysis in blood specimens caught the attention of the researchers as the number of studies concerning this analysis either in whole blood, plasma and serum have increased tremendously (Moeller, 1998). Today, studies on blood specimens are performed routinely due its advantages and advanced analyses have also been introduced to provide fast, sensitive, and accurate analysis for the detection of the illicit drugs.

Blood, or the blood fractions serum and plasma, has traditionally been the ideal specimen for drug testing. More information has been reported on the correlations between blood concentrations of the drug or active metabolites with pharmacodynamic functions when compared with other matrices (Moody, 2006).

Several highlights need to be considered when transferring methodology originally formulated for urine drug testing to blood drug testing. First, most drugs and drug metabolites present in blood samples at much lower concentrations compared to urine samples. Secondly, blood samples contain other cellular materials at much higher concentrations than in urine. Hence, analysis using blood samples required development of appropriate procedures to enhance the sensitivity and selectivity of the assay (or accept

the limitations of sensitivity and selectivity of the assay in regards to the matrix) as well as to transform the matrix to an accessible form for analysis.

Also, there is some post-mortem processes that need to take into consideration when analyzing biological specimens, *i.e.* degradation of compounds due to chemical instability or metabolic changes, redistribution and the appearance of decomposition products. In post-mortem specimens, peripheral blood is used as this type of specimen suffers the least changes in concentration compared to other specimens (Drummer, 2004), and routinely adapted in analysis.

2.1.3 Analytical Requirements

Following Orfila's maxim "*the presence of a poison must be proven in the blood and organs before it can be considered as a cause of death*" (Drummer, 2001), the four stages in toxicology analysis need to be fulfilled:

1. Detection - screening procedures as a preliminary test to detect any drugs or poisons in the submitted specimen.
2. Identification - confirmation procedures using specific relevant physicochemical test to conclusively identify the drugs, metabolites and other poisons.
3. Quantification - quantify accurately the drugs, metabolites and poisons present in order to measure the level of those compounds especially in the blood specimen.
4. Interpretation - interpret the analytical findings and determine the significance of the results in the context of the case for complete toxicological investigation.

2.1.4 Systematic Toxicological Analysis (STA)

Procedures for the identification of organic compounds in biological matrixes are numerous and the newest technology or latest instrument developments can definitively add significant benefits to the overall efficiency to the analytical strategy. Systematic Toxicological Analysis (STA) is an analytical procedure aiming to search for as many foreign compounds as possible within a specimen in order to identify any substances a person might have ingested or be exposed to in the course of medico-legal examinations.

STA is a major part of the examination in forensic and clinical toxicology. STA is aimed at detecting and identifying all toxicological relevance substances such as drugs, drugs of abuse, poisons, and their metabolites in biological materials through screening and confirmatory techniques. In forensic toxicology field, specimens tested positive, are often rerun by a second confirmation test.

STA stated that additional positive result by a second technique constitutes more compelling evidence showing specimen does, in fact, contain the drug in question. Toxicology findings are often legally or medically critical and the need for accuracy is paramount. Therefore, two techniques that reinforce each other are a powerful argument in favor of the drug's presence.

Although screening methods are usually more sensitive to reduce false negatives, but in some non-specific cases, false positive results may be obtained. Therefore, the confirmatory methods must have as high specificity/selectivity as possible. With the best confirmatory methods this near-absolute specificity/selectivity is achieved and any false presumptive positives from the initial screen, shall be eliminated by the confirmation test.

2.1.4.1 Screening Techniques

Initial screening to verify the presence of drugs in suspected biological specimens is a vital step before proceeding to the utilisation of confirmatory and advanced analytical techniques. Several screening techniques applied for the screening of DOA particularly ATS are discussed in the following sections.

2.1.4.1.1 Rapid Test Kits

Rapid test screening is often applied on-site such as during operations, raids and arrests as well as in the clinics and laboratories by law enforcers. The screening test rely on a set threshold cut-off value. The cut-off value is a concentration that is used to distinguish a positive or negative result. Specimens with drug concentrations of above or equal to the cut-off level are considered positive while specimens with drug concentrations lower than the cut-off level are considered negative.

There are numbers of commercially available rapid test kits offered in the market to screen for the illicit drugs such as Amphetamine Test Strip, Bionike System and Combo Test that are discussed in the following sections.

2.1.4.1.1.1 Amphetamine Test Strip

One of the examples for commercially available amphetamine test strip used in Malaysia is the Diagnostic Automation/Cortez Diagnostics (AMP Drug Test). This test is a one-step immunochromatography test, designed for qualitative determination of amphetamine drug substances in human urine specimens. This test strip is used for diagnostic and forensic setting.

The principle of the test strip is based on the specific immunochemical reaction between antibodies and antigens with target compounds in human urine specimen. The

assay relies on the competition for binding antibody between drug conjugate and free drug in the urine specimen. If amphetamine drug is present in the urine specimen, it will compete with drug conjugate for the limited amount of antibody-dye conjugate. When the amount of drug is equal or more than the cut-off, binding of drug conjugate to the antibody will be prevented. Hence, a positive urine specimen will not show a colored band on the test line zone, while the presence of a colored band indicates a negative result. A control line present in the test window works as a procedural control. This colored band should always appear on the control line zone if the test device is stored in good condition and the test is performed appropriately.

2.1.4.1.1.2 Bionike One Step Test

Bionike Step Tests (Bionike Inc, South San Francisco, CA, USA) is a type of rapid test kits that provide the detection of illicit drugs in urine specimen used in Malaysia during raids and arrests in past years. This assay typically performed as an on-site drug test analysis which is capable of providing rapid results (Rockville, 2005).

The test relies on the competition for binding antibody between drugs coated on the test-kit's membrane and drugs which possibly present in the urine being tested. When a drug is present in the urine, it competes with the membrane-bound drugs for the limited antibodies present as dye-antibody conjugates. When sufficient amount of drug is present, it will prevent the binding of dye-antibody conjugate to the membrane-bound drug. Similar to amphetamine test strip, a positive urine specimen will not generate a color band in the test region while the presence of a color band in the test region indicates a negative result.

Several authors have previously described the use of Bionike test in the screening of illicit drugs in urine specimens. Brown E.D and Jarvie (1997) had evaluated six

Bionike tests on urine specimens to screen for amphetamine, methamphetamine, benzodiazepines, cannabinoids, methadone and opiates. Tests were performed blindly and the results were compared with Syva enzyme multiplied immunoassay technique (EMIT) assays (Behring Diagnostics UK Ltd, Milton Keynes, UK) as well as confirmatory GC-MS techniques. The authors reported that the results of Bionike tests correlated well with amphetamines, methadone, and opiates detected in urine using GC-MS. Another report suggested that to increase the read time from 3–10 min (suggested by the manufacturer) to 15–30 min to overcome any ambiguity in the results (George *et al.*, 2002).

2.1.4.1.1.3 Combo Test

Fast-Screen Drug Combo Test is another rapid, competitive binding immunoassay for qualitative determination of multiple drugs and drug metabolites in human urine. The drugs can be custom-made in a minimum of any two-test combination to a maximum of ten-test combination. This assay is intended only for professional and laboratory uses. The test provides preliminary data which should be subsequently confirmed by other techniques, such as GC-MS (FastScreen, 2014).

The Combo Test is based on a chromatographic absorbent device in which drug or drug metabolites in a specimen compete with drug conjugate immobilized on a porous membrane support for limited antibody sites. Labelled antibody-dye conjugate mixes with the specimens and binds to the free drug or drug metabolites present to form an antibody-antigen complex. This complex competes with immobilized antigen conjugate in the test zone and preventing the formation of a purplish-pink color band when the drug is above the detection level. Unbound dye conjugate binds to the reagent in the control zone and produces a purplish-pink color band, which demonstrate that the reagents and device are functioning correctly.

A negative specimen produces two distinct color bands, one in the test zone and one in the control zone while a positive specimen produces only one-color band in the control zone (FastScreen, 2014).

In Malaysia, the Combo Test strips is routinely used for screening of DOA including ATS. The strips are used in the screening of urine specimen for Royal Armed Forces personnel prior to sending the specimen to the Department of Chemistry Malaysia for drugs testing confirmation. The technique is also applied by the Royal Police Forces during operations and raids for enforcement to be charged under 399 CPC.

2.1.4.1.2 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is commonly used in forensic toxicology laboratories as a tool for acute poisoning, checking abstinence, forensic purposes, and therapeutic drug screening and monitoring. This technique is the best choice during shortage of analysis time. ELISA with relative ease of use, growing potential for automation, and their adaptability for use with good sensitivity and accuracy, as well as capable to determine poisons concentrations in different kinds of biological fluids; has made the technique most suitable for screening purposes (Ewa Gomolka *et al.*, 2012).

Unlike many of their homogeneous immunoassay counterparts, ELISAs are amenable to whole blood specimens without the need for specimen pre-treatment. Other advantages of enzyme immunoassays include small specimen volumes, high specimen throughput, rapid turn-around times, long shelf lives, and the lack of radioisotopes (Perrigo *et al.*, 1995).

The choice of specimens for ELISA depends on the purpose of analysis and includes blood, urine or other alternative specimens such as saliva, sweat and hair. Blood and urine are common in clinical and forensic toxicology, while alternative specimens

are increasingly analyzed, as the results of hair or sweat determination, for example; are useful in the history of drugs overdosing or abusing research.

In general, ELISA combines the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies or antigens coupled to an easily-assayed enzyme. This technique provides useful measurement of antigen or antibody concentration. The two main variations on this technique include the detection for the presence of antigens that are recognized by an antibody or it can be used to test for antibodies that recognize an antigen.

Several highlights on the analysis of ATS by ELISA include the work of Matapatara *et al*. (2007) on the simultaneous screening of ephedrine with amphetamine or methamphetamine of drug abusers in Thailand. By using heterologous immunoassay, the sensitivity and specificity of analysis was increased and the problem on the determination of amphetamine with ephedrine or methamphetamine with ephedrine was successfully carried out. The detection of ephedrine by immunoassay was successfully demonstrated for the first time in this experiment.

Kupiec *et al.* (2002) reported an ELISA assay using Neogen microtiter plate enzyme-linked immunoassays for the screening of post-mortem blood for amphetamine and/or methamphetamine. The Neogen type ELISA has proven to have excellent precision and accuracy with low variation (%CV), and the absorbance for the calibrators was also very stable and repeatable. The assays identified the presence of amphetamines in the post-mortem blood specimens with less interference from sympathomimetic amines and thus lead to little false positive results.

Kerrigan *et al.* (2001) described comparative assessment on several ELISAs for the detection of six common classes of drug including ATS in blood and urine. The

commercially available ELISAs were evaluated in a side-by-side study, investigating the binding characteristics, dose–response curves, limits of detection, sensitivity, intra- and inter-assay imprecision, and lot-to-lot reproducibility. Assay performance was also compared using 855 forensic casework specimens. The results indicated the LOD for d-methamphetamine was 2 μg/L, with superior binding characteristics, detection limits, improved overall precision and lot-to-lot reproducibility as compared to previous study.

Montgomery *et al.* (2008) also reported the use of ELISA in umbilical cord tissue to detect fetal exposure in maternal illicit drug use. In this study, the deidentified umbilical cord specimens were analyzed for presence of five drug classes including ATS (methamphetamine) and the ELISA was compared with a 'gold standard' test, consisting of gas or liquid chromatography tandem mass spectrometry. From the assessment, the sensitivity and specificity of the ELISA to ATS is 97% and the authors concluded that performances of the ELISA-based tests are sufficient for clinical testing of fetal exposure to methamphetamine and other four parameters (benzodiazepines, cannabinoids, opiates and cocaine).

Most ATS assays are designed to detect amphetamine, racemic compounds such as dextroamphetamine and methamphetamine as well as illicit analogues such as MDEA, MDA, and MDMA. The challenges and limitations in ELISA screening are highlighted by Stout *et al.* (2004) on the cross-reactivity during interpretation of ATS results.

Other than targeted illicit drugs, results of ATS assays are often positive among individuals taking prescription stimulants for attention deficit and hyperactivity disorder, for narcolepsy, and as anorexiants because many of these stimulants contain amphetamines. Common over-the-counter cold medicines such as pseudoephedrine, ephedrine, phenylephrine, and decongestants were reported to cross-react with the assay (Stout *et al.*, 2004).

Some drugs used in psychotropic medications may attribute to the cross-reactivity of ATS drugs, which are not typically assessed in manufacturers' evaluations of immunoassays for interference. For example, Nixon *et al.* (1995) and Weintraub *et al.* (2000) reported bupropion to be positive in the assay while other drugs such as phenothiazines including chlorpromazine, promethazine, and thioridazine as reported by Melanson *et al.* (2006) could interfere the assay.

Tricyclic antidepressants (TCAs) such as desipramine and doxepin also showed positive result in the assay (Merigian *et al.*, 1993). In addition, other unique agents with similar structures with ATS were also reported to cross-react in the assay, *e.g.* labetalol, isometheptene, ranitidine, ritodrine, and trimethobenzamide (Gilbert *et al.*, 1995; Grinstead *et al.*, 1989; Colbert *et al.*, 1994; Levine *et al.*, 1987; Nice *et al.*, 1989).

Another confounding factor that contributes to the limitation in ATS assay is the inability to distinguish between the two enantiomers of methamphetamine, *D*-methamphetamine and *L*-methamphetamine. The *D*-isomer is reported to be more potent than *L*-isomer in stimulating central nervous system, as the *L*-isomer mainly works peripherally and does not possess euphoric effects (Romberg *et al.*, 1995).

Drugs like selegiline and deprenyl used for the treatment of Parkinson disease and depression, produce *L*-amphetamine and *L*-methamphetamine metabolites, which give a positive result on immunoassays. Overall, the chiral chromatography is required to differentiate between the *D*- and *L*-forms (Cody, 2002).

Additional limitation includes the low sensitivity for detection of MDMA as reported by Hsu *et al.* (2003). Detection of MDMA is normally attributed to cross-reactivity, however, sensitivity for MDMA is approximately 50% less than for amphetamine and methamphetamine and high concentrations of the drug are needed to

elicit positive results on ATS assay. Hsu *et al.* (2003) has further designed incorporating three monoclonal antibodies specific for amphetamine, methamphetamine, and MDMA which resulted in greater detection sensitivity MDMA.

Other than false positive challenges, false-negative results also add limitation to the technique. The false negative results can be caused by a variety of factors including the cross-reactivity of the antibody used by the assay, the cut-off concentration for a positive result, and length of time between drug ingestion and specimen acquisition which require additional confirmatory test to verify of results.

2.1.4.1.3 Fluorescence Polarization Immunoassay (FPIA)

Fluorescence polarisation immunoassays (FPIA) is a simple and reliable tool which yields useful information concerning the conformation and interactions of macromolecules including drugs. It is a fast screening technique based on the principles of immune competition and fluorescence polarisation (Nielsen *et al.*, 2000). FPIA is a homogeneous immunoassay which is very useful for rapid and accurate detection of antibody or antigen.

The detection in FPIA relies on the increase in fluorescence polarisation when small fluorescent-labelled antigen was bound to specific antibody. The advantages of FPIA includes homogeneous testing condition, unaffected by the solution colour, and high instrument sensitivity. Besides, no separation step is required in conducting the assay. However, the most notable advantage over other immunoassays is the simplicity of the assay which minimise the analysis time, make it highly suitable to screen large number of specimens (Wang *et al.*, 2007).

The principle behind the assay is that a fluorescent dye (attached to an antigen or an antibody fragment) is excited by plane-polarized light at its specific wavelength. Small

molecule rotates faster in a solution when compared to a larger molecule and the rate of rotation may be assessed by measuring the light intensity in the vertical and horizontal planes.

Normally, the time it takes for a molecule to rotate through a given angle is an indication of its size. Thus, when a small molecule which rotates rapidly is bound to a larger molecule, the rotation rate is decreased and the differences of rotation rate is measured. FPIA is a primary antigen antibody interaction; thus, the rate of reaction is very rapid and usually a result may be obtained in minutes (Nielsen *et al.*, 2000).

The fluorescence emission process is characterised by two spectra *i.e.* fluorescence emission spectrum and excitation spectrum. Basically, fluorescence occurs when a molecule is exposed to radiation in the portion of the excitation spectrum in which it is capable of absorbing radiation and results in an electronic transition starting from the ground state to an upper electronic state or an excitation state.

During the excited state, the electron is unstable and the duration of the excited state is very short, in the order of 10^{-8} to 10^{-4} s (Wang *et al.*, 2007). When the electron returns to its ground state, it released energy in the form of fluorescence emission. Hence, the greater the ability of the solution to absorb radiation, the greater is the fluorescence intensity (Nielsen *et al.*, 2000).

The example of instrument-dependent immunoassays commercially marketed for use in forensic toxicology for the screening of ATS using FPIA is *FPIA Abbott Diagnostics.* The performance of the instrument shows that the antibody has equal reactivity towards amphetamine and methylamphetamine drugs and has a much lower cross-reactivity towards ephedrine and phenylpropanolamine when compared with the enzyme multiplied immunoassay (EMIT) technique amphetamines antibody. The

instrument also does not require frequent calibration to maintain the efficacy in the operation (Braithwaite *et al.*, 1995).

Screening of illicit drugs such as ATS using immunoassays is commonly performed on urine specimens (Moeller *et al.*, 1998). However, there are a few attempts made by several authors that applied the respective methods on blood specimens to screen for the absence or presence of ATS, particularly using this FPIA.

One of the attempts was by Caplan *et al.* (1987), that analyzed amphetamine and methamphetamine in 249 urine specimens by *Abbott FPIA* and compared the results with those from the *Syva EMIT* and a gas chromatographic assay. They concluded that the *Abbott* Abused Drug amphetamine assay is an acceptable method for the screening of the presence of amphetamine and methamphetamine in urine specimens. The precision and sensitivity of the FPIA assay were also reported to be highly acceptable, in which its specificity exceeded that of the EMIT amphetamine assay.

Moreover, Bogusz *et al.* (1990) utilized immunoassay techniques of EMIT and FPIA to screen six groups of common drugs of abuse (cannabinoids, benzoylecgonine, opiates, barbiturates, benzodiazepines and amphetamines) in whole blood after acetone precipitation. Both methods which were initially designed for urine specimens were applied on the whole blood specimens, including post mortem specimens.

Turner *et al.* (1991) reported the production of an antiserum with a broad specificity for the amphetamine group of drugs. The antiserum produced in sheep using an immunogen linking amphetamine to keyhole limpet haemocyanin *via* an *N*-aminobutyl bridge was used for FPIA application in urine specimens. The 46 positive specimens from FPIA were confirmed by gas-liquid chromatography (GLC) and produced no false negative results.

Another attempts was by Simonick and Watts (1992) to determine the level of *D*-methamphetamine in haemolysed whole blood using the *FPIA Abbott* amphetamine/methamphetamine (Irving, TX, USA) method, originally designed for urine screening. The blood calibration curve was obtained from FPIA (linearity in a range from 25–100 ng/mL) and compared with the results obtained with radioimmunoassay (RIA) and GC–MS methamphetamine controls as well as on eight positive case specimens, FPIA has proven to be reliable for the screening of the ATS in haemolysed blood specimens under such experimental condition.

2.1.4.1.4 Radioimmunoassay (RIA)

Radioimmunoassay (RIA) is a type of heterogenous immunoassays that incorporated a radiolabeled antigen which competes for the antibody binding sites with the unlabeled drug in the specimen. Generally, the types of RIA used in forensic laboratories is ^{125}I-labelled antigens as ^{125}I capable to be detected by gamma counters or liquid scintillation counters.

Some RIAs utilize ^{3}H-labeled antigens for drug analysis. As the amount of drug in the specimen increases, more radiolabeled antigen will remain in the unbound state; thus, the amount of radioactivity in the pellet (attached to the tube) will decrease. The short half-life of ^{125}I-labeled antigens will result in rapid loss of counts per minute (cpm) from one day to the next (Moody, 2006).

Since RIA involves the use of radioactive materials, this technique requires special laboratory conditions for storage, handling, and disposal of the radioactive material safely. The half-life of the radiolabel, ^{125}I is 60 days which is also the shelf-life of the reagent. ^{3}H half-life is 12.4 years, it can be stored for a longer period (Moody, 2006).

RIA has been applied to drugs analysis in blood with few modifications. With the exception of cannabinoids analysis, blood can be directly analyzed by this technique (Altunkaya *et al.*, 1990; Hand *et al.*, 1998). Extractions of blood for RIA analysis have also been automated using a robotic system (Collins *et al.*, 1992) and enhanced the sensitivity for benzodiazepines (Huang *et al.*, 1993).

The development of commercial radioimmunoassay for amphetamine detection in urine have been around since the early 1970s such as *Abuscreen, Roche Diagnostics* (Braithwaite *et al*, 1995). In 1975, kits with ^{125}I labelled drug became available and subsequently followed by the introduction of double antibody technique in 1983 to replace saturated ammonium sulphate in separating bound drug. The antibody raised against *D*-amphetamine possessed a high degree of specificity, with the cross-reactivity reported with *p*-hydroxyamphetamine and *p*-methoxyamphetamine, an illicit hallucinogenic drug (Braithwaite *et al.*, 1995).

Cleeland *et al.* (1976), reviewed the status of RIA for its applicability to rapid screening and quantitation of morphine, barbiturates, methadone, methaqualone or benzoylecgonine and amphetamine in biological specimens, and evaluated the sensitivity, specificity and stability in terms of the shelf life of the reagents. The assays appeared to be equally applicable to detect these illicit drugs in urine, blood, saliva, and tissues, at ambient temperature and for emergency tests or mass screening.

Smith (1981) reported analysis of amphetamine in different types of biological specimens, *i.e.* dried blood stains, semen, seminal stains, saliva, and saliva stains using RIA technique. The author emphasized the purpose of utilizing RIA technique for amphetamine detection as it is relatively inexpensive to set up, simple to perform, and is a highly sensitive technique. The results showed the detection of low *i.e.* ng quantities of amphetamine in 100 µL specimens of dried blood stains using RIA. For the saliva, saliva

stains, semen, and seminal stains, RIA also responded a measurable quantity of the respective drugs.

Budd (1982) reported the analysis of frequently abused ATS drugs, comprises of amphetamine, methamphetamine, phentermine, ephedrine, phenylpropanolamine, phenmetrazine, and phendimetrazine, using three different analytical techniques, *i.e.* GC, EMIT and RIA in different types of biological specimens.

The purpose of the study was to evaluate the pros and cons of these three classes of techniques in the analysis of ATS and related drugs, in order to guide laboratories in choosing the best approach for their requirements. The author stated that RIA is most suitably to be utilized for large-scale analysis programs (more than 50 specimens at a time).

Another study was also performed to measure the concentration of ephedrine in the urine of users of a popular dietary supplement (Metabolife 356) and subsequently determined whether the urine concentrations observed after ingestion of the recommended doses of Metabolife 356 were sufficient to cause a positive screening test for rnethamphetamine using RIA screening technique.

The study was performed on urine specimens obtained from five healthy subjects interested in weight loss and planning on taking diet supplements. Each subject consumed the Metabolife 356 for five days and the positive results for methamphetamine (cross-reaction activity to norephedrine) obtained from the assays undergone confirmatory analysis using GC-MS (Levisky *et al.*, 2003).

2.1.4.1.5 Toxtyper

The latest and most advanced screening technique for the detection of illicit drugs is Toxtyper. This technique is popular in South East Asia, used for complement or replacement of the conventional immunoassay techniques. However, the use of Toxtyper is still depending on the background of cases and specimens, as well as the workloads in each forensic laboratory.

Toxtyper is an LC-MS^n library-based solution from Bruker Daltonics (Germany), using an ion-trap mass analyser (IT-MS) coupled to an ultra-high-performance-liquid-chromatography (UHPLC) device (Bruker, 2015; Ott *et al.*, 2017). In addition to the standard operational software that facilitates set-up of laboratory developed test (LDT), the technique is also equipped to enable operation by non-LC-MS experts for routine analysis, with ready-to-use assay protocols, and algorithms for automatic report generation.

The interpretation of MS spectra relies on the matching to reference spectra found in libraries that are either provided by the manufacturer (*i.e.* Drugs of Abuse Library, [DOAL] or Toxtyper Library) or commercial library (*i.e.* Maurer/Wissenbach/Weber; MWW or Wiley-VCH) (Wissenbach *et al.*, 2011). Customized libraries that include spectra of newly identified substances of interest can be developed, and the acquired spectra shared between laboratories to increase the ability of the lab network to address the appearance of novel illicit drugs (Ott *et al.*, 2017).

Toxtyper offers several advantages compared to other chromatographic techniques in the analysis of biological specimen, such as lower the cost, broad-based analysis, superior analytical capacity such as high sensitivity, ease of training, simple data evaluation, and most importantly, rapid specimen preparation time. For example, when

comparing the performance of GC-MS with Toxtyper, time required to analyze urine specimen using GC-MS is briefly 3 hours 25 minutes, and involve laborious preparation steps, *i.e.* enzymatic hydrolysis, extraction, derivatization and chromatographic run with cooling processes. While in the Toxtyper, the time reduced to 1 hour excluding the long hydrolysis and derivatization steps as well as cooling processes in the chromatographic run (Bruker, 2015).

Kempf *et al.* (2015) evaluated the feasibility of a previously developed automated LC-MS^n screening approach for the detection of xenobiotics (drugs and drugs of abuse including ATS) in vitreous humor and extending the application to post-mortem analysis. The report indicated that the screening approach was suitable for the detection of xenobiotics in vitreous humor. Besides the known physiological limitations of the matrix itself, the obtained limits of detection appeared to be sufficiently adequate for forensic casework.

Klima *et al.* (2016) reported the screening for the presence of medicinal and illicit drugs on post mortem dental hard tissues from three death cases using the Toxtyper and quantitatively analyzed with LC- MS/MS in MRM mode. The findings were then compared with the analytical results from different biological specimens, including cardiac blood, femoral blood, urine, stomach content and hair. The results from the dental hard tissues, 11 drugs (amphetamine, MDMA, morphine, codeine, norcodeine, methadone, EDDP, fentanyl, tramadol, diazepam, nordazepam, and promethazine) were detected and the concentrations showed a declination in the following order: carious material > root > crown.

Another study performed by Ott *et al.* (2017) involving the comparison of current screening strategy for 188 urine specimens for the abuse of cannabis, amphetamines, cocaine, opiates, benzodiazepines, methadone, sufentanil, and pregabalin, with Toxtyper

protocols provided by the manufacturer. The analytical performance of the instrument was determined against panel of selected drugs and the comparison on the drugs screened from the urine specimens were performed to establish the concordance between the authors currently established approach and the Toxtyper.

From the study, the authors established that Toxtyper LC-MS^n was suitable to be used for the screening of the majority of substances encountered in the urine specimens. Besides, they also established that the considerable portion of their current time-consuming protocol for screening drugs of abuse in urine, based on the combination of multiple analytical techniques, could be consolidated using Toxtyper.

The use of Toxtyper was also performed on dried urine spot (DUS) to screen for the presence of numbers of illicit drugs including amphetamine (Michely *et al.*, 2017). The authors developed and validated a DUS workup procedure for an established LC-MS^n approach for comprehensive screening of the metabolites. The workup procedure with on-spot conjugate cleavage was successfully validated with hydrolysis efficiencies comparable with the conventional cleavage procedure.

Toxtyper showed its applicability in comparison to established workup procedures with or without previous conjugate cleavage using 103 authentic urine specimens including six rat urine specimens taken after low dose administration of the corresponding substances each and two proficiency test specimens for systematic toxicological analysis.

2.1.4.2 Confirmation Techniques

The preliminary immunoassays test results are presumptive which can be influenced by external factors/variables. A confirmatory test is required before decisions can be made. Gas Chromatography Mass Spectrometer (GC-MS) and Liquid Chromatography Tandem Mass Spectrometer (LC-MS/MS) are routinely employed for confirmation of ATS drugs and are discussed in the following sections.

2.1.4.2.1 Gas Chromatography Mass Spectrometry (GC-MS)

GC-MS has been used as a routine analysis technique for identification of most illicit drugs in variety of biological specimen especially in whole blood specimen. The coupling of capillary gas chromatography (GC) (operated under a suitable temperature programme) with a mass spectrometer (GC-MS) allows the components of the drugs from most of the specimens to be individually separated and subsequently identified (Cooper *et al.*, 2013). Identification process can be performed by comparing the information obtained thru the retention time and mass-spectra from the drug's components with the commercial libraries. However, it is also important to apply quality assurance such as negative controls, positive controls and quality controls to ensure the validity of the results.

Generally, GC-MS begins with the volatilisation of specimen in the gas chromatograph. This process will effectively vaporise the specimen into gas phase and separates its various components by interacting with the capillary column packed with a stationary phase. The movement of compounds are propelled throughout the capillary column by a pure inert carrier gas such as argon, helium or nitrogen. As the components become separated, they will elute from the column at different retention times, which is known as time interval from injection to peak detector response. Compounds with greater

affinity for the stationary phase interact at longer time in that phase and consequently, require longer time to reach the detector (Cooper *et al.*, 2013).

The coupling of GC with MS allows the eluted components from the GC column to be ionised by the MS, commonly using electron or chemical ionisation sources. The ionised molecules are then accelerated through the instrument's mass analyser (such as quadrupole, ion trap), which separates the ions based on their different mass-to-charge (*m/z*) ratios. The obtained ion signals will be detected and analyzed, producing a mass chromatogram spectrum with compound peaks appearing as a function of their *m/z* ratios. In addition, the peak heights will reflect to the quantity of the corresponding compound in the specimen.

Comparing to LC technique, the GC is a cheaper instrument and provides generally faster separation. The coupling of GC with MS offers a higher separating power and efficient reproducibility as compared with LC-MS/MS. Consequently, the GC-MS is highly in demand and utilised for the identification of low molecular weight and heat resistance fragments volatile biomarkers. Nevertheless, the application of GC is only limited to gas soluble, volatile and heat resistance low molecular weight molecules, that often accompanied by the derivatisation step to produce thermally stable compounds, which often considered as a disadvantage (Simoes *et al.*, 2017; Zakaria *et al.*, 2016).

To date, very limited publications are reporting on the determination of ATS illicit drugs in the DBS specimen using GC-MS. Analysis of amphetamines, cocaine, methadone, and opiates in DBS specimen using GC-MS has been performed by Langel *et al.* (2011) and presented in the 49th Annual Meeting of The International Association of Forensic Toxicologists (TIAFT). Sadones *et al.* (2011) evaluated the validity of "microwave-assisted on-spot derivatization" method for the determination of two distinct polar low molecular weight compounds that are commonly abused, which are gamma-

hydro butyric acid (GHB) (date rape drug) and gabapentin (anti-epileptic drug) in DBS using GC–MS.

Despite of the limitation on the availability of the publications related to the ATS drugs in the DBS specimen with respect to GC-MS, the analytical instrument mentioned is indeed widely utilised in the determination of the ATS drugs in other biological specimens, particularly in whole blood specimen. Mercieca *et al.* (2018) developed a high-throughput and rapid method to determine 25 different classes of stimulants *i.e.* ATS, and new psychoactive substances (NPS) of cathinones, phenethylamines, and ketamine analogues from blood and urine specimens using GC–MS.

A study on the determination the ATS illicit drugs and structurally related common medicaments in blood and other biological specimens (serum, oral fluid, urine) using GC-MS was reported by Kankaanpa *et al.* (2004). A single-step GC-MS procedure for screening, identification, and quantitation a wide variety of ATSs was developed and involved derivatisation step using heptafluorobutyric anhydride (HFBA).

The illicit drugs investigated in the study were amphetamine, methamphetamine, MDA, MDMA, MDEA, 4-methylthioamphetamine (4-MTA), para-methoxyamphetamine (PMA), ephedrine, norephedrine (phenylpropanolamine), pseudoephedrine, cathinone, 1-(1′,3′-benzodioxol-5′-yl)-2-butanamine (BDB), N-methyl-1-(1′,3′-benzodioxol-5′-yl)-2-butanamine (MBDB), 4-bromo-2,5-dimethoxyphenethylamine (2C-B, “nexus”) and 1-benzylpiperazine. The method was found to be suitable for all of the objectives in all of the matrices examined.

Slightly different from the general method, Wozniak *et al.* (2018) proposed a GC–MS/MS method for the determination of amphetamine derivatives in blood and urine specimens. The applicability of the method was evaluated by the quantification of

amphetamine derivatives (amphetamine, methamphetamine, phentermine, MDA, MDMA and MDEA) in 22 cases wherein the use of drugs was suspected. The proposed protocol was successfully applied for the determination of ATS concentrations in blood and urine in several fatal and non-fatal intoxication cases as the method provided acceptable ranges of accuracy and repeatability as well as low limit of detections (LODs) and limit of quantitations (LOQs).

Gunnar *et al.* (2007) reported the use of GC-MS to analyse 11 types of ATS and their typical metabolites in urine, blood and hair specimens using the trifluoroacetylation reagent, *N*-methyl bis(trifluoroacetamide) (MBTFA) as the derivatising agent. They developed a two-step auto-injector for automated on-column derivatization and subsequent GC–MS of amine-type drugs and metabolites. This method was successfully applied for the determination of ATS in the blood and other respective biological specimens.

2.1.4.2.2 Liquid Chromatography Tandem Mass Spectrometer (LC-MS/MS)

To date, the introduction of LC-MS/MS has brought an enormous increase in reliability and sensitivity both in clinical toxicology as well as in forensic toxicology (Roman *et al.*, 2008). When comparing the GC and LC method in the analysis of illicit drugs in the biological specimens, LC is preferred for the analysis of thermally labile drugs since no heating is required and no limitation to the molecular sizes.

However, LC provides lower sensitivity when compared to GC since the drug compounds will co-interact with both mobile phase and solid phase (Miller, 2004). Despite, its coupling with MS/MS in DBS analyses has been reported to be more popular. The reasons are likely due to the enhanced specificity/selectivity and sensitivity afforded by LC-MS/MS for blood spot analysis; accompanied with a significantly faster (advanced

of LC technology) and better cost-effective process compared to GC-MS (Zhang *et al.*, 2012; Meesters *et al.*, 2013). Furthermore, advanced ultra-high-performance liquid chromatography (UHPLC) technology, which delivers substantial performance enhancements over conventional HPLC (Dong and Zhang, 2014) has boosted the run time and resolution of peak separation which is much more effective than GC.

Rapid technological advances over the last decade allowed the for higher levels of sensitivity and specificity in LC-MS analysis. In particular, introduction of the triple quadrupole detectors (QQQ) operating in multiple reaction monitoring mode (MRM), the new mass analysers, *i.e.* ToF (time-of-flight) and Orbitrap for high-resolution measurements and determination of exact masses, permits the identification and quantitation of drugs which are normally present in low concentrations in biological specimens (Dong and Zhang, 2014; Li and Tse, 2010; Thomas *et al.*, 2012). To date, QQQ remains the gold standard for quantification and is used in most of the drugs studies (Deglon *et al.*, 2015).

It was reported that the analysis of DBS specimens can be performed through direct MS methods and surface sampling techniques coupled with MS, which opposed to the pre-analytical clean-up and chromatographic front-end separation (Crawford *et al.*, 2011). Generation of ions from the surface utilizing desorption electrospray ionisation (DESI), direct analysis in real time (DART), and direct electro spray ionisation mass spectrometry (ESI-MS) eliminate the needs for purification or derivatisation process (Crawford *et al.*, 2011; Cody and Dane, 2010; Wang *et al.*, 2013; Wild *et al.*, 2001; Takats *et al.*, 2004; Wiseman *et al.*, 2014; Wiseman *et al.*, 2010). However, removal of the primary specimen purification and separation in the sample preparation steps may lead to the loss of sensitivity and precision due to metabolite interferences since no-clean up or special treatment performed prior to chromatographic analysis. (Wiseman *et al.*, 2010).

Saussereau *et al.* (2012) reported the application of LC–MS/MS for simultaneous detection of illicit drugs, including opiates, cocaines, and amphetamines in whole blood using DBS sampling method, with on-line extraction step. The illicit drugs are opiates (morphine and its 3- and 6-glucuronide metabolites, codeine, 6-monoacetylmorphine), cocaines (ecgonine methyl ester, benzoylecgonine, cocaine, cocaethylene) and amphetamines (amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), 3,4-Methylenedioxy-N-ethylamphetamine (MDEA). Significant correlations were obtained in authentic specimens of drivers suspected to drive under influence of drugs. Thus, DBS analysis has potential as an option for the illicit drugs determination in small blood volumes.

Jantos *et al.* (2011) compared the analysis results of MDMA and its metabolite of MDA from whole blood specimens and DBS using LC-MS/MS. 20 volunteers participated in a controlled experiment of driving under the influence of MDMA. They discovered that the statistical analyses results (mean concentration ratio of corresponding whole blood and DBS specimens, t-test, Bland-Altman difference plot) revealed that DBS method did not significantly differ from whole blood specimens with respect to the MDMA or MDA. Hence, DBS analysis has potential as a precise and inexpensive alternative to whole blood analysis of MDMA.

Subsequently, Jantos and Skopp (2011) also performed another similar study on the analysis of ATS in DBS specimen using LC-MS/MS. Besides MDMA and MDA, they widened the scope of illicit drugs by including dexamphetamine, alprazolam, risperidone and its active metabolite of 9-hydroxyrisperidone and zopiclone. The results portrayed that all analytes except zopiclone (degradation due to different storage temperature) from DBS precisely matched with those from the whole blood.

Versace *et al.* (2013) developed an automated on-line DBS extraction procedure prior to rapid Hilic/RP LC–MS/MS target screening of drugs as a complementary tool for systematic toxicological analysis (STA). 22 model illicit drugs that are commonly encountered in forensic investigations, *i.e.* cocaine, benzodiazepines, amphetamines, opioids, antidepressants, and antipsychotics were tested and runs on two different columns as well as on 34 authentic post mortem specimens. The findings showed that the coupling of DBS with LC–MS/MS for screening approach provided very satisfactory results when compared to the routine screening procedures.

Several other publications also reported on the utilisation of UHPLC-MS/MS to identify and quantify ATS drugs as well as other psychoactive drugs in the DBS specimens. Kyriakou *et al.* (2016) developed and validated an UHPLC–MS/MS method for simultaneous determination of 23 types of psychoactive drugs in the DBS, including cocaine, opiates, benzodiazepines, amphetamines, tetrahydrocannabinol (THC) and some of their metabolites. The proposed method has been applied in real cases of individuals admitted to the hospital emergency rooms for acute intoxication related to the consumption of principal psychoactive drugs.

Odoardi *et al.* (2014) also utilised UHPLC-MS/MS for the analysis of psychoactive drugs including ATS. They validated the UHPLC-MS/MS method for the analysis of drugs of abuse and their metabolites from different chemical classes *i.e.* opiates, methadone, fentanyl and analogues, cocaine, amphetamines and amphetamine-like substances, ketamine and LSD in post-mortem human blood specimens using DBS. Different from the general method, the blood specimens were mixed with internal standards prior to spotting on the card with the purpose to reduce the spotting variability that could affect quantitative results.

The method was validated and applied to authentic blood specimens from autopsy cases. A simultaneous determination method for 11 illicit drugs using the dried blood spot (DBS) sampling technique combined with the UPLC–MS/MS technology was developed to study its applicability within the forensic toxicology.

The application of LC methods in the analysis of illicit drugs in DBS specimens were highly demanded as another study analyzed 57 compounds using ultra-high-performance liquid chromatography-ion booster-quadrupole time-of-flight mass spectrometry (UHPLC-IB-QTOF-MS) by DBS sampling technique.

The list of targeted compounds in this study includes amphetamines, opioids, cocaine, benzodiazepines, barbiturates, and many other new and emerging abused drugs. The purpose of implementing IB with extra heated spray zone is to increase sensitivity for low-concentration drugs in the DBS specimens. The researchers also stated that this was the first study developed using simple and efficient UHPLC-IB-QTOF-MS for a wide range of abused drugs using a DBS sampling technique (Chepayala *et al.*, 2017).

While in the context of doping control, new type of MS combining a quadrupole mass filter, higher collision dissociation (HCD) cell and an Orbitrap detector was evaluated for the qualitative and quantitative analyses performed on the DBS specimens. 26 types of chemical compounds of interest in the context of doping control which include cocaine, MDA and MDMA were detected, providing an alternative sensitive assay to reveal the misuse of performance enhancing substances in sports.

Lee *et al.* (2015) validated the DBS procedure and LC–MS/MS conditions for 18 benzodiazepines, seven benzodiazepine metabolites, and one z-drug (zolpidem) in blood. Optimised specimen preparation prior to LC-MS/MS analysis (DBS card type, extraction solvent, extraction method and extraction time) was also performed.

While Antelo-Domínguez, Ángel Cocho (1998) utilised direct electrospray ionisation tandem mass spectrometry (ESI-MS/MS) for detection of cocaine, BZE, morphine, codeine, and 6-Monoacetylmorphine (6-MAM) from the whole blood on the DBS specimens after undergoing the designated, simple and low cost specimen pre-treatment method. The ESI-MS/MS has been selected as the preferred technique for screening and confirmatory studies due to the short acquisition time (approximately 90 s for each run).

2.2 Overview of Dried Blood Stain (DBS) Technique

Dried blood stain (DBS) technique is an emerging science in forensic field. It was first described by Ivar Bang a century ago and was popularized by Dr. Robert Guthrie in the early 1960s for the detection of phenylketonuria; a type of rare inherited disorder, based on the elevation of phenylalanine in the blood of new born infants (Guthrie and Susie, 1963). From 'cradle' *via* neonatal screening to 'grave' due to forensic purposes, the use of this technique has been widespread and accepted worldwide to complement the established whole blood specimen analysis.

The interest in using DBS for drugs detection in toxicology has recently increased significantly. From therapeutic drug monitoring (TDM) for drug identification and monitoring in clinical toxicology (therapeutic, toxic and lethal) (Li and Tse, 2010; Stolk and Edelbroek, 2011; Morrison *et al.*, 2015), toxico-pharmacokinetics study (Clark *et al.*, 2010; Crawford *et al.*, 2011) and diagnostic screening (Mercader *et al.*, 2006; Strasser *et al.*, 2007; Allard *et al.*, 2004), the wide application of DBS technique has made it highly desirable. The interests have expanded to determination of drugs and poisons in forensic toxicology (discussed in **analytical techniques** above), determination of biomarkers in alcohol abuse cases (Fuller *et al.*, 2009; Winkler *et al.*, 2011), trace-elements and metals analysis in blood and serum such as Pb (lead), Zn (Zinc), CuSO4 (Copper Sulphate), Fe (Ferum), *etc.* (Shen *et al.*, 2003).

This technique has also been applied to other types of biological fluids, such as DUS, cerebrospinal fluid (Rago *et al.*, 2011; Delaby *et al.*, 2014), amniotic fluid (Inoue and Ohse, 2011) or saliva (Sakhi *et al.*, 2015). Biological specimens deposited and dried on a filter paper received the generic name of dried matrix spots (DMS) (Resano *et al.*, 2017).

The advantages of DBS technique include the involvement of less invasive procedures, small blood volume, simple storage method, cheap specimen shipment, less risk of blood borne pathogens infection over conventional blood or plasma sampling since it is a dry matrix, ease of specimen collection, stable under ambient conditions and able to represent the stabilisation of various analytes compared with whole blood specimens despite of low temperatures storage (Sadler *et al.*, 2018; Demirev, 2013; Sharma *et al.*, 2014; Tretzel *et al.*, 2014; Andriamandimby *et al.*, 2013; Smit *et al.*, 2014).

For a forensic toxicology laboratory, the application of DBS provides an alternative to store small specimen volumes storage, especially advantages in closed cases when most of the evidences were discarded. Thus, a more economical, simple and less space of storing specimens for long period of time can be performed. As stated by Dubey and Caplan (Dubey and Caplan, 1996) when for one reason or another that a case is to be reopened, there is at least some material left, potentially allowing targeted analysis even though only stable analytes that remain for an extended period of time.

The DBS technique is in demand to overcome the high potential and frequency of viral infection occurred among the individuals that having a drug abuse history (Stove *et al.*, 2012). The dry matrix condition of DBS reduces enzyme activity and microbial degradation process and increase stabilisation for the substances. Hence, this technique offers longer storage periods of blood specimens (Alfazil and Anderson, 2008; Boy *et al.*, 2008).

2.3 Problem Statement: ATS drugs Analysis for Forensic Toxicology Cases in Malaysia

The high volume of blood is always available in post-mortem medico legal cases (Yarema and Becker, 2005), whereas in cases related to ante-mortem such as sexual assaulted cases, child and new-born abuses, *etc.* the volume obtained is always minute, *i.e.* less than 3 mL and as such, some of the analyses meet an inconclusive result in the court of justice. The need for full toxicology analysis is vital in the STA protocol. The value of the drugs and metabolites obtained from quantitative analysis is important for the interpretation of forensic toxicology cases, which describes the level of effects of the drugs in the body prior to incidence/death.

ATS drugs are normally stable and the level of the drugs in the blood are always high especially in the cases of drug-trafficking, suicide, murder and certain sudden death incidences (Dept of Chemistry Malaysia, 2016). The small volume of specimen in whole blood resulted in partial evaluation, where some cases managed to provide estimation without complete valid results.

The choice of analytical techniques and stability of drugs in biological specimens have raised some issues in relation to the qualitative and quantitative analyses. Problems with the post-mortem re-distribution of drugs, endogenous factors, instability of drugs and metabolites during transportation and storage and also the effects of external factors (*i.e.* light, temperature, pH, water) as well as suitability of the analytical techniques for detection low amounts of analytes (Wang *et al.*, 2015) are some of the challenges encountered by laboratories and forensic toxicology practitioners.

With the short turn-around-time in toxicology analysis, the higher confidence level was provided by LC-MS/MS using the Multi-Reaction-Monitoring (MRM) mode

(SANTE EU, 2015; SANCO EU, 2016), whereby other than relying on the qualifier ratio, the other mode such as library matching, MS^n, etc. is needed to support the evidence.

The importance of differentiating isomeric compounds in ATS drugs such as pseudoephedrine/ephedrine and phentermine/methamphetamine in the analysis is also a highlight due to different effects and charges/penalties from the use/abuse. For example, consuming phentermine to maintain body mass index (BMI) in the Malaysia Royal Armed Forces is permitted under prescription by physician, but having methamphetamine is totally prohibited under the Armed Forces Acts 1972.

Despite of the increasing trend of DBS technique in medical, the application of this convenient sampling technique to abused drugs is still rare (Chepyala *et al.*, 2017). On the other hand, even though a vast volume of dried blood stain methodologies has been reported in literatures, these analytical procedures when applied to the specimens obtained from local forensic cases as not be as effective as it reported and the results vary significantly due to several factors such as types of background cases, epidemiology and population, prevalence of drugs of abuse and instrumentation used.

Alternatively, method development and method optimization should be performed to establish a new methodology that is fit for drugs detection in DBS purpose. The methodology considered in this study emphasized the following:

1) Accuracy (following EU Guidelines for LC-MS/MS)

2) Simple and less training is required

3) Suitable for routine analysis

4) Economic and rapid

5) Repeatable and reproducible

6) Less specimen preparation effort

7) Small specimen size (100 μL) of blood specimen

The time and cost are the main concerns for effective implemented in real case situations. The study emphasizes in facilitating the numbers of drugs with the precursors, isomeric compound and metabolites in a single method, shorter analysis and cost effective. Minimum used of chemicals and disposable consumables are considered in this study as well. The methodologies are also planned in line with the availability of the required facilities.

2.4 Scope and Objective of Study

The scope of this study is to investigate the enhanced modes of Liquid Chromatography Tandem Mass Spectrometer (LC-MS/MS) for higher confidence level in the detection of ATS drugs in forensic toxicology field. The main purpose of this research is the development and validation of novel analytical methods, and subsequently applied in the analysis of ATS in dried blood stain (DBS) matrix as an innovative, simple and efficient technique for drugs detection.

The suitability of DBS as an alternative medium is evaluated and compared to that of whole blood specimen (WBS) medium. The reliability of the outcome for both the techniques and application of DBS medium were tested in order for these methodologies to be applied in the worldwide forensics field. The present research work is divided into four main tasks, from which independent conclusions are drawn.:

Task 1: Multi-period-multi-experiment of MRM-EPI-MRM^3 with library matching for simultaneous determination of ATS in WBS, urine and DBS.

Objectives: i) to develop a simple and rapid method for simultaneous determination of ATS drugs using liquid chromatography with electrospray ionization tandem mass spectrometer (LC-ESI-MS-MS).

ii) to create an experimental flow from normal Multi-Reaction Mode (MRM) to Multi-Reaction Mode-Enhanced Product Ion Mode-Multi-Reaction Mode 3 (MRM-EPI-MRM^3) for higher confidence level (utilizing EPI) and unique identification (utilizing MRM^3).

iii) to optimize the chromatographic conditions and the ESI parameters in the LC-MS/MS method.

iv) to apply the developed and validated method in WBS, DBS as well as urine specimens as well as to several real forensic cases in Malaysia.

Task 2: High-throughput flow injection analysis-MRM^3 (FIA-MRM^3) spectrometry for alternative fast screening of isomeric compounds in blood for forensic toxicology cases in Malaysia.

Objectives: i) to identify critical isomeric compounds related to ATS, *i.e.* methamphetamine and phentermine without LC separation.

ii) to optimize the experimental flow in LC part to shorten the analysis time and to set MRM^3 experiment in the MS part in order to identify the presence of methamphetamine or/and phentermine.

iii) to evaluate the performance and reliability of the technique using data/sample from real forensic cases in Malaysia.

Task 3: Bland-Altman statistical analysis and mean concentration ratio for the determination of ATS in DBS vs WBS by LC-MS/MS- Application to forensic toxicology cases in Malaysia.

Objectives: i) to apply Bland-Altman statistical analysis in the comparative study of DBS versus WBS.

ii) to complement the Bland-Altman with additional mean concentration ratio analysis to evaluate the reliability of DBS method versus WBS method.

iii) to apply both statistical analyses to external quality controls from Proficiency Testing (PT) samples as well as real cases samples to establish

the performance of DBS as an alternative and innovative method for forensic analytical purposes.

Task 4: Stability and efficiency of DBS medium for the analysis of ATS drugs during sample storage- Application to forensic toxicology cases in Malaysia.

Objectives: i) to evaluate the stability and efficiency of DBS medium for the analysis of ATS during 6-month sample storage which include the use of several conditions; *i.e* time and temperature in Malaysia as the variants.

ii) to apply the evaluation study to real cases samples to establish their stability and efficiency to be applied in the future.

CHAPTER 3: METHODOLOGY

Four specific tasks were undertaken in this study to investigate the enhanced mode of detection in LC-MS/MS for amphetamine type stimulants drugs in biological samples. The mediums for the study included WBS, urine as well as DBS as an alternative technique in the forensic toxicology field. In order to obtain maximum validity of the investigation, the following tasks were performed:

- **Task 1**: Multi-period-multi-experiment of MRM-EPI-MRM^3 spectrometry with library matching for simultaneous determination of ATS drugs in WBS, urine and DBS.
- **Task 2**: Flow Injection Analysis-MRM^3 (FIA-MRM^3) spectrometry for the alternative screening in the differentiation of critical isomeric compound for ATS drugs in WBS and DBS.
- **Task 3**: Evaluation of DBS study: Use of Bland-Altman analysis and Mean Concentration Ratio for the DBS versus WBS by LC-MS/MS.
- **Task 4**: Evaluation of DBS study: stability of DBS medium for ATS drugs during sample storage.

3.1 Multi-period-multi-experiment of MRM-EPI-MRM^3 spectrometry with library matching for simultaneous determination of ATS drugs in WBS, urine and DBS.

3.1.1 General

There are ample of methods for detecting ATS in biological specimens from the screening to qualitative and quantitative parameters. Most of the methods have been reviewed and discussed earlier in Chapter 2 with all the significances and limitations highlighted. The efforts have shown that serious concerns have been undertaken continuously from international communities worldwide to combat and overcome the abuse of drugs.

In recent times, LC-MS/MS techniques has been developed for ATS analyses, which involved simpler sample preparation procedures as well as lesser analytical concerns. MRM is one of the most common detection mode used for identification and quantification in LC-MS/MS analysis. Despite its popularity, very few studies were performed for the detection and identification of ATS in biological matrices. Hence, it provided a window of opportunity to develop the fast, robust and efficient LC-MS/MS method for the determination and confirmation of ATS in biological matrices.

The purpose of the present investigation is to establish and validate an efficient and robust method for the analysis of ATS- related drugs in biological matrices, including dried blood stain (DBS). A unique multi-period and multi-experiment method is developed and employed with higher degree of confidence for identification using library matching confirmation and has applied succesfully for real sample monitoring. ATS drugs investigated in this study includes the common methamphetamine and MDMA together with the precursors of pseudoephedrine and ephedrine, metabolites

(amphetamine, MDA, MDEA), and isomeric compound of phentermine. These ATS compounds are analyzed and discussed together throughout the study.

3.1.2 Materials and methods

3.1.2.1 *General*

Analytical balance for weighing chemical standards was from Sartorius MC210S (accuracy = 0.00001 g). 10 mL polypropylene centrifuge tubes with screw caps and 10 mL glass test tubes were purchased from Fischer Scientific (USA). 2 mL glass vials with inserts and caps were acquired from Agilent Tech (USA). Micropipettes were from Eppendorf (Germany) and Gilson (Illinois, USA) while Savant concentrator and centrifuge from Thermo Scientific (USA). pH meter was acquired from Mettler Toledo (Australia), roller mixer from Scientific Industries (USA) and multi-pulsed vortexer from Glas-Col, Terre Haute (USA). Whatman® FTA® card was purchased from Merck (Darmstadt, Germany) and LC column of C18 Luna Omega analytical column (100 mm x 2.1 mm x 1.6 µm i.d from Phenomenex (USA).

3.1.2.2 *Chemicals*

Certified reference materials (CRM): amphetamine (AMP), methamphetamine (MA), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxy-*N*-methylamphetamine (MDMA), 3,4-methylenedioxy-*N*-ethylamphetamine (MDEA), pseudoephedrine (PEP), ephedrine (EP), phentermine (PTM), methamphetamine-d_{14} (MA-d_{14}) and 3,4-methylenedioxy-*N*-methylamphetamine-d_5 (MDMA-d_5) were purchased from Lipomed (Switzerland). Acetonitrile and methanol were HPLC grade (Merck, Darmstadt, Germany). Chlorobutane was acquired from Fischer Chemical (Loughborough, Leicestershire, UK). Formic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was from arium® pro UV Ultrapure Water with a

specific resistance at > 18 MΩcm. Phosphate buffer (20 mM, pH 7.4) was prepared by dissolving monobasic sodium phosphate in water, followed by adding sodium hydroxide to adjust the pH (phosphate buffer releases Hb from the stain and lead to best recovery).

3.1.2.3 *Sample collection and pre-treatment*

Blank blood samples were obtained from bull's blood while blank urine samples were collected from laboratory personnel volunteers. Both blood and urine samples have been tested earlier and were used in the preparation of calibration solutions and matrix matched analysis.

Whole blood and urine samples, n=20 respectively, with positive identification of ATS drugs were obtained from various real cases submitted for forensic and medico-legal analysis from January 2016 until September 2017 to Forensic Division, Department of Chemistry Malaysia. The whole blood samples (n=20) were individually stained onto Whatman FTA cards for DBS analysis.

External Quality Controls (QC) consisted of whole blood sample tested for Proficiency Testing provided by College of American Pathologists (CAP), USA and Internal Proficiency Testing by Proficiency Testing Unit, Research and Quality Assurance Division, Department of Chemistry, Malaysia. While for urine sample, the QC acquired from previous UNODC external Proficiency Testing participated by the laboratory of Department of Chemistry, Malaysia. All samples were stored at 4º C until the time of analysis.

3.1.2.4 *UHPLC Conditions*

An Exion LC SCIEX Binary SL Series System (Toronto, Canada) consisting of a binary pump and an autosampler with cooling system set at 10 ºC. The injection volume was 5 µL. Mobile phase (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid with flow rate of 300 µL/min on a C18 Luna Omega reversed-phase analytical column (100 mm x 2.1 mm x 1.6 µm; Phenomenex, USA). Autosampler was set at 20 ºC and column temperature was at 40 ºC. The LC gradient was initially set at 5% of component B within 2 min; to 10% for 3 min and to 20% for 1 min. The gradient was increased linearly at 90% for 1 min and remained isocratic for another 1 min followed by equilibration for 2 min to initial condition resulting 10 min for total run time.

3.1.2.5 *Mass Spectrometry Conditions*

A SCIEX 5500 hybrid QTRAP tandem mass spectrometry system (Toronto, Canada) equipped with patented Turbo V source was used. Compound ionisation was performed using ESI and set at positive mode. Heating gas temperature was held at 550ºC and ion spray (IS) voltage of 5500 V. The nebulizing gas (GS1), heating gas (GS2) and curtain gas pressures were set at 40, 40 and 20 psi, respectively throughout the analysis. Purified nitrogen gas was used as collision and spray gas.

Analyst software version 1.6.3 together with MultiQuant version 3.0 were used. Mass spectrometry scan modes of MRM, enhanced product ion (EPI) and (MRM^3) experimental conditions for individual analytes were optimized by post-column infusion of working solution (100 ng/mL) using Analyst Software for all targeted analytes. The optimized parameters included AF2 voltage, Declustering Potential and Collision Energy to get the best ions (precursor ion, 2nd precursor) for each particular compound. The summary of the parameters is shown in Table 3.1.

Table 3.1: Detailed LC-MS/MS instrument parameter and settings on multi period and multi experiment of MRM – EPI – MRM^3

Compound		Experiment in Period 1			
		MRM^3			
	m/z	1st precursor	2nd precursor	AF2 Voltage	Collision Energy
Ephedrine	166.2	166.2	148.2	0.07	15
Pseudoephedrine	166.2	166.2	148.2	0.07	15
		EPI			
		Precursor Ion	Fragment Mass Scan Range	Declustering Potential	Collision Energy
		166.2	50-200	80	35
		166.2	50-200	80	35
		MRM			
		Precursor Ion	2nd precursor	Declustering Potential	Collision Energy
		166.2	148.2	70	15
		166.2	115.2	70	35
Compound		Experiment in Period 2			
		MRM^3			
	m/z	1st precursor	2nd precursor	AF2 Voltage	Collision Energy
Amphetamine	136.1	136.1	119	0.06	15
		EPI			
		Precursor Ion	Fragment Mass Scan Range	Declustering Potential	Collision Energy
		136.1	50-150	80	30

Table 3.1: Detailed LC-MS/MS instrument parameter and settings on multi period and multi experiment of MRM – EPI – MRM^3, cont.

		MRM			
		Precursor Ion	2nd precursor	Declustering Potential	Collision Energy
		136.1	119.1	70	11
		136.1	91.1	70	21
Compound		Experiment in Period 3			
		MRM^3			
	m/z	1st precursor	2nd precursor	AF2 Voltage	Collision Energy
MDA	180	180	163	0.07	25
		EPI			
		Precursor Ion	Fragment Mass Scan Range	Declustering Potential	Collision Energy
		180	50-200	80	25
		MRM			
		Precursor Ion	2nd precursor	Declustering Potential	Collision Energy
		180	105	70	29
		180	133	70	11
		MRM^3			
	m/z	1st precursor	2nd precursor	AF2 Voltage	Collision Energy
Methamphetamine	150.1	150.1	119.1	0.06	35
		EPI			
		Precursor Ion	Fragment Mass Scan Range	Declustering Potential	Collision Energy
		150.1	50-200	80	25

Table 3.1: Detailed LC-MS/MS instrument parameter and settings on multi period and multi experiment of MRM – EPI – MRM^3, cont.

		MRM			
		Precursor Ion	2nd precursor	Declustering Potential	Collision Energy
		150.1	119.1	100	15
		150.1	91	100	23
		MRM^3			
	m/z	1st precursor	2nd precursor	AF2 Voltage	Collision Energy
d_{14}-Methamphetamine	164	164	130	0.04	20
		EPI			
		Precursor Ion	Fragment Mass Scan Range	Declustering Potential	Collision Energy
		164	50-200	80	30
		MRM			
		Precursor Ion	2nd precursor	Declustering Potential	Collision Energy
		164	98	80	25
		164	130	80	15
Compound		Experiment in Period 4			
		MRM^3			
	m/z	1st precursor	2nd precursor	AF2 Voltage	Collision Energy
d_5-MDMA	199	199	165	0.06	14
		EPI			
		Precursor Ion	Fragment Mass Scan Range	Declustering Potential	Collision Energy

Table 3.1: Detailed LC-MS/MS instrument parameter and settings on multi period and multi experiment of MRM – EPI – MRM^3, cont.

		199	50-250	80	30
		MRM			
		Precursor Ion	2nd precursor	Declustering Potential	Collision Energy
		199	165	70	16
		199	135	70	20
		MRM^3			
	m/z	1st precursor	2nd precursor	AF2 Voltage	Collision Energy
MDMA	194	194	163.1	0.08	22
		EPI			
		Precursor Ion	Fragment Mass Scan Range	Declustering Potential	Collision Energy
		194	50-200	80	30
		MRM			
		Precursor Ion	2nd precursor	Declustering Potential	Collision Energy
		194	163.1	86	15
		194	105.1	86	31
		MRM^3			
	m/z	1st precursor	2nd precursor	AF2 Voltage	Collision Energy
Phentermine	150	150	91.1	0.16	44
		EPI			
		Precursor Ion	Fragment Mass Scan Range	Declustering Potential	Collision Energy
		150	50-200	80	30

Table 3.1: Detailed LC-MS/MS instrument parameter and settings on multi period and multi experiment of MRM – EPI – MRM^3, cont.

		MRM			
		Precursor Ion	2nd precursor	Declustering Potential	Collision Energy
		150	133	100	15
		150	91	100	22
Compound		Experiment in Period 5			
		MRM^3			
	m/z	1st precursor	2nd precursor	AF2 Voltage	Collision Energy
MDEA	208.1	208.1	163	0.06	20
		EPI			
		Precursor Ion	Fragment Mass Scan Range	Declustering Potential	Collision Energy
		208.1	50-250	80	30
		MRM			
		Precursor Ion	2nd precursor	Declustering Potential	Collision Energy
		208.1	163	70	17
		208.1	135	70	29

3.1.2.5.1 *Mass Spectrometry* Calibration

Calibration and optimization of the quadrupole in mass spectrometry is crucial in order to ensure that MS/MS is in a good condition prior to the analysis. The process was performed manually using common calibrant of polypropylene glycol (PPG) in direct infusion via syringe pump and was done in positive ion mode using Turbo Ion Spray. This is in conjunction with the tested analytes that all in positive mode and accurate masses for those analytes is needed in library matching. The measurement covered Q1 Scan (Q1 MS) and Q3 Scan with the masses (amu) setting of 59.1, 175.0, 616.5, 906.7, 1254.9 and 1545.1.

The PPG standard solution specifications for Q1 and Q3 quadrupoles in positive mode must meet the requirement as in the following:

Table 3.2: Intensity specifications for PPG standard solution in positive ion mode

Nominal m/z	Q1 (cps)	Q3 (cps)	FWHH (amu)
59.050	2.0×10^6	1.0×10^6	0.6 – 0.8
175.133	2.0×10^6	1.0×10^6	0.6 – 0.8
616.464	2.5×10^6	1.0×10^6	0.6 – 0.8
906.673	1.5×10^7	8.0×10^6	0.6 – 0.8
1254.925	2.0×10^6	1.0×10^5	0.6 – 0.8
1545.134	5.0×10^5	N/A	0.6 – 0.85

(Source: SCIEX University guidelines for LC-MS/MS)

3.1.2.5.2 *MRM-EPI-MRM^3 Workflow*

The workflow for multi-experiment of MRM-EPI-MRM^3 is summarized as follows:

a) <u>Multiple Reaction Monitoring (MRM)</u>

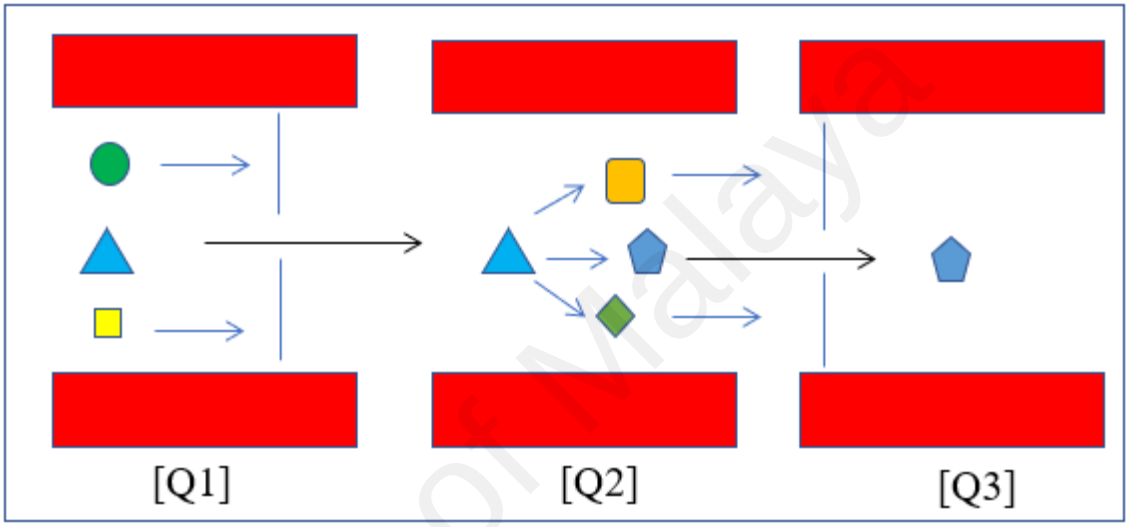


Figure 3.1: Multiple Reaction Monitoring (MRM)

MRM is normally applied in LC-MS/MS as a highly specific and sensitive mass spectrometry technique that can selectively identify and quantify compounds within complex mixtures. The experiment analyzed targeted compounds with product ions as quantifier and qualifier.

The processes include:

1) Precursor ions selected in Q1
2) Fragmentation of precursor occurs in Q2
3) Product ions selected in Q3

In MS/MS, the scan types include:

1) Product Ion Scan (Q1 is fixed at a certain mass, Q3 is scanning out all masses)
2) Precursor Ion Scan (Q1 is scanning out all masses, Q3 is fixed at a certain mass)
3) MRM

Note: Q2 is filled with N_2 gas for fragmentation.

b) <u>Enhanced Product Ion (EPI)</u>

EPI is applied in order to improve sensitivity for product ions and to achieve faster scanning speed that suits for high throughput and highly confident analysis. In this experiment, Q3 operated as Linear Ion Trap (LIT) that filtered and trapped product ion (figure 3.2).

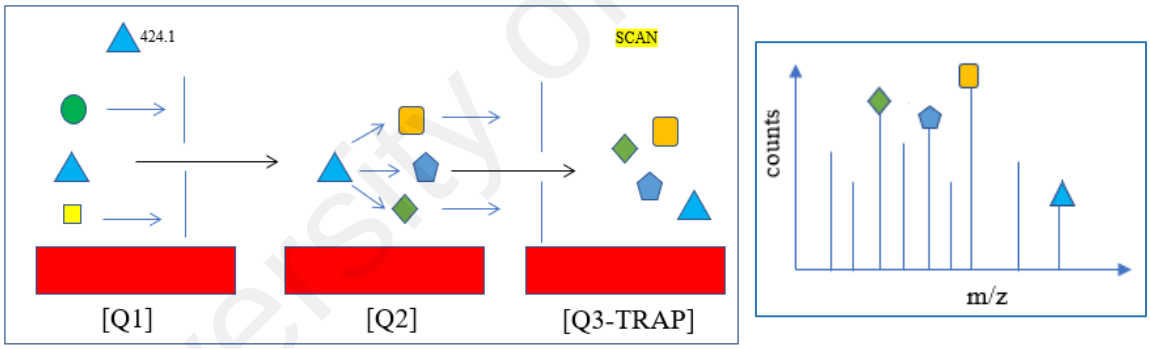


Figure 3.2: Enhanced Product Ion (EPI)

The processes include:

1) Precursor ions selected in Q1
2) Fragmentation of precursor occurs in Q2
3) Trap products in LIT
4) Mass scan out of LIT

In MS/MS, the scan types include:

1) Product Ion Scan (Q1 is fixed at a certain mass, Q3 is scanning out all masses)
2) Enhanced Product Ion Scan (Q1 is scanning out all masses, Q3 is fixed at a certain mass)

c) <u>MS^3 / MRM^3</u>

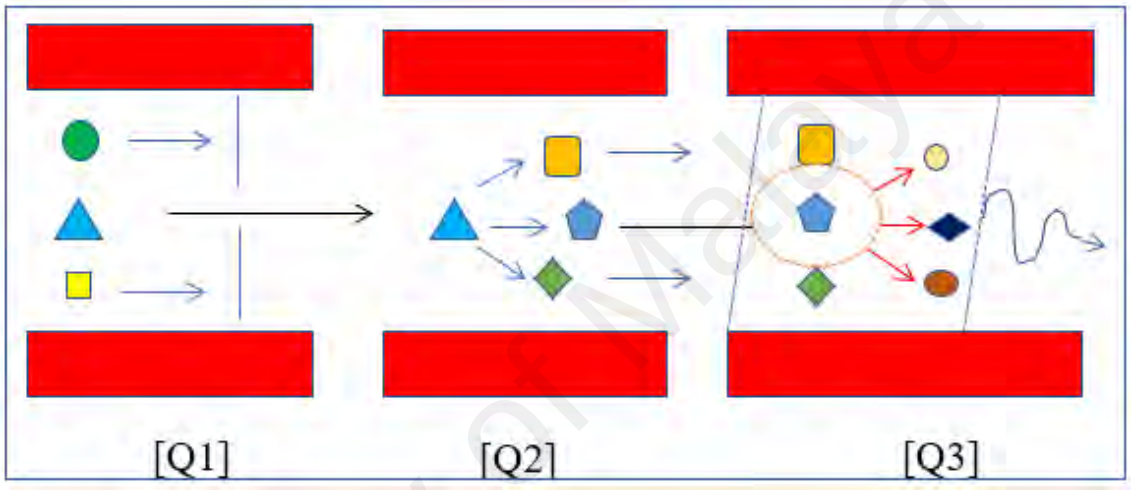


Figure 3.3: MS^3 / MRM^3

The experiment is employed for higher confidence level of detection especially for isomeric compounds in the study. The MS^3 provides the ability to further fragmentize product ions using TRAP fragmentation in MS/MS. As in EPI, Q3 also operated as a linear ion trap (LIT) that filtered and trapped product ions. LIT isolated second precursor ion and generated second generation of product ions.

The processes include:

1) Precursor ions selected in Q1
2) Fragmentation of precursor occurs in Q2
3) Trap products in LIT

4) Mass scan out of LIT (second generation of product ions)

In MS/MS, the scan types include:

1) Product Ion Scan (Q1 is fixed at a certain mass, Q3 is scanning out all masses)
2) MS^3 Scan (Q1 is scanning out all masses, Q3 is fixed at a certain mass)

3.1.2.6 Mixed standard and internal standard solutions preparation

3.1.2.6.1 Stock standard solutions in methanol - A (10 mg/mL)

Stock standard solutions consisted of AMP, MA, MDMA, MDA, MDEA, EP, PEP, and PTM. Each of the solutions was prepared in the concentration of 10 mg/mL.

3.1.2.6.2 Working mixed standards solution in methanol – B (1 µg/mL)

Working mixed standards solution was prepared by pipetting 1000 µL of each stock standard solutions (A) into a 10 mL volumetric flask and made up to volume with methanol (1mg/mL). Subsequently 1000 µL of mixed standard solution (1 mg/mL) was pipetted into a 10 mL volumetric flask and made up to volume with methanol (1 µg/mL).

3.1.2.6.3 Internal standard stock solutions in methanol – C (10 mg/mL)

Internal standard stock solutions consisted of methamphetamine-d_{14} and MDMA-d_5, each with the concentration of 10 mg/mL.

3.1.2.6.4 Working mixed internal standards solution in methanol – D (1 µg/mL)

Working mixed internal standards solution was prepared by pipetting 1000 µL of each internal standard stock solutions (C) into a 10 mL volumetric flask and made up to volume with methanol (1 mg/mL). The mixed internal standard solution (1 mg/mL)

was then pipetted into a 10 mL volumetric flask and made up to volume with methanol (1 μg/mL).

Calibration solutions for AMP, MA, MDA, MDMA, MDEA, PEP, EP, and PTM were prepared in blank blood and blank urine at six different concentrations of 5 ng/mL, 10, 20, 50, 100 and 200 ng/mL. Seven replicates with concentration of 20 and 100 ng/mL were prepared in blood, DBS and urine samples for method validation.

3.1.2.7 *Sample Preparation*

3.1.2.7.1 Whole Blood and Urine Sample

Whole blood and urine samples were subjected to liquid-liquid extraction (LLE). An aliquot (30 μL) of the mix internal standard; MA-d_{14} and MDMA-d_5 solution was added into 1 mL of the samples, followed by 0.5 mL of phosphate buffer solution and 3 mL of 1-chlorobutane. The samples were then equilibrated on a roller mixer for approximately 1 hour followed by centrifugation at 2000 rpm for 10 mins. The upper organic solvent layer was transferred to a clean tube and dried using rotary evaporator. The dried extract was re-constituted in 80 μL of 50% methanol solution and transferred to an autosampler vial for LC-MS/MS analysis.

3.1.2.7.2 Dried Blood Stain (DBS)

Blood stains were prepared by spotting 100 μL aliquot of blood onto Whatman FTA Card, which were subsequently dried at room temperature overnight. For the extraction procedure, the stained FTA Card were put into test tubes followed by adding 30 μL of the mix internal standard; MA-d_{14} and MDMA-d_5 solution. LLE also performed by adding 1 mL of water followed by 0.5 mL phosphate buffer and 3 mL of 1-

chlorobutane. The next steps followed whole blood and urine samples accordingly for LC-MS/MS analysis.

3.1.2.7.3 Application to real samples

In order to evaluate the applicability of the method for routine applications, 20 samples of each whole blood and urine that previously analyzed using routine LC-MS/MS with MRM mode were re-analyzed using the developed method. The method was subsequently applied to dried blood stain (DBS) to investigate the potential of the validated method in forensic toxicology study. The external quality controls (QC) consisted of whole blood sample tested for Proficiency Testing provided by College of American Pathologists (CAP), USA and Internal Proficiency Testing by Proficiency Testing Unit, Research and Quality Assurance Division, Department of Chemistry, Malaysia as well as the urine sample from UNODC Proficiency Testing also been tested for the study.

3.1.2.7.4 Method Validation

The method validation followed the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology (SWGTOX, 2013) and UNODC Guidance for the Validation of Analytical Methodology and Calibration of Equipment used for Testing of Illicit Drugs in Seized Materials and Biological Samples (UNODC, 2009) including linearity, specificity/selectivity and matrix effect, LOD and LOQ, accuracy and precision (within the laboratory repeatability and/or within the laboratory reproducibility conditions) to determine robustness and carry over.

Calibration curves were plotted to obtain the linearity of the system at six calibration levels ranging between 5 to 200 ng/mL. The curves were constructed using

the peak area ratio of the analyte and internal standard, plotted versus the corresponding concentration and determined by linear regression with a 1/x weighting factor. The reagent-only calibration standards and matrix-matched calibration standards were used to assess the matrix effects.

LOD is defined as the lowest concentration of the analyte that resulted in signal-to-noise ratio of 3:1 while the LOQ is defined as the lowest concentration of the analyte that resulted in signal-to-noise ratio of 10:1. Specificity of the proposed method was assessed by analysing the response in both blank and control samples.

The method accuracy was expressed in as average recoveries of spiked blank matrix at 20 and 100 ng/mL concentration levels. Precision of the method was represented as relative standard deviation (RSD%) of within-laboratory reproducibility analyses. Seven (7) replicates for each set were analyzed during each working day to test the intra-day precision and subsequently during three consecutive days for inter-day precision.

The matrix effects was assessed using standard mixture and matrix-matched or spiked calibration samples. A blank control (free of analytes and internal standard) was analyzed and no significant interfering peaks were observed from the blank matrix. The criteria for matrix effect are set at 75-125% with a 15% maximum allowable coefficient of variation (% CV) (Remane e*t al.*, 2010).

Matrix effect calculated using the following equation (Matuszewski , 2006) :

$$\%\ Matrix\ effect = \frac{Response\ post\text{-}extracted\ spiked\ sample}{Response\ non\text{-}extracted\ neat\ sample} \times 100$$

The post-extracted spiked sample consists of the analytes spiked into blood matrix while the non-extracted neat sample consists of analytes spiked into methanol solution.

While for application to real cases samples, the developed method was applied to whole blood, urine and DBS as well as the Proficiency Testing (PT) samples. 20 samples of each whole blood and urine that previously analyzed using routine LC-MS/MS in MRM mode were re-analyzed again using the developed method. The method was subsequently applied to DBS to evaluate the potential of the method thru the DBS medium for forensic toxicology study.

The external QC consisted of whole blood sample tested for PT provided by College of American Pathologists (CAP), USA and Internal Proficiency Testing by Proficiency Testing Unit, Research and Quality Assurance Division, Department of Chemistry, Malaysia as well as the urine sample from UNODC Proficiency Testing.

3.2 High-throughput Flow Injection Analysis-*MRM*^{3} (FIA-*MRM*^{3}) Spectrometry for Alternative Fast Screening of Isomeric Compounds in Blood for Forensic Toxicology Cases in Malaysia.

3.2.1 General

LC-MS/MS has increasingly been used in clinical and forensic toxicology for the determination and quantification of a wide range of compounds, including differentiation of isomeric compounds in biological samples (Maurer, 2005). Variation within these methods primarily focused on the LC conditions such as the type of column and the solvent selection for mobile phase system. The effect of modifier was also reported (Buse, 2013) to increase LC separation and better peak shape in quantitative analysis.

Flow injection analysis (FIA), in combination with MS/MS is a well-known technique firmly established in many biomedical laboratories. FIA, also termed loop injection, is an alternative approach to LC-MS/MS in which the analytical column is removed and detection or separation occurs within the MS instrument. The method simply utilises a small amount of sample introduced into a stream of carrier mobile phase solution which is delivered continuously into the mass spectrometer. In such cases, the analyte response bypassed chromatographic separation is used for quantification. The principle of the technique is a novel approach to the existing LC-MS/MS methods for the differentiation of isomeric compounds that requires relatively long run time and the use of specilized analytical column. The removal of the analytical column can substantially increase speed of analysis which is a valuable assets in analytical techniques.

The FIA-MS methods were successfully applied in many cases across the sciences including the work of Johnson (2010), Sander *et al.* (2015), and Niesser *et al.* (2012), who have independantly developed FIA-MS techniques for the analysis of endogenous

compounds in human body fluids for medical diagnostics and clinical applications. The capability of this technique has extended to the applications of forensic toxicology, *e.g.* determination of cocaine and opiates in human hair samples (Miguel-Framil *et al.*, 2011), organophosphorus pesticides in porcine (John *et al.*, 2010) as well as in the pharmaceuticals such as determination of metformin in dog serum after intravenous injection in which analysis time has reduced to 2 min (Michel *et al.*, 2015).

In this study, a simple, fast, economical and effective method was demonstrated for analysing critical isomeric compounds in ATS, *i.e.* methamphetamine and phentermine using a new dimension technique of FIA-MRM^3 spectrometry. The developed and validated method was successfully applied in whole blood and dried blood stain (DBS) medium for forensic toxicology cases in Malaysia.

3.2.2 Materials and methods

Apparatus, chemicals, sample collection and pre-treatment and sample preparation for WBS and DBS were similar to those described in section **3.1.2.1, 3.1.2.2**, **3.1.2.3** and **3.1.2.7.**

3.2.2.1 FIA-MRM^3 spectrometry conditions

FIA-MRM^3 spectrometry was performed using an Exion LC SCIEX Binary SL Series System interfaced to SCIEX 5500 Q TRAP system (Toronto, Canada) equipped with a Turboionspray^{TM} interface. MDS Sciex Analyst Software (Version 1.6.3) together with MultiQuant Software (Version 3.0.2) were used during method development, data acquisition, data processing and statistical analysis.

A sample volume of 2 µL was injected using the Exion LC SCIEX auto injector set to 10 ºC at sampling speed of 5.0 µL/s and was delivered thru union connector with

an isocratic mobile phase consisted of water/acetonitrile (50:50, v/v) with 0.1% formic acid at a flow rate of 0.2 mL/min for a run time of 2 mins. MRM^3 was achieved by using ESI in the positive ion mode. The method was performed via MRM- Information Dependent Acquisition (IDA) – MRM^3.

Optimization on the first and second MRM transitions, corresponding to the confirmation and quantification, were conducted via manual infusion to achieve maximum sensitivity. The parameters optimized for the MRM transitions were declustering potential (DP), entrance potential (EP), collision energy (CE) and collision exit potential (CXP). Similarly, on the MRM^3 experiment, the product ions were optimized using the second precursor ions of the MRM confirmation transition. Using the optimized compound settings from the MS/MS, the intensity for the product mass spectra from the second precursors were monitored and recorded.

The excitation energy (AF2) was ramped from 10 mV to 200 mV to achieve the highest product signal counts. Essentially, the AF2 parameters equates to the fragmentation voltage energy transferred towards the isolated second precursor ions in the linear ion trap (LIT). The MS/MS/MS (MS^3) spectra were also collected during MRM3 optimization and updated into the software database package. The monitored ion transitions in MRM^3 for the tested analytes were 150→ 119→ 91 Da (methamphetamine), 150→ 133→ 91 Da (phentermine) and 164→ 130 Da (ISTD methamphetamine-d_{14}). Optimization for sensitivity was also conducted on the source settings and the optimization parameters were summarized in Table 3.3.

Table 3.3: Optimization of source and parameters on MRM-IDA-MRM^3 settings.

		Methamphetamine	**Phentermine**
Source Settings	Heater temperature (^oC)	550	
	Ion Spray voltage (V)	5500	
	Nebulizing gas (Psi)	40	
	Heater gas pressure (Psi)	40	
	Collision gas pressure	Low	
Compound settings	Declustering potential, DP (V)	60	56
	Entrance potential, EP (V)	10	10
	Collision potential, CE (V)	14[a], 23[b]	12[c], 23[d]
	Collision exit potential, CXP (V)	9	9
MRM^3 settings	Excitation energy, AF2 (mV)	85	62

Note: MRM transition corresponding to: [a]150/119; [b]150/91; [c]150/133; [d]150/91.

3.2.2.2 Method Validation

The method validation followed guidelines of UNODC and SWGTOX as in 3.1.2.7.4 and the parameters tested were based on the qualitative and relative quantitation purposes. Several specific assessments for this study included linearity, additional investigation on the cross-analyte investigation as well as the application of this method to the external QCs and real cases samples.

For linearity test, a standard curve of six points (5 to 200 ng/mL) was constructed by determining the best fit of peak-height ratios (peak height ratio of the analyte to internal standard) versus the analyte concentration and running a linear regression analysis weighing the standard curve with 1/x. While in the additional investigation, the

presence of cross-analyte was evaluated since separation between the isomers was not performed in the FIA and the identification of analytes was entirely based on the strength of the MRM^3 mode. The investigation was performed using MultiQuant Software to cross-check the analyte in respective spiked sample.

For the application on forensic toxicology cases in Malaysia, the developed method was applied to whole blood (WBS) and dried blood stain (DBS) as well as the Proficiency Testing (PT) samples. 20 samples of WBS and DBS that were previously analyzed using routine MRM mode and advanced MRM-EPI-MRM^3 modes of LC-MS/MS in the laboratory were re-analyzed using this method.

3.3 Evaluation of DBS study: Use of Bland-Altman analysis and Mean Concentration Ratio for the DBS versus WBS by LC-MS/MS.

3.3.1 General

There are numerous ways to evaluate the methods in comparative study such as t-test, paired t-test, correlation coefficients, R, one-sided or two-sided test to measure the strength of a relation between two variables (Miller and Miller, 2000) in the clinical and forensic studies. A newly developed method needs to be compared against an established method to determine whether these methods can be used interchangeably or the new method can replace the established method (Myles *et al.*, 1996 & 1997; Opdam *et al.*, 2007; Niedhart *et al.*, 2006).

Bland-Altman statistical analysis compares two methods of measurement to determine 1) interchangeable between the methods and 2) replacement of old method by new method (Myles *et al.*, 1996 & 1997). The Bland – Altman method calculates the mean difference between two methods of measurement with 99% limits of agreement as the common mean difference (3SD) or more precisely (2.575 SD). It is estimated that the 99% limits include 99% of differences between the two measurement methods.

The Bland–Altman plots show the difference between method values (y-axis) against the average of method values (x-axis), and provide an assessment of the level of agreement between the two methods (Dewitte *et al.*, 2002). The presentation of the 95% limits of agreement is for visual judgement on how well the measurement of the two methods agrees. The smaller the range between these two limits the better is the agreement (Bland and Altman, 1986).

The preeminent way to use Bland-Altman plot system is to define the bias, limit of acceptable difference (limits of agreement), and determine whether the data points are

within or exceed the stated limits. In order to do so, the mean difference of each drugs analyzed in WBS vs DBS was calculated. Bias of the average difference of mean and the standard deviation was also determined. 99% confidence interval and its standard deviation (SD) was calculated prior to determining the lower limit of agreement and the upper limit of agreement; Bias + 3SD and Bias – 3SD.

The 99% confidence interval of mean difference and of the agreement limits were used in this study to described possible errors in the estimation, *e.g.* from sampling and analysis. The greater the number of samples used for the evaluation of the difference between the methods, the narrower the confidence interval, for the both mean difference and limits of agreement.

Following Bland-Altman method, the SD of the difference between the means of the repeated measurements was calculated based on the within-subject. The Bland-Altman plot for difference of WBS-DBS vs mean difference was constructed. Bias, lower limit, upper limit and each data point for the corresponding drugs was also plotted. The Bland-Altman plot showed the difference between individual measurement and the average of the means. The SD difference for each drug in WBS and DBS was further calculated using Microsoft Excel 2016.

The aim of this study was to compare the performance of dried blood stain (DBS) versus whole blood sample (WBS) methods using Bland-Altman analysis and mean concentration ratio for the determination of ATS drugs by MRM-EPI-MRM^3 method. In this study, the Bland-Altman plots were constructed for the spiked samples and real cases samples (reported positive for the presence of ATS drugs from Government Enforcement Laboratory, Forensic Division of Department of Chemistry, Malaysia) together with the external QCs from International Proficiency Testing of College of American Pathologists (CAP), USA. For the spiked samples, low and high level of ATS drugs spiked into the

WBS and DBS were analyzed, *i.e.* 20 and 100 ng/mL, respectively. The comparison was to determine the quantitative analysis of the drugs using DBS method is equivalent and has potential to be an alternative to WBS method.

3.3.2 Materials and methods

Apparatus, chemicals, sample collection and pre-treatment, sample preparation for WBS and DBS, standards preparation and instrumentation were similar to those described in section **3.1.2.1-3.1.2.7.**

Analyst software version 1.6.3 together with Multi-Quant version 3.0 were used during method development, data acquisition, data processing and statistical analysis. Further assessment for Bland-Altman plot (Bland and Altman, 1999) was done by Microsoft Excel.

Data were monitored with the following MRM transitions: AMP *m/z* 136.1 → 119.1* and 136.1 → 91.1; MA *m/z* 150.1 → 119.1* and 150.1 → 91.1; MDA *m/z* 180 → 105* and 180 → 133; MDMA *m/z* 194 → 163.1* and 194 → 105.1; MDEA *m/z* 208.1 → 163* and 208.1 → 135; PEP/EP *m/z* 166.2 → 148.2* and 166.2 → 115.2 (pseudoephedrine, PEP and ephedrine, EP were determined via chromatographic separation) ; PTM *m/z* 150.1 → 133* and 150.1 → 91.1; MA-d_{14} *m/z* 164 → 98 and MDMA-d_5 *m/z* 199 → 165. (Transitions marked with an asterisk were used for quantitation and Bland-Altman analysis).

3.4 Evaluation of DBS study: stability of DBS medium for ATS drugs during sample storage.

3.4.1 General

Dried blood stain (DBS) has been increasingly accepted worldwide due to its numerous advantages including that it is stable under ambient conditions and able to represent the stabilisation of various analytes compared with whole blood samples despite of low temperatures storage (Sharma *et al.*, 2014 and Smit *et al.*, 2014). Application of the technique in forensic toxicology field is also highly demanded, since there is high frequency of viral infection occurred among the individuals that having a drug abuse history (Stove *et al.*, 2012).

Besides the fact that DBS is a dry matrix, there is reduction in enzyme activity and microbial degradation which act as a form of stabilisation for many substances. Hence, this technique offers advantage for the storage of blood samples for prolonged periods of time. For instance, stabilisation of drugs with ester groups in its chemical structure, such as cocaine and 6-MAM were achieved by reducing the hydrolysis reactions in DBS, in contrast with traditional whole blood samples (Alfazil and Anderson, 2008; Boy *et al.*, 2008).

Also, the most significant use of DBS in forensic toxicology field as mentioned by Dubey and Caplan (1996) that DBS is possible to preserve small amount of sample in an economical way, where all other evidence needs to be collected or discarded. When for one reason or another that a case is to be reopened, there is at least some material left, potentially allowing targeted analysis even though only stable analytes that remain for an extended period of time.

Despite all the advantages possessed by DBS technique, however, an evaluation of the stability of drugs and metabolites in the medium is a critical element to ensure that the DBS medium is practical to be used in forensic field in Malaysia. In this project, the study has focused on two major factors including temperature and time for ATS drugs in DBS medium during sample storage. The investigation has been extended to external Proficiency Testing for drugs sample (CAP, USA) as well as to the real forensic toxicology cases in Malaysia

3.4.2 Materials and methods

Apparatus, chemicals, sample collection and pre-treatment, sample preparation for DBS, standards preparation and instrumentation were similar to those described in section **3.1.2.1-3.1.2.7.**

In this study, the two main variants of time and temperature (local measures) during sample storage were investigated to evaluate the stability of ATS drugs in the DBS matrix. Time measurement consisted of T_0 (time at baseline where sample was treated as real case, ≤ 2 weeks), T_1 (sample storage 3 months) and T_2 (sample storage 6 months). The measurement of time was based on the local forensic scenario in Malaysia for case handling. The time for sample storage was measured at month-3 (T_1), where the need to re-open the case ordered by court and hospitals especially for ante-mortem and sexual-assaulted cases often arise. While measurement at month-6 (T_2) was based on the significances of further forensic investigations especially for certain particular post-mortem cases.

While temperature measurement included C_0 (temperature of 4 ^oC at baseline where sample was treated as real case, ≤ 2 weeks), C_1 (temperature of 4 ^oC during sample storage 3 and 6 months) and C_2 (temperature of 25 ^oC during sample storage 3 and 6

months). The temperature stability was measured at 4 ^{o}C at storage of 3 and 6 months (C_1) similar to the normal storage of whole blood and other biological specimens for real case analyses. The measurement also performed at 25 ^{o}C (ambient temperature) during sample storage of 3 and 6 months (C_2) in the desiccator in order to prevent the samples from humidity interferences.

The stability of the analytes was determined by measuring the drug concentration in DBS (duplicate) and compared with the initial concentration (real time analysis). The investigation was extended to the external Proficiency Testing for drugs sample (CAP, USA) and Quality Assurance Div., Dept. of Chemistry, Malaysia as well as to the 20 real forensic toxicology cases in Malaysia.

Analyst software version 1.6.3 was used during data acquisition and data processing. Further assessment for comparison in stability testing was performed by determination of recovery (Microsoft Excel).

The MRM transitions of ATS drugs were similar to those described in section **3.3.2** for data monitoring and quantitative analysis.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Multi-period-multi-experiment of MRM-EPI-MRM^3 spectrometry with library matching for simultaneous determination of ATS drugs in WBS, urine and DBS.

4.1.1 General

Regulatory and law enforcement bodies around the world are increasingly demanding more development in detection, identification and confirmation of ATS especially in biological samples such as urine and blood samples with the growing number of ATS abuse cases. GC-MS techniques were routinely used for ATS analysis, however tedious and time consuming derivatization process were often required in the procedures. In addition, false positive and misleading results were sometime obtained using GC-MS analysis (Dasgupta, 2010).

In LC-MS/MS analysis, identification and confirmation are determined by the qualifier ions and the ratios of selective ions, respectively. The most abundance ion used for identification. Matrix-matched calibration solutions are used and ion ratio should not deviate more than 30% (relative).

Larger tolerances in the ratio may lead to a higher percentage of false positive results. Similarly, if the tolerances are decreased, then the likelihood of false negatives will increase. For a higher degree of confidence in identification, it is suggested to have further evidence that may be gained from additional mass spectrometric information. For example, evaluation of full scan spectra, isotope pattern, adduct ions, additional accurate mass fragment ions, additional product ions (in MS/MS), or accurate mass product ions (Table 4.1).

Table 4.1: Identification requirements for different types of mass spectrometers.

MS mode:	Single MS (unit masss resolution	Single MS of high mass accuracy	MS/MS
Typical systems (example)	Quadrupole, ion trap, time-of-flight (TOF)	High resolution: Q-TOF, Orbitrap. FTMS, magnetic sector	Triple quadrupole, ion trap, hybride MS (e.g. Q-TOF, Q-trap
Acquisition:	Full scan, Limited m/z range, Selected ion monitoring (SIM)	Full scan, Limited m/z range, Selected ion monitoring (SIM)	Selected/multiple reaction monitoring (SRM/MRM), full scan product-ion spectra
Requirements for ions:	≥ 3 ions	≥ 2 ions with mass accuracy < 5 ppm	≥ 2 product ions
Ion ratio:	Within ±30% (relative) of average of calibration standards from same sequence.		
Other:	S/N ⩾3Analyte peaks in the extracted ion chromatograms must full overlap.		

(Source: SANTE/11945/2015).

The ratio of quantifier to qualifier transition of the unknown sample is compared to the MRM ratio of standards for identification. However, it has been reported that relying only on MRM ratios for identification resulted in a significant number of false positive results for compound identification, especially if the targeted analytes have low fragmentation efficiency (many low intensity product ions).

According to the report from "EURL (European Union Reference Laboratories)-Pesticides Joint Workshop 2013", there is 8.1% false negative identification when using <±30% ion ratio criteria at signal-to-noise ratio (S/N) range from 3 to 15 (Table 4.2).

Table 4.2: False negatives vs ion ratio tolerance (data of spiked extracts from all combined laboratories (44,000 results)).

S/N range	3-5	15-45	45-135	135-405	405-1215	>1215
All 5 laboratories/instruments #pest/matrix/conc combinations	5884	6955	9253	9279	6829	5798
Ion ratio criterion	% false negatives					
<± 15%	26.0	12.5	5.7	3.5	3.0	5.6
<± 20%	16.3	6.4	2.2	1.1	1.1	2.5
<± 25%	10.9	3.6	1.0	0.4	0.3	1.2
<± 30%	8.1	2.2	0.5	0.2	0.1	0.6
<± 50%	2.9	0.3	0.1	0.0	0.0	0.1
<± 60%	2.2	0.3	0.1	0.0	0.0	0.1
<± 70%	1.6	0.2	0.1	0.0	0.0	0.0

(Source: EURL-Pesticides Joint Workshop 2013)

While for false positive identification, about 73% was reported (32 compounds were false positive from 44 compounds detection, which 12 compounds were known as true positive) when using <±30% ion ratio criteria at 2 ng/mL concentration of pesticides (Table 4.3).

Table 4.3: False positives vs ion ratio tolerance (All data blank extracts compiled (12,600 pesticide/matrix combinations).

RT criterion	#transitions	Ion ratio criterion	Detected ≥ 0.002 mg/kg	Detected ≥ 0.010 mg/kg
≤ ± 0.1 min	1	-	121	66
	2	None	61	13
		EU	40	12 → True positive
		≤ ± 20%	36	10
		≤ ± 20%	39	11
		≤ ± 30%	44	12
		≤ ± 50%	46	12

(Source: EURL-Pesticides Joint Workshop 2013)

This study has captured the issues and introduced a novel mass spectrometry detection technique in LC-MS/MS based on a multi period and multiple experiment (MRM-EPI-MRM^3) with library matching. The experiments were adopted to reduce the risks of false results in complicated matrix as reflected in the report of pesticides in food matrix to the forensic drugs in blood specimen. The method was performed in a single run for fast and rapid screening and identification of ATS- related drugs in WBS, urine and DBS. The technique introduced the detection of those drugs in higher level of confidence via EPI and MRM^3 experiment to complement MRM and thus reducing/eliminating the risk of false positive results in complex matrices.

The MRM acquisition method was improved by including EPI scanning experiment to acquire full scan MS/MS spectra for any MRM that was detected in an unknown sample and compared those results to MS/MS compound libraries to increase confidence in compound identification and confirmation. The expected benefits of this workflow included:

- **Improved selectivity** – multiple fragment ions are detected, greater than 2 MRM transitions, resulted in additional confidence in identification of positive findings
- **Improved sensitivity** – EPI scans are called 'enhanced' because fragments are accumulated in Q3 of your mass spectrometer, giving you better signal-to-noise for the detected MS/MS spectra
- **Improved data acquisition** – EPI spectra are acquired fully automatic using the logic provided by Information Dependent Acquisition (IDA), Dynamic Background Subtraction (DBS), and Dynamic Fill Time (DFT).
- **Improved data processing** – ability to compare results to MS/MS mass spectral libraries for better identification.

- **Improved confidence** – ability to automatically calculate ion ratios and compare results to MS/MS mass spectral libraries.

While for MRM^3 experiment, the advantages are also similar as reflected by EPI experiment in terms of the above-mentioned parameters. The MRM^3 experiment produces multiple fragment ions together with the second generation of product ions and enables full scan product ion spectra for library matching. The experiment provides a robust quantitative strategy for the quantitation of analytes with high background and interferences, which make standard MRM quantitation difficult. The MRM^3 removed the interferences and enables a much lower detection of the particular analyte. The MRM3 experiment has higher specificity and reduces the fractionation required for low level detection and increases sample throughput.

4.1.2 Method development of multi-period and multi-experiment (MRM-EPI-MRM^3) analysis with library search

In this study, all compounds were separated chromatographically in order for multi-period and multi-experiment workflow to be effectively implemented. Each period consisted of the MRM, EPI and MRM^3 (or MS^3) experiments. Period 1 [ephedrine and pseudoephedrine], period 2 [amphetamine], period 3 [methamphetamine, MDA and methamphetamine-d_{14} ISTD], period 4 [MDMA, phentermine and MDMA-d_5 ISTD] and the final period 5 [MDEA]. Refer Figure 4.1.

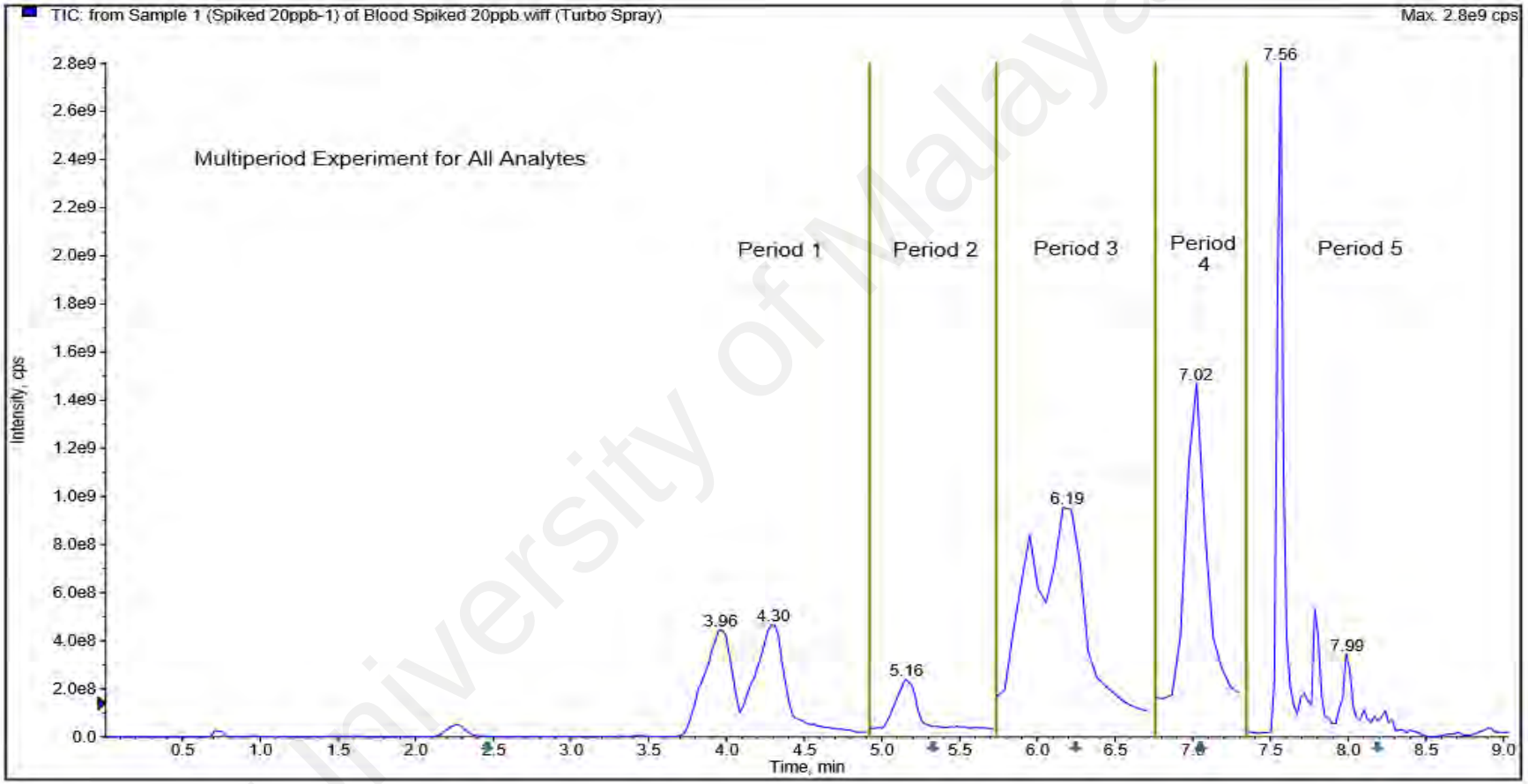


Figure 4.1: 5-period experiment in single chromatogram of targeted compounds.

The mass spectra of each analyte were recorded using a Q1 scan during manual infusion in method development process to determine the highest abundant mass to charge ratio (*m/z)* proton adducts of the molecular ion $[MH]^+$ as the precursor ion for all analytes. Subsequently, EPI scan was carried out to obtain the product mass spectra of the precursor ion. This step was to ensure that the correct compounds were being infused into the system before system parameters were optimized to set up the experimental method.

Two transitions were chosen for each analyte taking in conformity with the EU criteria. The most abundant precursor ion was used as quantifier in the first transition. The product ion was used as qualifier for confirmation purpose in the second transition. To obtain maximum sensitivity for the analytes, optimization of MS/MS parameters such as declustering potential (DP), collision energy (CE), entrance potential (EP) and collision exit potential (CXP) were conducted for each analyte using 0.1 μg/mL of pure compound in methanol.

The presence of precursor and product ions was investigated in MRM experiment mode with a dwell time of between 50-150 ms. In addition, the developed MRM experiment protocols were then uniquely setup and utilized under a multi period experiments workflow for enhanced compounds identification and confirmation (Figure 4.2).

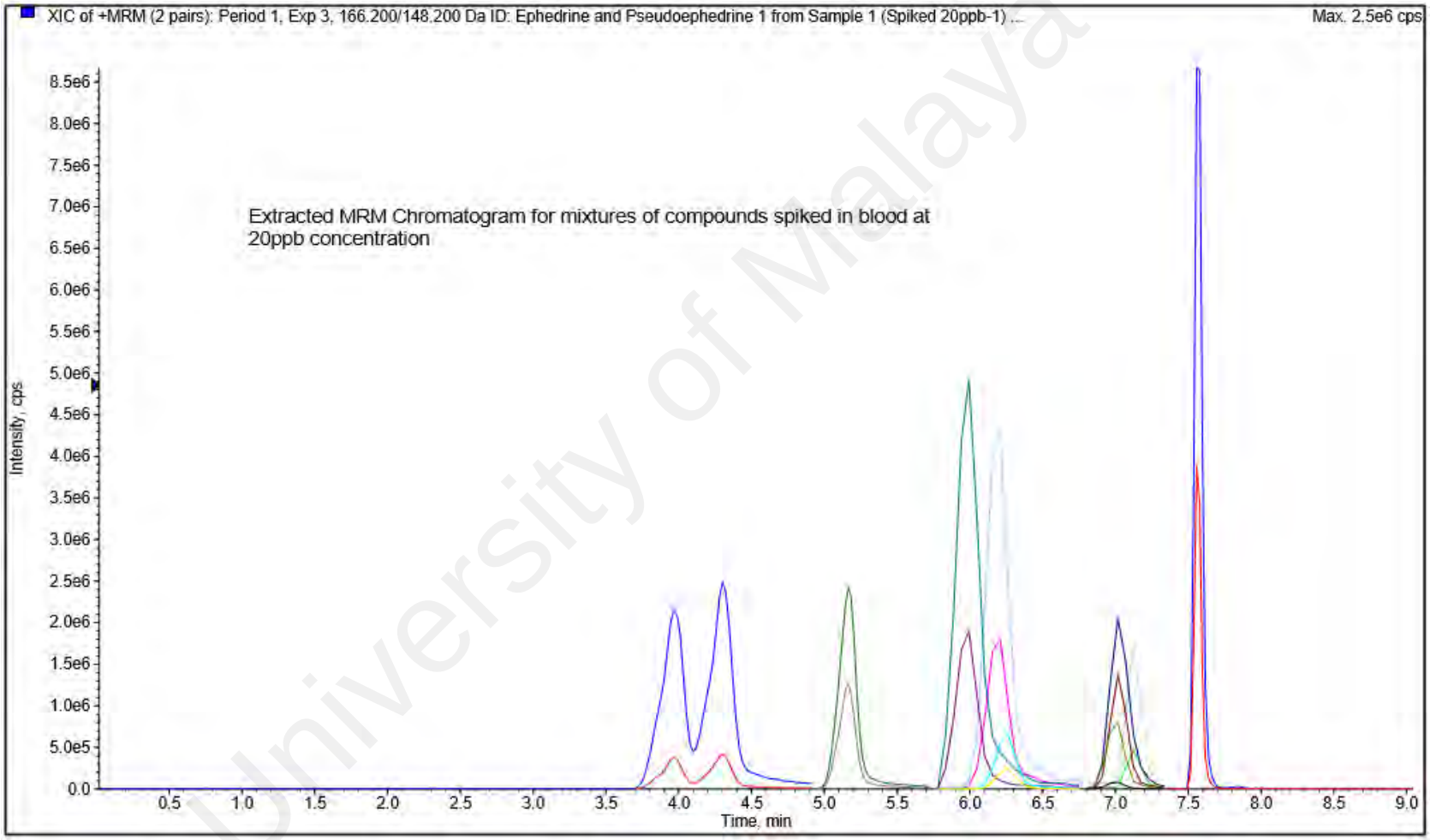


Figure 4.2: Extracted MRM scan mode chromatogram for all mixtures of the targeted compounds spiked in blood.

Utilizing the unique capability of the QTrap system, two additional experiments were added in a single experiment run, under the multi-period multi-experiment workflow, *i.e.* EPI and MRM^3 or MS^3 multistage fragmentation experiment. A MS/MS or fragmentation experiment under the EPI were included to obtain the unique fingerprint of each analytes and compared with a MS/MS spectra library, which provides another level on the identification of the analytes for positive identification.

The EPI experiment was set with a collision energy spread (CES), averaging three MS/MS spectrum at three different collision energies (low, medium, high) to collect the most representative mass fragment spectra. The declustering potential (DP), entrance potential (EP) and collision cell exit potential (CXP) were set at default value.

At the same time, an MS^3 experiment was also included in the experimental workflow. Collecting the MS^3 spectra or fragment of a fragment ion spectrum and comparing the spectrum with a MS^3 library provided further dimension on the interpretation and identification of the compound in the samples.

Using a built-in auto optimization script in the Analyst 1.6.3 software, the targeted analytes were selected and information such as targeted *m/z* and possible MS/MS target ion for further fragmentations (MS^3) were set. The optimized method consisted of the entrance exit barrier (EXB) which critically determined how the fragment ion will be further fragmented in the third quadrupole (Q3). In order to collect the MS^3 mass fragment spectrum, a scan range was set in the final MS^3 method.

The summary of ATS drugs analyzed by MRM, EPI and MRM^3 mode in the LC-MS/MS are illustrated below:

Period 1 [ephedrine and pseudoephedrine]:

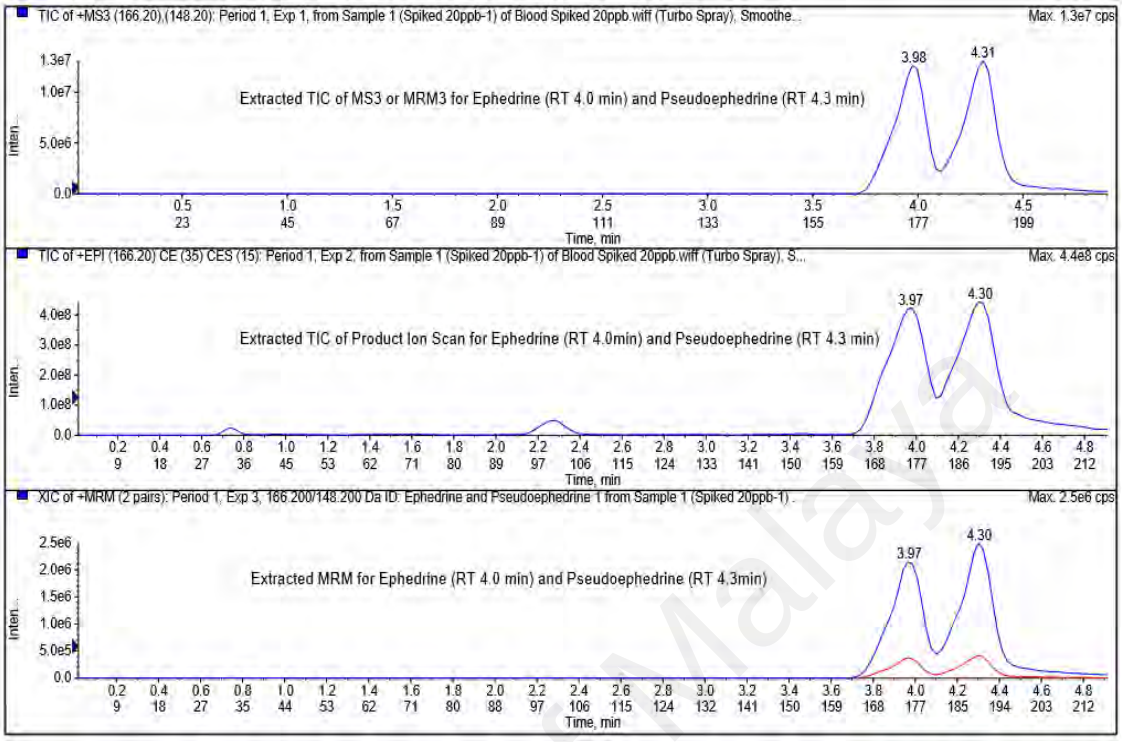


Figure 4.3a: Multi-experiment of ephedrine and pseudoephedrine from period 1.

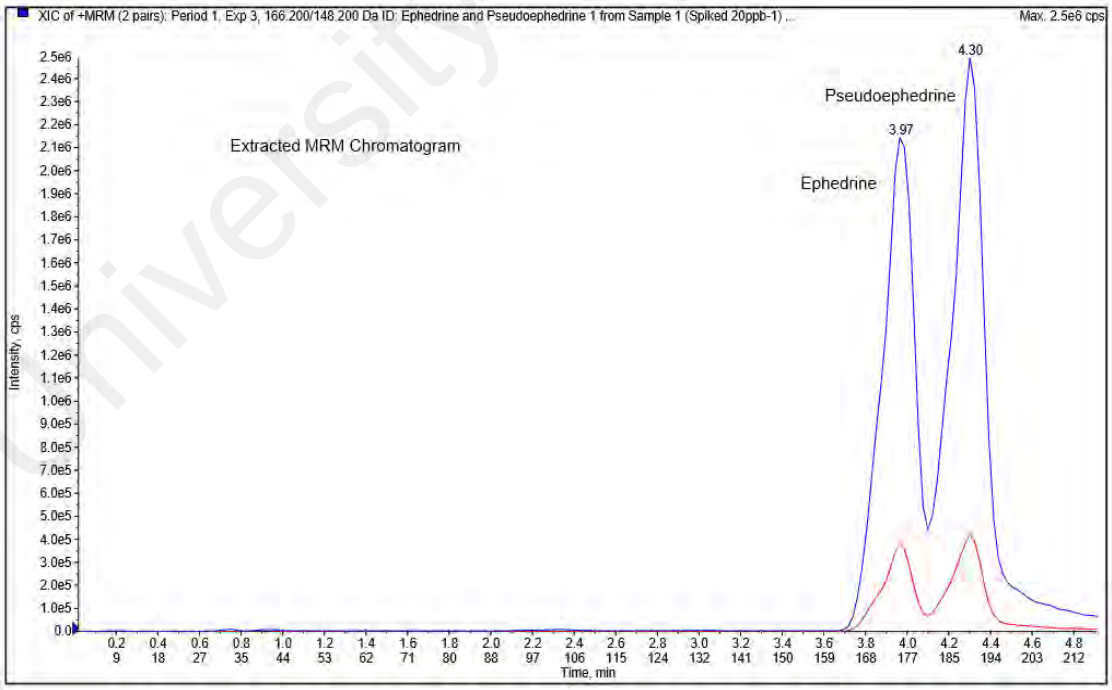


Figure 4.3b: MRM experiment - Extracted MRM chromatogram for ephedrine and pseudoephedrine.

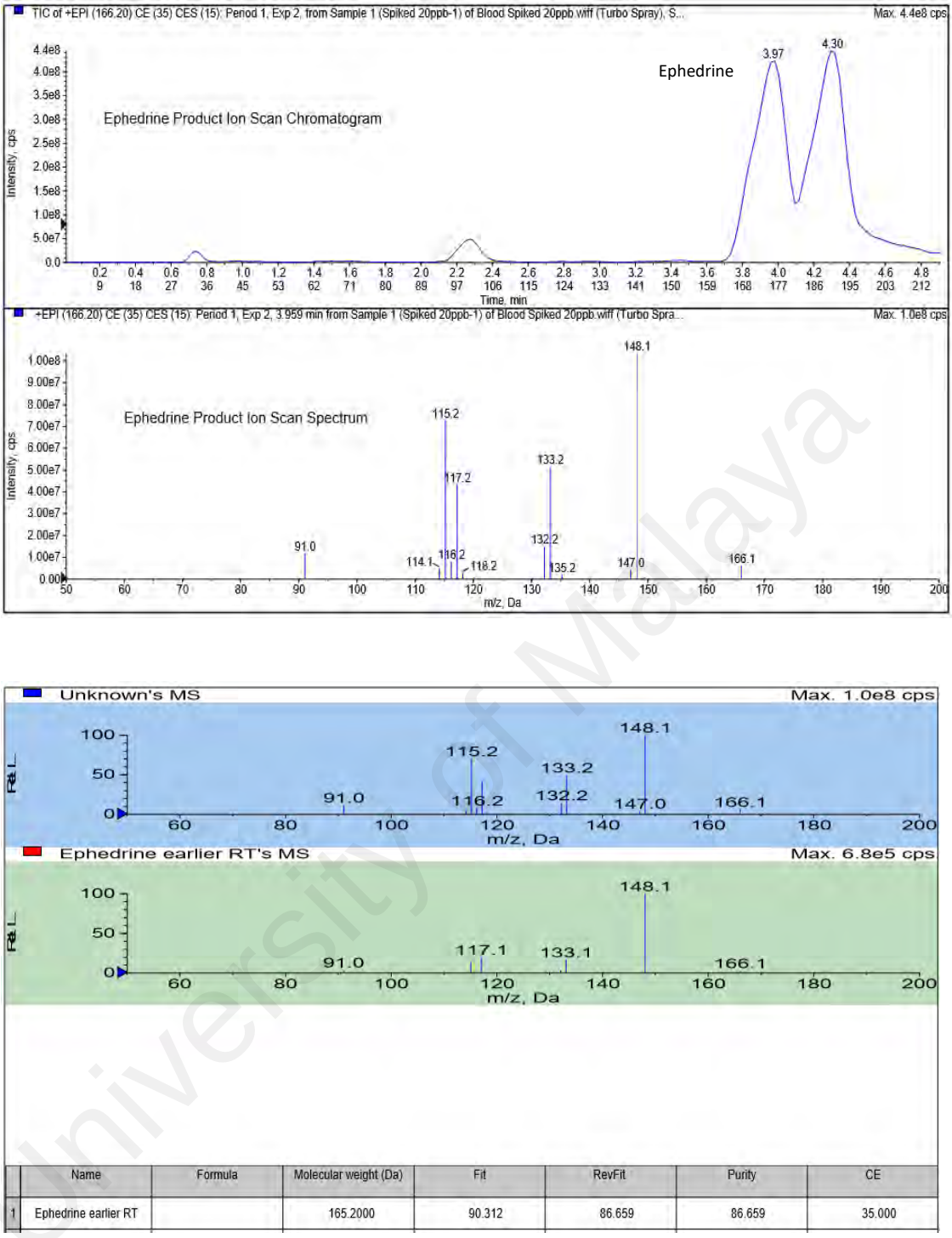
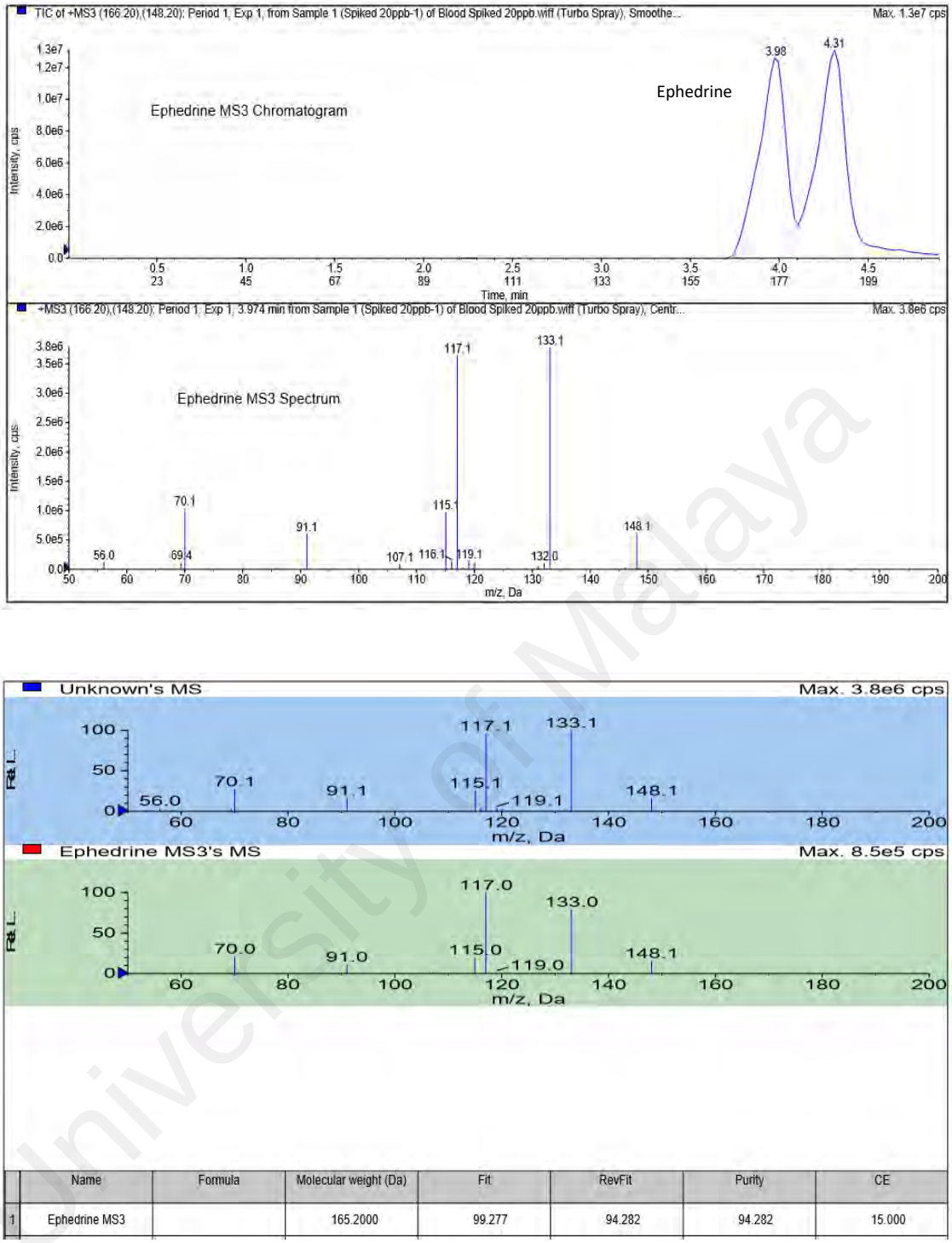
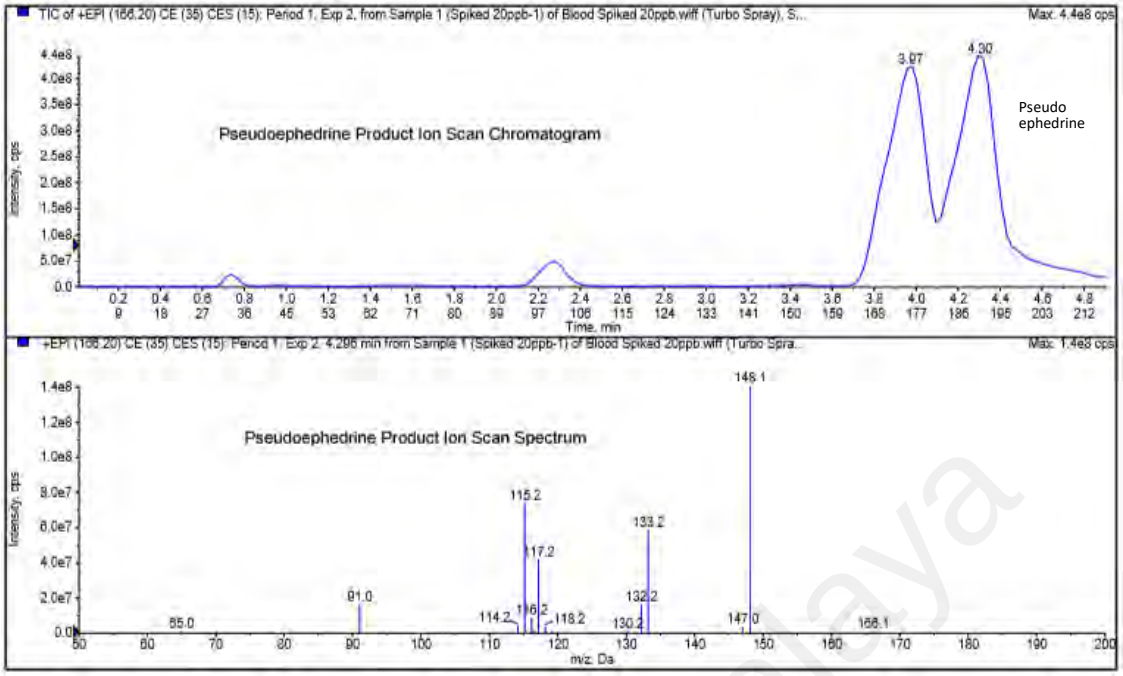


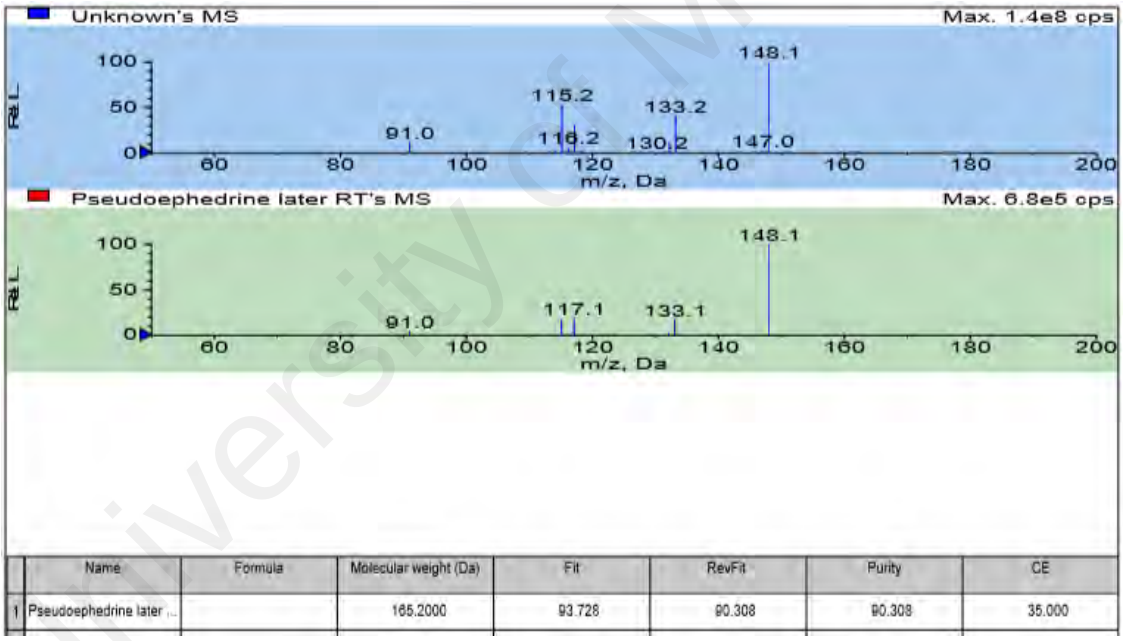
Figure 4.3c: EPI experiment - ephedrine Enhanced Product Ion Scan chromatogram and the scan spectrum with library matching.



	Name	Formula	Molecular weight (Da)	Fit	RevFit	Purity	CE
1	Ephedrine MS3		165.2000	99.277	94.282	94.282	15.000

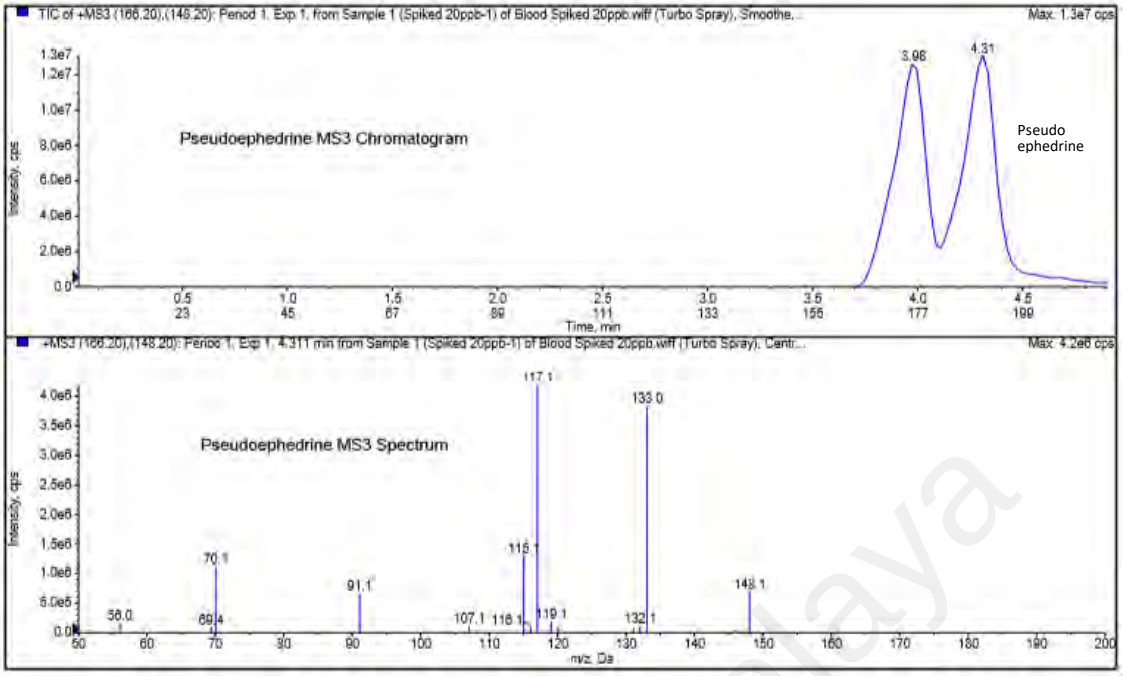
Figure 4.3d: MS^3 or MRM^3 experiment - ephedrine MRM^3 Scan chromatogram and the scan spectrum with further library matching.

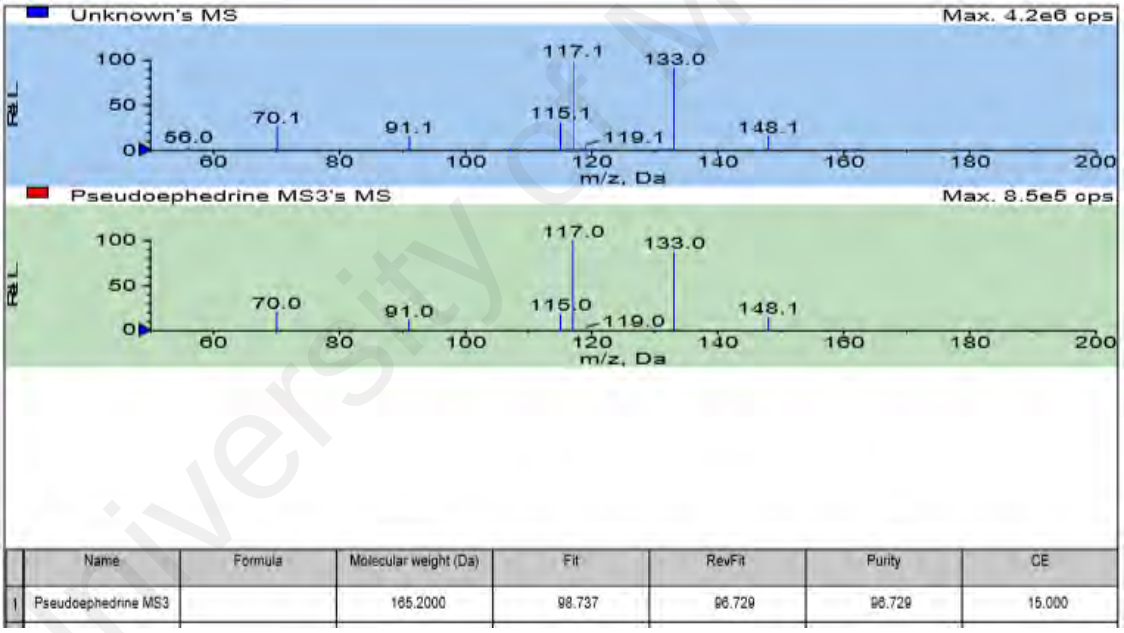




	Name	Formula	Molecular weight (Da)	Fit	RevFit	Purity	CE
1	Pseudoephedrine later ...		165.2000	93.728	90.308	90.308	35.000

Figure 4.3e: EPI experiment - pseudoephedrine Enhanced Product Ion Scan chromatogram and the scan spectrum with library matching.





	Name	Formula	Molecular weight (Da)	Fit	RevFit	Purity	CE
1	Pseudoephedrine MS3		165.2000	98.737	96.729	96.729	15.000

Figure 4.3f: MS^3 or MRM^3 experiment - pseudoephedrine MRM^3 Scan chromatogram and the scan spectrum with further library matching.

Period 2 [amphetamine]:

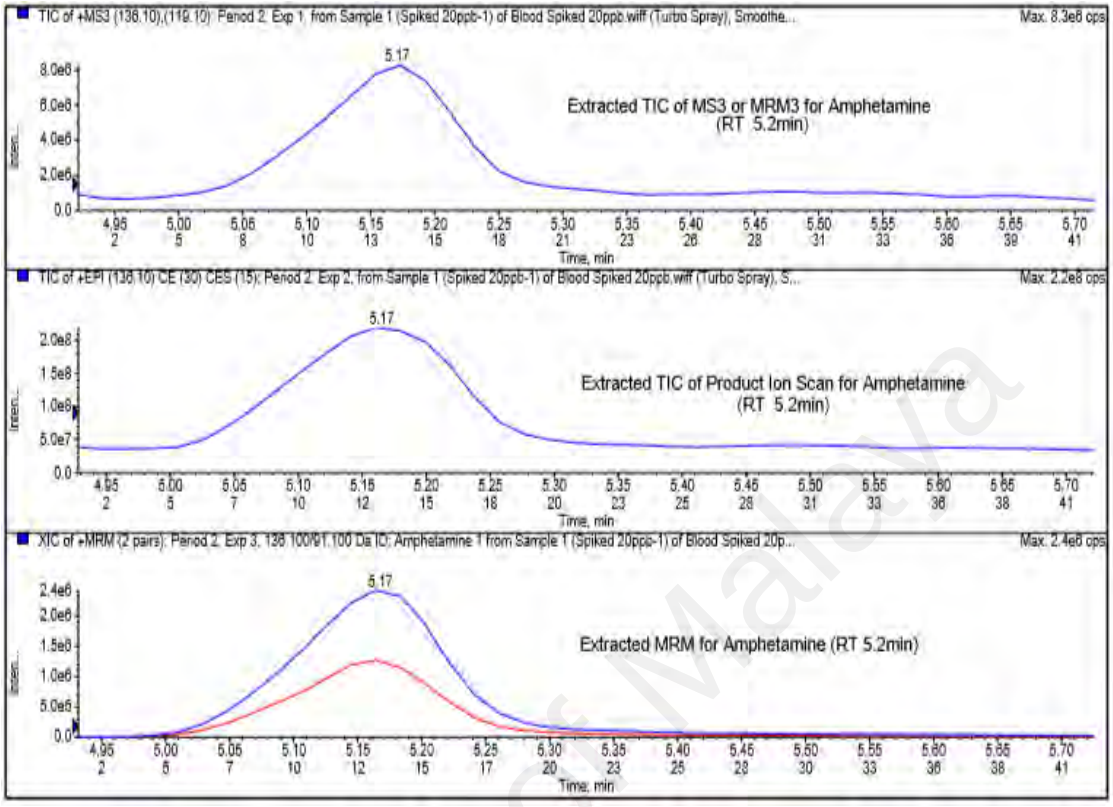


Figure 4.4a: Multi-experiment of amphetamine from period 2.

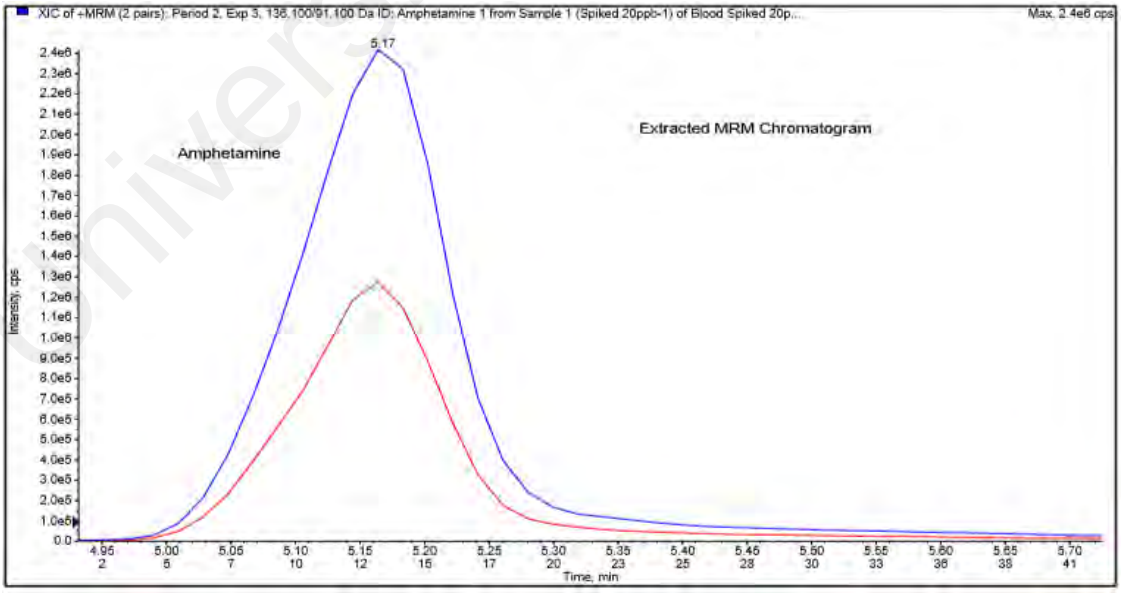
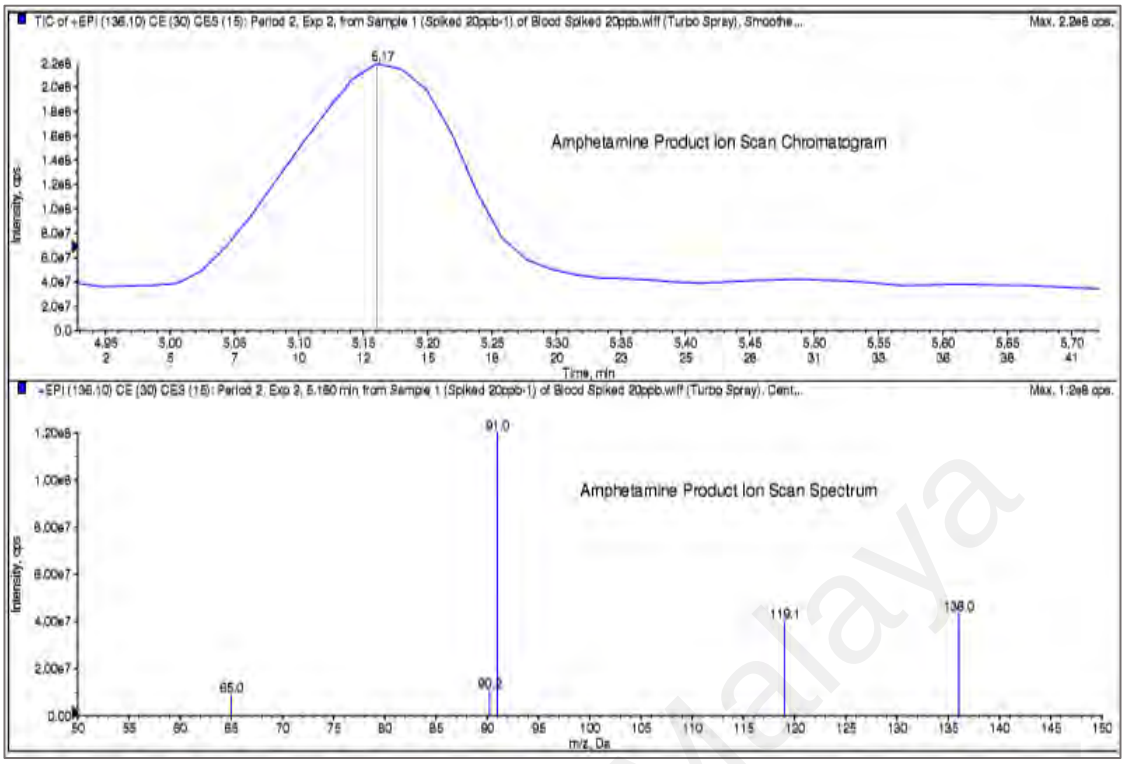
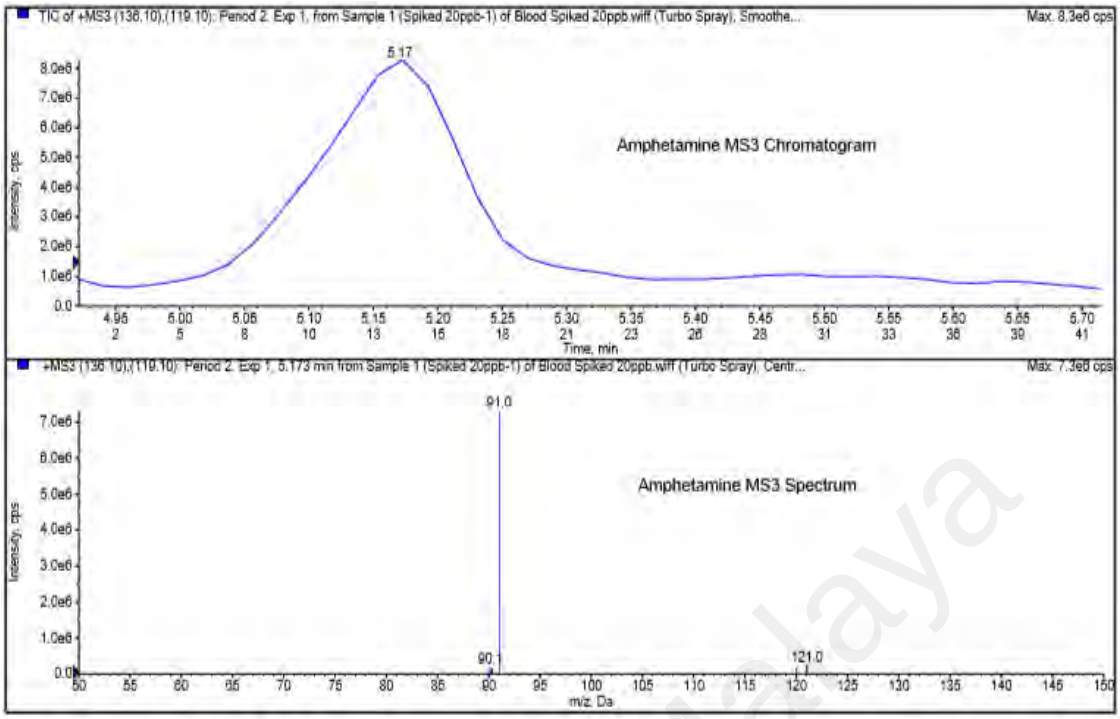


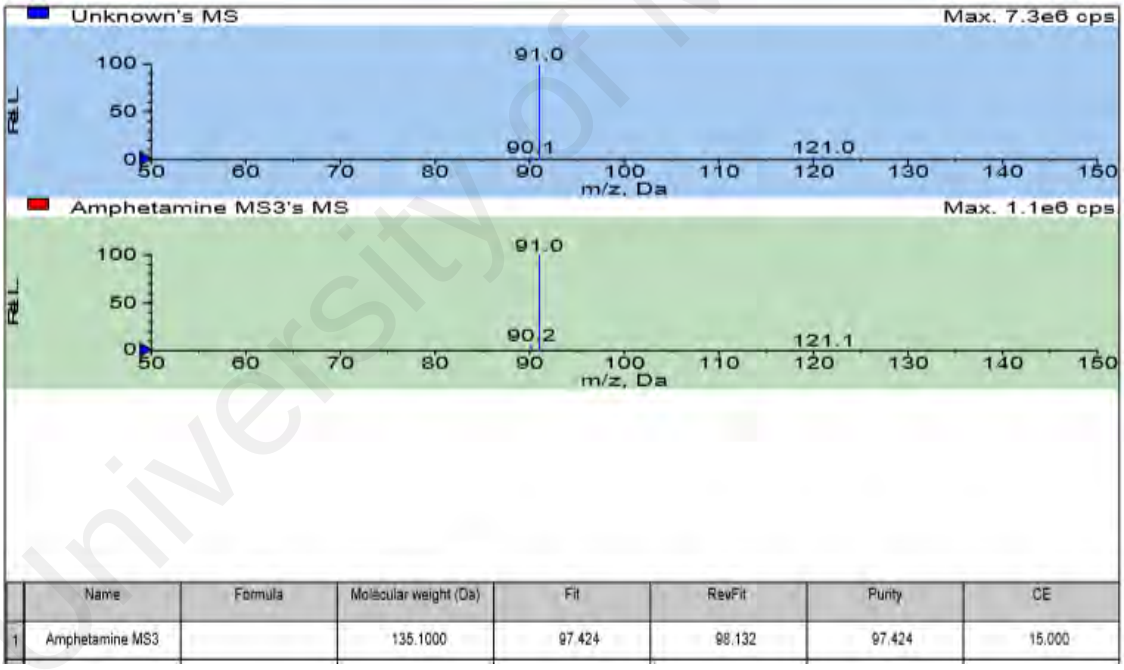
Figure 4.4b: MRM experiment - Extracted MRM chromatogram for amphetamine



	Name	Formula	Molecular weight (Da)	Fit	RevFit	Purity	CE
1	Amphetamine		135.1000	92.054	95.405	92.054	30.000
2	Amphetamine		135.1000	92.219	92.219	92.219	30.000

Figure 4.4c: EPI experiment - amphetamine Enhanced Product Ion Scan chromatogram and the scan spectrum with library matching.





	Name	Formula	Molecular weight (Da)	Fit	RevFit	Purity	CE
1	Amphetamine MS3		135.1000	97.424	98.132	97.424	15.000

Figure 4.4d: MS^3 or MRM^3 experiment - amphetamine MRM^3 Scan chromatogram and the scan spectrum with further library matching.

Period 3 [methamphetamine, MDA and methamphetamine-d_{14} ISTD]:

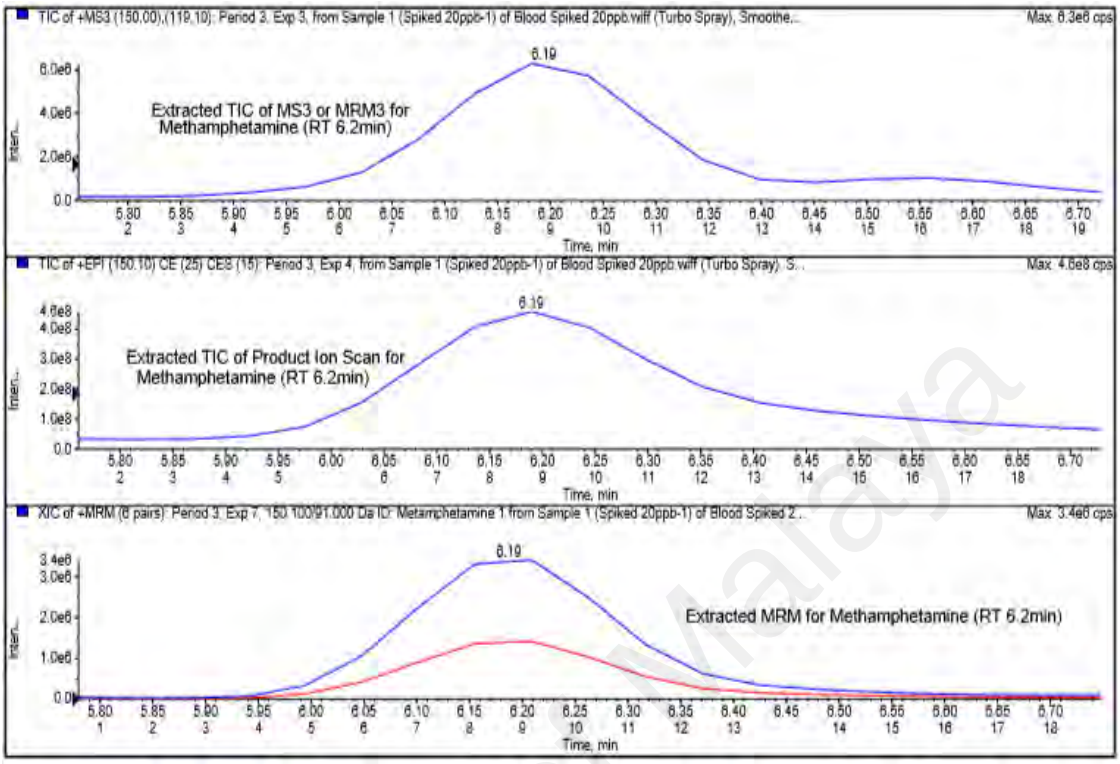


Figure 4.5a: Multi-experiment of methamphetamine from period 3.

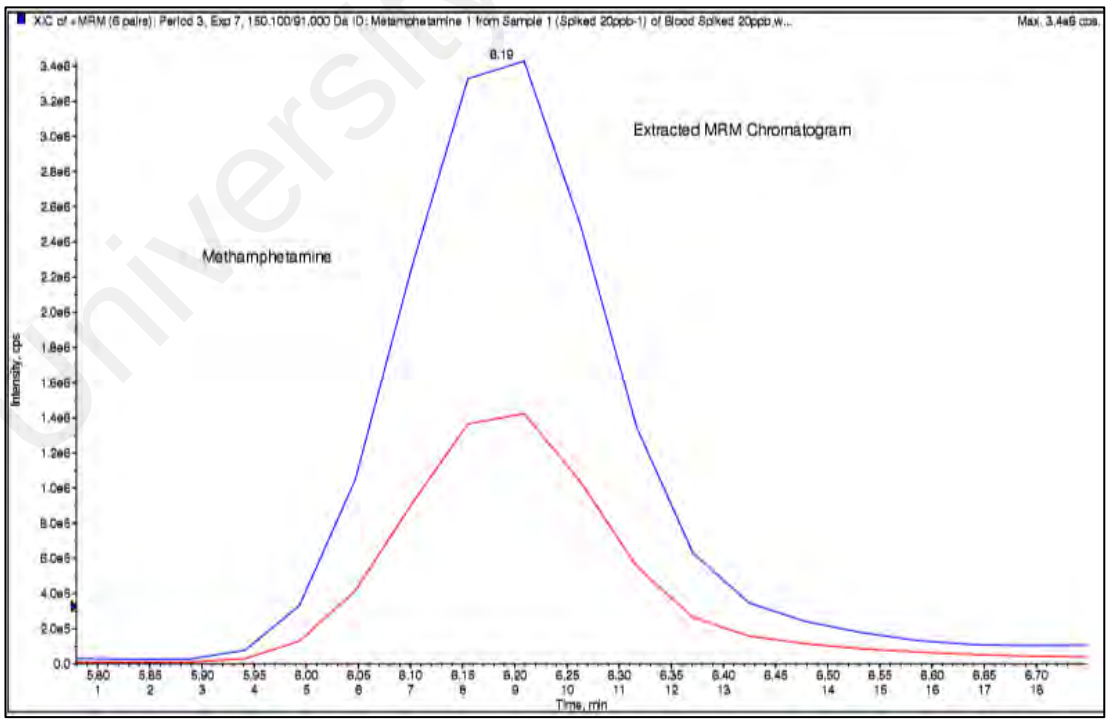
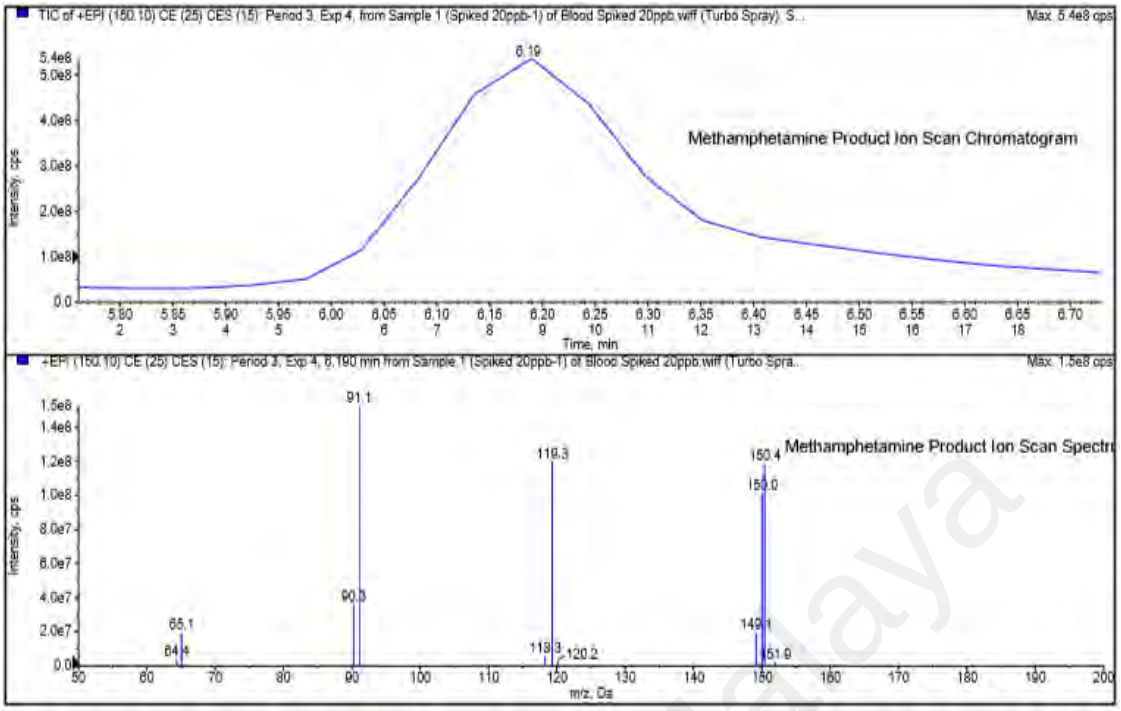
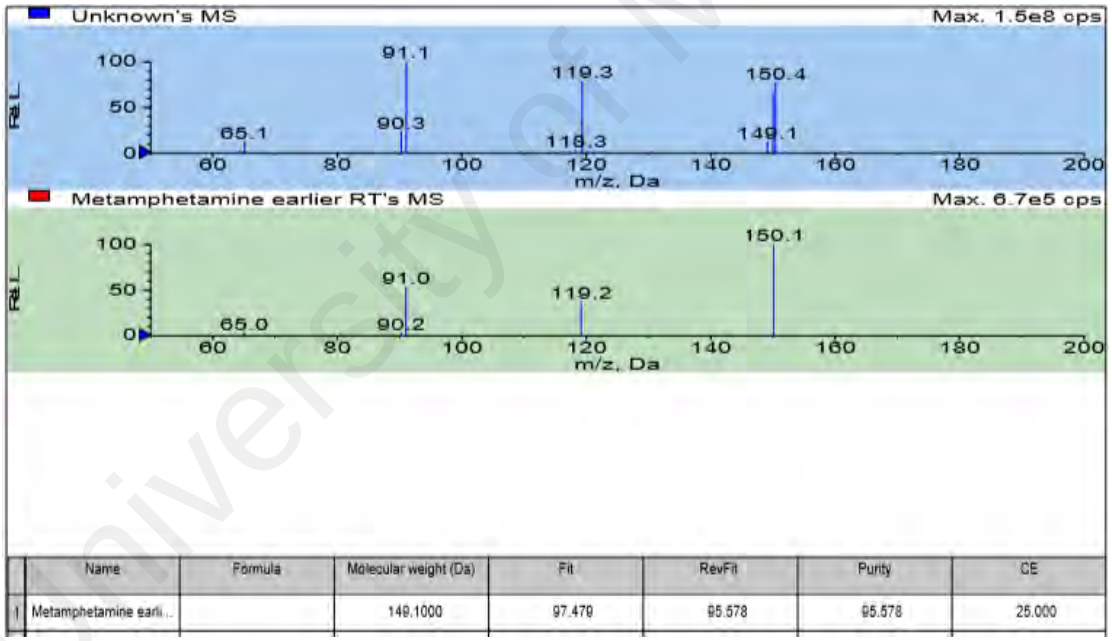


Figure 4.5b: MRM experiment - Extracted MRM chromatogram for methamphetamine.





	Name	Formula	Molecular weight (Da)	Fit	RevFit	Purity	CE
1	Metamphetamine earli...		149.1000	97.479	95.578	95.578	25.000

Figure 4.5c: EPI experiment - methamphetamine Enhanced Product Ion Scan chromatogram and the scan spectrum with library matching.

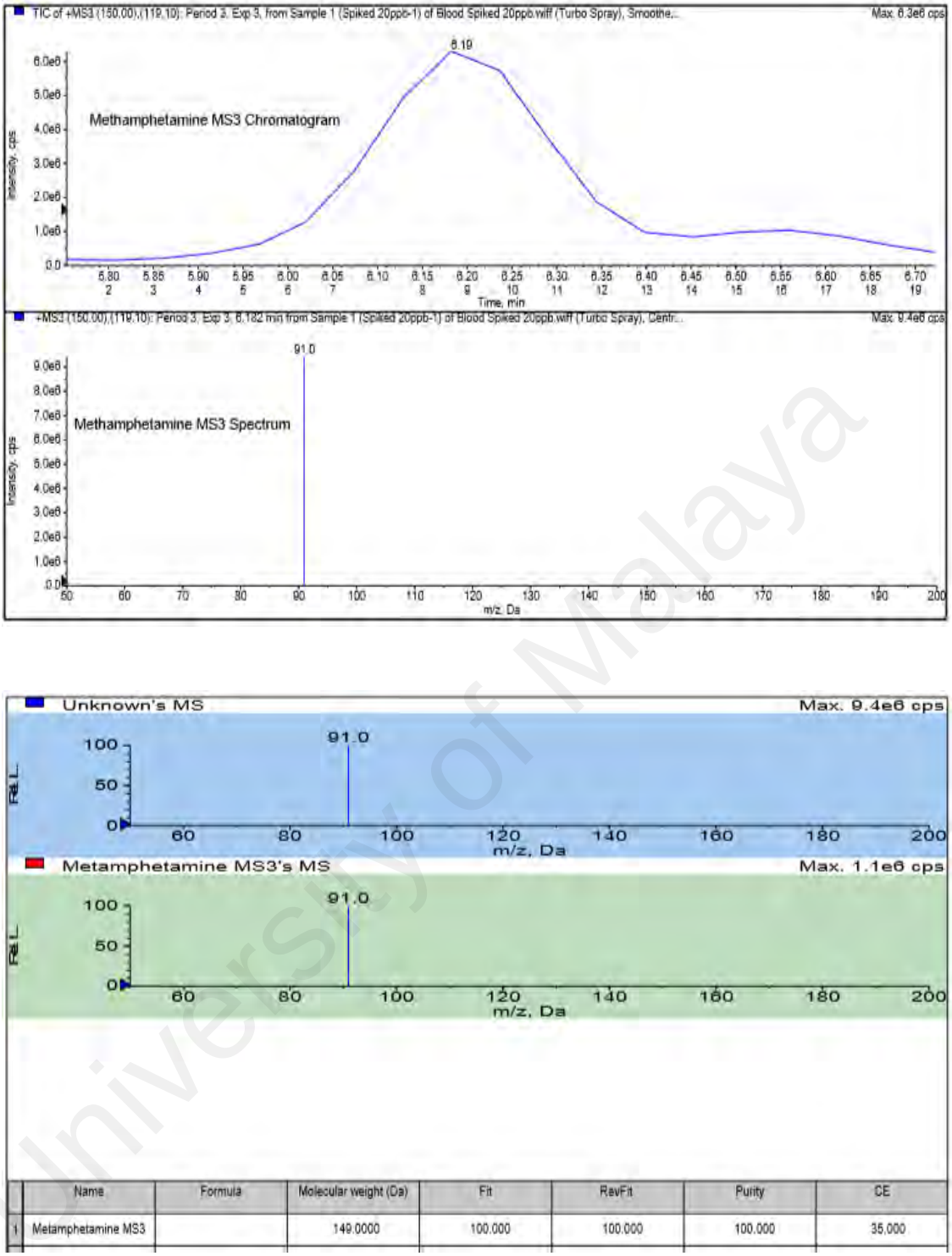


Figure 4.5d: MS^3 or MRM^3 experiment - methamphetamine MRM^3 Scan chromatogram and the scan spectrum with further library matching.

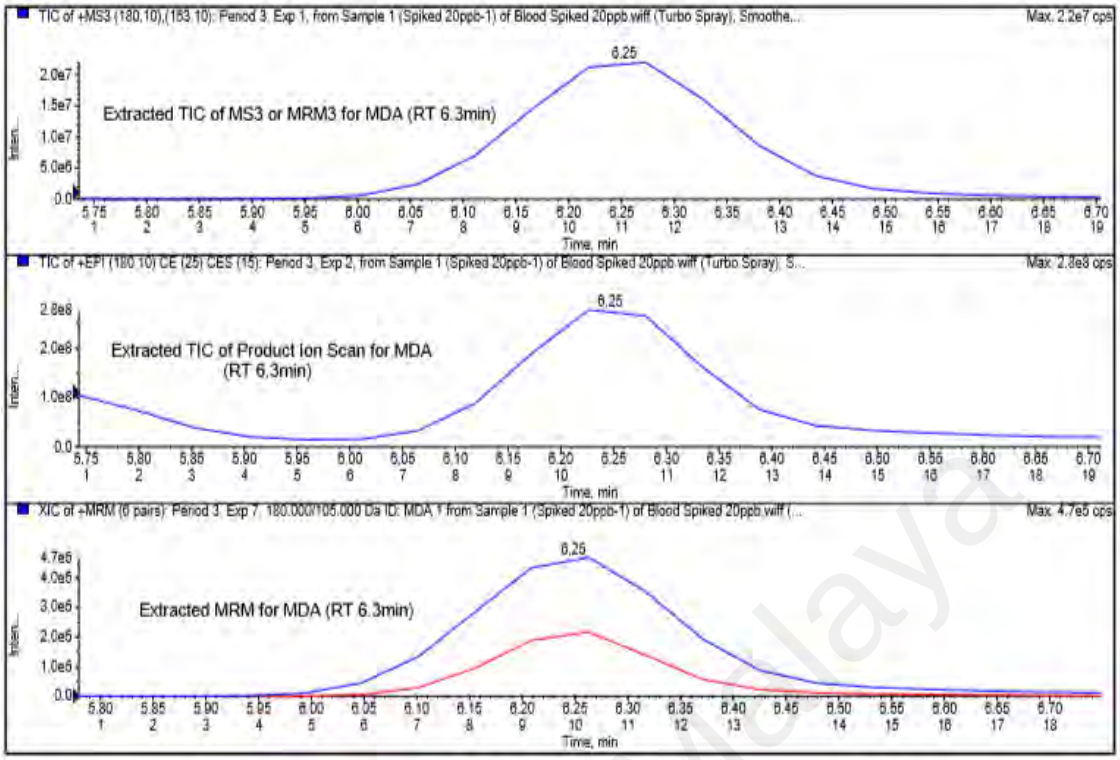


Figure 4.5e: Multi-experiment of MDA from period 3.

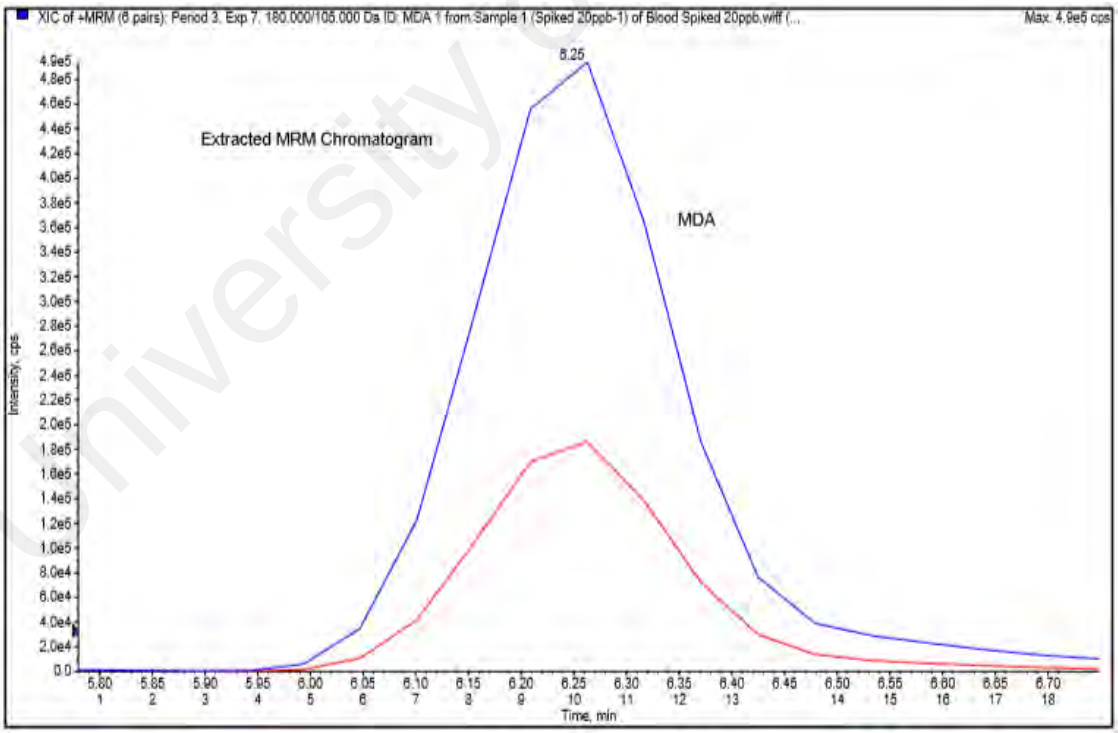
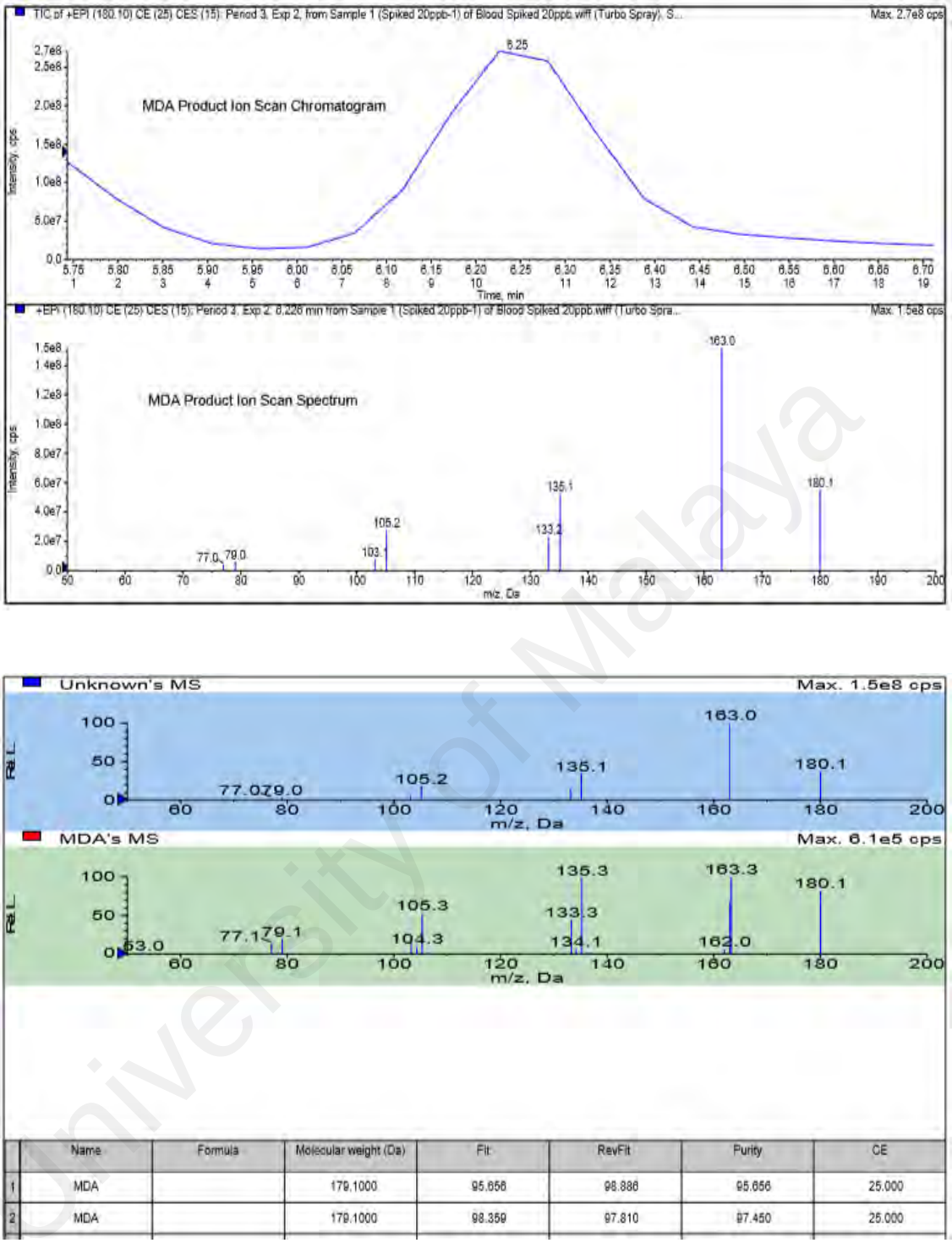


Figure 4.5f: MRM experiment - Extracted MRM chromatogram for MDA.



	Name	Formula	Molecular weight (Da)	Fit	RevFit	Purity	CE
1	MDA		179.1000	95.656	98.886	95.656	25.000
2	MDA		179.1000	98.359	97.810	97.450	25.000

Figure 4.5g: EPI experiment - MDA Enhanced Product Ion Scan chromatogram and the scan spectrum with library matching.

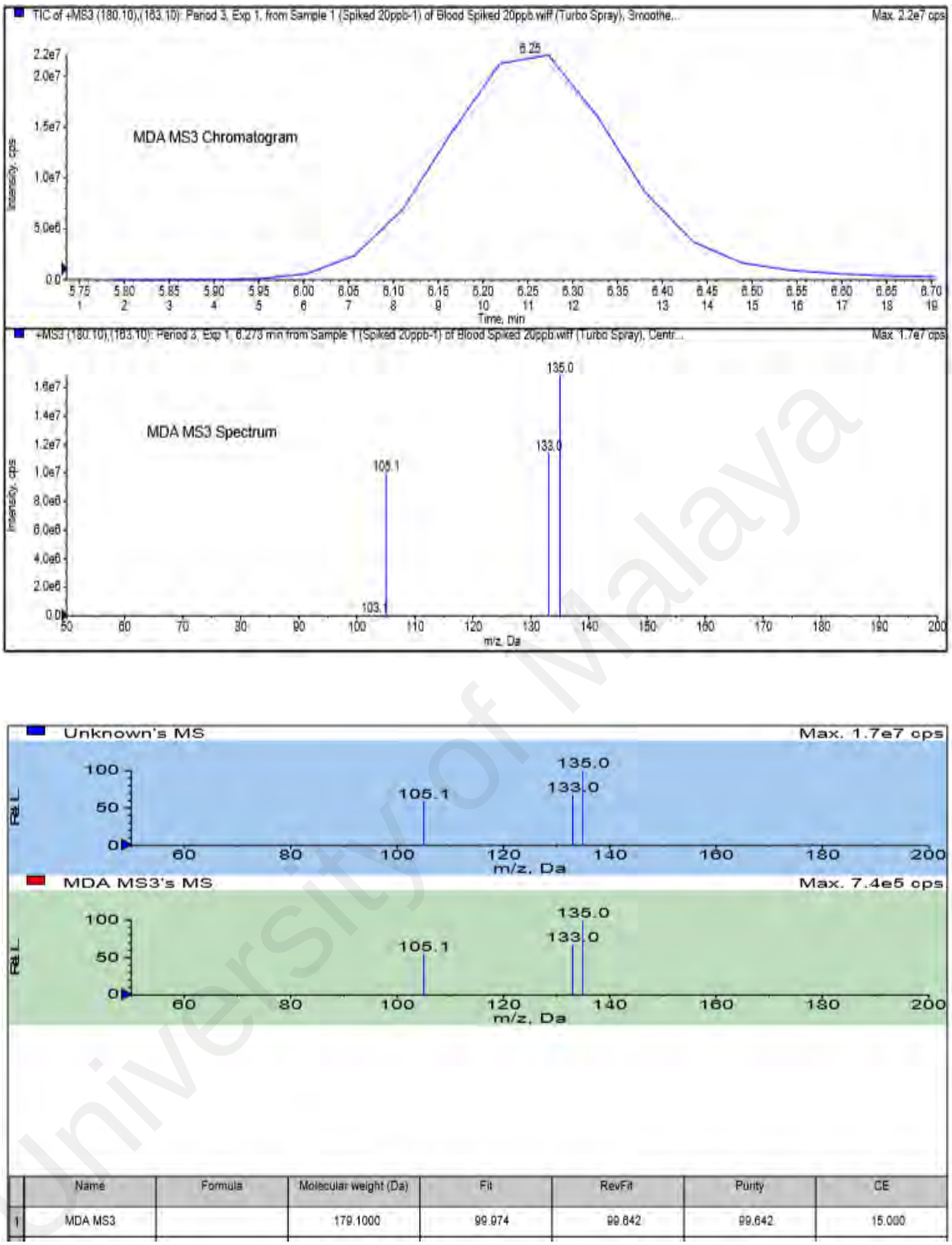


Figure 4.5h: MS^3 or MRM^3 experiment - MDA MRM^3 Scan chromatogram and the scan spectrum with further library matching.

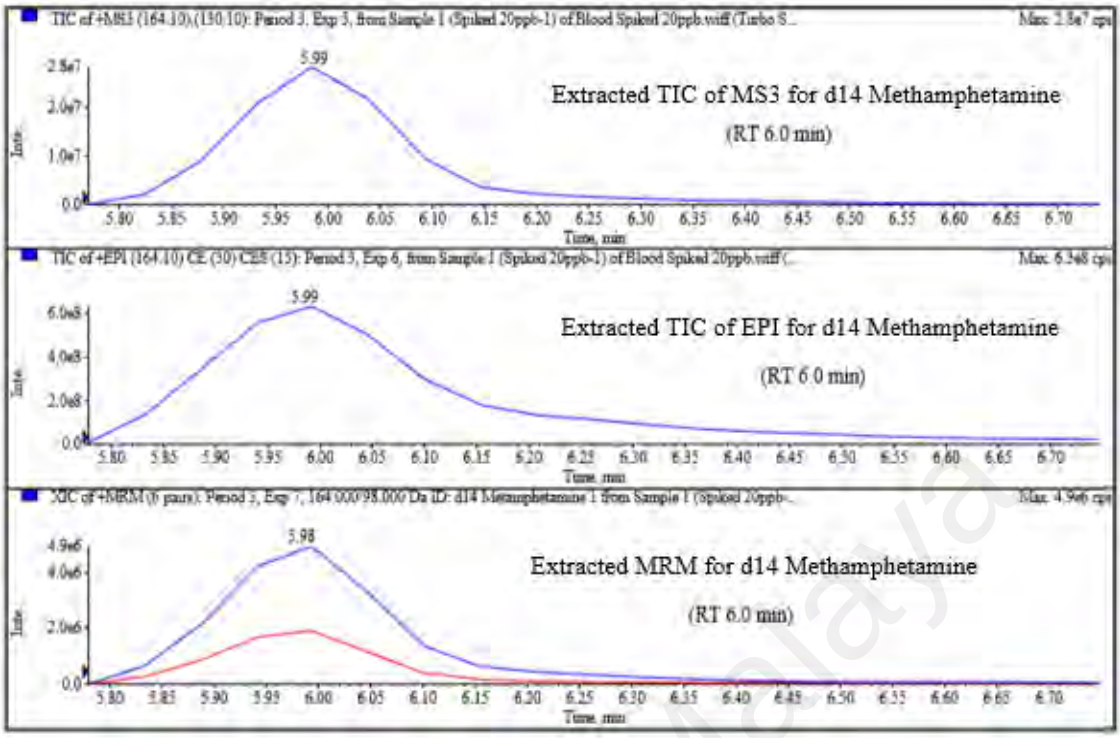


Figure 4.1.5i: Multi-experiment of methamphetamine-d_{14} ISTD from period 3.

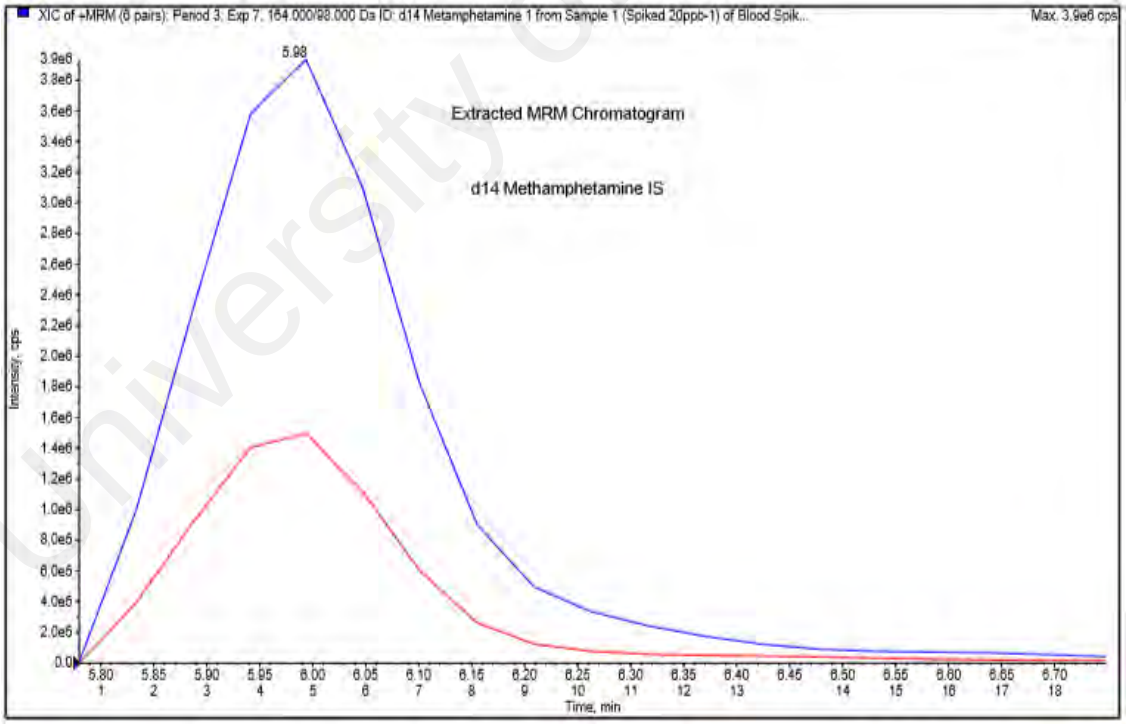
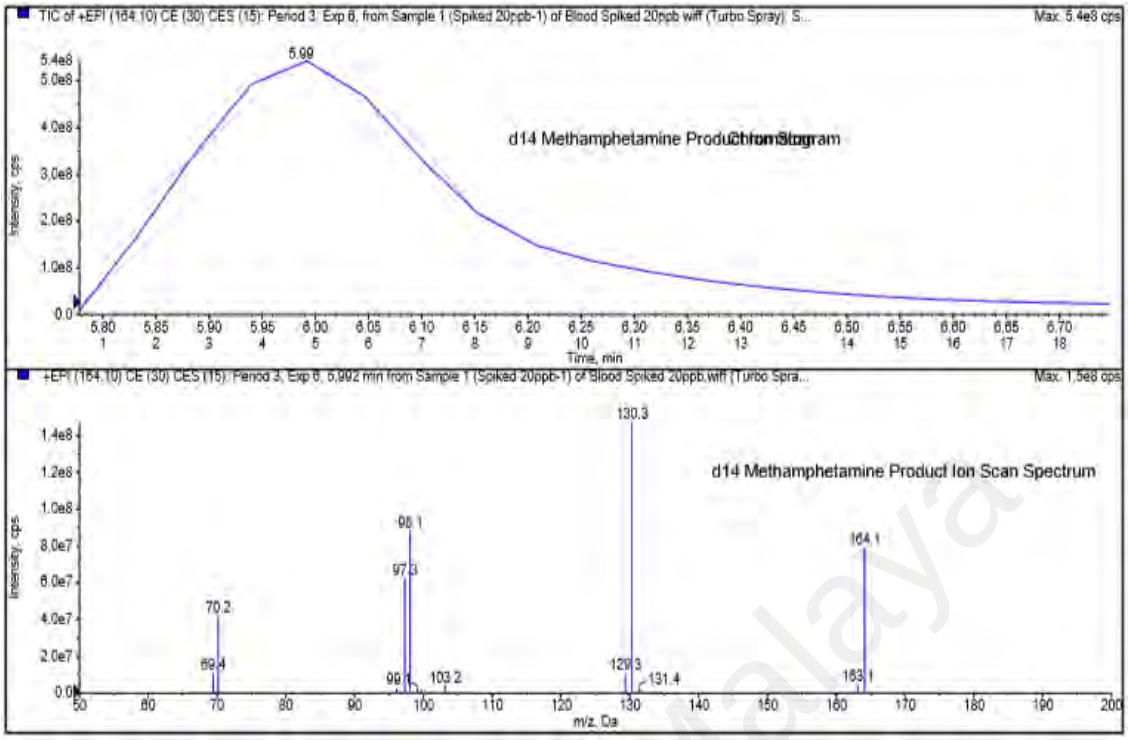
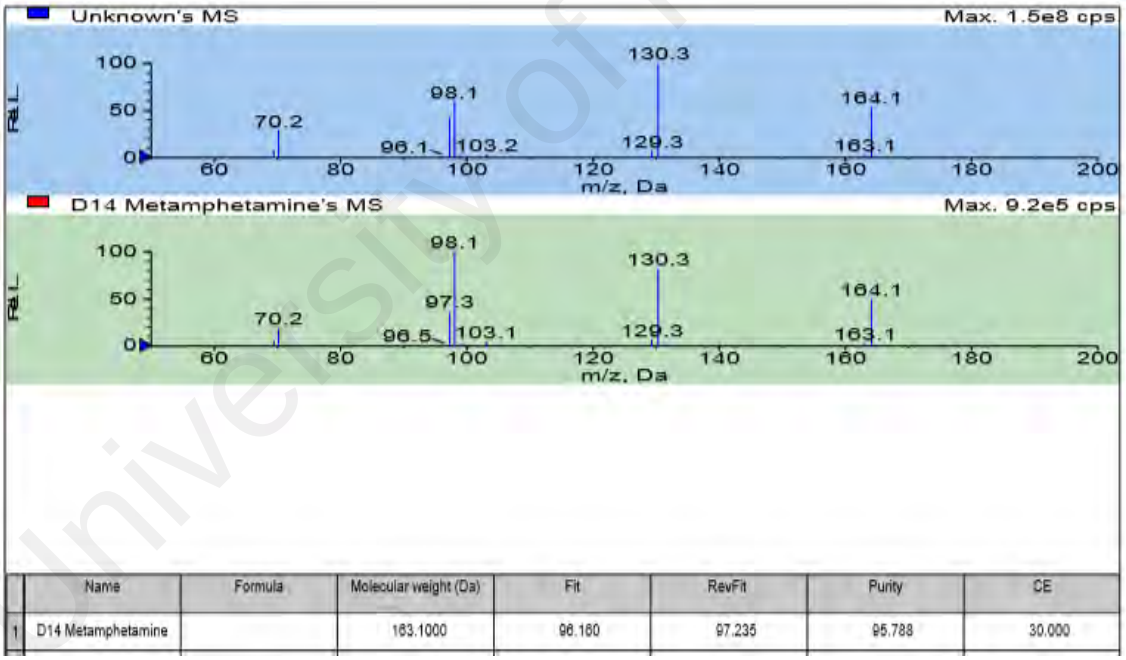


Figure 4.5j: MRM experiment - Extracted MRM chromatogram for methamphetamine-d_{14} ISTD.





	Name	Formula	Molecular weight (Da)	Fit	RevFit	Purity	CE
1	D14 Metamphetamine		163.1000	96.160	97.235	95.788	30.000

Figure 4.5k: EPI experiment - methamphetamine-d_{14} Enhanced Product Ion Scan chromatogram and the scan spectrum with library matching.

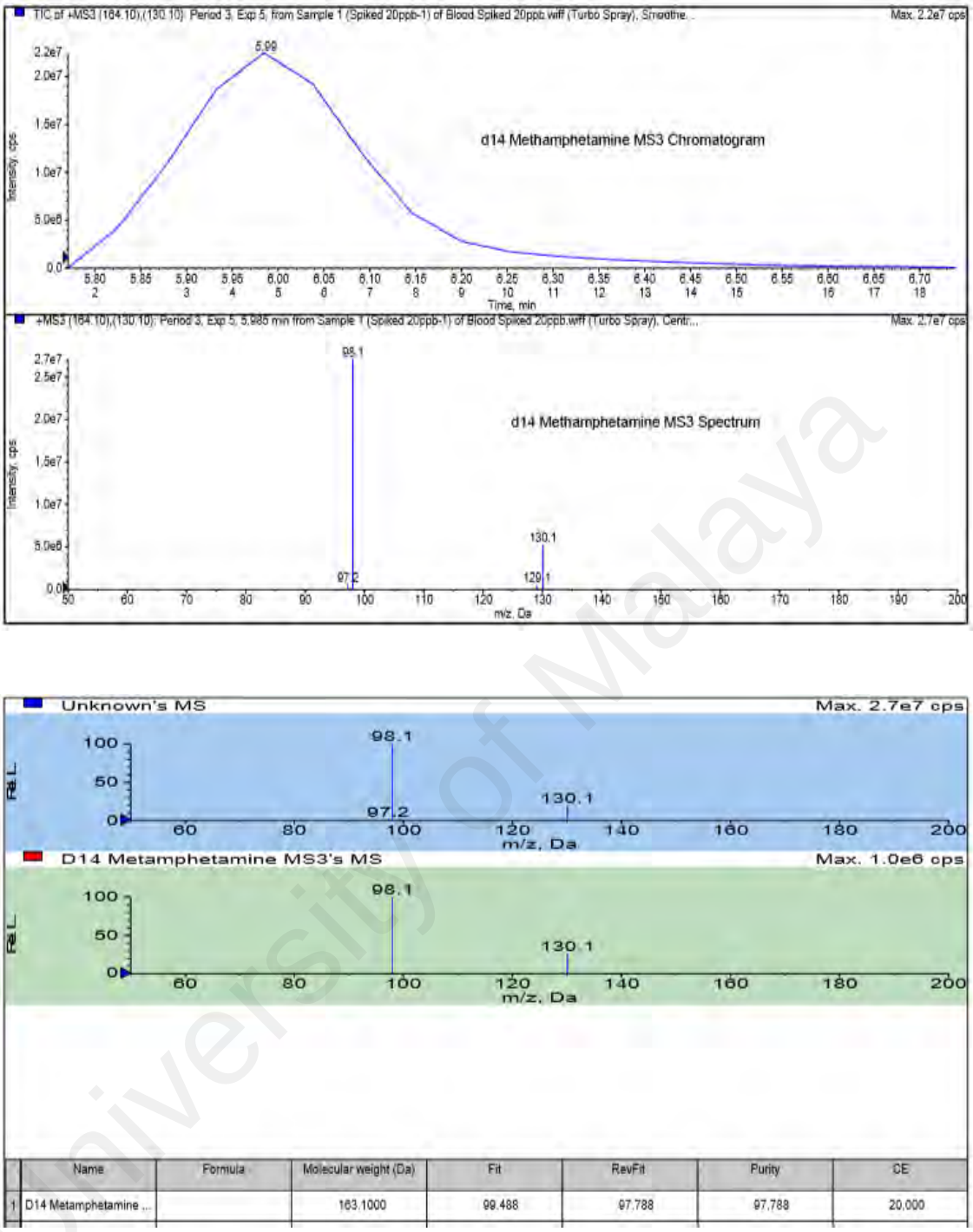


Figure 4.5l: MS^3 or MRM^3 experiment - methamphetamine-d_{14} MRM^3 Scan chromatogram and the scan spectrum with further library matching.

Period 4 [MDMA, phentermine and MDMA-d_5 ISTD]:

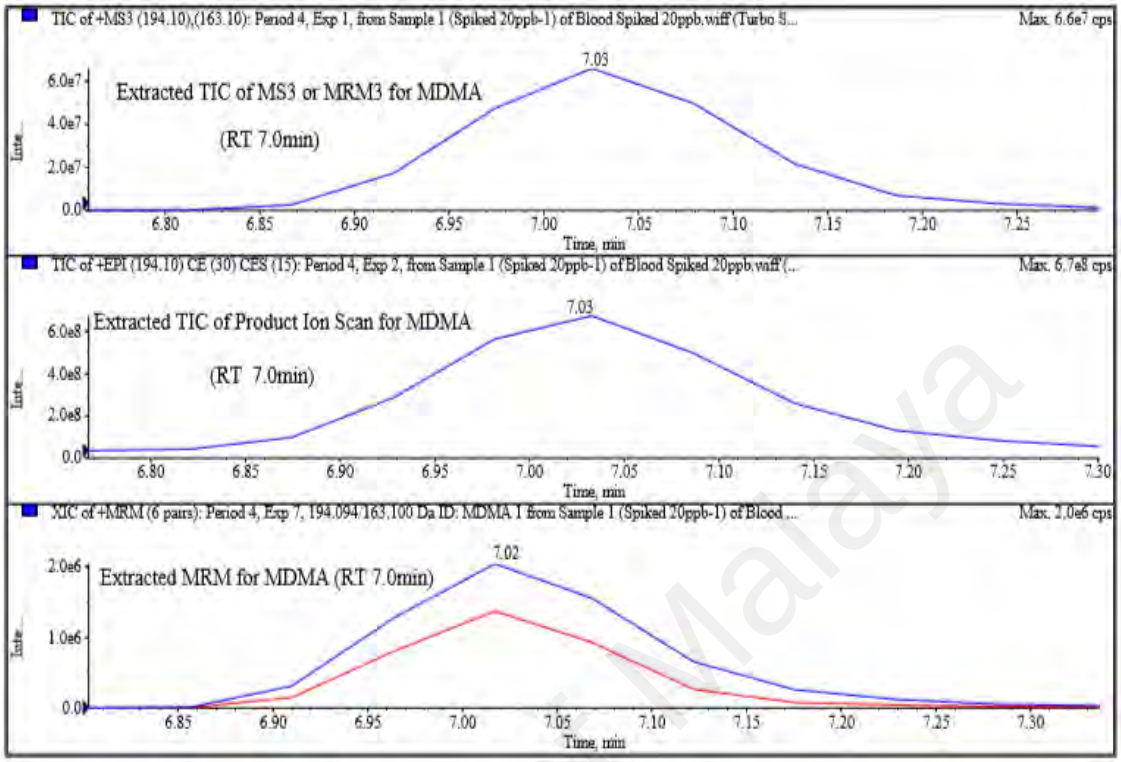


Figure 4.6a: Multi-experiment of MDMA from period 4.

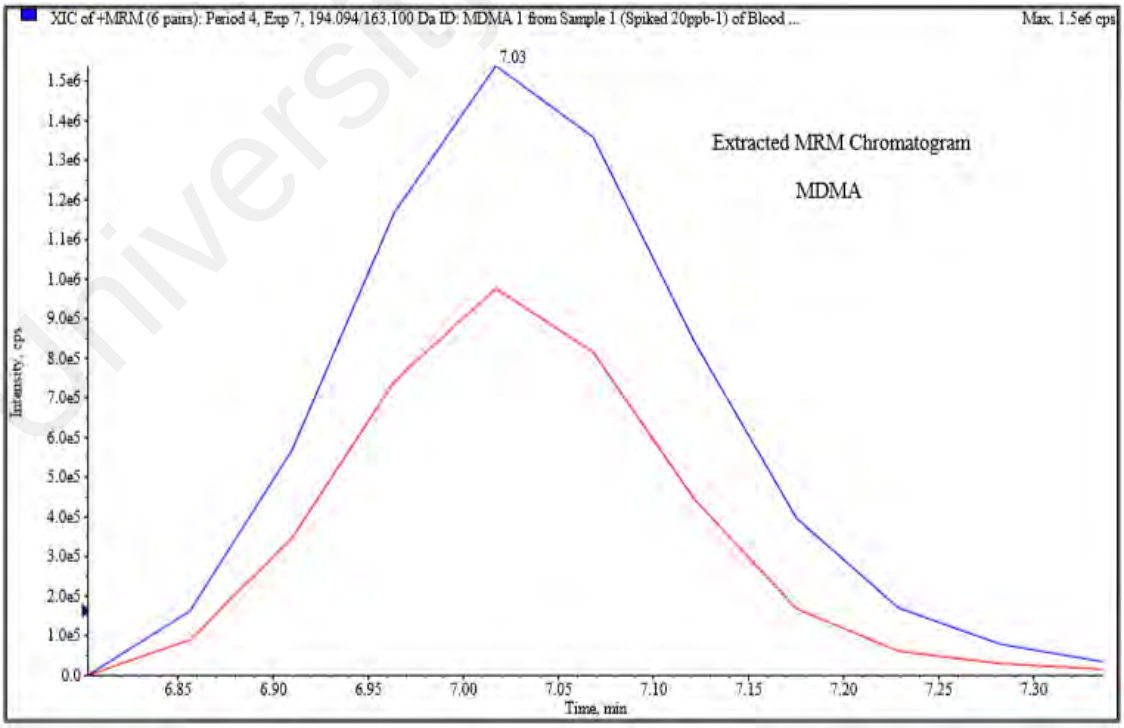
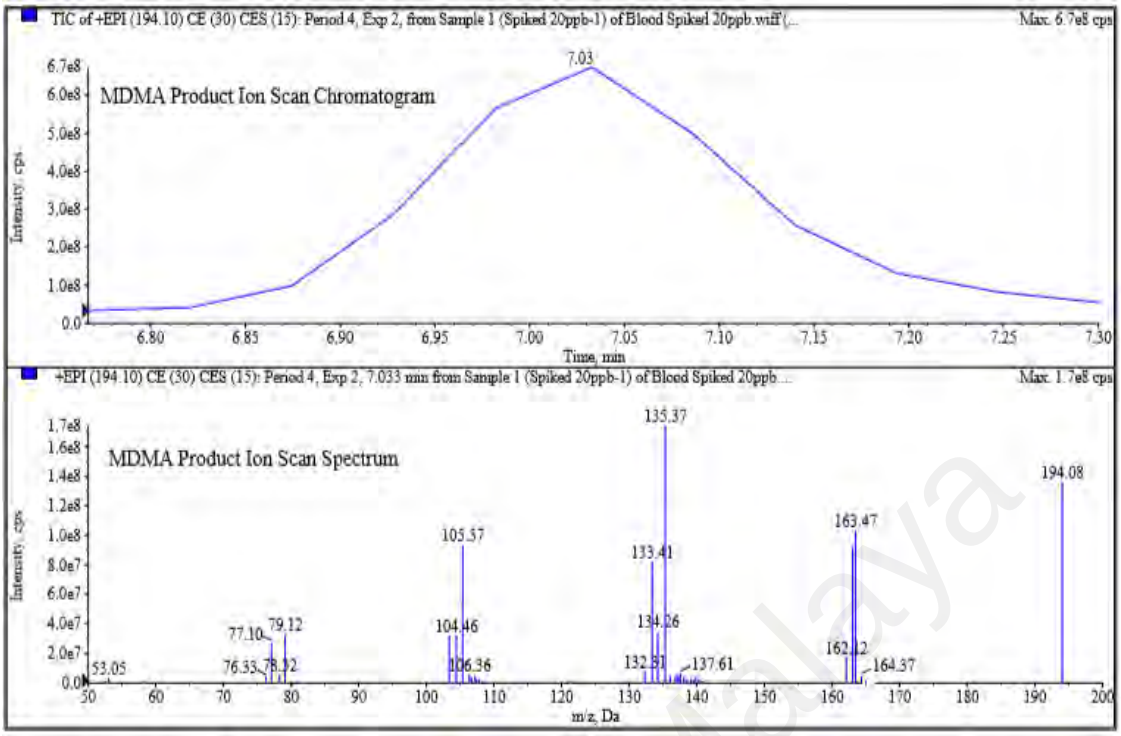
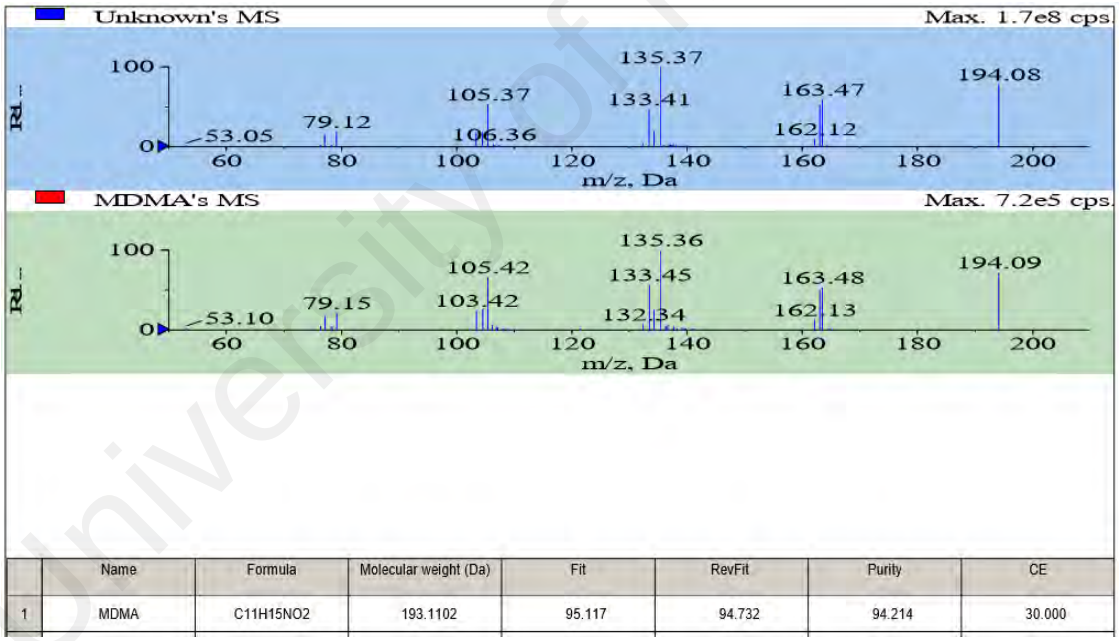


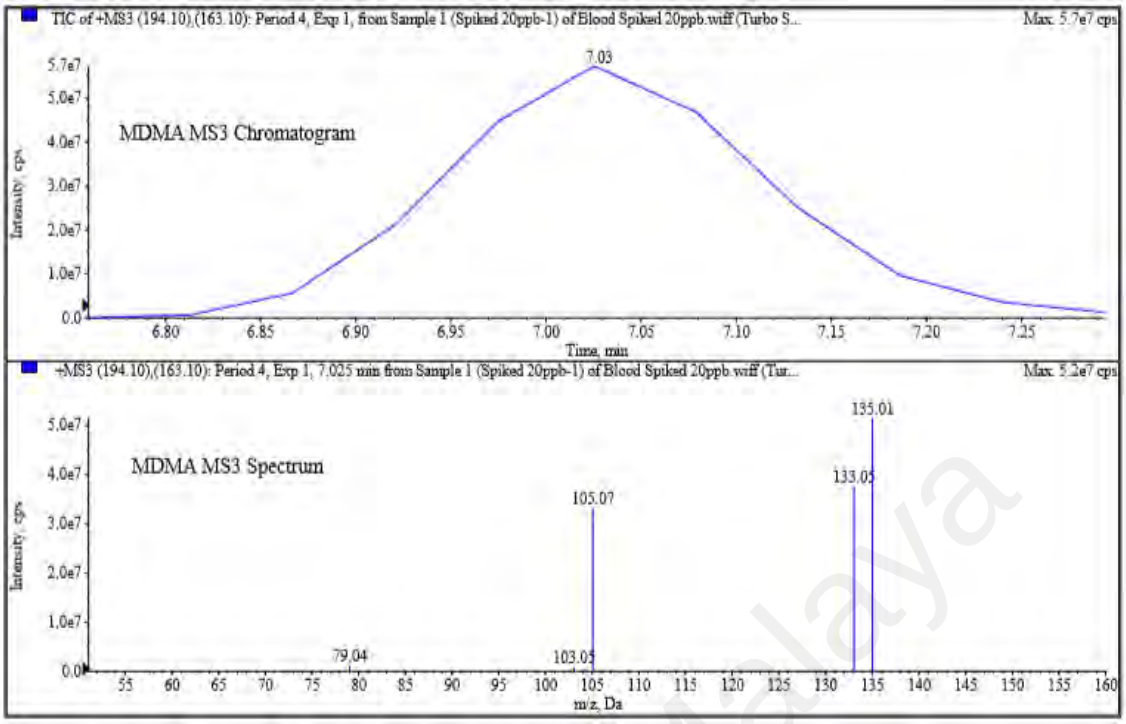
Figure 4.6b: MRM experiment - Extracted MRM chromatogram for MDMA.

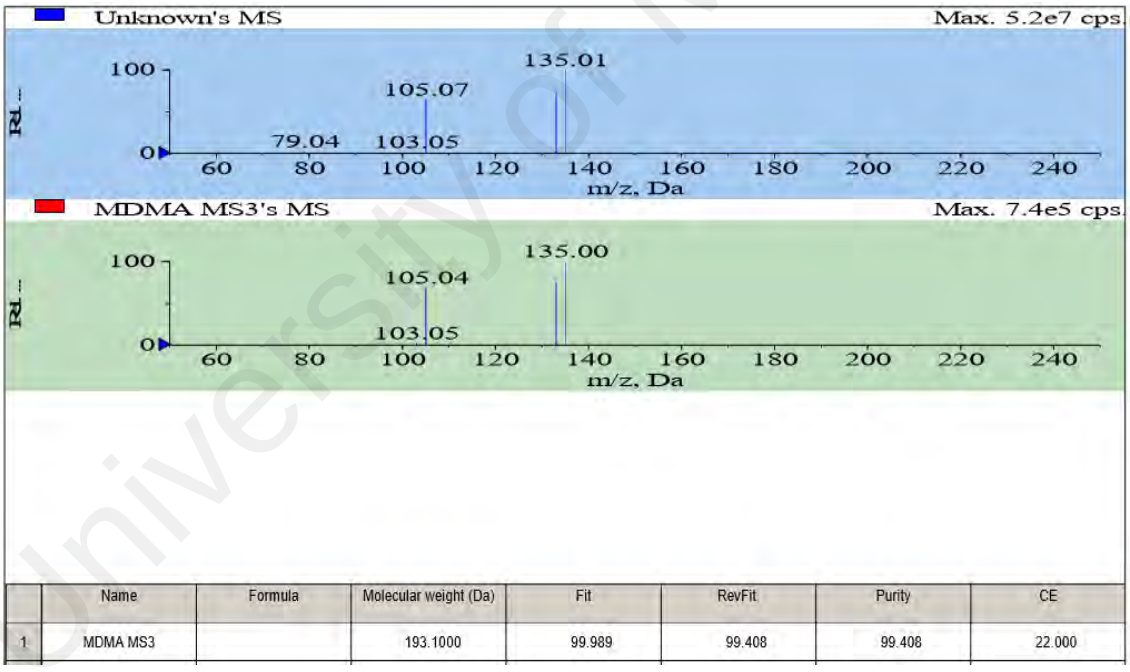




	Name	Formula	Molecular weight (Da)	Fit	RevFit	Purity	CE
1	MDMA	C11H15NO2	193.1102	95.117	94.732	94.214	30.000

Figure 4.6c: EPI experiment - MDMA Enhanced Product Ion Scan chromatogram and the scan spectrum with library matching.





	Name	Formula	Molecular weight (Da)	Fit	RevFit	Purity	CE
1	MDMA MS3		193.1000	99.989	99.408	99.408	22.000

Figure 4.6d: MS^3 or MRM^3 experiment - MDMA MRM^3 Scan chromatogram and the scan spectrum with further library matching.

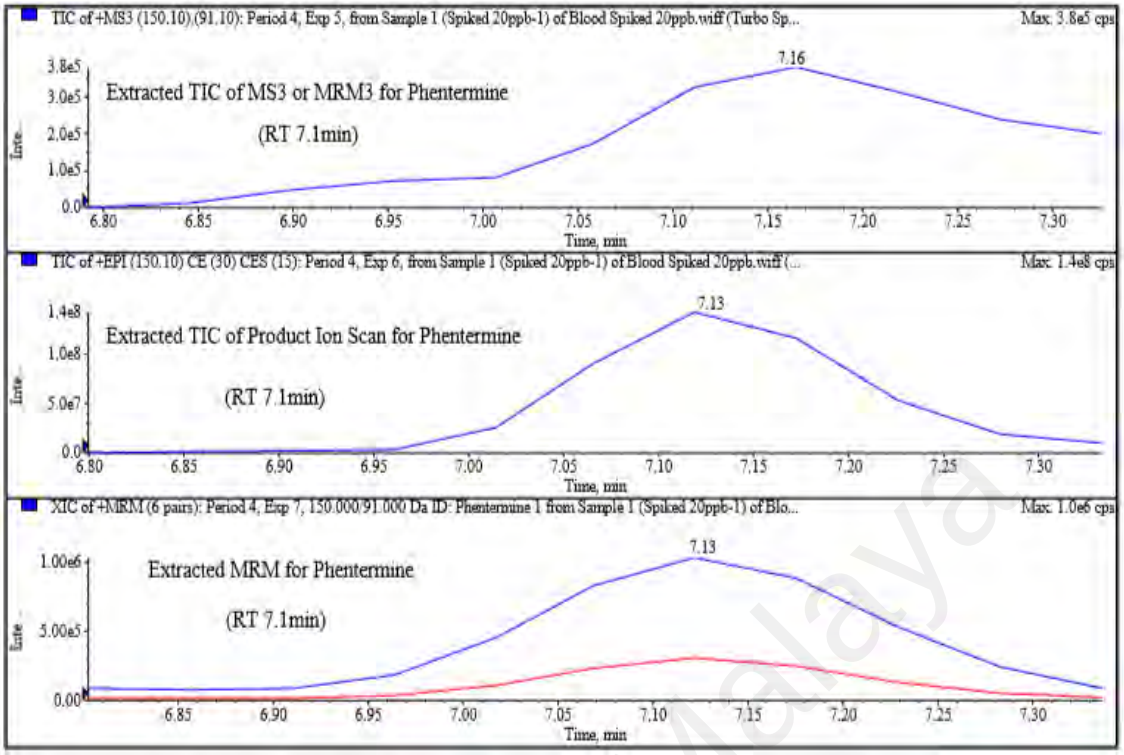


Figure 4.6e: Multi-experiment of phentermine from period 4.

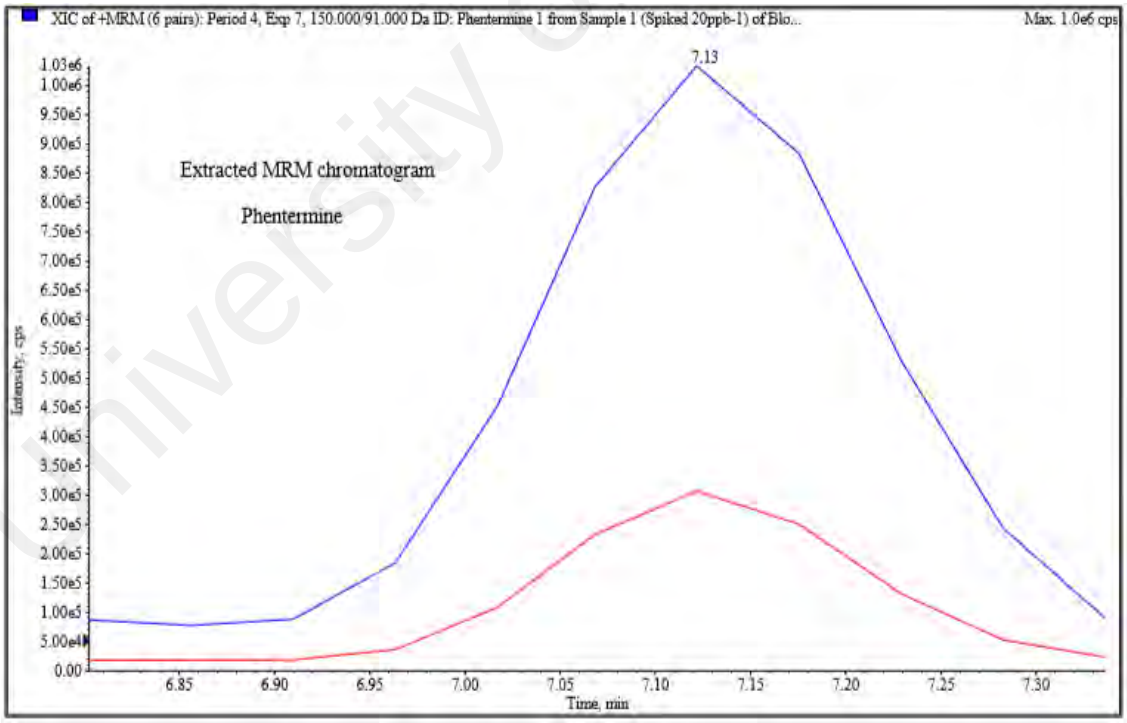
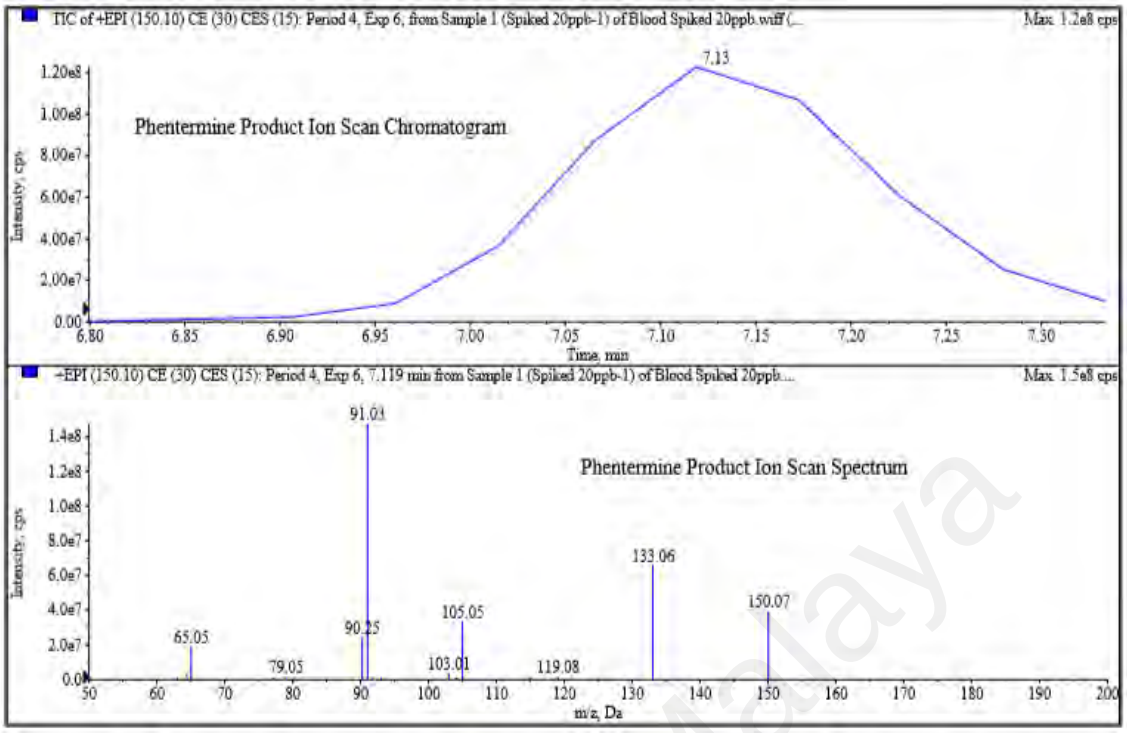
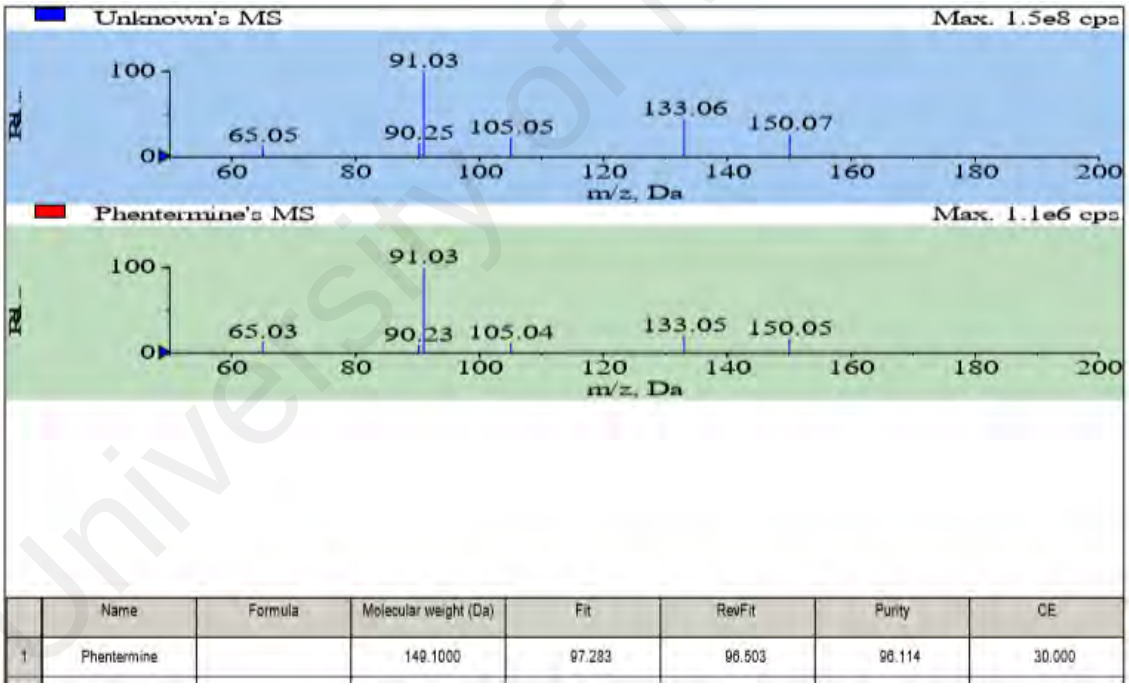


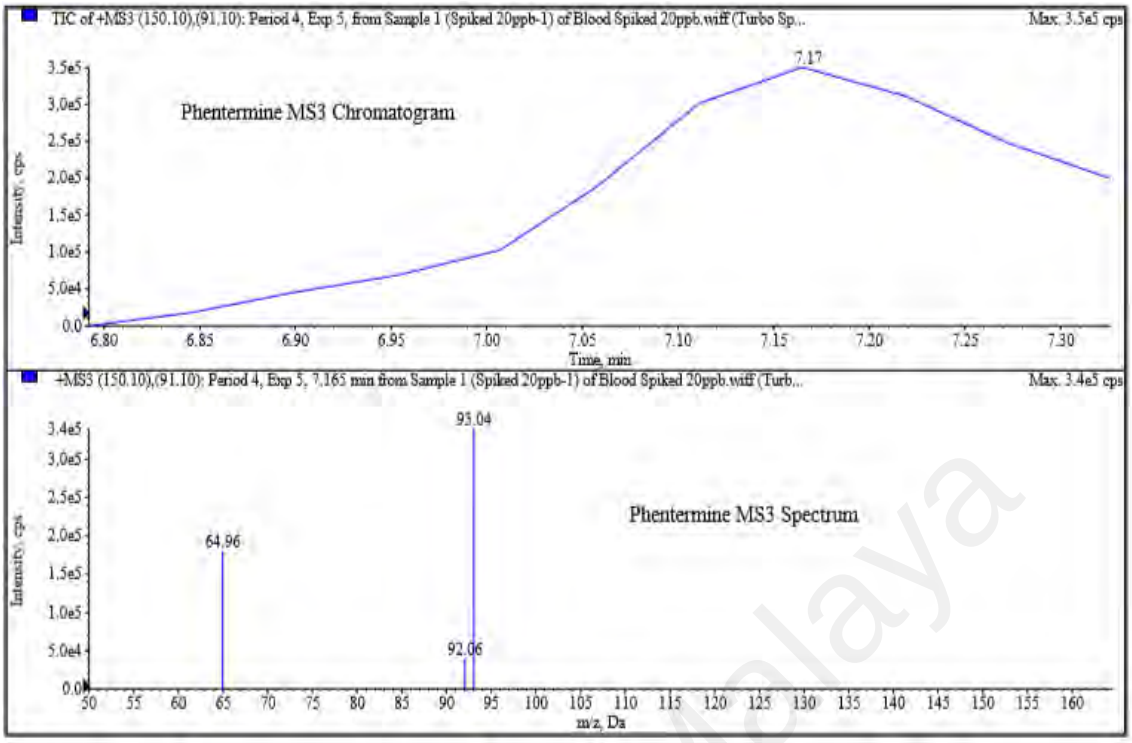
Figure 4.6f: MRM experiment - Extracted MRM chromatogram for phentermine.





	Name	Formula	Molecular weight (Da)	Fit	RevFit	Purity	CE
1	Phentermine		149.1000	97.283	96.503	96.114	30.000

Figure 4.6g: EPI experiment - phentermine Enhanced Product Ion Scan chromatogram and the scan spectrum with library matching.



	Name	Formula	Molecular weight (Da)	Fit	RevFit	Purity	CE
1	Phentermine MS3		149.1000	87.679	94.301	87.679	44.000

Figure 4.6h: MS^3 or MRM^3 experiment - phentermine MRM^3 Scan chromatogram and the scan spectrum with further library matching.

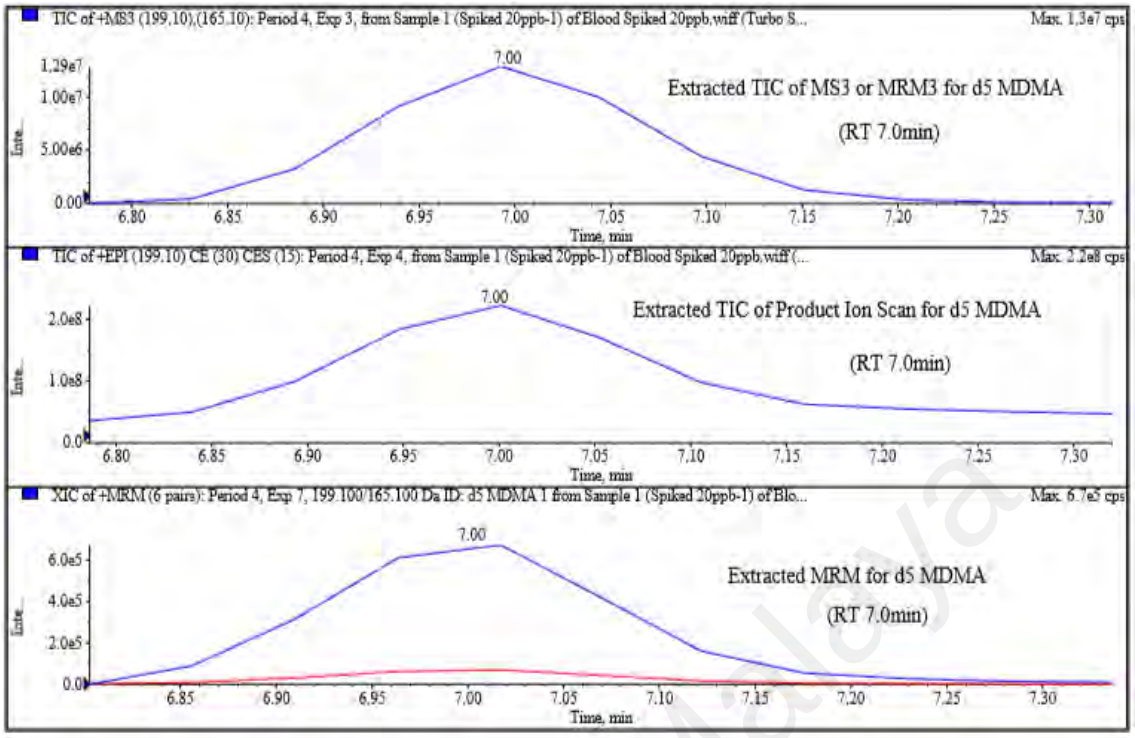


Figure 4.6i: Multi-experiment of MDMA-d_5 ISTD from period 4.

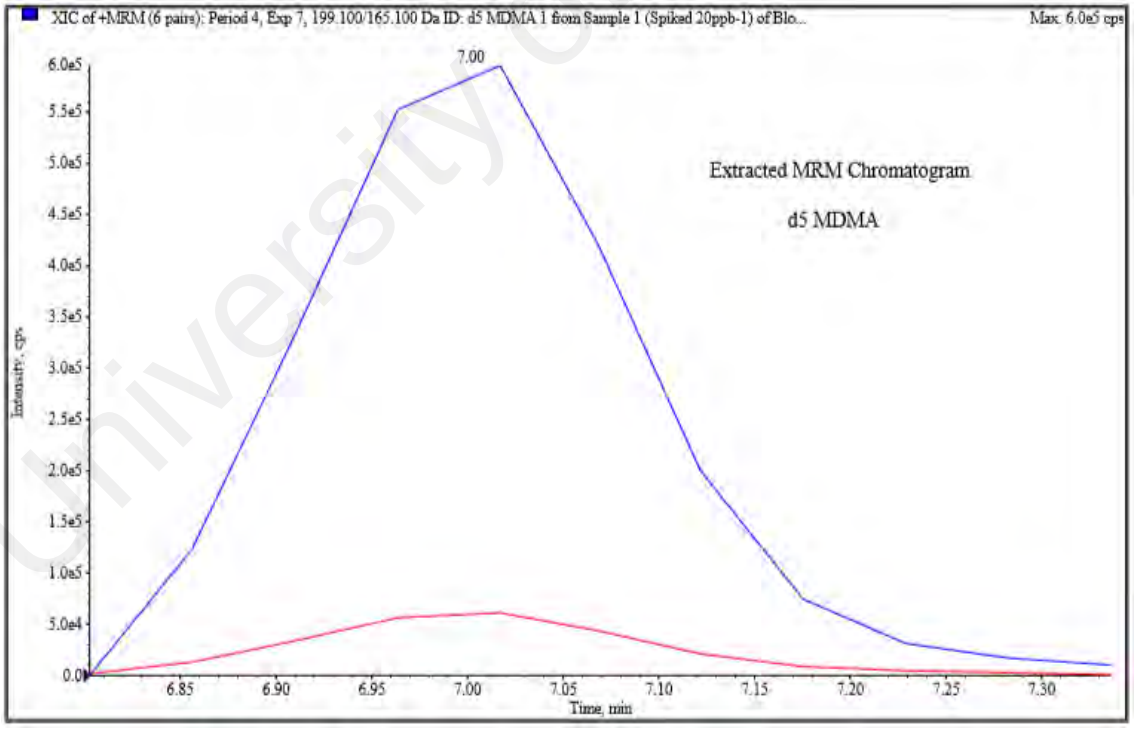
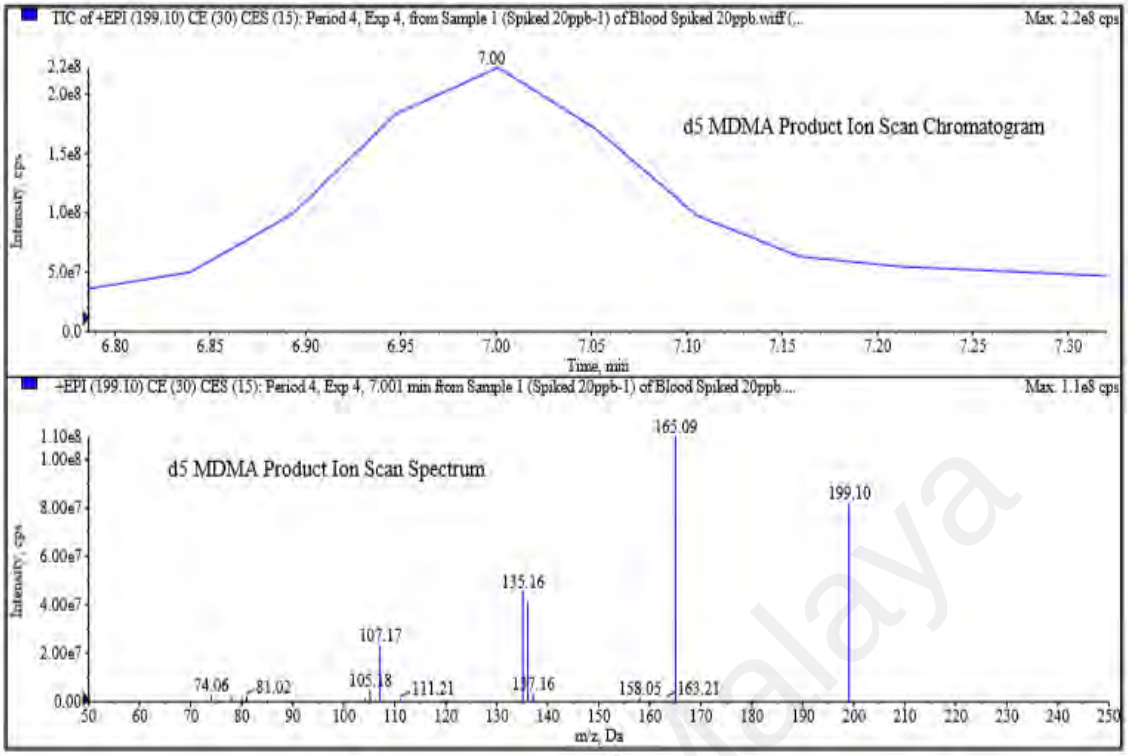
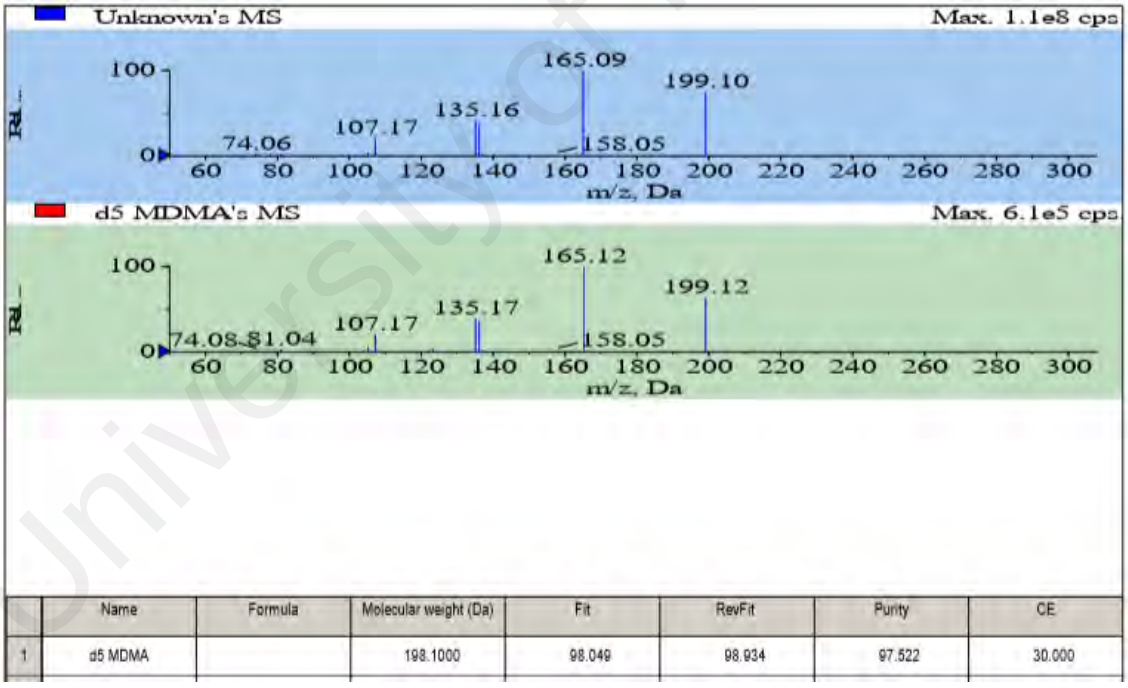


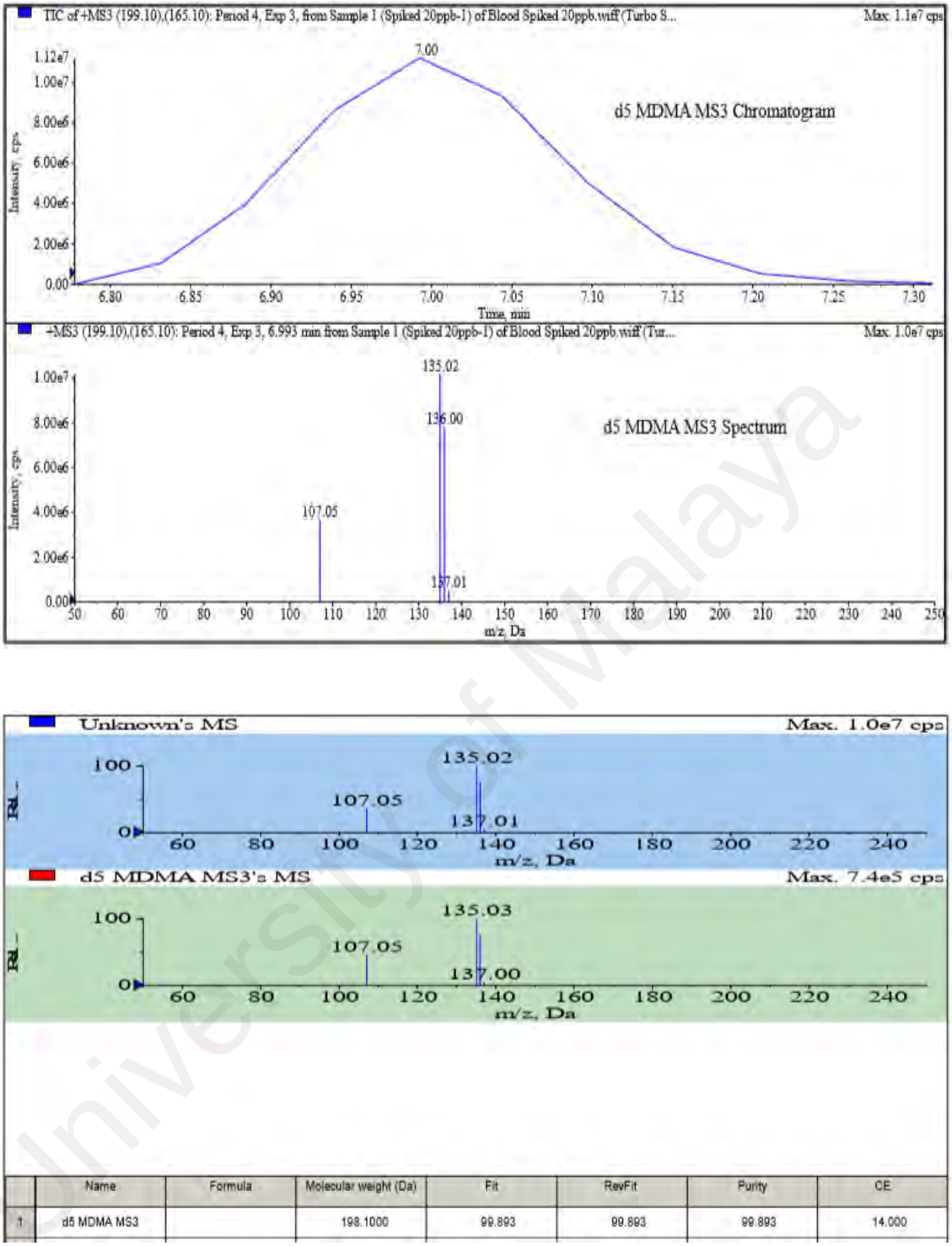
Figure 4.6j: MRM experiment - Extracted MRM chromatogram for MDMA-d_5 ISTD.





	Name	Formula	Molecular weight (Da)	Fit	RevFit	Purity	CE
1	d5 MDMA		198.1000	98.049	98.934	97.522	30.000

Figure 4.6k: EPI experiment - MDMA-d_5 Enhanced Product Ion Scan chromatogram and the scan spectrum with library matching.



	Name	Formula	Molecular weight (Da)	Fit	RevFit	Purity	CE
1	d5 MDMA MS3		198.1000	99.893	99.893	99.893	14.000

Figure 4.6l: MS^3 or MRM^3 experiment - MDMA-d_5 MRM3 Scan chromatogram and the scan spectrum with further library matching.

Period 5 [MDEA]:

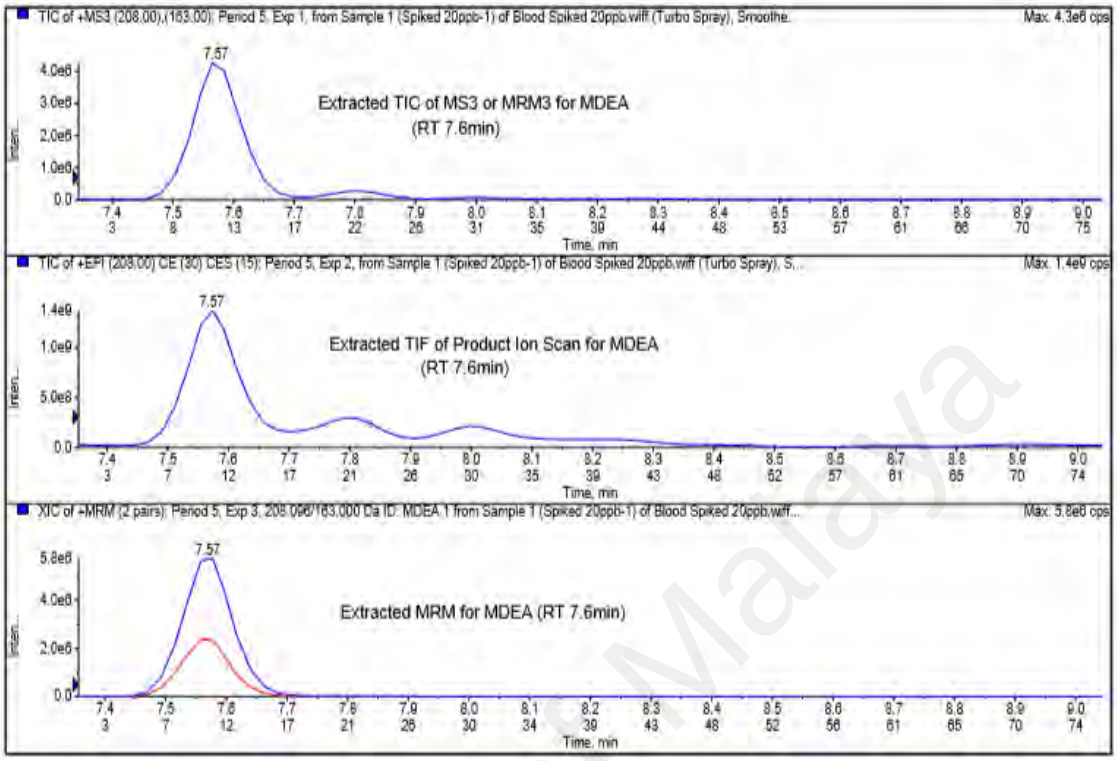


Figure 4.7a: Multi-experiment of MDEA from period 5.

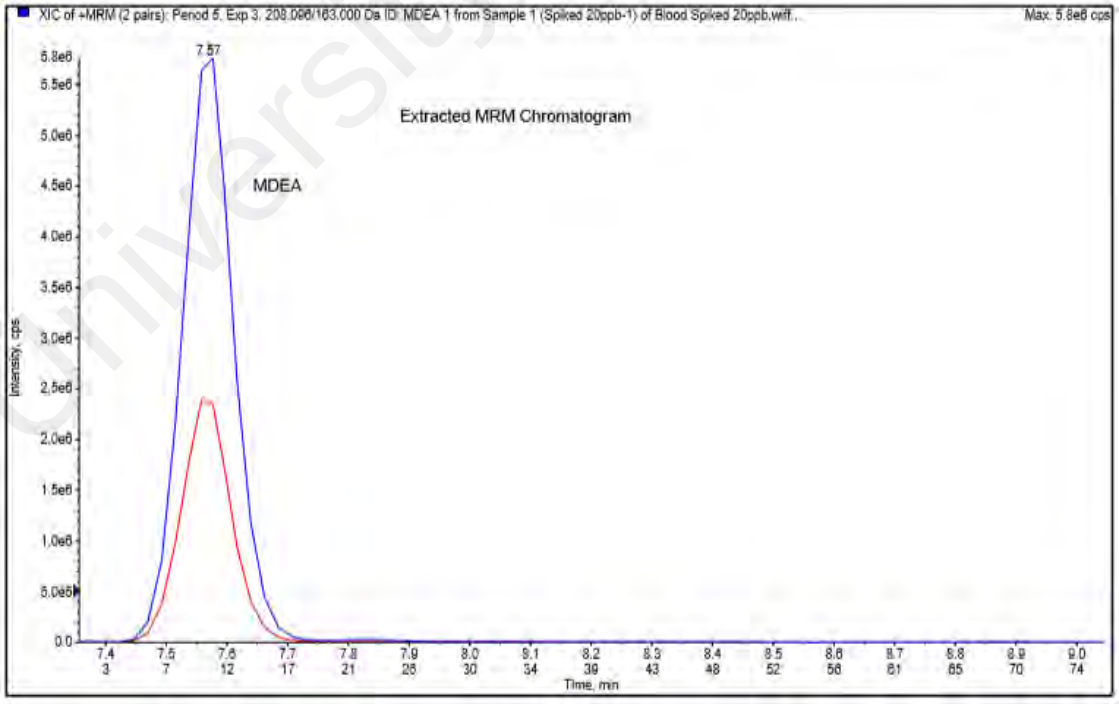
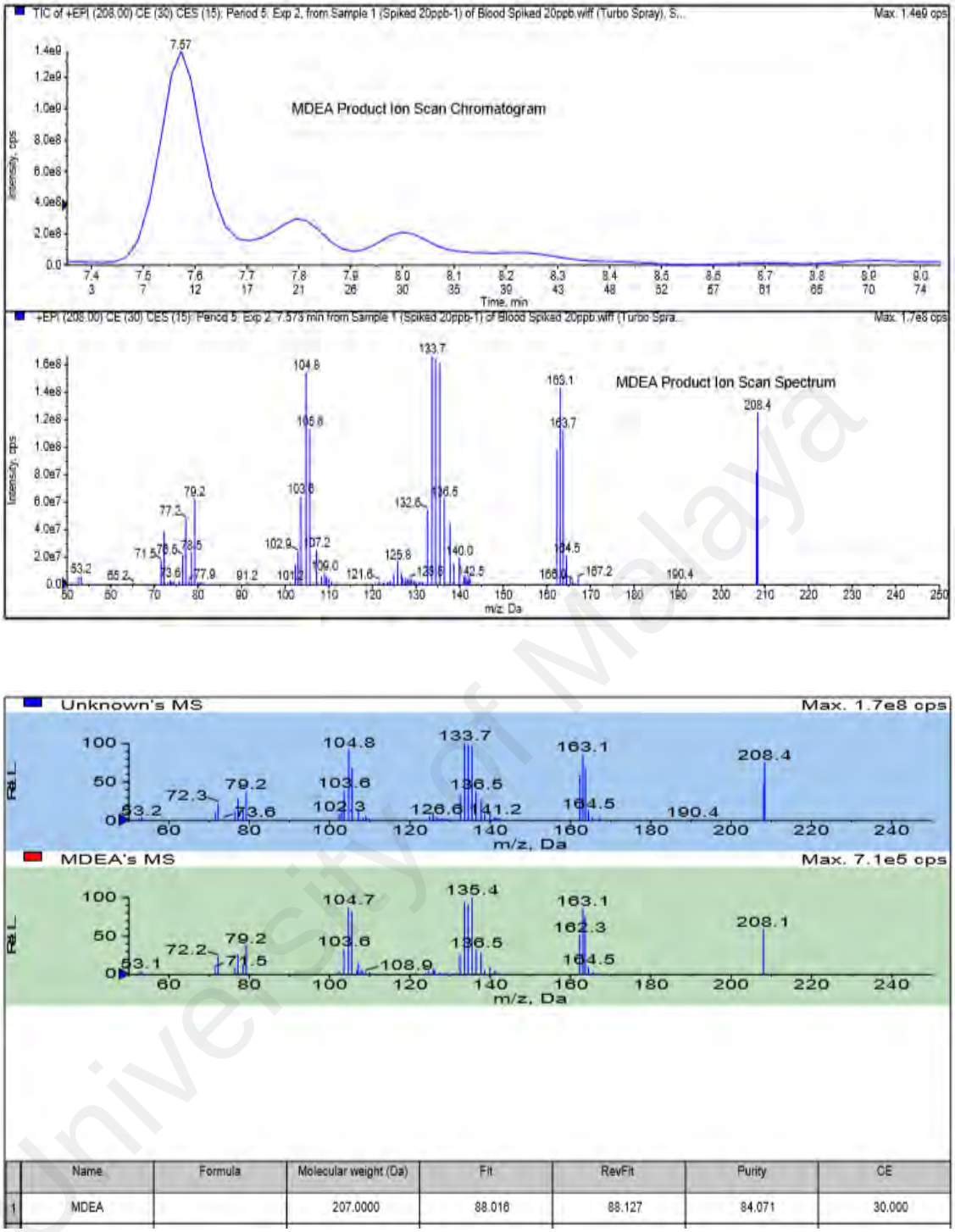
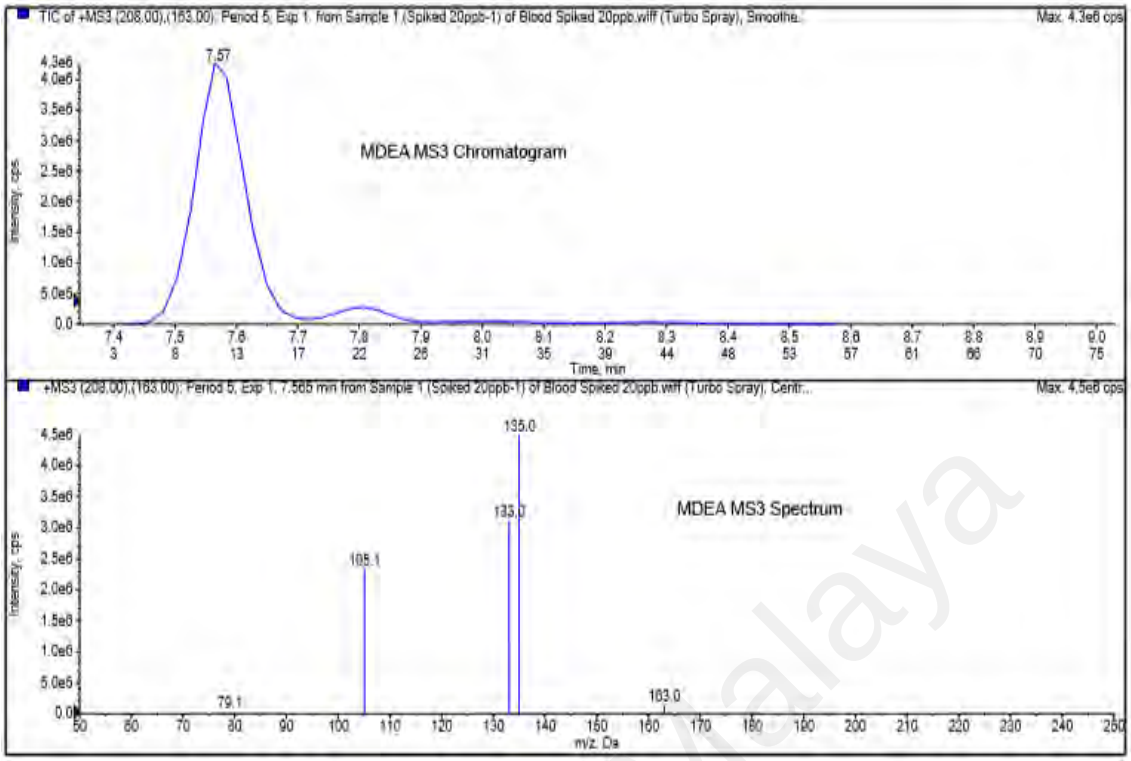


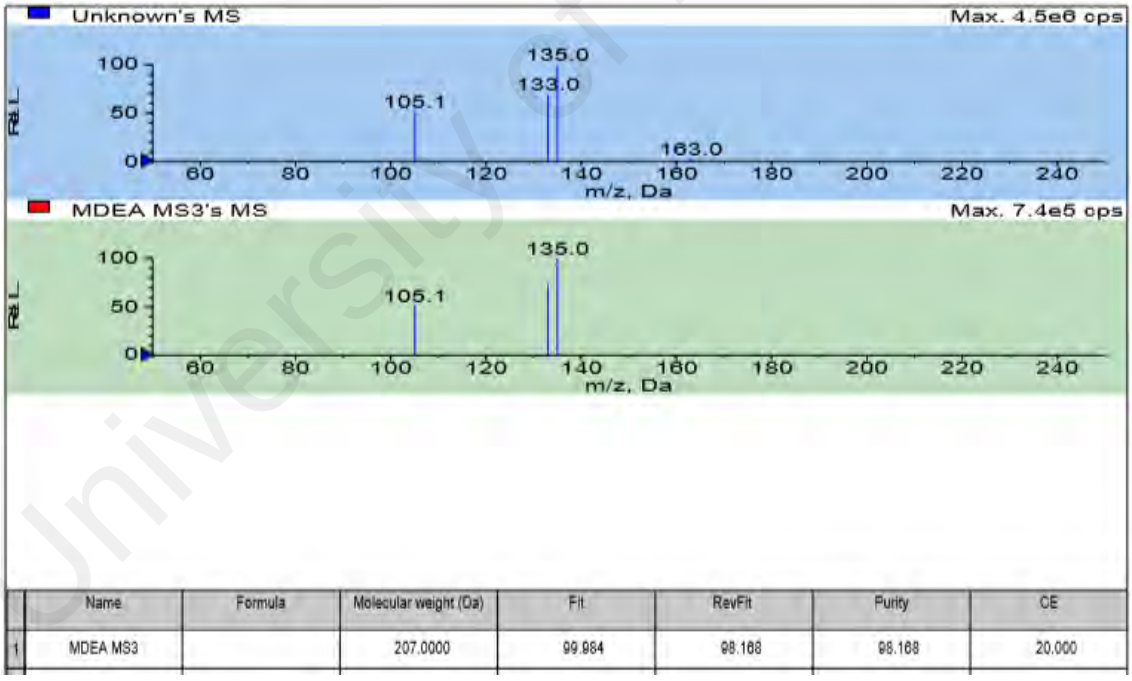
Figure 4.7b: MRM experiment - Extracted MRM chromatogram for MDEA.



	Name	Formula	Molecular weight (Da)	Fit	RevFit	Purity	CE
1	MDEA		207.0000	88.016	88.127	84.071	30.000

Figure 4.7c: EPI experiment - MDEA Enhanced Product Ion Scan chromatogram and the scan spectrum with library matching.





	Name	Formula	Molecular weight (Da)	Fit	RevFit	Purity	CE
1	MDEA MS3		207.0000	99.984	98.168	98.168	20.000

Figure 4.7d: MS3[3] or MRM3[3] experiment - MDEA MRM3[3] Scan chromatogram and the scan spectrum with further library matching.

In summary, all the tested ATS drugs were successfully determined using MRM-EPI-MRM^3 experiment and all of the compounds have shown good purity matching to the library for high confidence level of detection (Table 4.4)

Table 4.4: Purity matching of compounds spiked in matrices showing high confidence level for compound identification.

Purity matching of compounds spiked in matrices		
Compound	**% Purity Matching with standards**	
	Enhanced Product Ion Scan	**MS^3 or MRM^3**
Ephedrine	87	94
Pseudoephedrine	90	97
Amphetamine	92	97
Methamphetamine	96	100
MDA	96	99
MDMA	94	99
Phentermine	97	87
MDEA	84	98

4.1.3 Method Validation

The linearity, specificity/selectivity and matrix effect, LOD and LOQ, accuracy and precision (within the laboratory repeatability and/or within the laboratory reproducibility conditions) were investigated during method validation.

4.1.3.1 Linearity, LOD, LOQ and carry over

The results showed good linearity relationships between peak areas and analytes concentration with correlation coefficients greater than 0.994 for all analytes. Dynamic linear ranges, LOD, LOQ and calibration results were shown in Table 4.5 and 4.6 as follows:

Table 4.5: Instrument limit of detection and limit of quantitation for all analytes level at 3 SD

Compound	LOD (ng/mL)	LOQ (ng/mL)
AMP	0.006	0.021
MA	0.156	0.521
MDA	0.150	0.500
MDMA	0.150	0.500
MDEA	0.003	0.009
PEP	0.017	0.017
EP	0.005	0.005
PTM	0.150	0.500

Table 4.6: Calibration curve dynamic range and compound extraction protocol limit of detection and limit of quantitation.

Analyte	LOD (ng/mL)	LOQ (ng/mL)	Slope	Intercept	R^2
Whole Blood (ng/mL)					
AMP	3.4	10.2	0.0348	0.0104	0.9975
MA	4.4	13.3	0.0353	0.1750	0.9975
MDA	4.3	13.0	0.6384	0.6341	0.9955
MDMA	3.7	11.2	1.2582	-2.1548	0.9945
MDEA	5.1	15.4	0.0319	0.0834	0.9943
PEP	3.6	10.8	0.0074	0.0359	0.9997
EP	3.6	11.0	0.0063	0.0340	0.9959
PTM	3.9	11.7	0.0028	-0.0106	0.9973

Table 4.6: Calibration curve dynamic range and compound extraction protocol limit of detection and limit of quantitation; cont.

Analyte	LOD (ng/mL)	LOQ (ng/mL)	Slope	Intercept	R^2
Dried Blood Stain (ng/mL)					
AMP	3.5	10.6	0.0187	0.0181	0.9991
MA	5.3	15.9	0.0182	0.1402	0.9957
MDA	2.1	6.5	0.1035	0.3026	0.9985
MDMA	5.2	15.6	0.2153	-0.0057	0.9973
MDEA	2.4	7.3	0.0050	0.0260	0.9975
PEP	4.7	14.2	0.0047	0.0267	0.9975
EP	1.5	4.5	0.0037	0.0249	0.9976
PTM	5.3	16.1	0.0013	-0.0044	0.9946

Table 4.6: Calibration curve dynamic range and compound extraction protocol limit of detection and limit of quantitation cont;

Analyte	LOD (ng/mL)	LOQ (ng/mL)	Slope	Intercept	R^2
Urine (ng/mL)					
AMP	4.1	12.5	0.0061	0.0034	0.9990
MA	7.2	21.8	0.0034	0.0776	0.9951
MDA	3.6	10.9	0.0201	0.0315	0.9972
MDMA	5.1	15.3	0.0332	0.0580	0.9964
MDEA	1.8	5.5	0.0095	0.0529	0.9952
PEP	3.3	10.0	0.1788	0.6104	0.9968
EP	3.1	9.3	0.1249	0.2950	0.9954
PTM	4.8	14.6	0.0183	-0.1054	0.9994

Scientific Working Group for Forensic Toxicology (SWGTOX) recommended to evaluate analyte's carry over to eliminate risk of false positive results. For this evaluation, blank sample with ISTD and blank solvents were analyzed after injection of 200 ng/mL calibrator and after each of three replicates injection of 100 ng/mL in a batch run. The results have shown the absence of carry over effect.

4.1.3.2 Recovery, accuracy and Precision

Method validation results for accuracy and precision are summarized in Table 4.7. The recoveries for all tested analytes were all within $\pm$ 20% error while the intra-day and inter-day precision results have shown acceptable precision of which all were less than 15% RSD. The results revealed good precisions and accuracies as well as established the reliability of the method.

Table 4.7: Method accuracy and precision

Analyte	Conc. (ng/mL)	Mean	Recovery	Standard Deviation	Percent CV
Whole Blood					
AMP	20.0	19.8	99.0	1.140	5.75
	100.0	91.5	91.5	4.416	4.82
MA	20.0	22.2	111.2	1.479	6.65
	100.0	102.0	101.9	4.708	4.62
MDA	20.0	18.8	94.4	1.451	7.68
	100.0	95.4	95.4	7.729	8.09
MDMA	20.0	18.2	91.0	1.247	6.85
	100.0	106.5	106.4	1.039	9.76
MDEA	20.0	22.9	114.8	1.717	7.47
	100.0	108.0	108.2	6.222	5.75
PEP	20.0	20.2	101.0	1.210	5.98
	100.0	96.7	96.7	7.545	7.80
EP	20.0	19.4	97.2	1.233	6.34
	100.0	90.6	90.6	5.406	5.96
PTM	20.0	17.2	86.1	1.302	7.55
	100.0	85.1	85.1	4.019	4.72

n= 7 replicates of each 20.0 and 100.0 ng/mL respectively

Table 4.7: Method accuracy and precision. cont;

Analyte	Conc. (ng/mL)	Mean	Recovery	Standard Deviation	Percent CV
DBS					
AMP	20.0	21.9	109.9	1.182	5.37
	100.0	99.1	99.2	5.089	5.13
MA	20.0	21.8	109.0	1.775	8.14
	100.0	108.9	108.7	6.942	6.39
MDA	20.0	22.1	110.9	0.727	3.27
	100.0	100.5	100.5	8.753	8.70
MDMA	20.0	20.4	102.1	1.747	8.55
	100.0	108.9	108.9	9.141	8.39
MDEA	20.0	21.6	108.0	0.815	3.77
	100.0	106.6	106.6	6.487	6.08
PEP	20.0	19.3	96.5	1.581	8.19
	100.0	95.9	95.9	2.943	3.07
EP	20.0	18.5	92.7	0.504	2.71
	100.0	94.3	94.3	3.538	3.75
PTM	20.0	18.4	92.0	1.790	9.72
	100.0	93.0	93.0	8.269	8.88

n= 7 replicates of each 20.0 and 100.0 ng/mL respectively

Table 4.7: Method accuracy and precision. cont;

Analyte	Conc. (ng/mL)	Mean	Recovery	Standard Deviation	Percent CV
Urine					
AMP	20.0	19.0	95.1	1.396	7.34
	100.0	102.0	102.1	3.765	3.69
MA	20.0	19.5	97.5	2.430	12.45
	100.0	115.3	115.3	2.664	2.31
MDA	20.0	17.9	89.6	1.219	6.80
	100.0	103.4	103.4	2.794	2.70
MDMA	20.0	18.9	94.7	1.718	9.07
	100.0	105.6	105.5	7.776	7.37
MDEA	20.0	24.5	122.5	0.620	2.53
	100.0	93.8	93.8	12.04	12.83
PEP	20.0	19.4	97.2	1.119	5.75
	100.0	85.2	85.2	4.329	5.08
EP	20.0	20.0	100.0	1.040	5.20
	100.0	102.3	102.2	2.581	2.52
PTM	20.0	16.9	84.7	1.632	9.63
	100.	82.2	82.2	8.643	10.51

n= 7 replicates of each 20.0 and 100.0 ng/mL respectively

4.1.3.3 Matrix Effects

The evaluation was performed in seven (n=7) replicates and the results were 80–110% with CV less than 10% for the entire set of analytes. Addition of the isotopically labelled internal standards in the MRM mode for quantitative analysis and the use of highly selective mode of MRM^3 have helped in reducing the matrix effects for higher confidence level of confirmation with library matching. The observed reduced matrix effect would be helpful for the differentiation of isomeric compounds and quantification of the compounds for the desired forensic application.

4.1.3.4 Application on Forensic Toxicology Cases in Malaysia

From the results of the 20 real cases samples, the measured responses and intended responses (previous case samples) are listed in Table 4.8 and 4.9. Comparison of the measured and tested results indicated the method accuracies of 81.0-106.7%. The values were within 80-120% as stated in the guidelines and established the performance of the method.

Table 4.8: Measured response and accuracy for ATS drugs in Whole Blood Sample (WBS; n=20) and Dried Blood Stain (DBS; n=20)

Blood (n=20)	Intended Response (ng/mL) -WBS	Measured Response (ng/mL) -WBS	Recovery (%) WBS	Measured Response (ng/mL) -DBS	Recovery (%) DBS
1	MDMA (600.0), MDA (50.0)	MDMA (591.2), MDA (48.6)	MDMA (98.5), MDA (97.2)	MDMA (585.3), MDA (48.0)	MDMA (97.5), MDA (96.0)
2	MDMA (200.0), MDA (35.4) MA (40.1)	MDMA (190.4), MDA (31.2) MA (33.4)	MDMA (95.2), MDA (88.1) MA (83.3)	MDMA (192.6), MDA (30.1) MA (33.4)	MDMA (96.3), MDA (85.0) MA (83.3)
3	MA (105.8), AMP (16.8)	MA (100.5), AMP (14.2)	MA (95.0), AMP (84.5)	MA (98.8), AMP (13.6)	MA (93.3), AMP (81.0)
4	PEP (350.6)	PEP (352.3)	PEP (100.4)	PEP (348.6)	PEP (99.4)
5	MDMA (635.2), MDA (56.3)	MDMA (621.3), MDA (52.1)	MDMA (97.8), MDA (92.5)	MDMA (612.5), MDA (48.7)	MDMA (96.4), MDA (86.5)
6	PTM (485.6), PEP (205.8)	PTM (451.0), PEP (215.2)	PTM (92.8), PEP (104.5)	PTM (447.9), PEP (216.0)	PTM (92.2), PEP (104.9)
7	PTM (151.2)	PTM (130.4)	PTM (86.2)	PTM (132.2)	PTM (87.4)
8	MDMA (1130.2), MDA (60.8)	MDMA (1100), MDA (65.8)	MDMA (97.3), MDA (108.2)	MDMA (1020), MDA (60.2)	MDMA (90.2), MDA (99.0)
9	MA (245.8), AMP (30.2)	MA (241.7), AMP (28.6)	MA (98.3), AMP (94.7)	MA (230.8), AMP (25.6)	MA (93.8), AMP (84.7)
10	MA (297.6), AMP (58.0)	MA (291.6), AMP (55.0)	MA (97.9), AMP (94.8)	MA (281.2), AMP (60.0)	MA (94.4), AMP (103.4)
11	PTM (157.8)	PTM (160.2)	PTM (101.5)	PTM (155.8)	PTM (98.7)
12	MA (275.0), AMP (70.3)	MA (278.3), AMP (67.4)	MA (101.2), AMP (95.8)	MA (270.2), AMP (60.0)	MA (98.2), AMP (85.3)
13	MA (680.9), AMP (86.2)	MA (678.0), AMP (85.4)	MA (99.5), AMP (99.0)	MA (690.0), AMP (90.2)	MA (101.3), AMP (104.6)
14	MA (450.0), AMP (40.6)	MA (445.1), AMP (38.6)	MA (98.9), AMP (95.1)	MA (435.0), AMP (40.2)	MA (96.7), AMP (99.0)
15	MA (370.7), AMP (45.0)	MA (375.1), AMP (43.7)	MA (101.1), AMP (97.1)	MA (350.9), AMP (37.8)	.MA (94.6), AMP (84.0)
16	PEP (57.8)	PEP (56.8)	PEP (98.2)	PEP (60.2)	PEP (104.1)
17	MDMA (50.8), MDA (42.5)	MDMA (51.6), MDA (44.6)	MDMA (101.5), MDA (104.9)	MDMA (48.7), MDA (41.5)	MDMA (95.8), MDA (97.6)
18	MDMA (60.0), MDA (45.0)	MDMA (59.9), MDA (40.3)	MDMA (99.8), MDA (89.5)	MDMA (62.5), MDA (43.7)	MDMA (104.1), MDA (97.1)
19	PEP (55.0)	PEP (57.0)	PEP (103.6)	PEP (50.8)	PEP (92.3)
20	MA (218.7), AMP (23.8)	MA (222.4), AMP (25.4)	MA (101.6), AMP (106.7)	MA (212.9), AMP (21.5)	MA (97.3), AMP (90.3)
QC 1	MA (769.42), AMP (150.10)	MA (750.20), AMP (135.50)	MA (97.5), AMP (90.3)	MA (753.3), AMP (138.6)	MA (97.9), AMP (92.3)
QC 2	MA (395.65), AMP (75.84) MDMA (1220.50) MDA (201.93)	MA (360.10), AMP (78.3) MDMA (1300) MDA (185.80)	MA (91.0), AMP (103.2) MDMA (106.5) MDA (92.0)	MA (356.8), AMP (80.2) MDMA (1280) MDA (178.9)	MA (90.1), AMP (105.7) MDMA (104.8) MDA (88.6)
QC 3	MA (299.66), AMP (49.44)	MA (275.80), AMP (51.70)	MA (92.0), AMP (104.5)	MA (268.4), AMP (52.31)	MA (89.6), AMP (105.8)
QC 4	PTM (248.50)	PTM (240.25)	PTM (96.6)	PTM (235.9)	PTM (94.9)

*QC: College of American Pathologists (CAP) and Quality Assurance Div., Dept. of Chemistry, Malaysia.

Table 4.9: Measured response and accuracy for ATS drugs in urine sample.

Urine(n=20)	Intended Response	Measured Response
1	PTM	PTM
2	PTM, PEP	PTM, PEP
3	MDMA, MDA	MDMA, MDA
4	MA, AMP, EP	MA, AMP, EP
5	MA, AMP, MDMA, MDA	MA, AMP, MDMA, MDA
6	PEP	PEP
7	MDEA	MDEA
8	MA, AMP	MA, AMP
9	MA, AMP	MA, AMP
10	MDMA, MDA	MDMA, MDA
11	MDA	MDA
12	MA, AMP, MDMA, MDA	MA, AMP, MDMA, MDA
13	MDMA, MDA	MDMA, MDA
14	MA, AMP	MA, AMP
15	MA, AMP	MA, AMP
16	MA, AMP	MA, AMP
17	MA, AMP, MDMA, MDA	MA, AMP, MDMA, MDA
18	PTM	PTM
19	MA, AMP	MA, AMP
20	MDMA, MDA	MDMA, MDA
QC 1	PEP, EP	PEP, EP
QC 2	MDA	MDA
QC 3	MDA	MDA
QC 4	MA	MA
QC 5	MA, AMP	MA, AMP
QC 6	MDMA	MDMA

*Urine sample is subjected to qualitative analysis based on the routine laboratory practice.

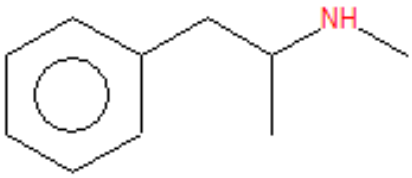
4.2 High-throughput Flow Injection Analysis-*MRM*^{3} (FIA-*MRM*^{3}) Spectrometry for Alternative Fast Screening of Isomeric Compounds in Blood for Forensic Toxicology Cases in Malaysia.

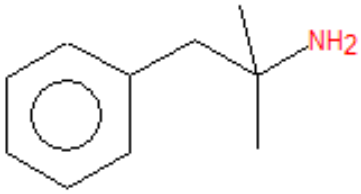
4.2.1 General

Isomeric compounds have similar molecular formula but different chemical structures. Isomeric compounds gave similar parent and fragment *m/z*. Prime example of critical isomeric AMPHETAMINE TYPE stimulants are methamphetamine and phentermine, which often co-exist in biological specimen in toxicology cases. The summary of physical properties and chemical structures for both compounds is summarized as follows (Table 4.2.1 and Figure 4.2.1):

Table 4.10: Physical properties of Methamphetamine and Phentermine

Compound	Molecular Formula	Molar Mass	Monoisotopic Mass
Methamphetamine	$C_{10}H_{15}N$	149.24 g/mol	149.12 g/mol
Phentermine	$C_{10}H_{15}N$	149.24 g/mol	149.12 g/mol





Methamphetamine **Phentermine**

Figure 48: Chemical structure of Methamphetamine and Phentermine

In Malaysia, methamphetamine is a drug of abuse and is listed under both Poison Act (PA) 1952 and Dangerous Drugs Act (DDA) 1952. While phentermine is a prescribed medicine for treating obesity (Wai *et al.*, 2009) as part of a complete weight management programme and listed under the PA 1952 as a controlled substance. The substances which are listed under DDA 1952 carries heavier penalties chargeable under Criminal Procedure Code 399 in the legislation for forensic and medicolegal cases. In Royal Malaysia Armed Forces, methamphetamine and other drugs abusers can be charged under Armed Forces Act 1972. The toxicological summary of methamphetamine and phentermine are as in Table 4.11:

Table 4.11: Summary of Methamphetamine and Phentermine (use, effect, toxicology blood level).

Methamphetamine	**Phentermine**
- strong Central Nervous System stimulant	- psychostimulant drug
- recreational drug	- appetite suppressant
- rarely prescribed due to potential of neurotoxicity, aphrodisiac & euphoriant	-prescribed by physician to treat obesity
- Adverse Effects: psychosis, breakdown of skeletal muscle, seizures, bleeding in the brain, delusions, violent behavior, high addiction liability	- Adverse Effects: overstimulation, restlessness, nervousness, insomnia, tremor, dizziness, headache
- *(Toxicology Blood-Level)* *	- *(Toxicology Blood-Level)* *
Normal level : 0.01 -0.05μg/mL	Therapeutic level : 0.09-0.51 μg/mL
Toxic level : 0.60 – 5.00 μg/mL	Toxic level : not available
Lethal level : > 10 μg/mL	Lethal level : 1.50 – 7.60 μg/mL

(Source: i) Winek's Drug & Chemical Blood-Level Data (2001); ii) Clarke's Analytical Forensic Toxicology. Pharmaceutical Press (2013)).

Andre *et al*. (2011) reported on the use of a Phenomenex Kinetex™ C18 column with water containing 10 mM ammonium acetate and methanol buffer system to separate isomeric phthalates in food and beverages . The C18 material with a neutral buffer of ammonium acetate was found to give good separation and methanol which is organic modifier was highly efficient in separating isomers.

Sharon Lupo *et al.* (2015) used Raptor™ Biphenyl 2.7 µm, 100 x 2.1 mm column using a linear scouting gradient program and water, methanol, and acetonitrile mobile phases each modified as follows: 1) acidic - addition of 0.1% formic acid 2) neutral - addition of 5 mM ammonium acetate 3) buffered acidic - addition of 0.1% formic acid and 5 mM ammonium formate in the Shimadzu Nexera UHPLC equipped with a SCIEX API 4500™ MS-MS. The column was used for improved resolution of barbiturate isomers amobarbital and pentobarbital in 6 minutes run time.

The work has been extended to the separation of 43 opioids and metabolites, which able to completely separate 15 opioids isomers in less than 3.5 minutes run time (Sharon Lupo *et al,* 2015). The group of isomers include 1) *m/z* 286 (morphine, hydromorphone, norcodeine, norhydrocodone, pentazocine); 2) *m/z* 302 (oxymorphone, dihydrocodeine, noroxycodone); 3) *m/z* 328 (naloxone, 6-acetylmorphine, butorphanol); 4) *m/z* 300 (codeine, hydrocodone); 5) *m/z* 250 (O-desmethyltramadol, N-desmethyltramadol).

Hypersil Pentafluorophenyl (PFP) reversed phase column with addition of ammonium acetate in the mobile phase has been used in the separation of thirty perfluorinated compounds (PFC) and their isomers as reported by Kadar *et al*. (2010). The PFC are contaminants present in anti-sticking or amphoteric material commercial products such as carpet, clothes, fire-fighting-foams and detergents. The PFCs together with the isomers of perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid

(PFOA), which are the main degradation products of PFC have been successfully separated and analyzed in good resolution and in a short time analysis.

The previous work on MRM-EPI-MRM^3 has also described the use of Luna C18 Omega column which has 100% aqueous stability and enhanced selectivity or retention for polar analytes without diminishing useful non-polar retention for the separation and identification of common AMPHETAMINE TYPE stimulants and their metabolites together with the isomeric phentermine in the analysis.

The FIA spectrometry has been used worldwide and the principle has been described earlier in Chapter 3. Other than the applications in the clinical, pharmaceuticals and forensic toxicology (Chapter 3), this technique has also been applied in various other fields, *e.g.* in the agricultural and environment chemistry especially on pesticide residue analysis which has been consistent over a decade (Nanita *et al.*, 2009, 2011, 2013; Mol and Van Dam, 2014; Ito *et al.*, 2003).

Geerdink *et al.* (2006) used the FIA-thermo-spray tandem mass spectrometry to analyze twelve triazine herbicides and eleven of their degradation products in surface water with acceptable analytical characteristics. In the comparative studies, the obtained results are closely similar to those by means of other chromatographic procedures and illustrates the reliability of the procedure.

Metobolomics is another field with emerging application of FIA-MS where the technnique targets complex problems of high throughput analysis in medical research (Gonzalez *et al.*, 2015). From the study described by Kuldeep *et al.* (2015), Kang *et al.* (2011) and Koulman *et al.* (2014), the technique has been successfully applied in lipid profiling. The infusion MS/MS workflow on the SCIEX TripleTOF 5600 shown a novel data independent acquisition strategy for qualitative and quantitative molecular

characterization of complex lipid samples. The simple workflow enables easy data acquisition and post acquisiton data analysis for lipid identification. The method was succesfully applied DBS medium for analysis with some modifications in the preparation techniques.

Additional applications also include in the dietary supplements and medications for product quality and counterfeit detection. The works include analysis of ingredients in multivitamin tablets and some other to address the authenticity of dietary supplements and counterfeiting of medications used to treat life threatening ilnesses (Fernandez *et al.*, 2014) such as counterfeit anti-malarial medications.

The FIA-MS technique has become one of the best practices when looking for a short-time analysis even with adequate selectivity that this technique may offer. FIA-MS eliminated of sample analysis component associated with retention time measurement. This limitation has become the subject of consideration especially while dealing with isomeric compounds. At this point, the MRM^3 spectrometry technique is also investigated to add another dimension in identifying the isomeric compounds by adding the level of selectivity and using second generation fragments of MRM in MRM^3 mode.

There are several studies performed using this MRM^3 experiment such as in the quantification of Prostate Specific Antigen (PSA) in non-depleted human serum (Fortin *et al.,* 2009 and 2015) showing that MRM^3 analysis is a robust quantitative strategy for peptides in complex matrices when significant background or interferences are present. The MRM^3 with higher specificity also reduces the fractionation required for low level detection and increases sample throughput.

The value of MRM^3 also been proven in the study performed by Xu *et al.* (2015) in the work on quantification of the Therapeutic Peptide Exenatide in human plasma. The

increased selectivity using the technique allows for the elimination of baseline noise and chromatographic interference, resulting in superior analytical performance compared to traditional MRM. MRM^3 has also demonstrated the potential for excellent linearity range tested while the accuracy and reproducibility tested also compatible with the requirements for a development stage bioanalytical assay.

MRM^3 was implemented for the quantitation of crustacean allergens derived from shrimp and lobster with applicability demonstrated at trace concentrations in complex food matrix (Korte *et al.*, 2016). The technique has also been extended in pesticides analysis of malathion in fruit samples. Andre *et al*. (2010) used both traditional MRM and MRM^3 mode and the methods were compared regarding selectivity, sensitivity, accuracy and reproducibility. The results showed that MRM^3 eliminated background and matrix interference, resulting in better data quality due to its higher selectivity. MRM^3 also gave comparable data versus MRM for the quantifier signal but much better sensitivity, accuracy, and reproducibility for the qualifier signal in fruit matrix.

The study performed by Besa (2011) on the selective detection of tetrahydrocannabinol (THC) Carboxylic Acid direct from hair specimens. The advantage of the selectivity of MRM^3 experiment was obvious as THC carboxylic acid was able to be identified in high intensity and resolution despite various intense matrix induced peaks as well as high background on principle. The background noise in the study was decreased while the signal-to-noise ratio was increased made the analysis more efficient especially in the case of lower concentrations.

To date, the application of MRM^3 is not being investigated further in the analysis of isomeric compounds in forensic field especially if there is no separation in the LC part. This study has highlighted the unique identification using this MRM^3 for the differentiation of critical isomeric compounds as mentioned above. The experiment

conduct using MRM^3 mode has been described in the previous work and the similar principle is applied in this study for spectrometry design.

4.2.2 Method development of FIA-MRM^3 Spectrometry

The main goal in this study was to develop and establish a simple method which complement existing analysis methods on isomeric compounds, focusing analysis time since forensic toxicology studies require high throughput analysis with short period of time. To ensure simplicity and covers the requirement in Systematic Toxicological Analysis (STA) especially for minute volume of sample in ante-mortem cases, the study has adopted a simple preparation method in DBS medium as described earlier in Chapter 3 with some modification in sample preparation to support the drugs analysis.

The design of the study applied a quick and effective screening of isomeric methamphetamine and phentermine utilizing the advantages of FIA and MRM technique. Without column separation, the retention time as matching parameter was not exploited, capitalized solely on the MS sensitivity towards the analyte *via* MS infusion. The principle of analysis in FIA is different when compared to routine MS infusion. In routine MS infusion, only compounds parameters are optimized. While in the FIA technique, the optimization includes compounds parameters, as well as source and gas parameters.

Under positive ESI mode, both methamphetamine and phentermine were found to form the $[MH]^+$ adducts and had the same nominal mass of 150.1 m/z. Upon MS/MS fragmentation, both compounds had comparable product ions (Figure 4.2.2).

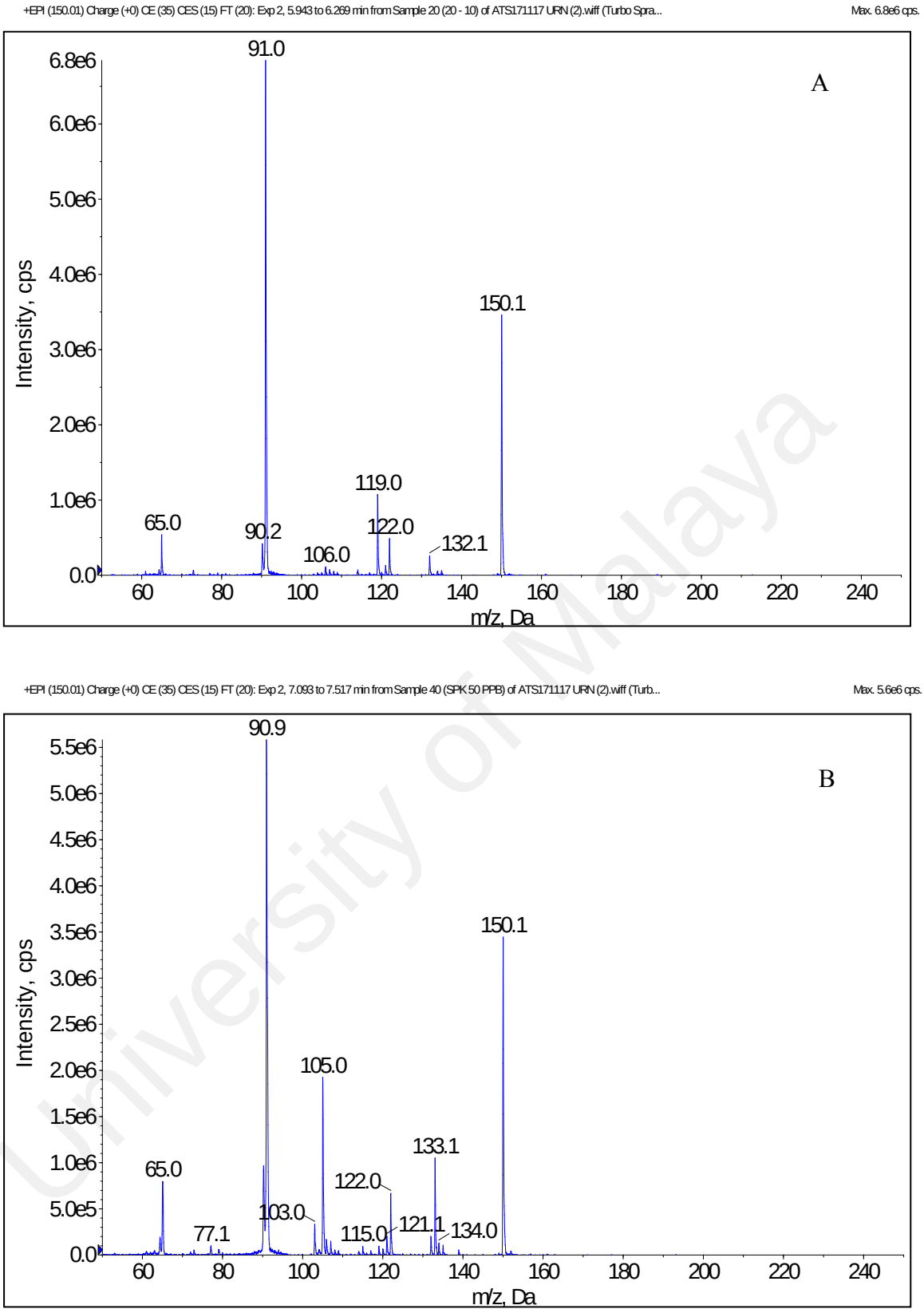


Figure 4.9: MS/MS spectrum showing comparable fragmented product ions for A) methamphetamine and, B) phentermine.

Commonly, at least two transition selected reaction monitoring (SRMs) for the relative abundance ratio matching are utilized in a confirmation and quantitation method as provided in analytical guidelines (SWGTOX, 2013 and UNODC, 2009). In the present case, any SRM combinations for methamphetamine also coincide with SRM for phentermine due to similar fragment ions.

Moreover, the quantitation trace 150→91 gives a higher signal regardless of either single spiked methamphetamine and/or phentermine in the sample (Figure 4.10).

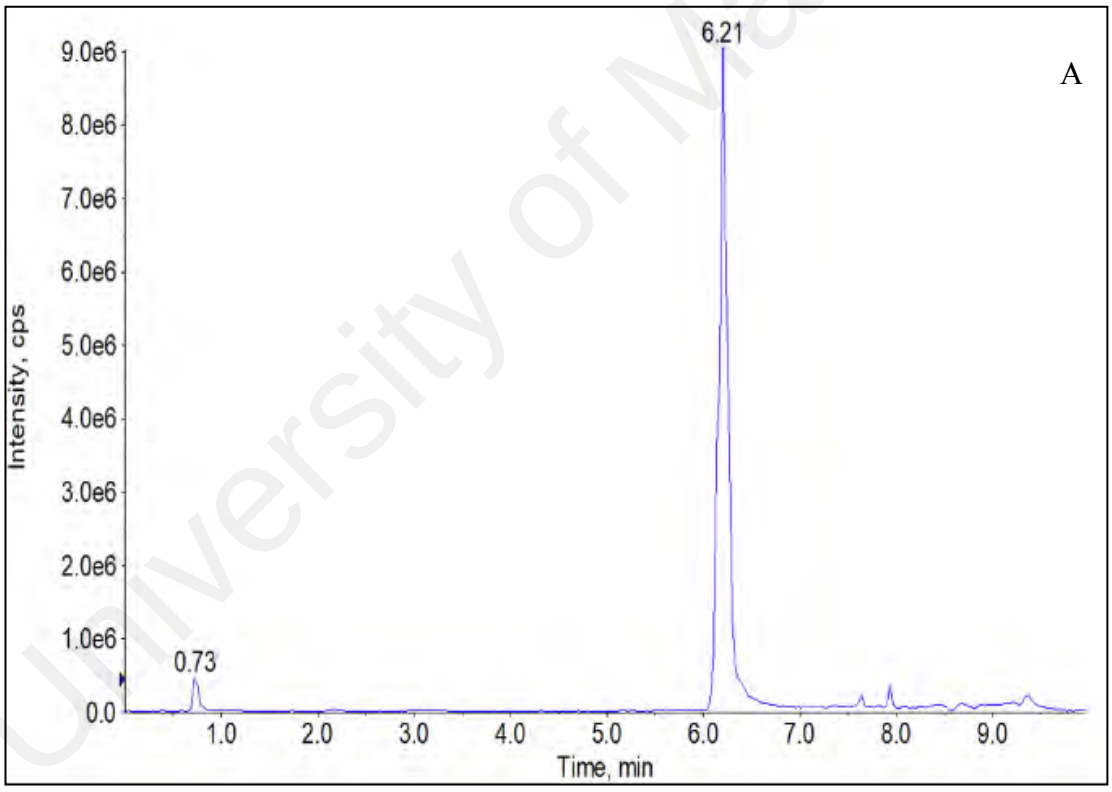


Figure 4.10: Chromatogram for monitored transition of 150>91 m/z for: A) Single 50ng/mL methamphetamine injection, B) Single 50ng/mL phentermine injection and; C) mixture of 50ng/mL methamphetamine and phentermine injection; cont.

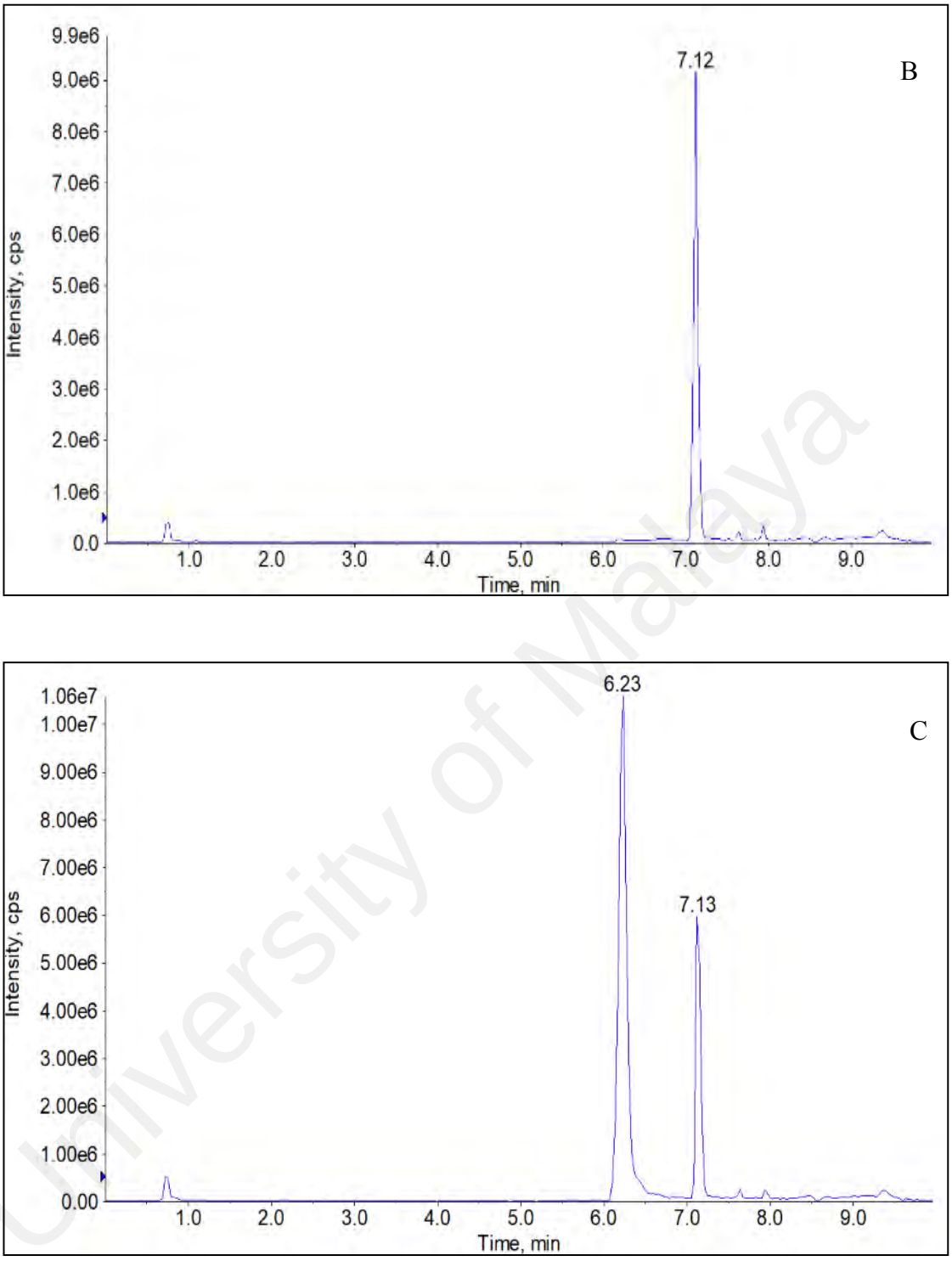


Figure 4.10: Chromatogram for monitored transition of 150>91 m/z for: A) Single 50ng/mL methamphetamine injection, B) Single 50ng/mL phentermine injection and; C) mixture of 50ng/mL methamphetamine and phentermine injection.

Further disadvantage in the pairing FIA with conventional MRM method is observation of substantial signal for methamphetamine 150>119 m/z even in blank specimens spiked with phentermine (Figure 4.11).

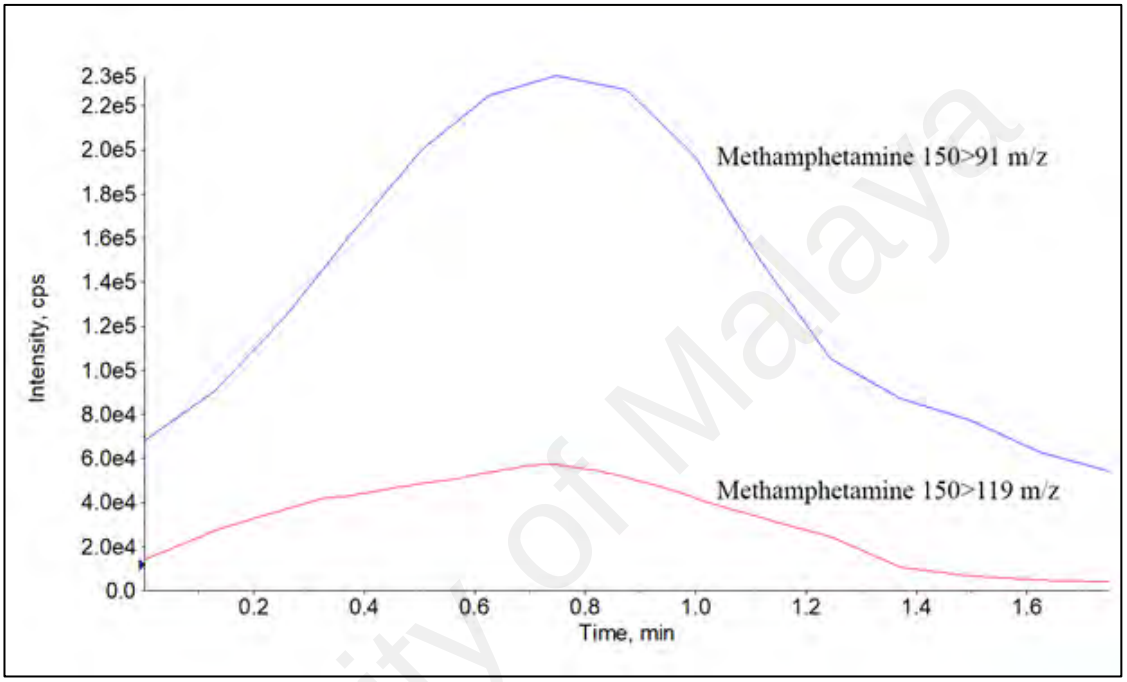


Figure 4.11: Disadvantage of FIA-MRM technique showing a positive signal for methamphetamine on a known blank whole blood specimen that was spiked with 20 ng/mL phentermine.

Another example when using the two transitions in MRM technique, the quantitation trace 150→91 have shown a signal regardless of the presence of either methamphetamine and/or phentermine in the sample (Fig 4.12).

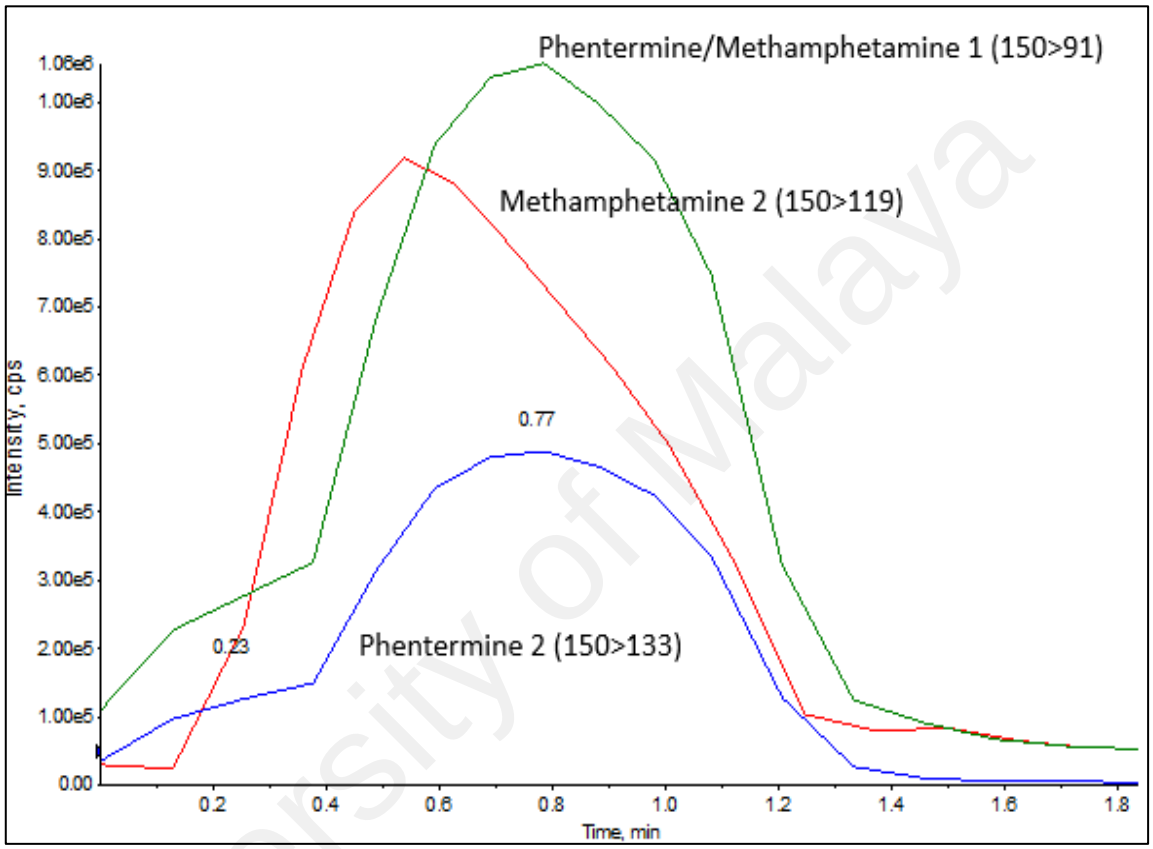


Figure 4.12: MRM transitions for methamphetamine and phentermine standards spiked in whole blood sample.

In the FIA-MRM^3 technique, the MRM^3 selectivity towards targeted transitions has additional identification dimension to substitute the absence of retention time information. Furthermore, similar concept of MS^3 technique was described with the purpose of library matching using the scanned mass ranges of the second product ions (Cesari *et al.*, 2010). Thus, the qualifying transitions, *i.e.* 150>119 and 150>133 was chosen as the differentiation criteria in determining the presence of the isomeric methamphetamine and phentermine confidently.

However, this technique might be compromised in the case of endogenous matrix interferences and trace concentration for practical adoption reason. The sequential delivery of samples into the MS via FIA has resulted in the increase of background signal noise level and could introduce interferences that bear similar MRM transitions. Moreover, sample with low concentration risk having false negative result due to the monitored qualifier transition signals falling below the threshold level. The effect of biological matrix interferences in methamphetamine and phentermine screening methodologies were also reported in previous enantiomer studies (Ward *et al.*, 2016; Adamowicz and Kala, 2015)

From the investigation, these issues can be eliminated effectively using the MRM^3 technique while capitalizing on the FIA technique (Fig 4.13). The MRM^3 technique have an improved sensitivity and specificity due to the reduced probability of common product ions. Additional selection criterion towards the fragmented second precursor ions effectively eliminates the matrix interferences since monitored product transition for interfering ions are different than target analyte. Moreover, an additional confirmatory experiment through collection of MS/MS/MS (MS^3) spectra also allowed extended confidence level.

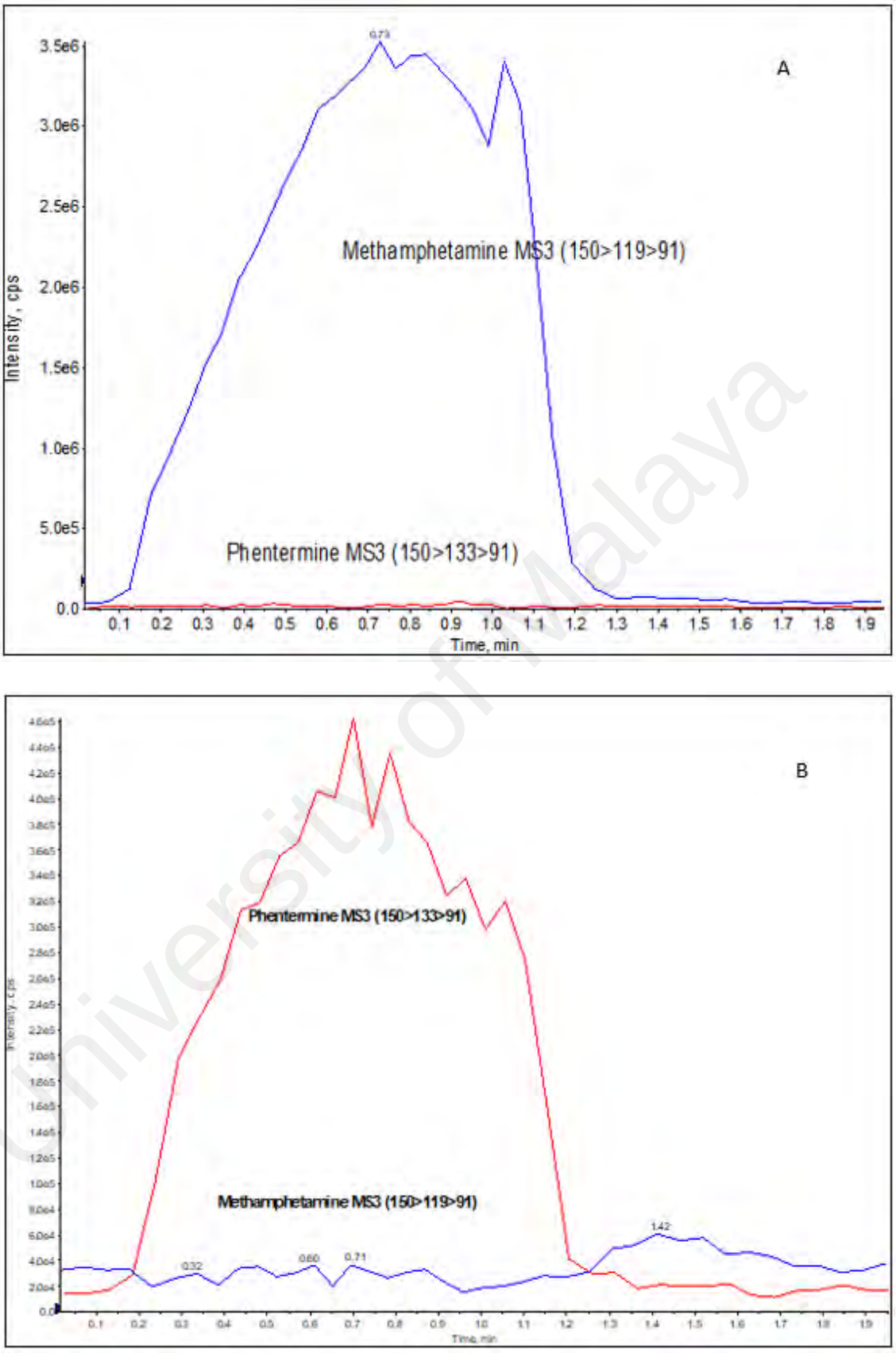


Figure 4.13: MRM^3 of (A) methamphetamine and (B) phentermine standards spiked in whole blood sample.

In the previous studies, MS^3 (Cesari N. et al, 2010; Hopfgartner G. et al, 2004) and MRM^3 (Wright M.J. et al., 2015; Schmidlin T. et al., 2016) was performed in tandem with MRM acquisition method to support retrospective analysis. The developed FIA-MRM^3 method offers high throughput information for screening purposes through large samples quantity before submitting to chromatographic-MS acquisitions. Such screening method cuts analysis duration time down to 80% and saves solvent consumptions as well as column lifetime without compromising on sensitivity and selectivity. Therefore, the actual confirmation and quantitation method can then be proceeded for the positive samples identified in the FIA-MRM^3 method.

4.2.3 Method Validation

The validation was based on the qualitative and relative quantitation purposes. The parameters invloved will be discussed further in the following sections.

4.2.3.1 Linearity, LOD, LOQ and carry over

Dynamic linear ranges, LOD, LOQ and calibration results were shown in Table 4.12. The results showed good linear relationships with correlation coefficients greater than 0.990 for all the targeted analytes.

Table 4.12: Calibration curve dynamic range and compound extraction protocol limit of detection (LOD) and limit of quantitation (LOQ). MA - Methamphetamine, PTM - Phentermine.

Analyte	LOD (ng/mL)	LOQ (ng/mL)	Slope	Intercept	R^2
Whole Blood (20.0 ng/mL)					
MA	2.2	7.4	20794.30	20103.02	0.994
PTM	2.0	6.9	19752.60	265.73	0.995
Dried Blood Stain (20.0 ng/mL)					
MA	3.4	11.3	20200.99	21896.81	0.990
PTM	2.8	9.5	22074.71	62103.69	0.990

For the assessment of carry over, no analyte was detected in both blank sample and blank solvent injected immediately following of the-above mentioned analysis in a batch run indicating the absence of carry over effect.

4.2.3.2 Accuracy and precision

Method validation results for accuracy and precision are summarized in Table 4.13. The recoveries for both tested analytes were all within ± 20% error while the intra- and inter-day precision results have shown acceptable precision of which all were less than 10% RSD. The results revealed good accuracies as well as established robustness of the method.

Table 4.13: Accuracy and Precision. MA- Methamphetamine, PTM –Phentermine, CV-Coefficient of Variation

Analyte	Conc. (ng/mL)	Mean (ng/mL)	Recovery (%)	Standard deviation	CV%
Whole Blood					
MA	20.0	20.0	100±17	3.33	9.34
	100.0	100.0	100±8	8.12	9.01
PTM	20.0	19.9	100±20	3.93	9.55
	100.0	99.9	100±15	10.06	9.55
Dried Blood Stain					
MA	20.0	22.2	111.1±27	5.08	7.17
	100.0	100.0	100.0±27	9.08	9.07
PTM	20.0	19.9	105.1±22	7.86	7.98
	100.0	99.9	108.5±24	10.38	8.40

n= 7 replicates of each 20.0 and 100.0 ng/mL respectively

4.2.3.3 Matrix effects

Signal for the double blank control (free of analytes and internal standard) is shown in Figure 4.14. No interfering peaks from endogenous compounds were observed at the retention times of the analytes or the internal standard from the blank matrix.

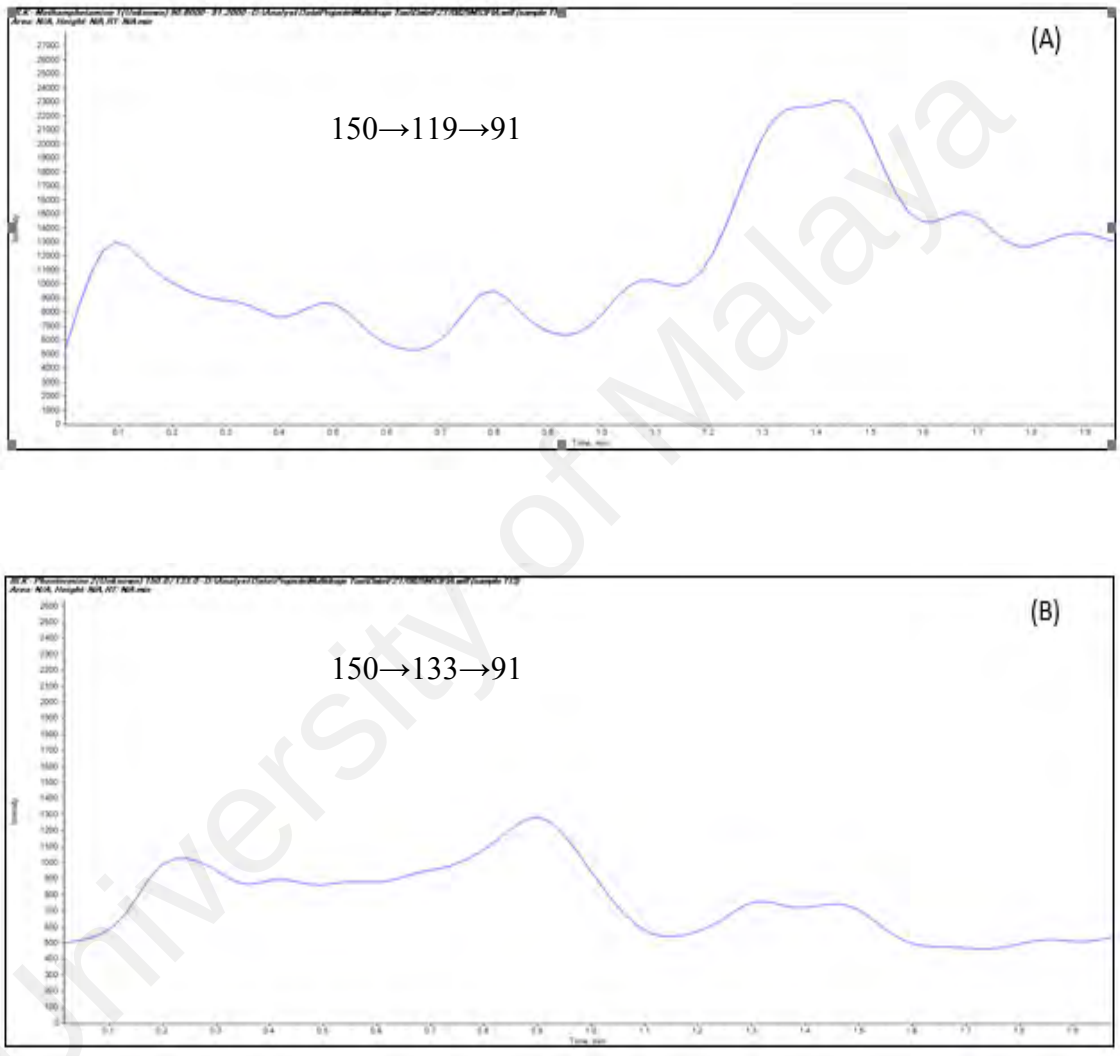


Figure 4.14: Signals (A) double blank (tested for methamphetamine) and (B) double blank (tested for phentermine).

While Figure 4.15 showed the presence of both analytes (methamphetamine and phentermine) with a total data acquisition time (*i.e.* run time) of 2 min.

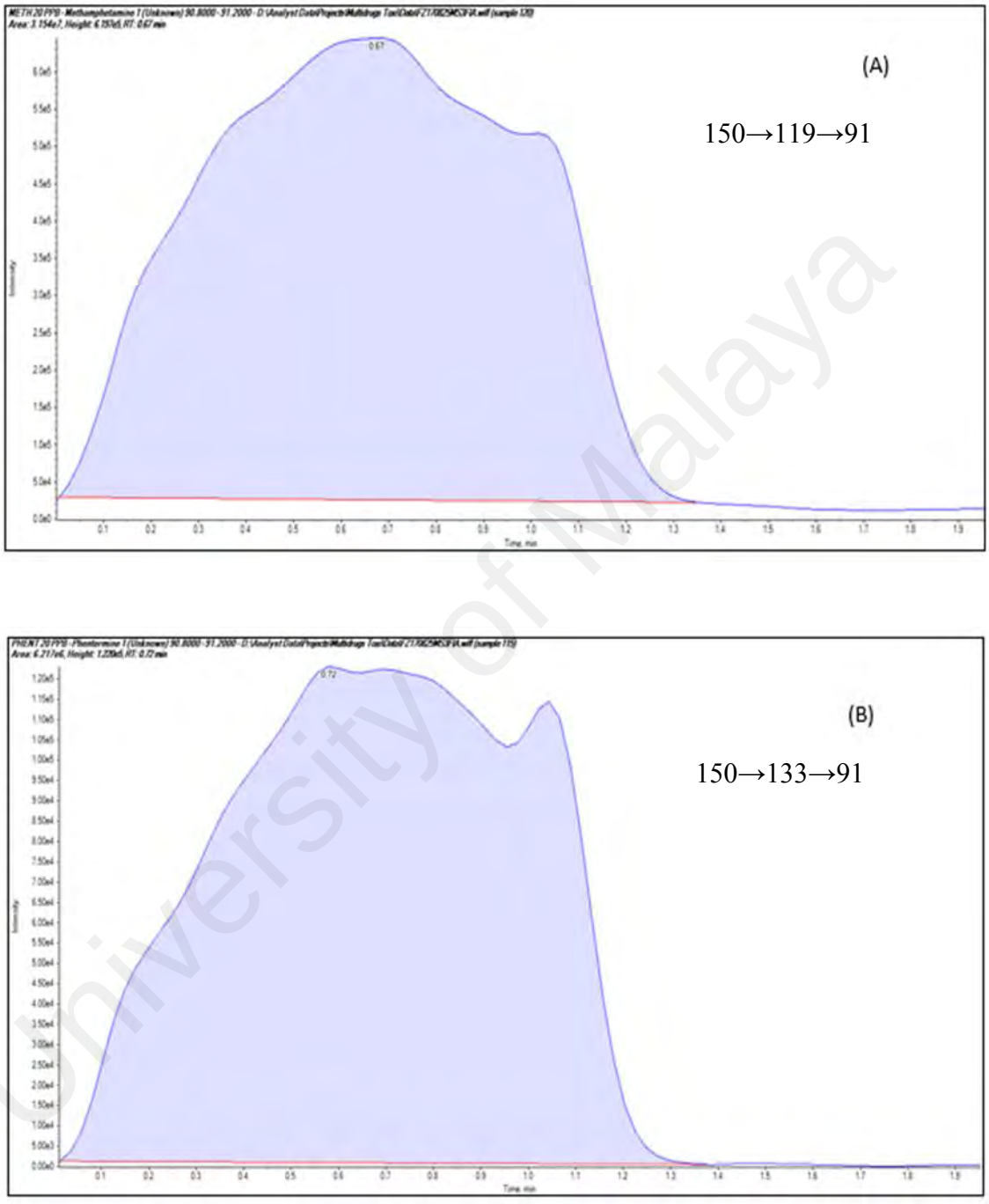


Figure 4.15: Signals (A) spiked methamphetamine 20 ng/mL and (B) spiked phentermine 20 ng/mL.

The evaluation study was performed in seven (N=7) replicates and the results were 80–110% with CV less than 10% for the both analytes. Addition of the isotopically labelled internal standard of MA-d_{14} coupled with the MRM^3 with unique identification and highly selective mode, helped in reducing the matrix effects. MRM^3, as an enhanced MS detection mode, provided an effective scan solution when high background and interferences make standard MRM difficult. The mode removed the interference and enabled a much lower detection of the particular analyte thru additional structural information from second generation product ion that made this mode more selective for detection. The observed reduced matrix effect would be helpful for the differentiation of isomeric compounds for the desired forensic application.

4.2.3.4 Cross-analyte Examination

The cross-analyte examination was performed to cross-check the analyte in respective spiked sample. By using the MultiQuant Software, the analyte was investigated by comparing the dedicated analyte pane, *i.e.* methamphetamine with the peak review corresponded to the analyte. The results showed that phentermine was not present in the singly methamphetamine spiked sample and *vice versa* (Figure 4.16) confirming that no cross-analyte occurred during analysis and thus, eliminating the possibility of false positive results. In addition, the Table 4.14 below briefly illustrated the FIA detection including when the analytes presented/absented in the sample as an alternative fast screening method for both drugs.

Table 4.14: F1A Cross-analyte examination with library matching

Analyte/FIA with library matching	Methamphetamine	Phentermine
Methamphetamine	+	-
Phentermine	-	+
Methamphetamine + /- Phentermine	+/-	+/-

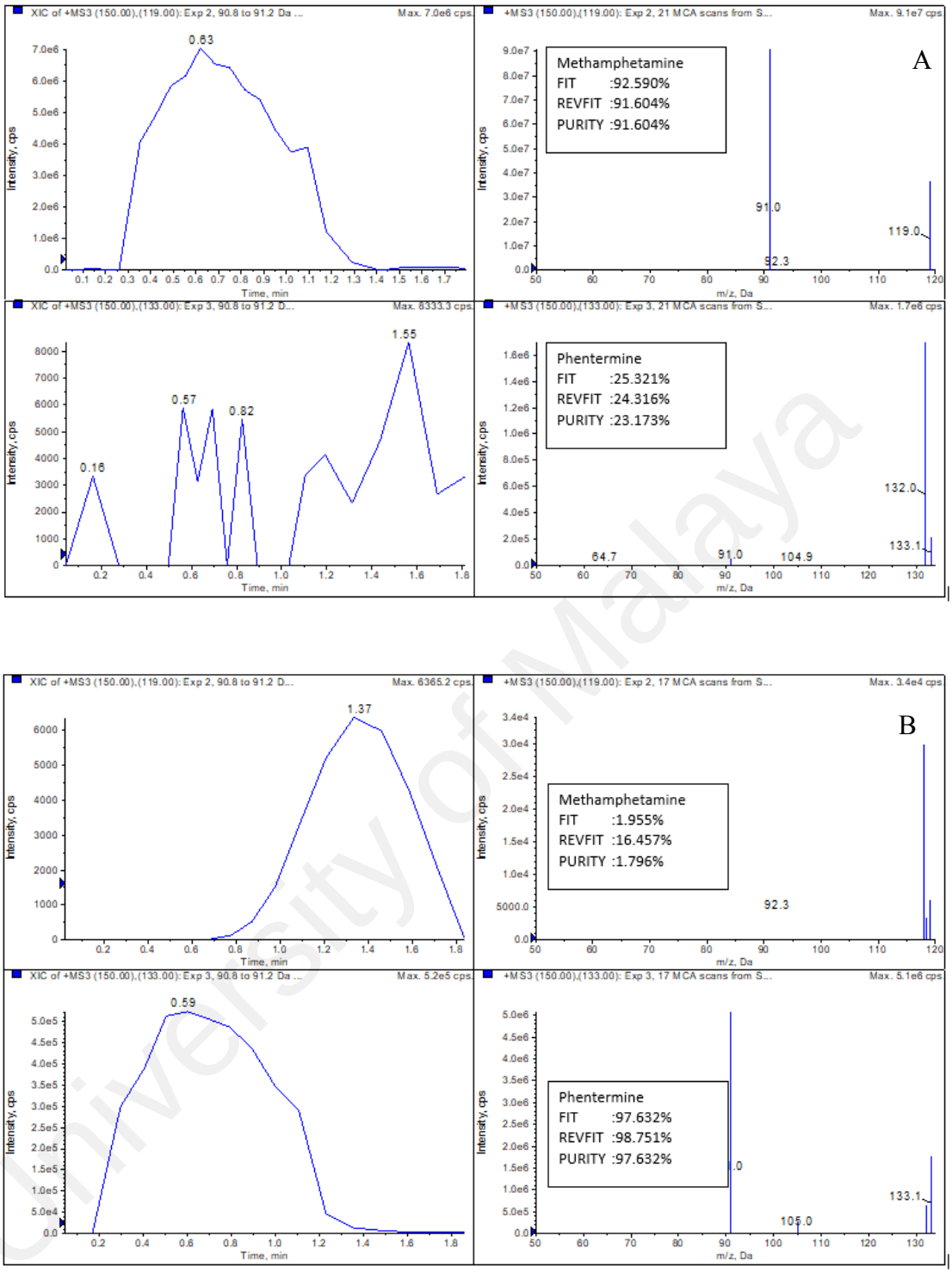


Figure 4.16: Cross analyte examination of methamphetamine and phentermine with MS^3 library matching. (A = spiked methamphetamine; 20 ng/mL – phentermine negative; B = spiked phentermine; 20 ng/mL – methamphetamine negative).

4.2.3.5 Application on forensic toxicology cases in Malaysia

For the assessment on the real cases and external QCs, the laboratory responses and intended responses (previous cases samples) were listed in Table 4.15. The findings were found to be satisfactory whereby the laboratory responses for all tested samples were matched with intended responses. All of the results were subjected to qualitative analysis for the purpose of discriminating the isomeric methamphetamine and phentermine in monitoring scheme.

Table 4.15: Intended and laboratory response for methamphetamine (MA) and phentermine (PTM) in Whole Blood Sample (WBS; n=20) and Dried Blood Stain (DBS; n=20).

Blood (n=20) WBS & DBS	Intended Response	Laboratory Response
1	PTM	PTM
2	PTM	PTM
3	MA	MA
4	MA	MA
5	MA	MA
6	MA	MA
7	MA	MA
8	PTM	PTM
9	PTM	PTM
10	MA	MA
11	MA	MA
12	MA	MA
13	PTM	PTM
14	PTM	PTM
15	PTM	PTM
16	MA	MA
17	MA	MA
18	MA	MA
19	PTM	PTM
20	PTM	PTM
QC 1	MA	MA
QC 2	MA	MA
QC 3	MA	MA

QC: College of American Pathologists (CAP), USA. (Sample is subjected to qualitative analysis for monitoring scheme).

4.3 Bland-Altman Statistical Analysis and Mean Concentration Ratio for the Determination of AMPHETAMINE TYPE Stimulants-Related Drugs in Dried Blood Stain (DBS) versus Whole Blood Sample (WBS) by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)- Application to Forensic Toxicology Cases in Malaysia.

4.3.1 General

The use of DBS as an alternative matrix has been gaining popularity as samples can be collected easily with minimum chances for adulteration, greater stability over other matrices including blood, as well as have the potential to identify recent drugs consumption (Jantos *et al*, 2011 and 2013; Batterman *et al.*, 2016; Saussereau *et al.*, 2012; Ellefsen *et al.*, 2015). Although DBS has been gaining acceptance in therapeutic drug monitoring in recent years, however, applications to forensic samples have not received similar attentions.

In the clinical and forensic studies, a newly developed method needs to be compared against an established method to determine whether these methods can be used interchangeably or the new method can replace the established method (Myles *et al.*, 1996 and 1997; Opdam *et al.*, 2007; Niedhart *et al.*, 2006). In most cases, the 'true' value of the measured quantity is unknown.

Investigations of DBS and WBS are often analyzed using correlation coefficients, r which measures the strength of a relation between two variables, not the agreement between them (Giavarina, 2015; Myles and Cui, 2007; Dewitte *et al.*, 2002). The magnitude of the correlation coefficient can be reduced almost equal to 1 by measuring samples that are similar to each other, and larger by measuring samples that are very different from each other (Giavarina, 2015; Myles and Cui, 2007; Dewitte *et al.*,

2002). Hence, the magnitude of the correlation does not indicate the differences between the two methods being measured.

If the new method agrees sufficiently with the existing method, hence, it is possible to replace the existing method with the new method. In cases where the comparison of two methods does not provide the absolute compatible measurements, the degree of agreement will be assessed (Niedhart *et al.*, 2006; Giavarina, 2015).

The limit of agreement allows estimation of the closeness between the new and old method measurements, carried out by the same analyst. If these limits are within satisfactorily limits and suggested similar conclusions about the measured quantity, it can be concluded that the methods agree sufficiently well for the two methods to be used interchangeably (Myles and Cui, 2007).

Bland-Altman statistical analysis is well established in the clinical studies in order to compare the performance of new method with the existing one. The advantages of using Bland-Altman plot compared to other type of statistical analysis such as t-test and correlation coefficient, is that Bland-Altman uses graphical techniques and simple calculations to measure the mean difference, relation between magnitude of the compared analysis and the assessment of repeatability, in which t-test and correlation coefficient method can only measure one parameter at one time.

Bland-Altman emphasizes on the comparative agreement rather than correlation coefficient and linear regression for the degree of agreement between two methods as both correlation coefficient and linear regression measurements have its shortcoming. The correlation coefficient and linear regression values can be as close as 1.0 with presence of significant bias in the measured methods. For example, regression value can be closed to 1.0 in cases where calibration measurement is 7 units

higher than the intended value, as long as the minimum points are considered for calibration curve. As a consequence, the significant bias cannot be assessed accurately when using Bland-Altman analysis (Giavarina, 2015; Myles and Cui, 2007; Dewitte *et al.*, 2002).

Applications of Bland–Altman comparison methods included the detection of low-level lead in child whole blood in clinical setting using LeadCare® System (LCS) to exchange the 'gold standard' of Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Sobin *et al.*, 2011). The system was reported to be compatible to the ICP-MS method in terms of repeatability and reproducibility, supporting the suitability of the device for the clinical evaluation and monitoring of blood lead levels among individual children.

Jantos *et al.* (2011) reported on the use of mean concentration ratio, *t*-test and Bland-Altman in the evaluation of DBS versus WBS for the determination of 3,4-methylenedioxymethamphetamine (MDMA) and its main metabolite 3,4-methylendioxyamphetamine (MDA) in a controlled driving experiment under the influence of MDMA. Statistical analyses revealed that the bias in mean concentration values were too small and methods did not significantly differentiate MDMA from MDA. Thus, DBS analysis has proven to be reliable technique and potential as a precise and inexpensive alternative to whole blood analysis of MDMA.

Ellefsen *et al.* (2015) also adopted Bland-Altman besides Spearman's correlation in the cocaine and metabolite concentrations in DBS and venous blood after controlled intravenous cocaine administration. The purpose was to quantify cocaine and metabolites in DBS and in venous blood by LC–HRMS and 2D GC–MS to determine if capillary DBS concentrations correlated well with blood concentrations. From the analysis, the plot indicated incompatibility between the two methods, in other words,

scatter increased as the mean concentrations of blood and median DBS increased especially for the metabolite, benzoylecgonine (BZE).

The statistical data illustrated poor agreement between blood and DBS cocaine and BE concentrations. Even though DBS are an alternative matrix to blood for detecting recent cocaine consumption; however, the authors suggested that DBS variability must be addressed and further investigation of differences between capillary and venous blood following controlled drug administration is warranted.

Craft *et al.* (2000) also reported the use of Bland-Altman graphical analysis to evaluate the relation between the difference in retinol concentrations obtained from venous serum (VS) and capillary dried blood spots (CDBS) as well as the average values from the both methods, for the vitamin A deficiency (VAD) cases which is the cause of high mortality rate in children. The difficulties in obtaining samples via serum retinol to assess VAD have been reported to hamper the detection, intervention, and surveillance of VAD and instead, the use of dried blood spots (DBS) was expected to ameliorate many problems of vitamin A assessment.

The results obtained from Bland-Altman statistical analysis indicated that the DBS retinol method was compatible with serum retinol with several advantages with less hassle in terms of acceptance, safety, and logistics to the case assessment.

In the present study, the performance of dried blood stain (DBS) versus whole blood sample (WBS) method was evaluated and compared using Bland-Altman analysis. The mean concentration ratios for ATS drugs were determined to allow both methods to be used interchangeably. The evaluation included the spiked samples of ATS drugs in low and high concentration. The scope has been extended to the real forensic cases and

international proficiency testing, which will then be allowed application of QC samples in the future.

4.3.2 Mean Concentration Ratio for WBS versus DBS

The mean concentration ratio of 1.0 between two method of measurements indicates the exact agreement of both methods (Jantos *et al*, 2011). The mean ratio of ATS drugs concentrations for WBS/DBS was determined at 20 and 100 ng/mL. The study revealed WBS/DBS ratios ranged from 0.91 to 1.05 for all targeted analytes suggesting that the two methods were comparable and the low relative standard deviation (% RSD) of 0.97 to 2.33 indicates insignificant differences between the WBS and DBS values (Table 4.16 and Table 4.17).

Table 4.16: Mean concentration ratio of 20 ng/mL, pooled from 21 spiked for WBS and DBS

Mean Concentration Ratio of 20 ng/mL

DRUGS	EPHED-WBS	EPHED-DBS	PSEUDO-WBS	PSEUDO-DBS	AMPHE-WBS	AMPHE-DBS	MDA-WBS	MDA-DBS	METH-WBS	METH-DBS	MDMA-WBS	MDMA-DBS	PHENT-WBS	PHENT-DBS	MDEA-WBS	MDEA-DBS
mean (pool of 21 data)	20.067	19.112	18.189	19.480	19.575	18.723	19.538	19.506	20.476	20.507	18.947	20.792	19.597	20.139	21.463	21.122
mean concentration ratio	1.050	1.050	0.934	0.934	1.046	1.046	1.002	1.002	0.998	0.998	0.911	0.911	0.973	0.973	1.016	1.016

Table 4.17: Mean concentration ratio of 100 ng/mL, pooled from 21 spiked for WBS and DBS

Mean Concentration Ratio of 100 ng/mL

DRUGS	EPHED-WBS	EPHED-DBS	PSEUDO-WBS	PSEUDO-DBS	AMPHE-WBS	AMPHE-DBS	MDA-WBS	MDA-DBS	METH-WBS	METH-DBS	MDMA-WBS	MDMA-DBS	PHENT-WBS	PHENT-DBS	MDEA-WBS	MDEA-DBS
mean (pool of 21 data)	96.426	93.607	95.440	98.631	100.200	100.063	97.786	98.483	93.236	102.185	99.846	97.362	96.085	95.737	100.089	100.705
mean concentration ratio	1.030	1.030	0.968	0.968	1.001	1.001	0.993	0.993	0.912	0.912	1.026	1.026	1.004	1.004	0.994	0.994

4.3.3 Bland-Altman Analysis for WBS versus DBS

Results from Bland-Altman difference plots (Figure 4.17 and Figure 4.18) suggested that ATS drugs could be identified and quantified from DBS as precisely as WBS for forensic interests. In forensic toxicology, the estimation of concentration of drugs in biological samples especially blood is paramount; to indicate the level of the drugs in the body that might influence the incidence. The rationale is, the value of the drugs is not necessarily to be exact as most of the drugs will have their range levels to indicate their effects to the person. Besides, some other contributors such as pharmacokinetic and pharmacodynamic factors could also affect the true value of the drugs. As explained earlier in Chapter 1, the level of the drugs in the body is interpreted as normal or therapeutic level, toxic level and lethal level that might cause the death of an individual.

In this study, the tested samples; spiked WBS and DBS as well as external QCs and real cases samples have given insignificant differences between both tested mediums as all data points were within limits of agreement and 99% of the points were within 3SD of the mean difference. Even though the data distribution were not precise, but all of the tested values were in acceptable range and those suggested that both of the results in WBS and DBS were comparable and can be used interchangeably. The results suggested that such in the long run, 99% of future mean differences between measurements made on the same instrument will also lie within the limit of agreement.

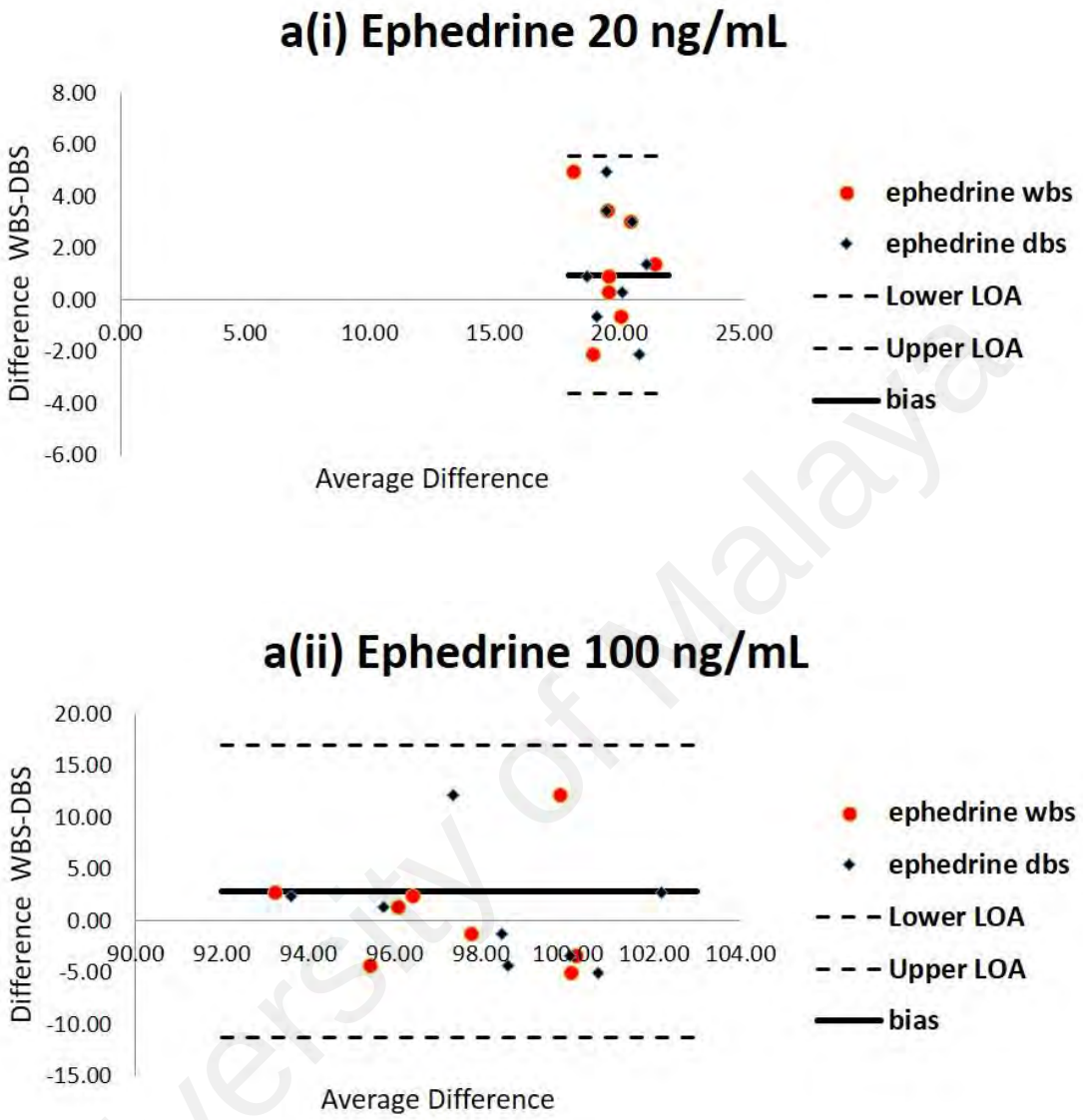


Figure 4.17 : Bland-Altman difference plots (a(i) and a(ii)- Ephedrine; b(i) and b(ii)- Pseudoephedrine; c(i) and c(ii)- Amphetamine; d(i) and d(ii)- MDA; e(i) and e(ii)- Methamphetamine; f(i) and f(ii)- MDMA; g(i) and g(ii)- Phentermine; h(i) and h(ii)- MDEA) of the differences between whole blood specimen (WBS) and dried blood stain (DBS) method respectively, against the average obtained by the two assays for ATS drugs (spiked at 20 and 100 ng/mL). The solid lines illustrate the mean differences; the dotted lines indicate the limits of agreement. Lower LOA (bias-2.575 SD), Upper LOA (bias+2.575 SD).

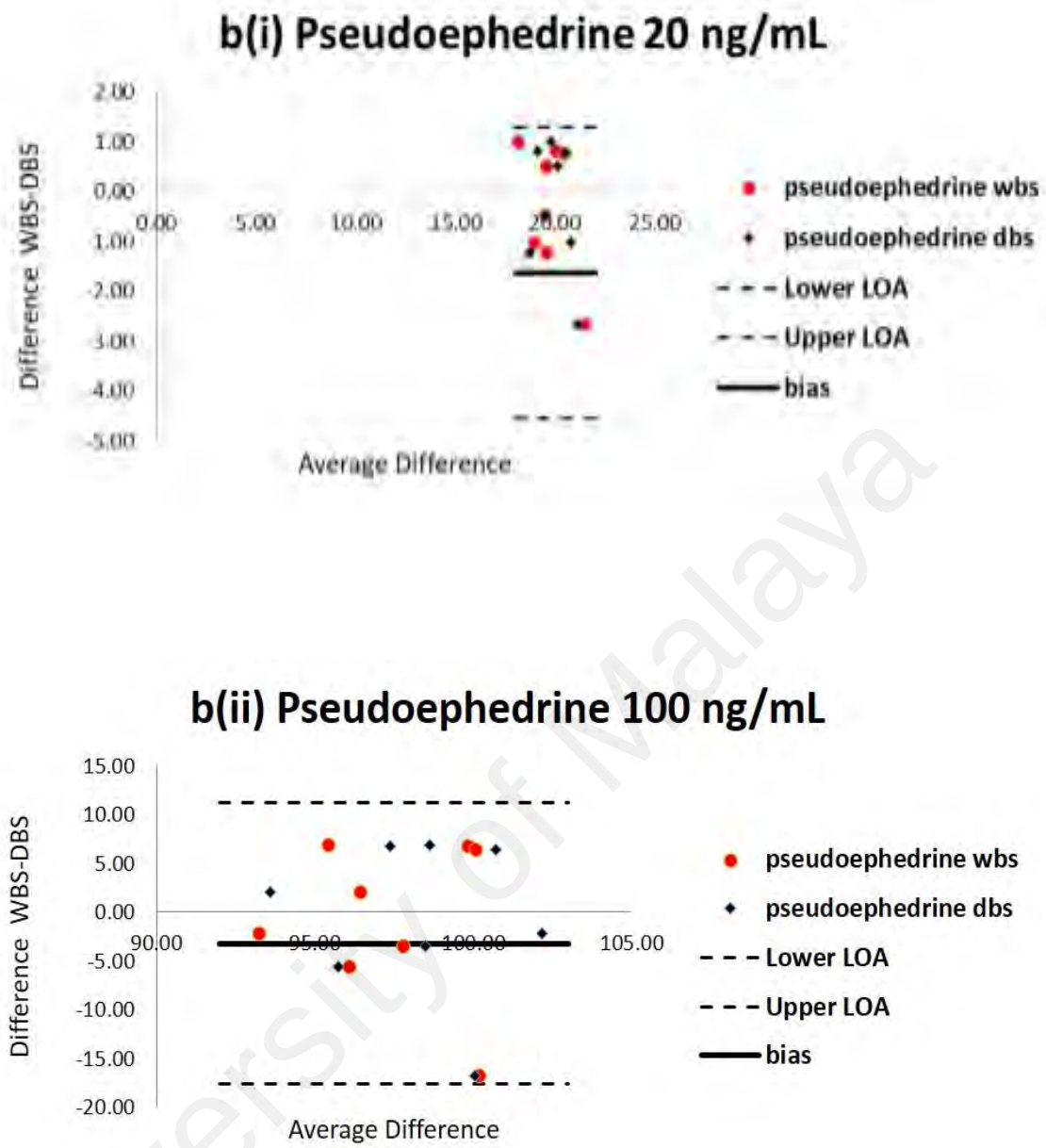


Figure 4.17 : Bland-Altman difference plots (a(i) and a(ii)- Ephedrine; b(i) and b(ii)- Pseudoephedrine; c(i) and c(ii)- Amphetamine; d(i) and d(ii)- MDA; e(i) and e(ii)- Methamphetamine; f(i) and f(ii)- MDMA; g(i) and g(ii)- Phentermine; h(i) and h(ii)- MDEA) of the differences between whole blood specimen (WBS) and dried blood stain (DBS) method respectively, against the average obtained by the two assays for ATS drugs (spiked at 20 and 100 ng/mL). The solid lines illustrate the mean differences; the dotted lines indicate the limits of agreement. Lower LOA (bias-2.575 SD), Upper LOA (bias+2.575 SD) cont:

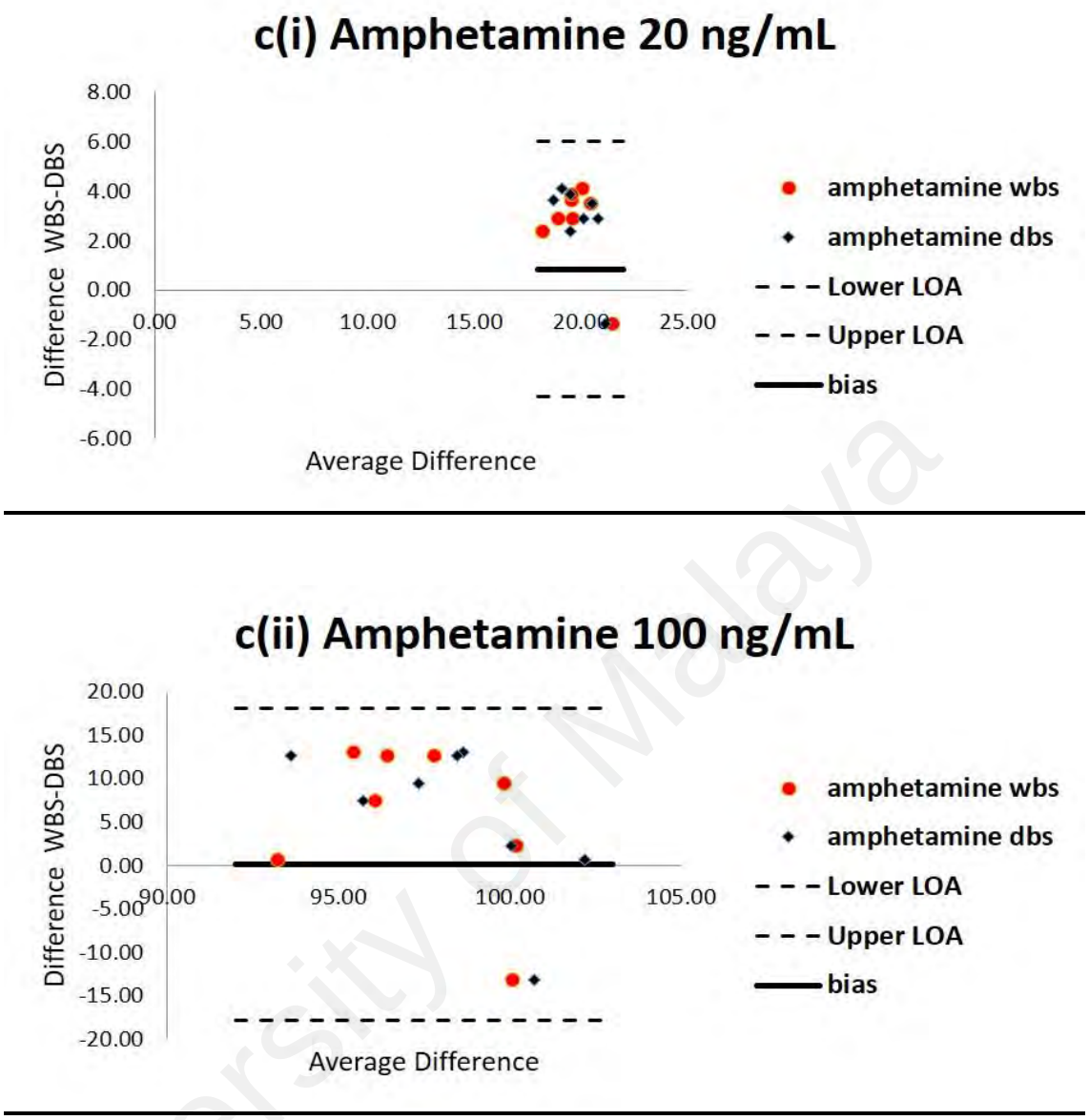


Figure 4.17 : Bland-Altman difference plots (a(i) and a(ii)- Ephedrine; b(i) and b(ii)- Pseudoephedrine; c(i) and c(ii)- Amphetamine; d(i) and d(ii)- MDA; e(i) and e(ii)- Methamphetamine; f(i) and f(ii)- MDMA; g(i) and g(ii)- Phentermine; h(i) and h(ii)- MDEA) of the differences between whole blood specimen (WBS) and dried blood stain (DBS) method respectively, against the average obtained by the two assays for ATS drugs (spiked at 20 and 100 ng/mL). The solid lines illustrate the mean differences; the dotted lines indicate the limits of agreement. Lower LOA (bias-2.575 SD), Upper LOA (bias+2.575 SD) cont:

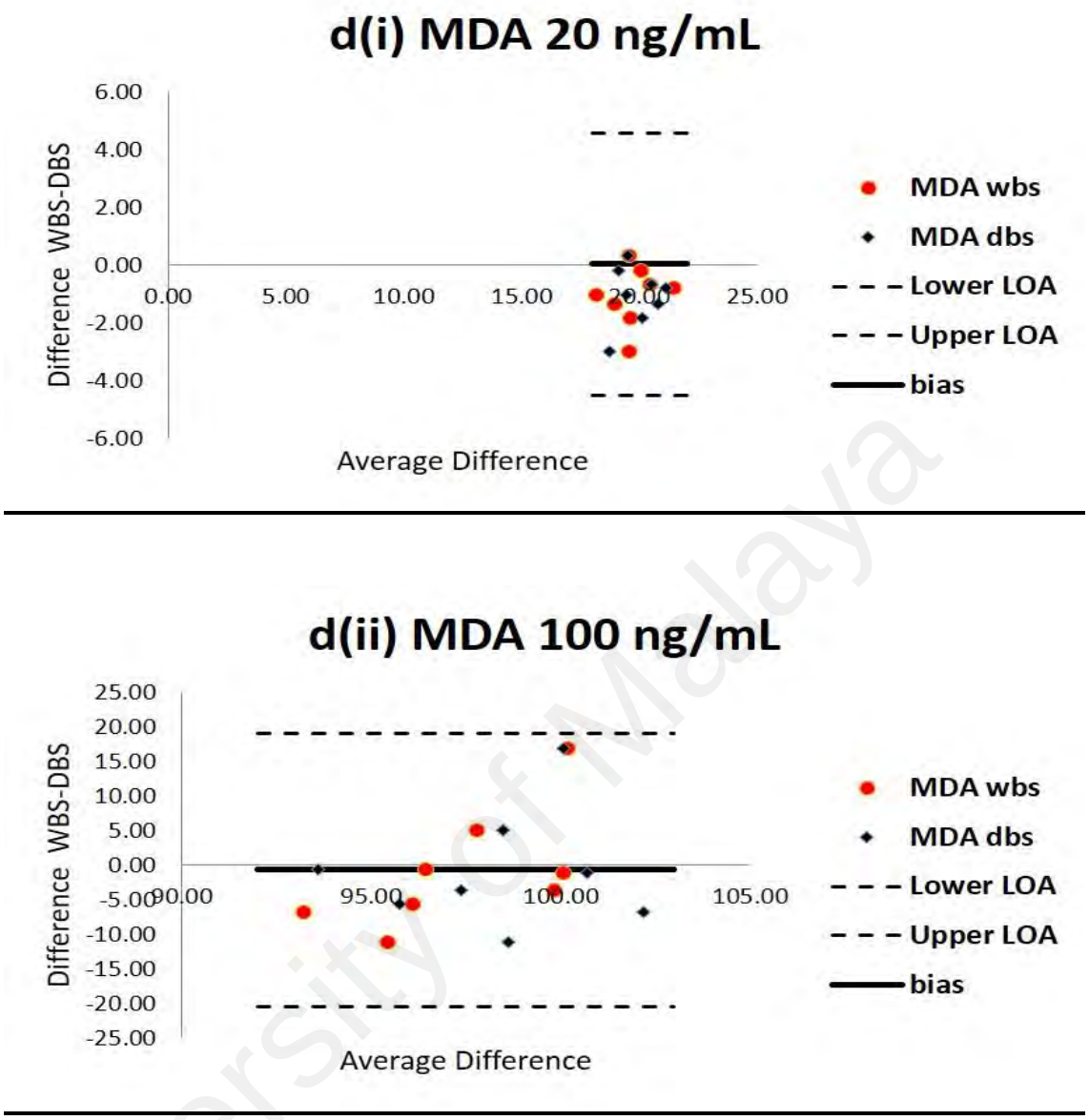
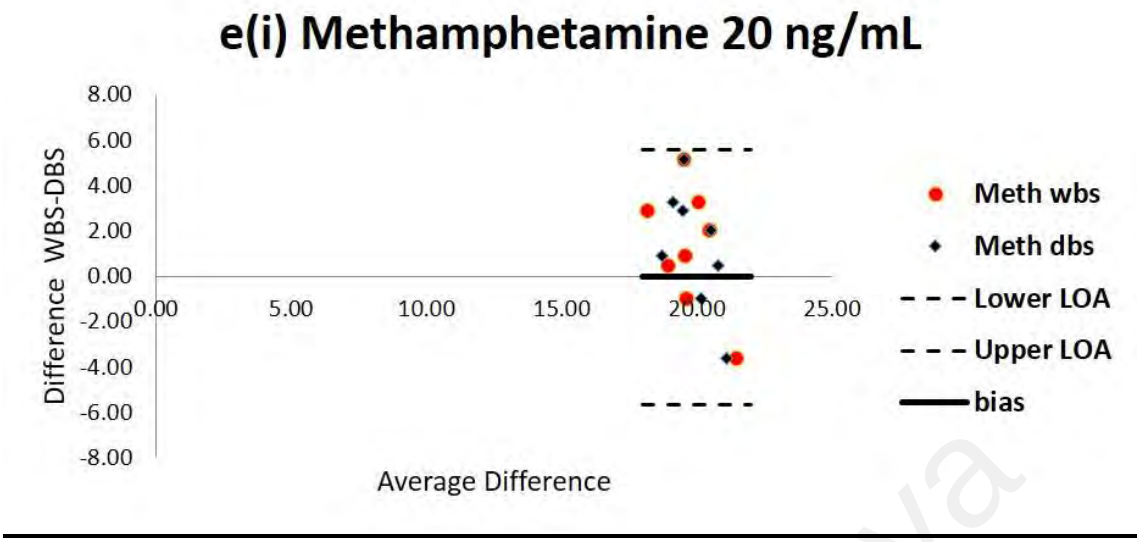


Figure 4.17 : Bland-Altman difference plots (a(i) and a(ii)- Ephedrine; b(i) and b(ii)- Pseudoephedrine; c(i) and c(ii)- Amphetamine; d(i) and d(ii)- MDA; e(i) and e(ii)- Methamphetamine; f(i) and f(ii)- MDMA; g(i) and g(ii)- Phentermine; h(i) and h(ii)- MDEA) of the differences between whole blood specimen (WBS) and dried blood stain (DBS) method respectively, against the average obtained by the two assays for ATS drugs (spiked at 20 and 100 ng/mL). The solid lines illustrate the mean differences; the dotted lines indicate the limits of agreement. Lower LOA (bias-2.575 SD), Upper LOA (bias+2.575 SD) cont:



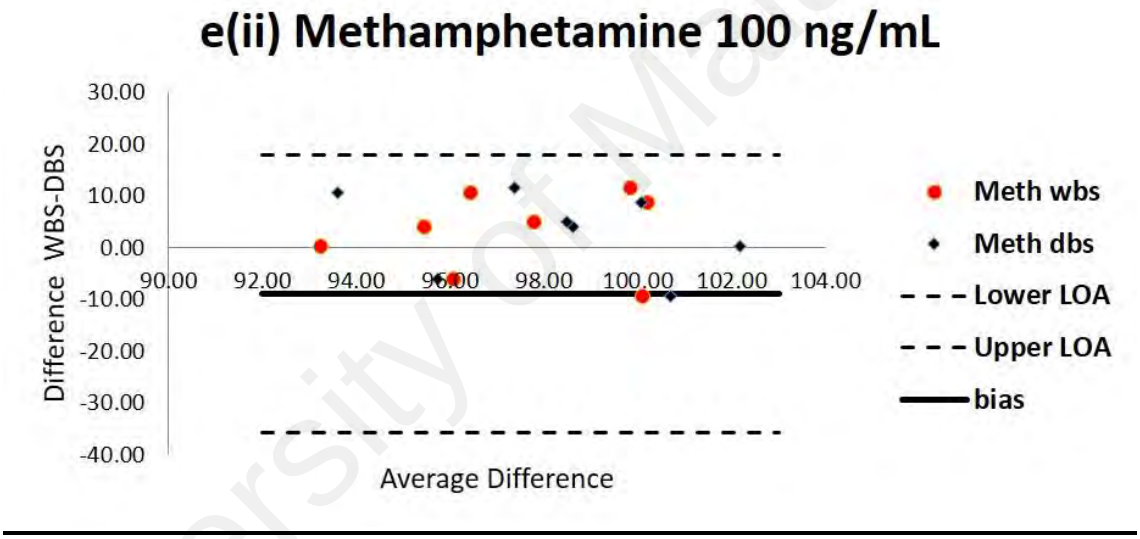


Figure 4.17 : Bland-Altman difference plots (a(i) and a(ii)- Ephedrine; b(i) and b(ii)- Pseudoephedrine; c(i) and c(ii)- Amphetamine; d(i) and d(ii)- MDA; e(i) and e(ii)- Methamphetamine; f(i) and f(ii)- MDMA; g(i) and g(ii)- Phentermine; h(i) and h(ii)- MDEA) of the differences between whole blood specimen (WBS) and dried blood stain (DBS) method respectively, against the average obtained by the two assays for ATS drugs (spiked at 20 and 100 ng/mL). The solid lines illustrate the mean differences; the dotted lines indicate the limits of agreement. Lower LOA (bias-2.575 SD), Upper LOA (bias+2.575 SD) cont:

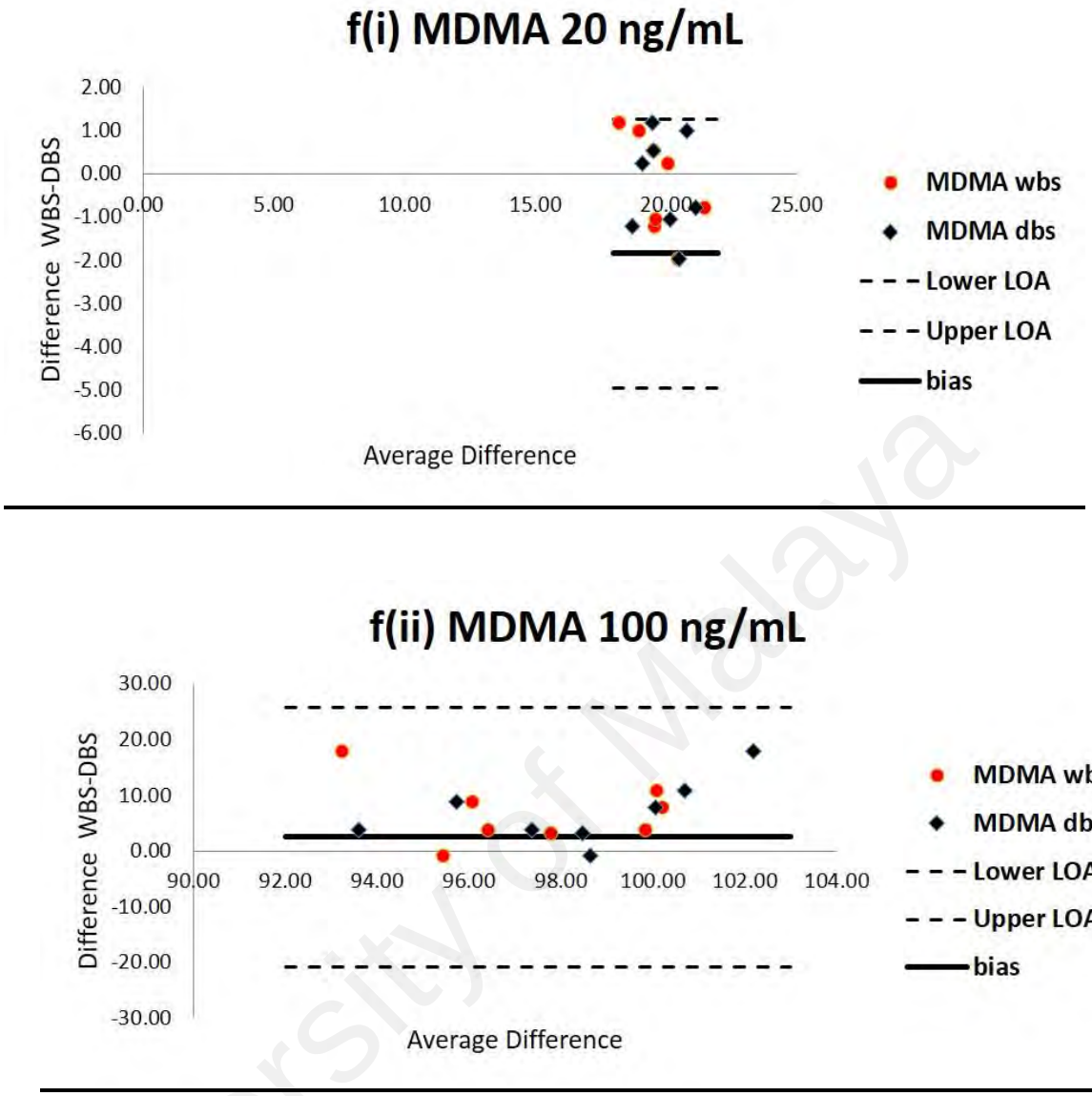
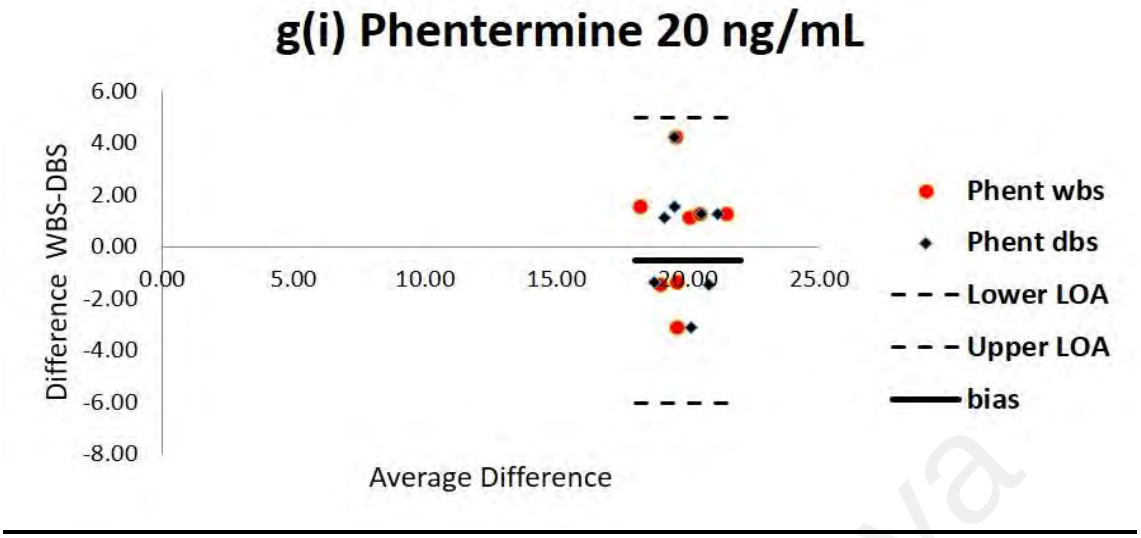


Figure 4.17 : Bland-Altman difference plots (a(i) and a(ii)- Ephedrine; b(i) and b(ii)- Pseudoephedrine; c(i) and c(ii)- Amphetamine; d(i) and d(ii)- MDA; e(i) and e(ii)- Methamphetamine; f(i) and f(ii)- MDMA; g(i) and g(ii)- Phentermine; h(i) and h(ii)- MDEA) of the differences between whole blood specimen (WBS) and dried blood stain (DBS) method respectively, against the average obtained by the two assays for ATS drugs (spiked at 20 and 100 ng/mL). The solid lines illustrate the mean differences; the dotted lines indicate the limits of agreement. Lower LOA (bias-2.575 SD), Upper LOA (bias+2.575 SD) cont:



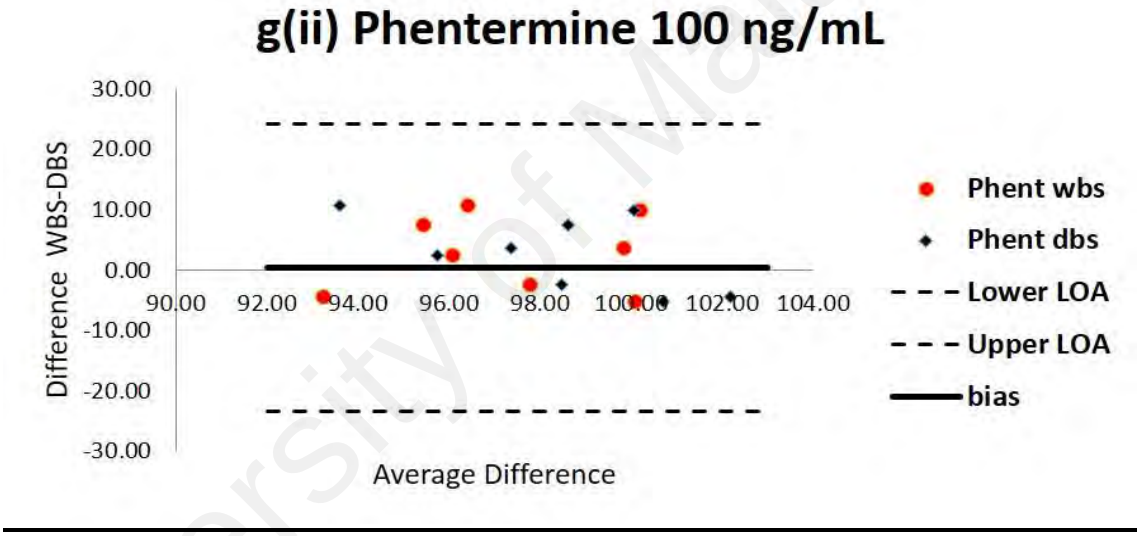


Figure 4.17 : Bland-Altman difference plots (a(i) and a(ii)- Ephedrine; b(i) and b(ii)- Pseudoephedrine; c(i) and c(ii)- Amphetamine; d(i) and d(ii)- MDA; e(i) and e(ii)- Methamphetamine; f(i) and f(ii)- MDMA; g(i) and g(ii)- Phentermine; h(i) and h(ii)- MDEA) of the differences between whole blood specimen (WBS) and dried blood stain (DBS) method respectively, against the average obtained by the two assays for ATS drugs (spiked at 20 and 100 ng/mL). The solid lines illustrate the mean differences; the dotted lines indicate the limits of agreement. Lower LOA (bias-2.575 SD), Upper LOA (bias+2.575 SD) cont:

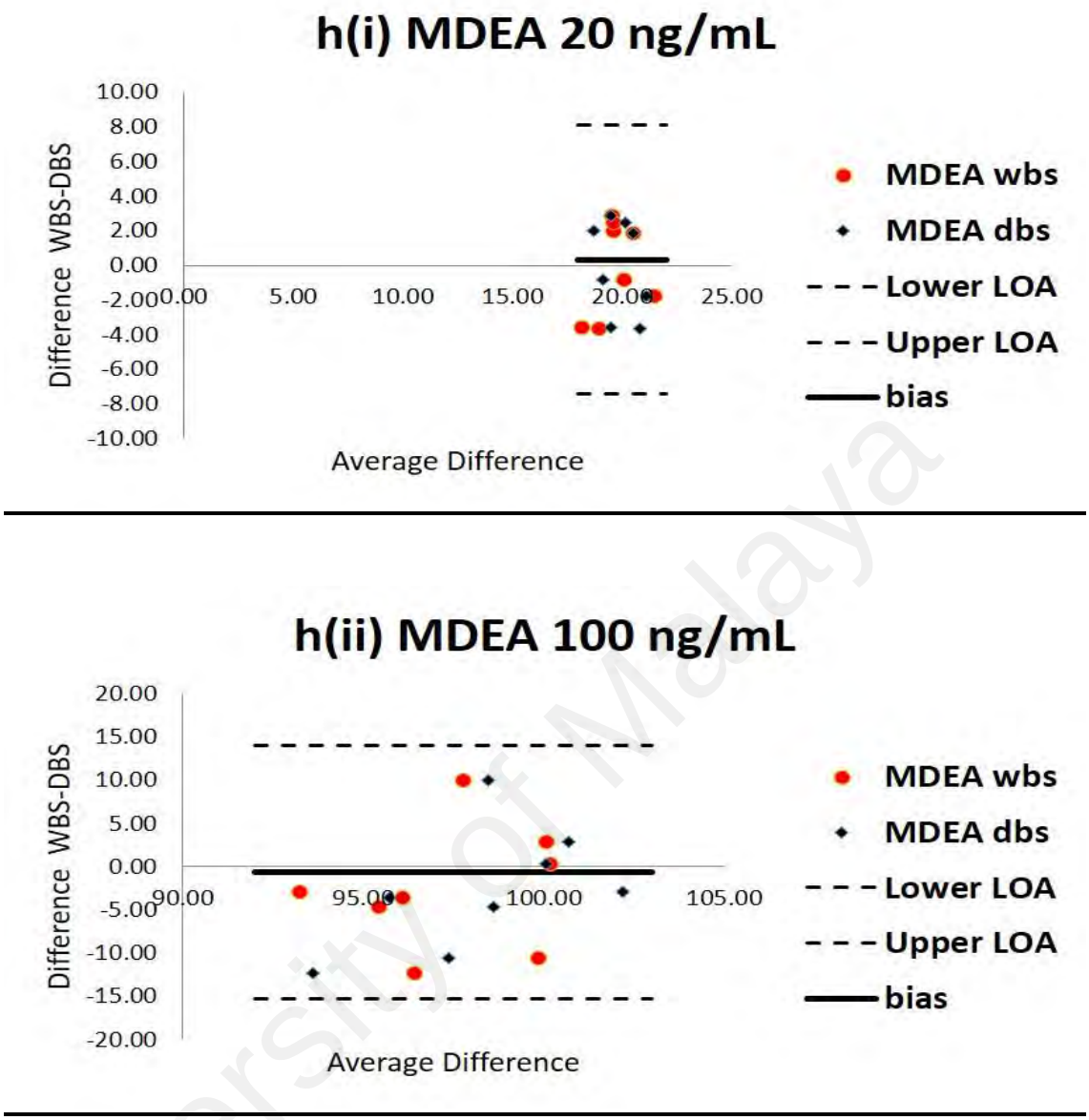


Figure 4.17 : Bland-Altman difference plots (a(i) and a(ii)- Ephedrine; b(i) and b(ii)- Pseudoephedrine; c(i) and c(ii)- Amphetamine; d(i) and d(ii)- MDA; e(i) and e(ii)- Methamphetamine; f(i) and f(ii)- MDMA; g(i) and g(ii)- Phentermine; h(i) and h(ii)- MDEA) of the differences between whole blood specimen (WBS) and dried blood stain (DBS) method respectively, against the average obtained by the two assays for ATS drugs (spiked at 20 and 100 ng/mL). The solid lines illustrate the mean differences; the dotted lines indicate the limits of agreement. Lower LOA (bias-2.575 SD), Upper LOA (bias+2.575 SD) cont;

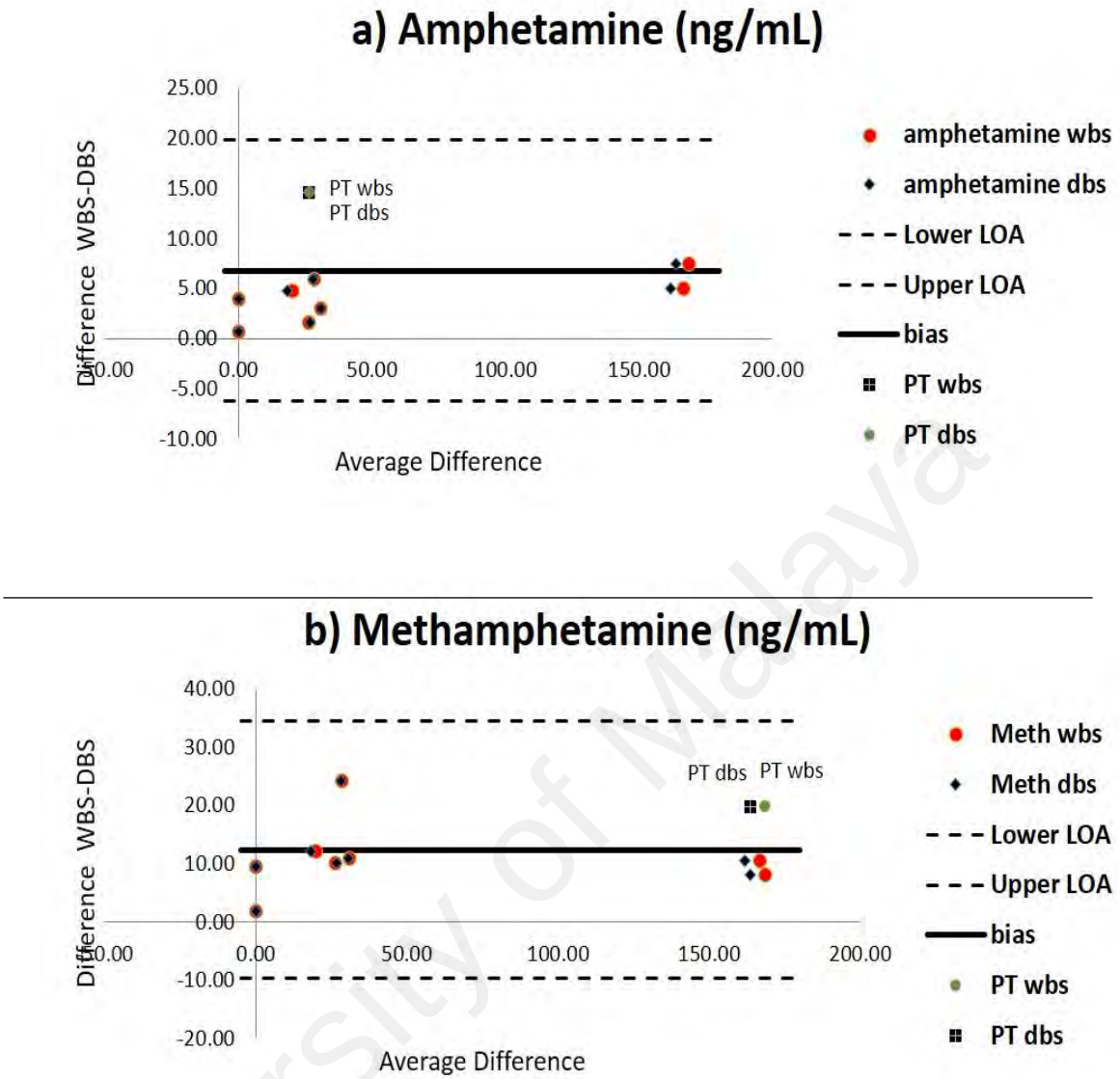
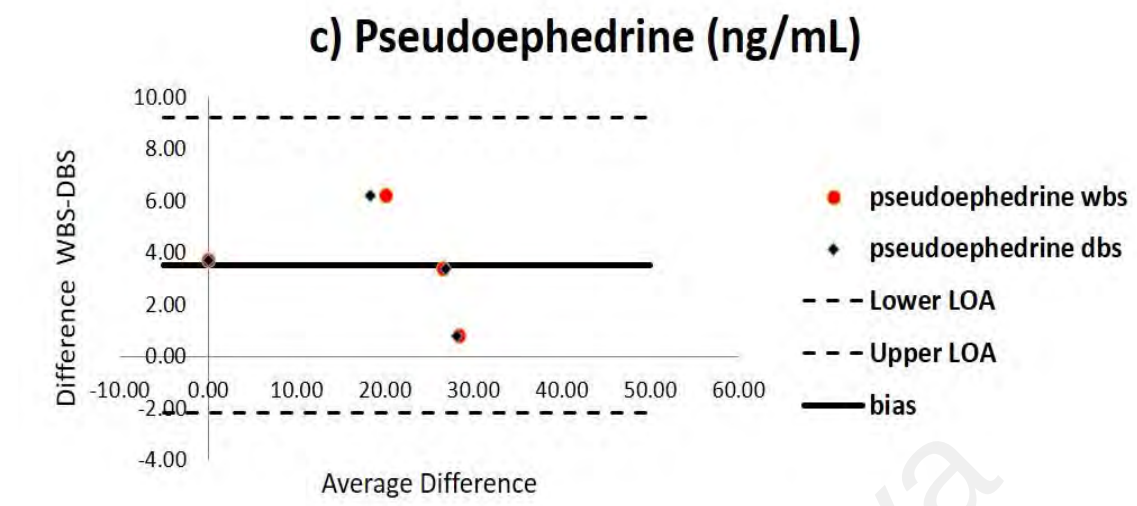


Figure 4.18: Bland-Altman difference plots a) Amphetamine, b) Methamphetamine, c) Pseudoephedrine, d) MDA, e) MDMA, f) Phentermine of the differences between whole blood specimen (WBS) and dried blood stain (DBS) method against the average obtained by the two assays for ATS drugs in 20 real cases samples and 4 external QCs (CAP, USA).



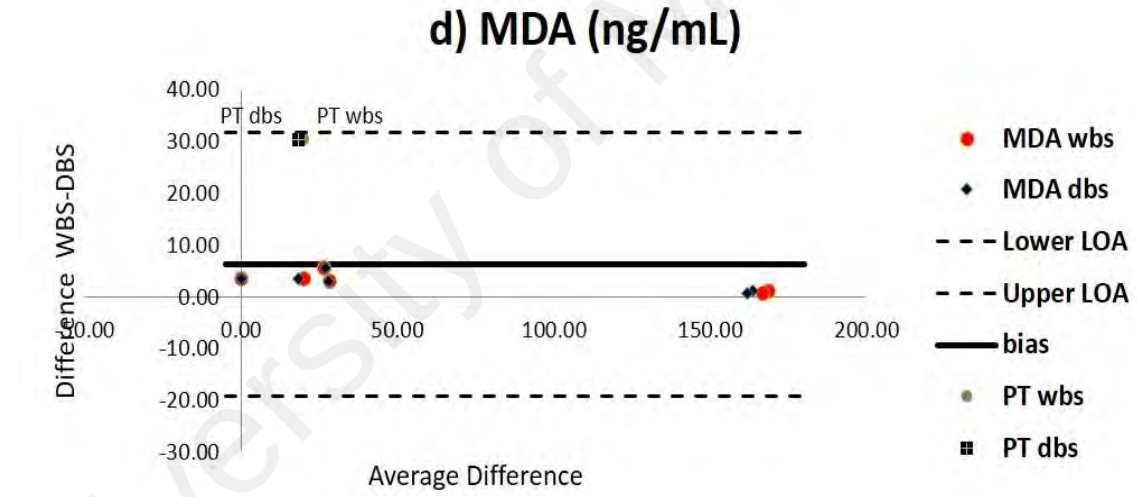
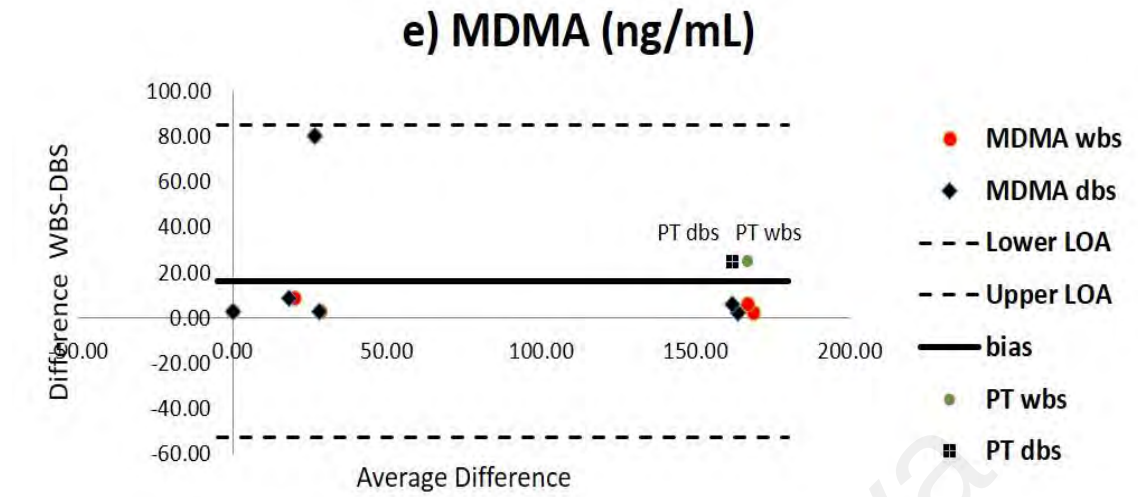


Figure 4.18: Bland-Altman difference plots a) Amphetamine, b) Methamphetamine, c) Pseudoephedrine, d) MDA, e) MDMA, f) Phentermine of the differences between whole blood specimen (WBS) and dried blood stain (DBS) method against the average obtained by the two assays for ATS drugs in 20 real cases samples and 4 external QCs (CAP, USA) cont;



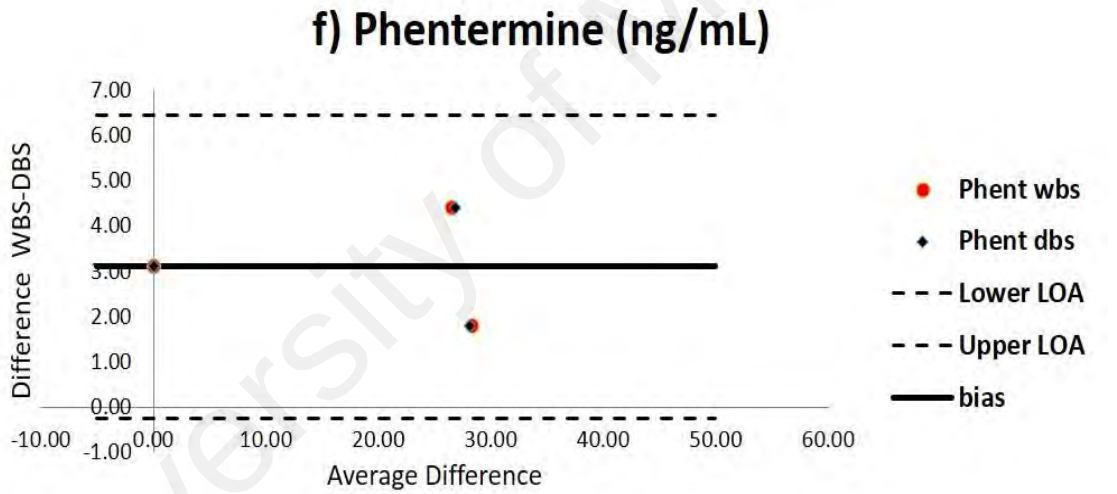


Figure 4.18: Bland-Altman difference plots a) Amphetamine, b) Methamphetamine, c) Pseudoephedrine, d) MDA, e) MDMA, f) Phentermine of the differences between whole blood specimen (WBS) and dried blood stain (DBS) method against the average obtained by the two assays for ATS drugs in 20 real cases samples and 4 external QCs (CAP, USA) cont;

4.4 Stability and Efficiency of Dried Blood Stain (DBS) medium for the analysis of ATS drugs during Sample Storage- Application to Forensic Toxicology Cases in Malaysia.

4.4.1 General

The instability of drugs in biological samples during transportation and storage has presented a challenge to the forensic toxicologists especially in the interpretation of the results of analysis. When focusing on the stability of the DBS, very limited studies have been conducted to understand the effect of these factors towards the qualitative and quantitative analyses of the illicit drugs under the class of ATS.

At present, data on the stability of drugs and metabolites on DBS samples under different temperatures and storage conditions are inadequate. Thus, with the exception of the availability of the analyte's data, it is important to perform full validation experiments which comprise of short-term stability in room temperature, long-term stability in a wide range of temperature including extreme temperatures and post-extraction stability (Antunes *et al.*, 2016).

4.4.1.1 Temperature

Transportation and storage temperature are one of the major variables in determining the stability of the drugs or analytes in the collected DBS samples. Chepyala *et al.* (2017) investigated the stability of 57 ATS drugs from various classes, including amphetamines, opioids, cocaine, benzodiazepines, ketamine, lysergic acid diethylamide (LSD) and metabolites of norketamine, norephedrine, 7-aminoflunitrazepam and nordiazepam. DBS cards stored in sealed bags containing desiccants were kept at two distinct temperatures; room temperature and −80 °C. The DBS cards were evaluated after

one and six months by comparing their responses with those from a freshly prepared DBS card.

The results revealed that a few drugs, including amphetamine, methamphetamine and aminorex showed poor stability (below 50%) after one-month storage at room temperature. In contrast, DBS cards stored at −80 °C for one month showed improved compound stability. Among all of the compounds, approximately 7% of the compounds showed poor stability (below 50%) after 6 months of storage at −80 °C.

The stability of the illicit drugs of opiates, cocaine and amphetamines on the DBS samples (Saussereau *et al.,* 2012) was determined at 4 and −20 °C storage, by comparing the concentration of drugs (in duplicate) after storing for 48 h, 1 month, 3 months and up to 6 months with the initial concentrations of 10 and 50 ng/mL. The results showed that the drugs are stable at −20 °C up to 6 months of storage (>80%).

Prieto *et al.* (2011) performed a study to identify the temperature stability of the NTBC or nitisinone (2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione) drug in the collected DBS samples. The paper with dry blood spots was kept at room temperature (20-25 °C) to investigate whether normal ordinary mail with this temperature is suitable for the sample's transportation. It was observed that the concentration of NTBC remains constant at least during a month, were the paper cards with blood spots were kept tightly closed in a plastic bag.

The stability of chemical compound other than drugs through the utilisation of DBS sampling method was also performed. Kato *et al*. (2009) discovered that the stability of polyfluoroalkyl chemicals (PFC) in DBS samples were considerably stable when stored in the designed temperatures up to 61 days. Both spiked and un-spiked DBS samples containing PFC were wrapped in the aluminium foil and stored at room

temperature and 37 °C (surrogate for storage under non-optimal conditions). The integrity and stability of the DBS are maintained even exposed to higher temperatures, which could be possibly encountered during shipping or other unforeseen circumstances.

In the context of doping control, Thomas *et al*. (2012) stored fortified whole blood samples of 10 ng/mL after spotted on a card at 2–8 °C for 0, 1, 3 and 7 days prior to the analysis. From the 26 types of chemical compounds of interest in the context of doping control which include cocaine, MDA and MDMA, the results portrayed that all selected model compounds (exception: formoterol) were shown to be stable for at least one week when stored at 2–8 °C. Referring to the chemical compounds stability outcomes, it was proven that the benefits of DBS sampling in the stability of the compounds on the cards was reflected.

4.4.1.2 Humidity

The presence of moisture in the DBS samples may promote bacterial growth when the conditions are sufficiently fulfilled. It has been verified that rapid drying and storage in low humidity conditions improved the stability of DBS samples (McDade *et al.*, 2007). Thus, proper drying before transportation and storage are highly required and it is recommended to dry the blood spot samples at minimum three hours over an open non-absorbent surface with temperature range between 15–22 °C (Mei *et al*, 2001). During the drying process, the paper must be avoided from direct sunlight and should not be heated, stacked or allowed to have contact with other surfaces. It is also recommended that the filter paper containing the dried blood specimen to be protected by a sturdy paper overlay such as glassine paper (weighing paper) (Mei *et al.*, 2001).

In order to understand the relationship between humidity and stability of analytes in the DBS, Tretzel *et al.* (2016) performed a study to investigate the stability aspects of

nicotine and its metabolites as well as alkaloids and its metabolites by exposing DBS samples in low humidity environment. The samples were stored in the plastic bags with desiccant (i.e. silica gel) at room temperature for different time periods up to 30 days and subsequently analysed. The purpose of designing this temperature was to apply simulated conditions of shipping and storage for authentic samples In the particular case of nicotine, stability could be demonstrated for at least 30 days indicated by a degradation of less than 20%. Based on these works, it is proven that there has been a close link between free-moisture conditions with the DBS samples in maintaining the stability of the analytes being measured.

4.4.1.3 DBS matrix

In addition to the storage conditions, type of DBS cards is also found to have profound impact on the stability of the analytes in the DBS samples based on the interaction between analyte with the substrate matrix. Different types of DBS cards possess different surface properties, paper thickness as well as having different degree of moisture loss (Denniff and Spooner, 2010). Thus, suitable choice of paper matrix is essential in providing optimum condition to ensure the stability and integrity of the analytes.

The most common matrix for DBS is cellulose-based papers, which are mostly differentiated based on its composition, thickness and resistance to the spread ability of blood. Variation of these characteristics may contribute to the differences in extraction recovery, matrix effects, analyte stability and chromatographic, haematocrit and volume effects (Wagner *et al*, 2016).

At present, there are two main types of commercially available paper cards that are suitable for DBS; chemically untreated and treated papers. The untreated papers are

the most widely used type, particularly the pure cellulose Whatman 903® and Ahlstrom 226®, which are registered by the US Food and Drug Administration (USFDA) (Antunes *et al.*, 2016).

While the chemically treated type of matrices is made of cellulose papers treated with different proprietary chemicals with the purpose to lyse cells, inactivate pathogens and cause denaturation of enzymes and other proteins from the blood samples and other biological specimens. The most commonly used types are Whatman FTA®, FTA Elute®, FTA DMPK-A® and FTA DMPK-B®. Alternatively, untreated paper can also be impregnated with chemicals in order to improve the stability of some analytes (Wagner *et al.*, 2016).

The correlation between DBS matrix with the stability of the analytes has been studied by Wang *et al.* (2015). They conducted a study to understand the effect of storage conditions and paper substrates on the stability and analysis sensitivity on seven types of therapeutic drugs, namely amitriptyline, clozapine, amisulpride, quetiapine, risperidone, aripiprazole and verapamil.

In the study, three types of paper substrates were utilised and based on the observation on the colour variations and concentration of drugs in the cards, silica coated paper of Whatman grade SG81 provides best results when compared to grade ET31 and grade 1. The results are attributed by the good separation between the compound of interest and blood sample as well as the favourable elution of the target from the paper substrate (Denniff and Spooner, 2010).

The effect of paper substrates on the stability of the drugs in the DBS samples can be more understandable when the prepared DBS samples were stored in a tightly closed zip-foiled bag. Wang *et al.* (2015) discovered that the degradation of the drugs was

effectively prohibited and among the three paper substrates, the drugs on grade SG81 demonstrated a more stable property than that on grade ET31 and 1 respectively. The drugs on the grade SG81 showed smaller data variation compared to the other two papers, which can be attributed to the difference in surface properties (Zhang *et al.*, 2017).

4.4.1.4 Other Potential Interferences

In addition to the interferences of temperature, humidity and DBS matrix discussed above, light exposure and the nature of the targeted analyte can be potentially affecting the stability of the analytes on the DBS matrix. For instance, rapidly lowering the pH of the spotted blood sample with the purpose of extending stability, some structurally unstable drugs may undergo degradation.

Another example is the use of ethylenediaminetetraacetic acid (EDTA) as an anticoagulant to preserve and stabilise DBS samples for enzyme activity determination may undermine the stability of the analytes (Liu *et al.*, 2011; Elbin *et al.*, 2011). As a result of variations in stability, analyte specific protocols need to be administered for the collection as well as the storage of the DBS samples.

4.4.2 Evaluation of Spiked Samples in DBS

The data were evaluated based on the recovery of the drugs in the DBS samples based on the parameters measured. The residual drug amounts of 20 ng/mL for 3-month and 6-month period are shown in Figure 4.19a, and 4.19b respectively. All analytes were measured against T_0/C_0 (real-time analysis) and changes in the concentrations over a storage period of 3-month and 6-month were less than 25% and 50% respectively.

After a 3-month storage at 4°C, AMP, MA, MDA, MDMA, MDEA, PEP, EP, and PTM concentrations decreased by 11, 12, 22, 5, 13, 19, 23, and 5%, respectively, from

the original concentrations at day zero. After storage for 3-month at 25°C, AMP, MA, MDA, MDMA, MDEA, PEP, EP, and PTM concentrations decreased by 22, 25, 30, 10, 30, 35, 33, and 15, respectively, from the original concentrations at day zero.

After the 6-month storage at 4°C, AMP, MA, MDA, MDMA, MDEA, PEP, EP, and PTM concentrations decreased by 15, 16, 25, 15, 25, 29, 28, and 7, respectively, from the real time analysis. While at 25°C, AMP, MA, MDA, MDMA, MDEA, PEP, EP, and PTM concentrations were decreased by 40, 47, 42, 23, 48, 56, 61, and 25, respectively, from the initial concentrations.

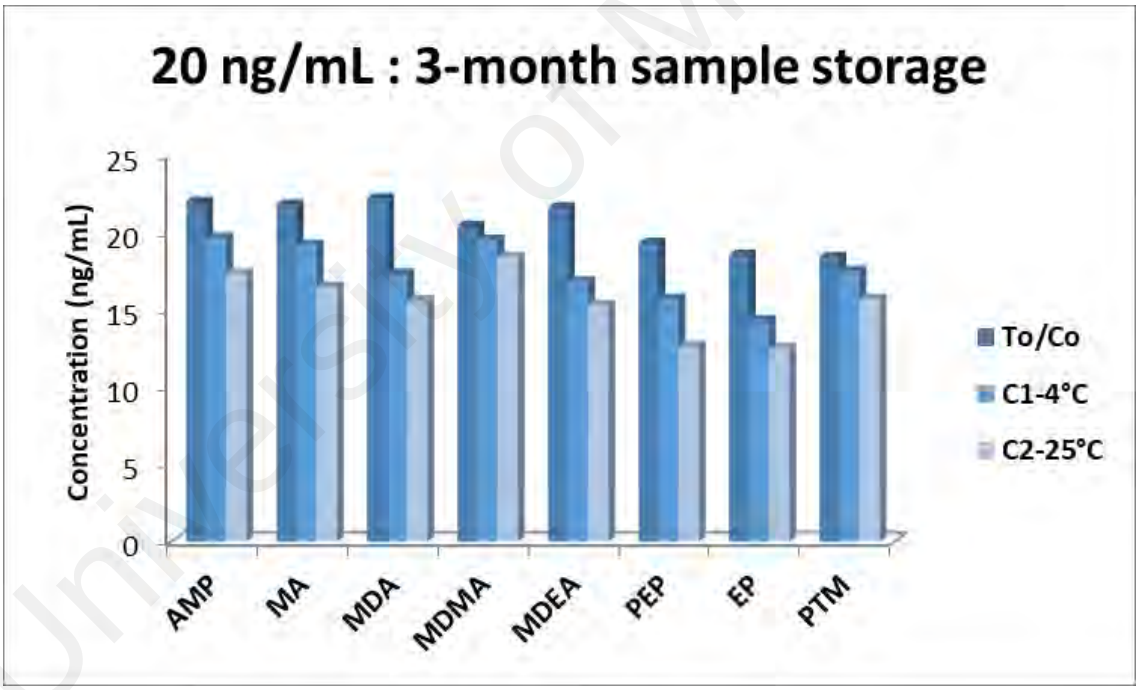


Figure 4.19a: Stability of ATS drugs in DBS for 3 months. Initial concentrations were 20 ng/mL.

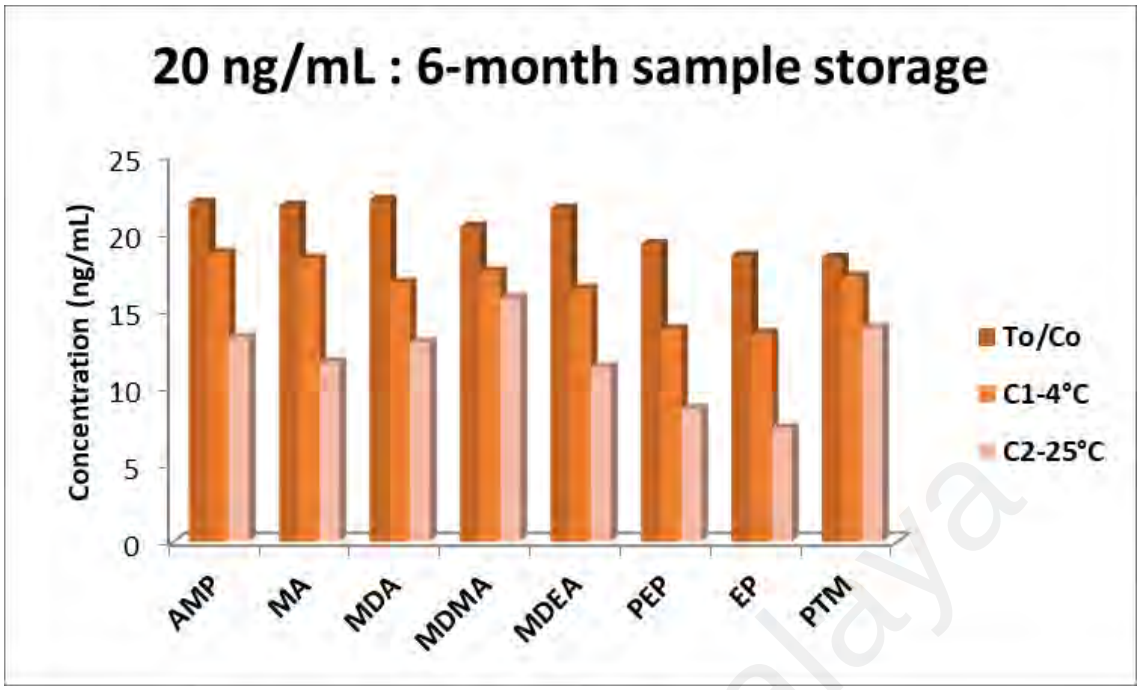


Figure 4.19b: Stability of ATS drugs in DBS for 6 months. Initial concentrations were 20 ng/mL.

The residual drug amounts of 100 ng/mL for 3-month and 6-month period are shown in figure 4.20a 4.20b respectively. The changes in the concentrations over a storage period of 3-month and 6-month were less than 10 and 20% respectively. After storage of 3-month at 4 °C, AMP, MA, MDA, MDMA, MDEA, PEP, EP, and PTM concentrations decreased by 2, 5, 5, 5, 3, 7, 9 and 1%, respectively, from the initial concentrations. While at 25°C, AMP, MA, MDA, MDMA, MDEA, PEP, EP, and PTM concentrations were decreased by 5, 8, 10, 10, 8, 16, 15 and 9%, respectively.

After up to 6-month storage at 4°C, AMP, MA, MDA, MDMA, MDEA, PEP, EP, and PTM concentrations decreased by 6, 8, 12, 10, 11, 21, 23 and 12%, respectively, from the real time analysis. At 25°C, AMP, MA, MDA, MDMA, MDEA, PEP, EP, and PTM concentrations were decreased by 14, 17, 17, 13, 19, 35, 38 and 20% respectively, also from the initial concentrations at the real time analysis.

From the evaluation, the stability of most of the drugs spiked in low and high concentrations were acceptable at 4 °C. The observed data were consistent across all time points. Dehydration of the samples on the FTA card minimized the hydrolysis of drugs which are liable to this type of degradation and as such, the stability of the drugs in DBS was enhanced compared to WBS.

Also, the higher concentration of the spiked samples (100 ng/mL) have advantages in term of stability where the recoveries of the drugs were found to be >70% in room temperature for up to 6 months storage. This could be due to the less systematical errors during samples conduct. The samples were protected from potential interferences that would come from humidity and moisture by keeping them in the desiccator during the testing period. Water present in the blood samples plays a very important role in hydrolysis reactions as an active reagent that cleaves drug molecules and by keeping the sample dry (FTA card in the desiccator), the stability of the drugs was maintained.

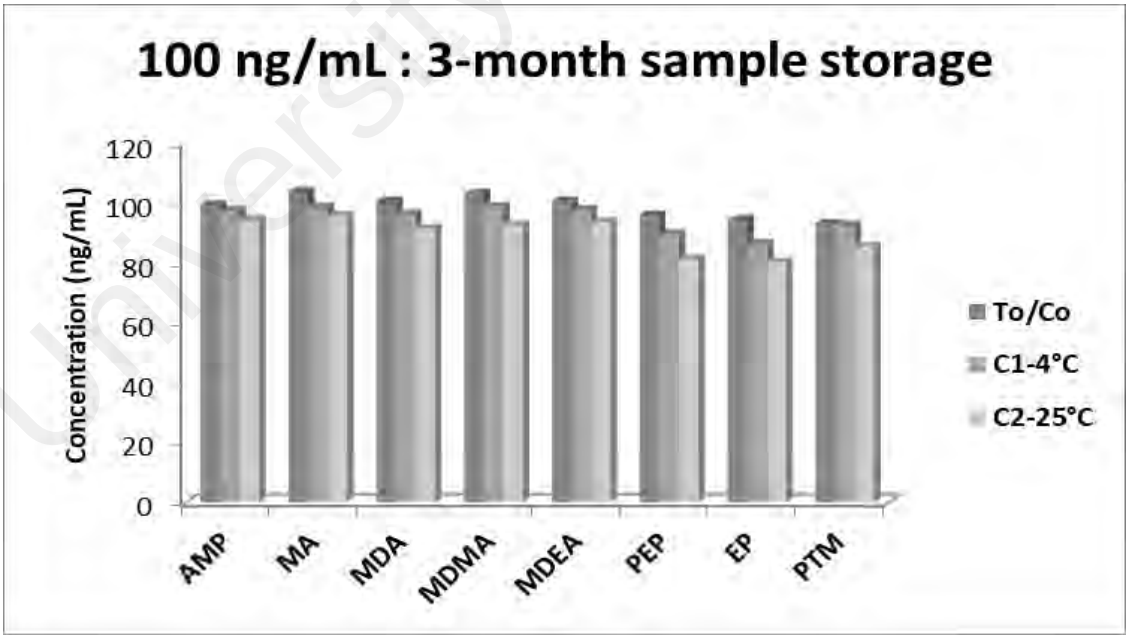


Figure 4.20a: Stability of ATS drugs in DBS for 3 months. Initial concentrations were 100 ng/mL.

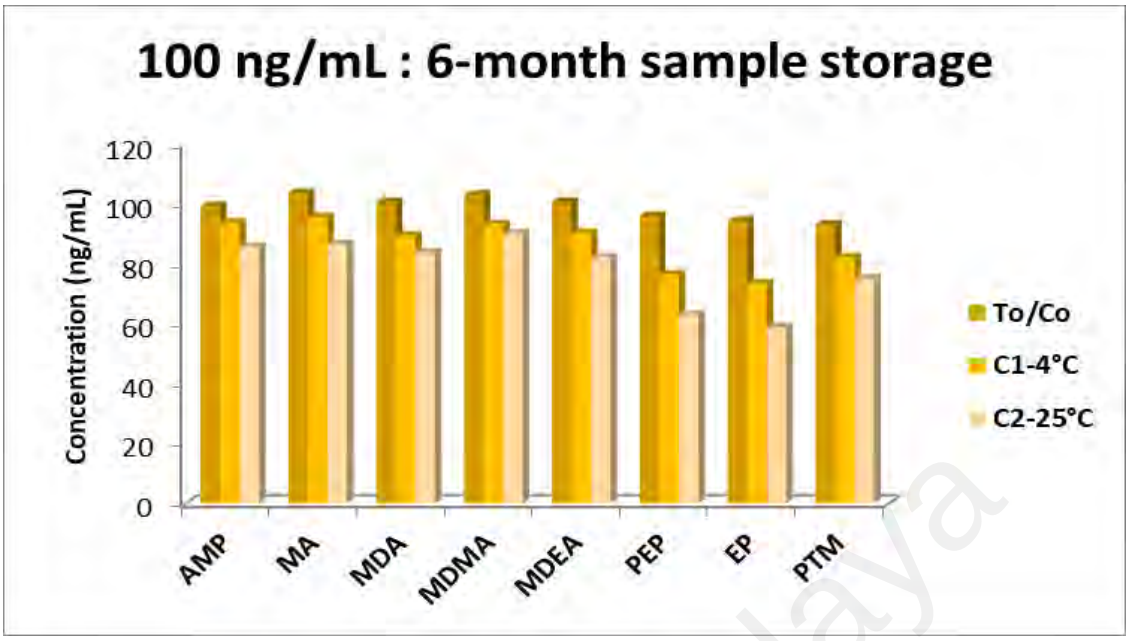


Figure 4.20b: Stability of ATS drugs in DBS for 6 months. Initial concentrations were 100 ng/mL.

4.4.3 Evaluation of Real Case Samples in DBS

The stability of the analytes in the real case samples and QC samples were determined at similar parameter as per in spiked samples. The residual drug amounts of 20 case samples and QC samples for 3-month and 6-month period are shown in Figure 4.21a and 4.21b respectively.

After the storage of 20 case samples for up to 3 months at 4 °C, decrement of AMP concentration was up to 24% (Sample 4), followed by MA up to 16% (Sample 13), MDA up to 19% (Sample 2), MDMA up to 12% (Sample 17), PEP up to 25% (Sample 16), and PTM up to 20% (Sample 7) compared to the real time analysis. While at 25 °C, decrement of AMP concentration was observed to be up to 50% (Sample 3), MA up to 22% (Sample 2), MDA up to 48% (Sample 5), MDMA up to 27% (Sample 17), PEP up to 34% (Sample 16), and PTM up to 28% (Sample 7) from the initial concentrations; T_0/C_0.

Further evaluation of the case samples was performed for the storage up to 6-month at 4 °C. The decrement of AMP concentration was up to 48% (Sample 15), MA up to 27% (Sample 20), MDA up to 52% (Sample 5), MDMA up to 36% (Sample 18), PEP up to 58% (Sample 16) and PTM up to 24% (Sample 7). While at 25 °C sample storage, decrement of AMP concentration was found to be up to 64% (Sample 3), MA up to 44% (Sample 2), MDA up to 64% (Sample 2), MDMA up to 43% (Sample 17), PEP up to 67% (Sample 16) and PTM up to 31% (Sample 7) from the real time analysis.

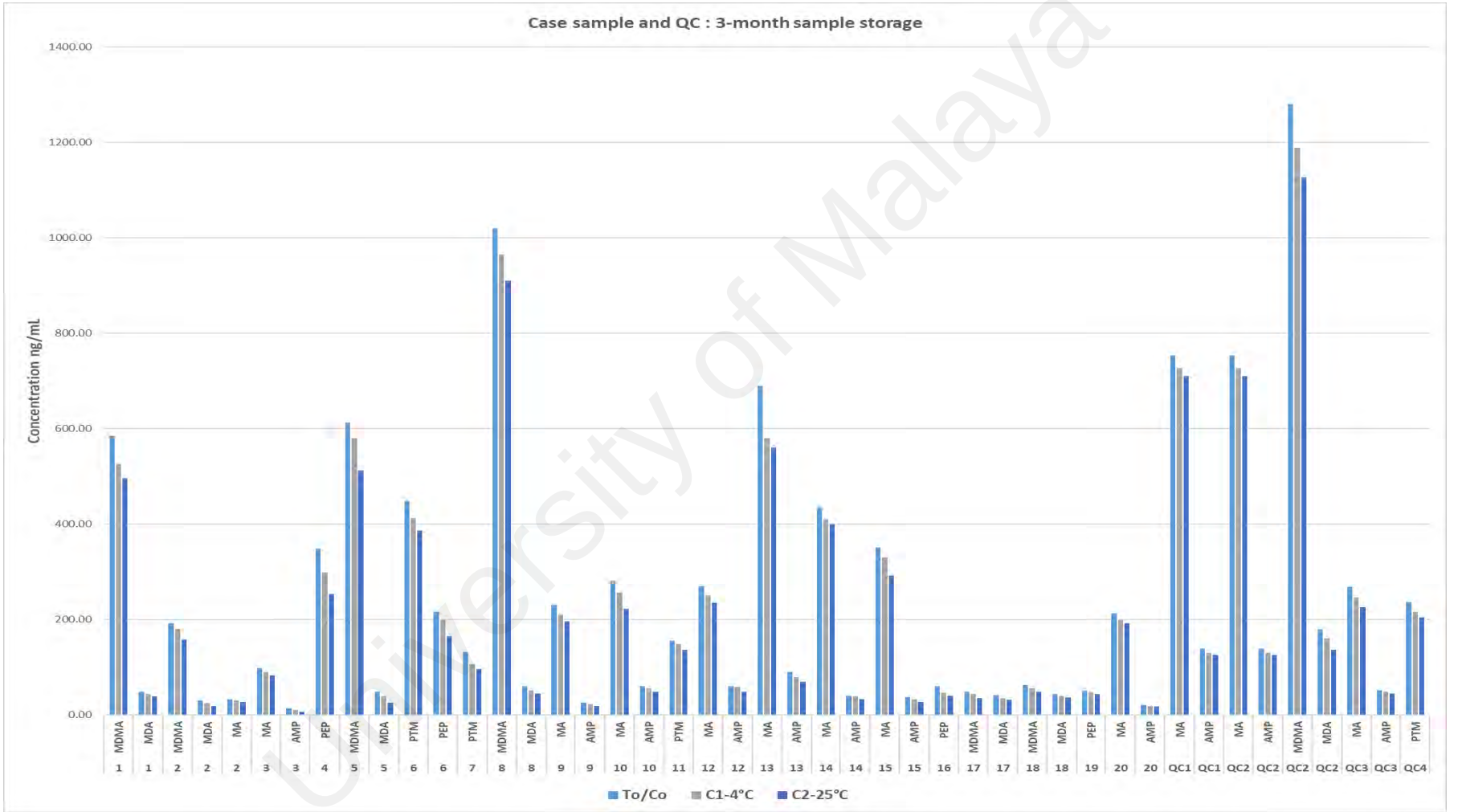


Figure 4.21a: Stability of ATS drugs in DBS (real cases and QCs) for 3 months storage.

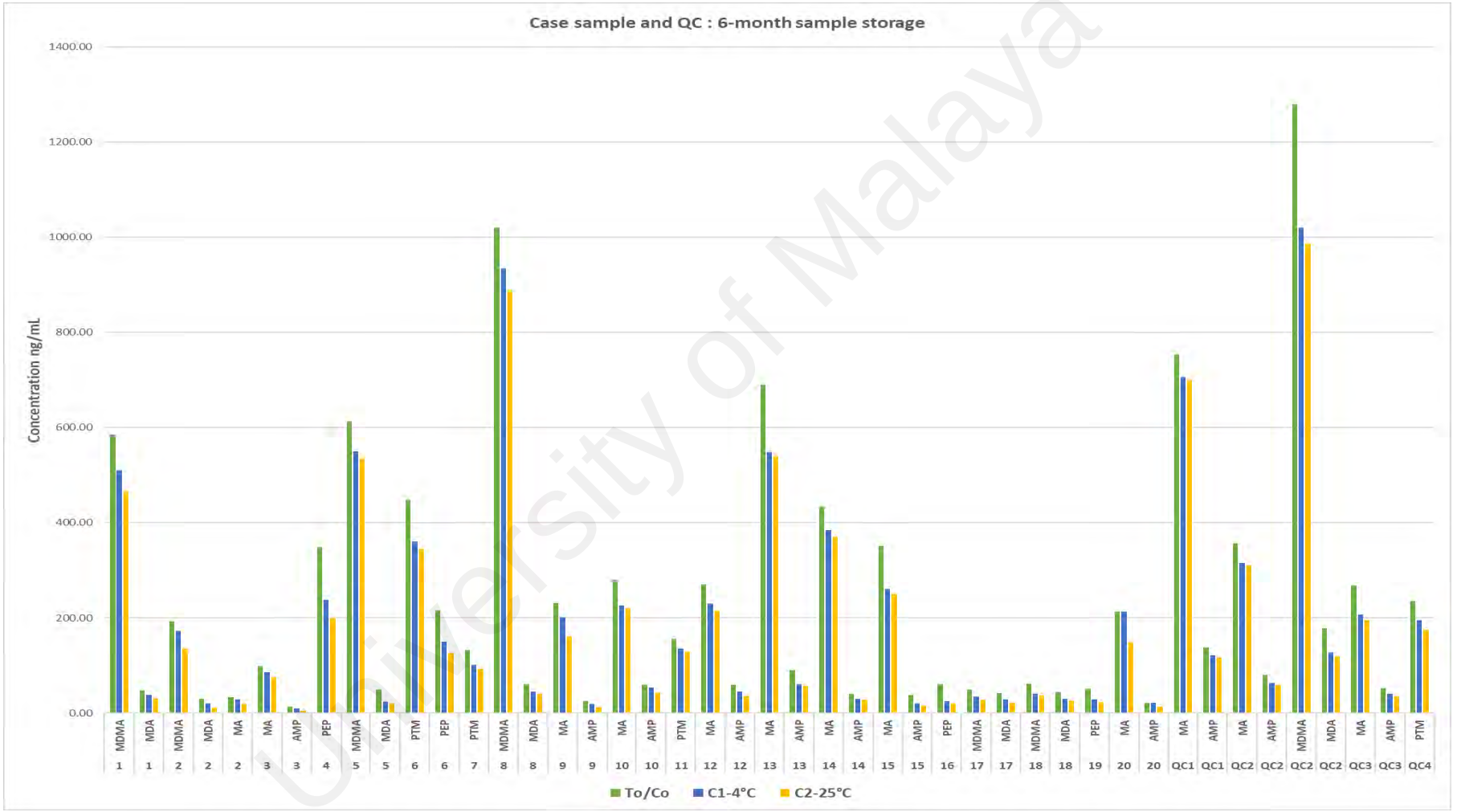


Figure 4.21b: Stability of ATS drugs in DBS (real cases and QCs) for 6 months storage.

CHAPTER 5: CONCLUSION

5.1 Summary of the Findings

From the investigation of the analysis of ATS drugs for forensic purposes, a rich amount of forensic information has been obtained which include the type or mode of analyses until the reliable specimens to be tested. Often, the analysis of ATS in biological specimens uses LC-MS/MS in MRM mode considering the high-end features and advantages offered by the instrument. Also, the normal biological specimens tested for the drugs included whole blood and urine that in-line with the significances highlighted for forensic interests.

In the further investigation of the ATS in biological specimens, four major tasks have been performed to provide new dimensions on the technique of analyses as well as the specimens tested. The technique involved was the advanced and enhanced MRM that capable to give higher confidence level of analysis during interpretation of the results. The application to the specimens was also extended to DBS as an innovative, simple and efficient technique for drugs detection to complement the established WBS method.

Task 1: The investigation of a novel method that is simple and rapid for the simultaneous determination of ATS drugs using LC-ESI-MS/MS was started due the high prevalence of ATS drugs abuse together with the complicated background of toxicology cases, that urged for a rapid and simple confirmatory approach. In this study, a novel mass spectrometry detection technique based on a multi-period and multi- experiment (MRM-EPI-MRM^3) with library matching in a single run for fast and rapid screening and identification of amphetamine type stimulants (ATS) drugs in whole blood, urine and dried blood stain was developed and validated.

The ATS drugs analyzed in this study includes, *i.e.* ephedrine, pseudoephedrine, amphetamine, methamphetamine, MDMA (3,4-Methylenedioxymethamphetamine), MDA (3,4-Methylenedioxyamphetamine), MDEA (3,4-Methylenedioxy-N-ethylamphetamine) and phentermine. The method was enhanced from normal Multi-Reaction Mode (MRM) to Multi-Reaction Mode-Enhanced Product Ion Mode- Multi-Reaction Mode 3 (MRM-EPI-MRM^3) for higher confidence level (EPI) and unique identification (MRM^3). The source and drugs optimization, the chromatographic conditions and the ESI parameters were taken into consideration.

The relative standard deviation for inter and intraday was less than 15% while recoveries ranged from 80-120% for all three matrices, *i.e.* whole blood, urine and dried blood stain. All compounds gave library matching percentage of more than 85% based on the purity. This unique multi-period and multi-experiment method was proven to be simple and robust as well as provided high confident results and has been applied succesfully for real sample monitoring.

Task 2: Further investigation for ATS detection involved a study on Flow Injection Analysis-MRM^3 (FIA-MRM^3) spectrometry in LC-MS/MS as a quick and effective screening method to distinguish the critical isomeric methamphetamine and phentermine utilizing the advantages of FIA and MRM^3 technique. The robust and high throughput FIA-MRM^3 was successfully developed and validated without LC separation in order to shorten the time of analysis and the detection was solely based on the strength of unique identification of MRM^3 in the MS part.

As mentioned earlier, the dependency on high-resolution mass spectrometer (HRMS) and special chiral stationary phase column impedes screening throughput especially in meeting high sampling demand. In the absence of such equipment for identification, the FIA-MRM^3 technique with the capabilities for quick identification of

both isomeric compounds to substitute the absence of retention time information and through the addition of selectivity criteria in MRM^3 mode has given new dimension to meet the objective.

From the analysis, the peaks from the two isomeric compounds were successfully discerned for all the tested specimens of WBS and DBS. The LOD and LOQ quantified relatively for MA and PTM were comparable to normal LC-MS/MS runs at 2.2 and 2.0ng/mL respectively for WBS, while for DBS, the LOD and LOQ were 3.4 and 2.8 ng/mL, respectively. The accuracy and inter-day precision for DBS and WBS were within 99.9 to 111.1% and 7.1 to 9.5%, respectively.

The developed and validated FIA-MRM^3 method offers high throughput information for screening purposes through large samples quantity before submitting to chromatographic-MS acquisitions. Such screening method cuts analysis time down to 80% and saves solvent consumptions as well as column lifetime without compromising on sensitivity and selectivity. Therefore, the actual confirmation and quantitation method can then be proceeded for the positive samples. In summary, MRM^3 in combination with the versatile FIA technique has successfully identified and confirmed the identity of isomeric compounds of similar masses in the simplest, economic and rapid analysis, which can be applied as routine analysis in forensic toxicology.

Task 3: The evaluation of DBS study applied Mean Concentration Ratio and Bland-Altman statistical in order to compare the reliability of DBS to be used interchangeably with WBS for the ATS drugs to be applied into the real cases samples. Based on the principle, the mean concentration ratio of 1.0 between two method of measurements indicates the exact agreement of both methods and the study revealed WBS/DBS ratios ranged from 0.91 to 1.05 for all targeted analytes suggesting that the two methods were

comparable. In addition, the low relative standard deviation (% RSD) of 0.97 to 2.33 indicated insignificant differences between the WBS and DBS values.

While for the Bland-Altman statistical analysis, the determination was performed via calculation of bias on the average difference of mean and the standard deviation for each drug. The values were determined whether the data points were within or exceed the limits of agreement. All data points tested were within limits of agreement and 99% of the points were within 3SD of the mean difference. The results suggested that such in the long run, 99% of future mean differences between measurements made on the same instrument will also lie within the limit of agreement.

In summary, the study has successfully demonstrated the use of both mean ratio and Bland- Altman plots to test hypothesis of equality between two mediums; *i.e.* WBS vs DBS, in the analysis of ATS drugs. Estimation of the both mediums using the methods have shown that the methods were not significantly different from each other and both were sufficiently well for them to be used interchangeably.

Task 4: Further evaluation of DBS study was performed on the stability and efficiency of DBS medium for ATS drugs and this included the time and temperature in Malaysia during of DBS sample storage. The tested samples included the spiked drugs, external QCs and real cases to be applied in the future. The stability of the analytes was determined by measuring the drug content remaining in DBS (in duplicate) per condition mentioned above and compared with the initial concentration (real time analysis).

The results suggested that the stability of all ATS drugs stored at 4 °C up to 3-month were considered acceptable (>70%) from the initial concentration. Most of the drugs with lower concentration especially stored at 25 °C started to degrade when stored

up to 3-month and the concentrations were gradually lowered especially for the two main precursors of pseudoephedrine and ephedrine at up to 6-month of storage (<50%).

The stability of higher concentration of ATS were acceptable at both temperatures up to 6 months storage due to the advantages of dehydration in the FTA card and the protection from moisture after keeping the samples in the desiccator with minimal error in sample preparation.

In summary, DBS technique has been proven to provide suitable procedure for the storage and analysis of samples in forensic toxicology field; with the advantages of easy to handle, transport and store in the laboratories, even in the absence of refrigerators, etc. However, several potential interferences such as UV and normal light exposure, slow drying process, use of anticoagulant and preservatives, seal storage bags, etc need to be considered in order to improve the stability of the drugs during sample storage. It is also highly recommended to store the DBS under cold temperature (<20 °C) to improve the stability and maintain the efficiency for future use.

Several suggestions for future works to provide a comprehensive investigation on the potential of DBS in forensic toxicology field would be as follows:

1) to extend the scope of detection to other common drugs of abuse such as benzodiazepines, opioids and cannabinoids as well as other common drugs in medications.
2) to study other matrices of DBS such as garments and tools for drugs especially in the cases of drug trafficking, drug related sexual assault as well as drugs in crime scene.

3) to widen the DBS applications to heavy metals by ICP-MS as the similar problem also happened in the heavy metals analysis in minute volume of blood samples.
4) to improve the sample preparation steps in DBS such as automated sampling systems and online SPE systems.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

PUBLICATIONS

1. **Fathiah A. Zubaidi**, Yeun-Mun Choo, Guan-Huat Tan, Hamimah Abd. Hamid, Yap Ken Choy (2019). A Novel Liquid Chromatography Tandem Mass Spectrometry Technique using Multi-Period-Multi-Experiment of MRM-EPI-MRM^3 with Library Matching for Simultaneous Determination of Amphetamine Type Stimulants Related Drugs in Whole Blood, Urine and Dried Blood Stain (DBS) - Application to Forensic Toxicology Cases in Malaysia. *Journal of Analytical Toxicology*, *43*(7),528-535.

2. **Fathiah A. Zubaidi**, Yeun-Mun Choo, Guan-Huat Tan, P. Myron, CS. Cornelia (2019). High-throughput Flow Injection Analysis-MRM^3 (FIA-MRM^3) Spectrometry for Alternative Fast Screening of the Isomeric Methamphetamine and Phentermine in Blood for Forensic Toxicology Cases in Malaysia. *Journal of Toxicology Research and Application*,*3*,1-10.

3. **Fathiah A. Zubaidi**, Syaiful Izwan Ismail, Yeun-Mun Choo, Guan-Huat Tan (2019). Bland-Altman Statistical Analysis and Mean Concentration Ratio for the Determination of Amphetamine Type Stimulants-related Drugs in Dried Blood Stain (DBS) Versus Whole Blood Sample (WBS) by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) - Application to Forensic Toxicology Cases in Malaysia. *Journal of Forensic Research*,*10*(1), 2157-7145.

PAPERS PRESENTED

1. **Fathiah A. Zubaidi,** Guan-Huat Tan, Yeun-Mun Choo (2012). The Simultaneous Determination of Common Drugs of Abuse in Dried Blood Stain (DBS) by LC-MS/MS. Paper presented at the 17th Malaysian Chemical Congress (17MCC), 15-17th October 2012, Putra World Trade Centre, Kuala Lumpur, Malaysia.

2. **Fathiah A. Zubaidi**, Guan-Huat Tan, Yeun-Mun Choo, Kamaruddin, A. (2013). Screening of Common Drugs of Abuse in Dried Blood Stain (DBS). Paper presented at the 51st Annual Meeting of the International Association of Forensic Toxicologists (TIAFT), 2-6th September 2013, Funchal, Madeira, Portugal.

3. **Fathiah A. Zubaidi**, Guan-Huat Tan, Yeun-Mun Choo (2014). Development and Validation of Liquid Chromatography Tandem Mass Spectrometer (LC-MS/MS) for the Simultaneous Determination of Common Drugs of Abuse in Forensic Cases in Malaysia: Application to Dried Blood Stain (DBS). Paper presented at the 9th Mathematics and Physical Sciences Graduate Congress, 8-9th January 2014, Faculty of Science University Malaya Kuala Lumpur, Malaysia.

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