Determination of MDMA, MDA and MA in Ecstasy Tablets and Urine Samples of Suspects Seized in Songkhla Province Using GC-FID Technique

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ชื่อวิทยานิพนธ์ การตรวจวัดปริมาณของ MDMA MDA และ MA ในเม็ดยาอีและตัวอย่างปัสสาวะของผู้ต้องสงสัยที่ถูกจับได้ในจังหวัดสงขลาโดยใช้เทคนิค GC-FID

ผู้เขียน นายอภิชัย พลชัย

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บทคัดย่อ

ได้พัฒนาเทคนิคที่ง่ายและรวดเร็วในการวิเคราะห์หา 3,4-methylenedioxymeth amphetamine (MDMA) 3,4-methylenedioxyamphetamine (MDA) และ methamphetamine (MA) ในตัวอย่างเม็ดยาอีและปัสสาวะของคนโดยใช้เทคนิคแก๊สโครมาโทกราฟีที่มีตัวตรวจวัดชนิดเฟลมไอออไนเซชัน ระบบโครมาโทกราฟีใช้คอลัมน์ชนิด CP-SIL 24 CB WCOT (ความยาว 30 เมตร เส้นผ่านศูนย์กลางภายใน 0.32 มิลลิเมตร และความหนาของฟิล์ม 0.25 ไมโครเมตร) อัตราการไหลของแก๊สพาเท่ากับ 2.6 มิลลิเมตรต่อนาที อัตราการไหลของแก๊สเชื้อเพลิง (แก๊สไฮโดรเจน) และ Make-up แก๊ส (แก๊สไนโตรเจน) เท่ากับ 30 มิลลิเมตรต่อนาที และอัตราการไหลของแก๊สออกซิแดนท์ (แก๊สออกซิเจน) เท่ากับ 300 มิลลิเมตรต่อนาที อุณหภูมิของหัวฉีดและตัวตรวจวัดเท่ากับ 290 และ 300 องศาเซลเซียส ตามลำดับ อุณหภูมิของคอลัมน์เริ่มต้นที่ 80 องศาเซลเซียส เป็นเวลา 0 นาทีและเพิ่มอุณหภูมิด้วยอัตรา 20 องศาเซลเซียสต่อนาที ถึงอุณหภูมิสุดท้ายที่ 270 องศาเซลเซียส และคงไว้เป็นเวลา 1 นาที สารละลายมาตรฐานของสารทั้งสามชนิดรวมทั้ง diphenylamine (DPA) ซึ่งเป็น

สารมาตรฐานภายในเตรียมโดยใช้เมทานอลบริสุทธิ์ ตัวอย่างเม็ดยาอีถูกสกัดด้วยเมทานอลบริสุทธิ์โดยปราศจากการใช้เทคนิคคลื่นเหนือเสียง ผลการทดลองพบว่าสารทั้งหมดแยกออกจากกันได้ดีภายใน 10.0 นาที กราฟเทียบมาตรฐานมีความเป็นเส้นตรงอยู่ในช่วงความเข้มข้น 3.125-200 ไมโครกรัมต่อมิลลิลิตร สำหรับ MDMA และ 6.25-200 ไมโครกรัมต่อมิลลิลิตร สำหรับ MDA และ MA โดยมีค่าสัมประสิทธิ์สหสัมพันธ์ที่ดี ($r \geq 0.9980$) ค่าความเที่ยงของการวิเคราะห์ภายในวันเดียวกันและระหว่างวัน ซึ่งแสดงในรูปค่าเบี่ยงเบนมาตรฐานสัมพัทธ์ (%RSD) มีค่าอยู่ในช่วง 2.92-10.38% และ 1.56-7.08% สำหรับ MDMA 2.32-3.59% และ 1.15-9.15% สำหรับ MDA และ 3.06-6.58% และ 2.51-9.77% สำหรับ MA ค่าความถูกต้องของการวิเคราะห์ซึ่งแสดงในรูปค่าการเบี่ยงเบน (%DEV) อยู่ในช่วง (-) 7.11 ถึง (+) 17.51% สำหรับ MDMA (-) 7.18 ถึง (+) 11.72% สำหรับ MDA และ (-) 19.79 ถึง (+) 6.91 สำหรับ MA ค่าความเข้มข้นต่ำสุดที่สามารถหาปริมาณได้มีค่า 3.125 ไมโครกรัมต่อมิลลิลิตร สำหรับ MDMA และ 6.25 ไมโครกรัมต่อมิลลิลิตร สำหรับ MDA และ MA เทคนิคนี้ได้นำมาใช้ในการวิเคราะห์หาความบริสุทธิ์ของยาอีที่ตรวจจับได้ในจังหวัดสงขลา ตัวอย่างยาอีส่วนใหญ่มี MDMA เพียงอย่างเดียวโดยมีความบริสุทธิ์ของสาร MDMA อยู่ในช่วง 1.65-80.90%

สำหรับตัวอย่างปัสสาวะ ได้ศึกษาสภาวะที่เหมาะสมของการเตรียมตัวอย่างด้วยเทคนิคตัวดูดซับของแข็ง เพื่อใช้ในการวิเคราะห์หา MDMA MDA และ MA ตัวอย่างปัสสาวะถูกปรับสภาพให้เป็นเบสก่อนใส่ในตัวดูดซับของแข็งชนิด Oasis® HLB cartridge ตัวดูดซับถูกล้างด้วยของผสม 5% เมทานอล-น้ำ ซึ่งมี 2% แอมโมเนียมไฮดรอกไซด์ และถูกชะด้วยของผสม 70% เมทานอล-น้ำ ซึ่งมี 2% กรดอะซิติก กราฟเทียบมาตรฐานมีความเป็นเส้นตรงอยู่ในช่วงความเข้มข้น 1-20 ไมโครกรัมต่อมิลลิลิตร สำหรับ MDMA MDA และ MA โดยมีค่าสัมประสิทธิ์สหสัมพันธ์ที่ดี ($r \geq 0.9990$) ค่าความเที่ยงของการวิเคราะห์ภายในวันเดียวกันและ

ระหว่างวัน ซึ่งแสดงในรูปค่าเบี่ยงเบนมาตรฐานสัมพัทธ์ (%RSD) มีค่าอยู่ในช่วง 2.97-17.31% และ 10.45-14.20% สำหรับ MDMA 6.99-16.54% และ 6.28-12.98% สำหรับ MDA และ 8.43-17.18% และ 8.17-11.64% สำหรับ MA ค่าความถูกต้องของการวิเคราะห์ซึ่งแสดงในรูปค่าการเบี่ยงเบน (%DEV) อยู่ในช่วง (-) 16.68 ถึง (+) 4.28% สำหรับ MDMA (-) 17.62 ถึง (+) 14.94% สำหรับ MDA และ (-) 8.72 ถึง (+) 0.60 สำหรับ MA ค่าความเข้มข้นต่ำสุดที่สามารถหาปริมาณได้มีค่า 1 ไมโครกรัมต่อมิลลิลิตร สำหรับ MDMA MDA และ MA ค่าร้อยละของการได้กลับคืนอยู่ในช่วง 84.17-95.01% สำหรับ MDMA 92.56-107.60% สำหรับ MDA และ 80.35-90.74% สำหรับ MA เทคนิคนี้ได้นำมาประยุกต์ใช้ในการวิเคราะห์หาปริมาณของ MDMA MDA และ MA ในตัวอย่างปัสสาวะของผู้ต้องสงสัย ซึ่งพบว่าความเข้มข้นของ MDMA MDA และ MA ในปัสสาวะอยู่ในช่วง 1.36-20.59 1.65-4.12 และ 1.80-19.98 ไมโครกรัมต่อมิลลิลิตรตามลำดับ

สรุปได้ว่า เทคนิคแก๊สโครมาโทกราฟีเป็นเทคนิคที่ง่าย มีความไว ความเที่ยงและความถูกต้องของการวิเคราะห์ที่ยอมรับได้ สามารถนำไปวิเคราะห์สาร MDMA MDA และ MA ได้พร้อมกันทั้งในเม็ดยาอีและปัสสาวะ เทคนิคนี้สามารถใช้ได้ทั่วไป มีต้นทุนในการวิเคราะห์ต่ำและช่วยให้งานวิเคราะห์ที่ทำประจำเร็วขึ้น เทคนิคนี้น่าจะเป็นประโยชน์ต่องานวิเคราะห์ของห้องปฏิบัติการของกองวิทยาการหรือหน่วยงานที่เกี่ยวข้องกับการวิเคราะห์สารเสพติดในประเทศไทย

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Abstract

A simple and rapid method for direct and simultaneous determination of 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA) and methamphetamine (MA) in ecstasy tablets and human urine samples using gas chromatography with a flame ionization detector (GC-FID) were developed and validated. The chromatography used a CP-SIL 24 CB WCOT fused silica capillary column (30 m × 0.32 mm i.d., 0.25 μm film thickness). Flow rate of carrier gas (N_2) was 2.6 mL min^{-1}. Flow rate of both fuel gas (H_2) and make-up gas (N_2) were 30 mL min^{-1}. Oxidant gas (O_2) flow rate was 300 mL min^{-1}. Temperature of the injector and the detector were 290^{o}C and 300^{o}C, respectively. Temperature of the column was programmed initially at 80^{o}C (0 min) and increased with a rate of 20^{o}C min^{-1} to 270^{o}C where it was held for 1 min. The standard solutions of all analytes including diphenylamine (DPA), an internal standard, were prepared in pure methanol. Sample of ecstasy tablets were extracted with pure methanol without ultrasonication. All analytes were well separated within 10.0 min. Calibration curves were linear over the concentration ranges of 3.125-200 μg mL^{-1} for MDMA and 6.25-200 μg mL^{-1} for MDA and MA with good correlation coefficient ($r \geq 0.9980$). The intra- and inter-day precisions of the method were 2.92-10.38%RSD and 1.56-7.08%RSD for MDMA, 2.32-3.59%RSD and 1.15-9.15%RSD for MDA, and 3.06-6.58%RSD and 2.51-9.77% RSD for MA. The accuracy ranged from (-) 7.11 to (+) 17.51%DEV for MDMA, (-) 7.18 to (+) 11.72%DEV for MDA, and (-) 19.79 to (+) 6.91 for MA. The lower limits of quantification (LLOQ) were 3.125 μg mL^{-1} for

MDMA and 6.25 μg mL^{-1} for MDA and MA. It was shown to be useful for determining the purity of MDMA in ecstasy tablets seized in Songkhla province. Among different tablets, almost all of them contained only MDMA as an active ingredient with varying purity ranging from 1.65-80.90%.

For human urine samples, solid phase extraction (SPE) method was developed for the determination of MDMA, MDA and MA. The samples were alkalinized before loading to Oasis® HLB cartridges. The cartridges was washed with 5% methanol-water mixture containing 2% ammonium hydroxide and eluted with 70% methanol-water mixture containing 2% acetic acid. Calibration curves were linear over the concentration range of 1-20 μg mL^{-1} for MDMA, MDA and MA with good correlation coefficient ($r \geq 0.9990$). The intra- and inter-day precisions of the method were 2.97-17.31%RSD and 10.45-14.20%RSD for MDMA, 6.99-16.54%RSD and 6.28-12.98%RSD for MDA, and 8.43-17.18%RSD and 8.17-11.64% RSD for MA. The accuracy ranged from (-) 16.68 to (+) 4.28%DEV for MDMA, (-) 17.62 to (+) 14.94%DEV for MDA, and (-) 8.72 to (+) 0.60 for MA. The lower limits of quantification (LLOQ) were 1 μg mL^{-1} for all analytes. The percentages of recovery were 84.17-95.01% for MDMA, 92.56-107.60% for MDA, and 80.35-90.74% for MA. Application of the method for determining MDMA, MDA and MA in urine of suspects revealed that the urinary concentrations of MDMA, MDA and MA ranged from 1.36 to 20.59, 1.65 to 4.12 and 1.80 to 19.98 μg mL^{-1}, respectively.

In conclusion, the present GC-FID method is simple, sensitive, precise and accurate to simultaneously determine MDMA, MDA and MA in ecstasy tablets and urine. This technique is universal and cost-saving and facilitates routine analytical work. It would be useful for most laboratories of the Forensic Science Division or any departments responsible for identifying narcotic substances in Thailand.

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Apichai Phonchai

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List of Abbreviations

A	Amphetamine
amu	Atomic mass unit
ATS	Amphetamine-type stimulants
CE	Capillary electrophoresis
CNS	Central nervous system
DEV	Deviation
DHA	3,4-Dihydroxyamphetamine
DHMA	3,4-Dihydroxymethamphetamine
DPA	Diphenylamine
ESI	Electrospray ionization
etc	Et cetera
FDA	Food and drug administration
GC	Gas chromatography
GC-FID	Gas chromatography-flame ionization detector
GC-MS	Gas chromatography-mass spectrometry
HETP	Height equivalent to a theoretical plate
HMA	4-Hydroxy-3-methoxyamphetamine
HMMA	4-Hydroxy-3-methoxymethamphetamine
HPLC-UV	High performance liquid chromatography-ultraviolet detection
HS	Headspace
HLB	Hydrophilic-lipophilic balanced

List of Abbreviations (cont.)

ICP	Inductively coupled plasma
IUPAC	The international union of pure and applied chemistry
K	Ketamine
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantification
MA	Methamphetamine
MBDB	*N*-methyl-1-(3,4-methylenedioxyphenyl)-2-butamine
MDA	3,4-Methylenedioxyamphetamine
MDEA	3,4-Methylenedioxyethylamphetamine
MDMA	3,4-Methylenedioxymethamphetamine
NIR	Near infrared spectroscopy
no	Number
NPD	Nitrogen-phosphorus detector
PDHID	Pulsed discharge helium ionization detector
r	Correlation coefficient
RSD	Relative standard deviation
SD	Standard deviation
SEM	Standard error of mean
SME	Solvent microextraction
SPE	Solid phase extraction
SPME	Solid phase microextraction

CHAPTER 1

Introduction

1.1 Background and rationale

Amphetamine-type stimulants (ATS) are defined by the United Nations Office on Drugs and Crime (UNODC) as consisting of amphetamine (A), methamphetamine (MA), 3,4-methylenedioxymethamphetamine (MDMA) and related products, such as 3,4-methylenedioxyamphetamine (MDA). ATS also includes a number of other synthetic stimulants, such as phentermine and methcathinone. In recent years, ATS abuses have become the main problem in a number of countries in East and South-East Asia, particularly Thailand, the Philippines, Japan, the Republic of Korea and Taiwan.

The growth of the illicit Drug market, especially for ATS such as MDMA, ketamine (K) and crystalline methamphetamine (ice), has been noted in the second half of the 1990s. MDMA or Ecstasy is a dangerous narcotic drugs and increasingly used as a recreational substance among adolescents in many countries including Thailand. Endemic areas of Thailand are the entertainment places in the big cities such as Bangkok, Chiangmai, Chiangrai, Suratthani, Phuket and Songkhla. MDMA is the remarkably threatening substance which causes social, economic and health problems. For social problem, narcotics somehow increase criminal rate and this is a cause of other social problems in a vicious circle manner. This problem is too chronic to solve and has an impact upon social security and socio-economic order.

MDMA was first developed and patented by Merck in 1914 as an appetite suppressant. Since its safety is unimpressive, the drug has never been marketed. By 1985, it was produced illegally for recreational use (Gahlinger, 2004) and became one of the popular club drugs used at dance parties, raves, and nightclubs. As the rave and club scenes expand to metropolitan and suburban areas across the several countries, MDMA use and distribution are increasing as well. MDMA is frequently used in combination with other drugs. MDMA most frequently exists in tablet form, although it is occasionally sold in capsules or as powder. It is most frequently used orally and

rarely snorted or intravenously. Street names for MDMA include ecstasy, adam, XTC, hug, beans, and love drug (National Institute on Drug Abuse, 2008).

MDMA ingestion produces both desired and undesired physical and mental effects such as wakefulness, sense of energy, euphoria, muscular tension, tooth grinding (bruxism), increase in body temperature, heart rate and blood pressure, mild hallucination, depersonalization, and depression. Chronic use of MDMA causes serious toxic effects including brain serotonergic neurotoxicity, deleterious effects on mood and cognition, liver failure, cardiovascular toxicity, cerebral toxicity, and hyperpyrexic toxicity. All the severe forms of toxicity are capable of causing death (Kalant, 2001).

In Thailand, MDMA is classified as a category I narcotic substance according to the Narcotics Act of B.E. 2522 (1979) (ONCB, 2007a). Any person who produces, imports or exports these category I narcotics is in violation of Section 15 (for amphetamine and derivatives; 375 mg or more of a pure substance or narcotic substance of 50 doses or more or a pure weight of 1.5 g or more), shall be liable to imprisonment for life and to a fine of one million to five million baht. If the commission of the offence is committed for the purpose of disposal, *i.e.* pure substance of 20 g or more, the offender shall be liable to the death penalty. Any person shall be a suspect if the level of a substance in urine is above the cut-off value which is 1 μg mL^{-1} for MDMA, MDA and MA. This is recommended by the Narcotics Act of B.E. 2543 (2000) (No.3) of Thailand (Provincial Police Region 7, 2008).

Ecstasy tablets may contain various chemicals other than pure MDMA, including MDA, MA, A, 3,4-methylenedioxyethylamphetamine (MDEA), caffeine, ephedrine, diphenhydramine, cocaine and diazepam. Some tablets contain no active drugs. The information about purity of MDMA and other substances in ecstasy tablets and human urine samples is often necessary to verify drug ingestion of suspects. This can be used as an evidence to support the trial case, narcotic offence, punishment and narcotic seizure reward. The penalties are determined by not only the purpose of possession but also the amount of pure substance. Therefore, a precise and accurate analytical technique is very important.

In the clinical toxicology and forensic laboratories and law enforcement, detection of MDMA and other amphetamine analogues in ecstasy tablets and urine samples is often necessary to verify drug addiction of suspects and in clinical test used in drug rehabilitation therapy and sport doping control programs (Martinis *et al.*, 2007). These investigations often carried out using non-specific techniques, such as color test, radioimmunoassay (RIA), enzyme multiplied immunoassays (EMIT), fluorescence polarization immunoassays (FPIA) and partical immunoassays (PIA). However, those techniques need to be confirmed the methods for isolating the analyte from the organic matrix (Centini *et al.*, 1996). Several techniques such as thin-layer chromatography (TLC) (Kala and Madej, 1997; Zakrzewska *et al.*, 2007), capillary electrophoresis (CE) (Fanga *et al.*, 2002), near infrared spectroscopy (NIR) (Schneider and Kovar, 2003), high performance liquid chromatography (HPLC) (Costa *et al.*, 2004), gas chromatography (GC) (Mitrevski and Zdravkovski, 2005), and liquid chromatography coupling mass spectrometry (LC-MS) (Costa *et al.*, 2004; Cheng *et al.*, 2006) have been shown to successfully detect MDMA and other amphetamine analogues in ecstasy tablets and biological samples. Recently, the gas chromatography coupled to a mass spectrometry (GC-MS) and liquid chromatography-atmospheric pressure ionization electrospray mass spectrometry (LC-APCI-MS) have been shown as a powerful technique for identification and confirmation of MDMA and metabolites (Huang and Zhang, 2003; Pichini *et al.*, 2008). However, most laboratories of the Forensic Science Division or Regional Medical Science Center in Thailand cannot afford such an expensive instrument. The gas chromatography-flame ionization detector (GC-FID) is considered to be more possible for determining the purity of a major ingredient in ecstasy tablets and biological samples.

1.2 Literature review

1.2.1 Drug situation in Thailand

Drugs which are concerned as a significant problem in Thailand include opium, heroin, cannabis, cocaine, methamphetamine or Ya Ba (including crystalline form, 'ice'), ketamine and volatile substances. Thailand is served as a producing, trading, distributing and transporting areas. Currently as a producing area, the amounts of drugs, especially opium, heroin and cannabis, are reducing since the Thai Government has strictly controlled the opium poppy cultivation mostly being in the mountainous areas of the Northern part of the country. However, the neighboring countries are also the drug source. The drug problem is therefore transnational.

While opium is in control, amphetamine-like stimulants such as methamphetamine and ecstasy are increasingly distributed. These drugs are not produced in Thailand but smuggled from other countries including neighboring areas. Ecstasy is usually smuggled from the Netherlands via the Bangkok airport and transported to the other parts of the country. The endemic areas for ecstasy are big towns and tourist cities such as Bangkok, Chiangmai, Suratthani, Songkhla (Hat Yai), Phuket and Chonburi (ONCB, 2007b). It is widely distributed among teenagers at well educated and high status and individuals working or spending their nightlife for dancing party in clubs and entertaining places. Since ecstasy has to be imported, it is more expensive than methamphetamine. To reduce the cost, there has been tried to make it in Thailand.

In 2003, the seizure of ecstasy was reported at the first time in Bangkok. The exhibits were 3,300 of ecstasy tablets with various logos and materials for forming tablet. The suspects bought the concentrated form of active ingredient from the Netherlands and mixed with some other substances to increase in quantity. In addition, one Singaporean and one Thai suspects were arrested in Bangkok together with 7,400 ecstasy tablets and other producing materials. The drug was smuggled from Indonesia (ONCB, 2007b). In 2004 and 2005, the Department of Medical Science reported the presence of an active compound of ecstasy in urine of 310 and 190 individuals attending the entertaining places, respectively. The figures were the highest in Southern Thailand, *i.e.* 233 individuals (75%) and 159 individuals (84%),

respectively. Methamphetamine and ketamine were also found in the samples investigated by the Bureau of Drugs and Narcotics (Angkana *et al*., 2006).

In 2006, the Bureau of Drugs and Narcotics and the Regional Medical Science Center Songkhla conducted the survey of drug abuse in the entertaining places in Songkhla by collecting the urine of the customers (196 samples). The samples were analyzed at the Regional Medical Science Center Songkhla. The results revealed that MDMA was the most popular (88.7%) while ketamine was the second majority (43.5%) among the users. Methamphetamine and Tetrahydrocannabinol (THC) were also reported as the minority. Morphine and cocaine were not a significant problem (Angkana *et al*., 2006).

According to the reports of drug seizure, this indicates the increasing distribution of ecstasy in Thailand. If it is successfully made within the country, its cost will be lowed, trafficking will be more often and the number of the users will be increased. Therefore, it is the responsibility of the government and private sectors as well as the society to seriously control and monitor the situation (ONCB, 2007b).

1.2.2 Chemical properties of MDMA, MDA and MA

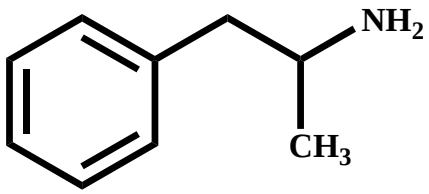
3,4-methylenedioxymethamphetamine ($C_{11}H_{15}NO_2$; m.w. 193.25) or MDMA (Figure 1.1) is a secondary amine and a methylenedioxy substituted analogue of methamphetamine (MA; Figure 1.1). Alternative chemical names include *N*-methyl-3,4-methylenedioxyphenylisopropylamine and *N*-methyl-3,4-methylenedioxy amphetamine. MDMA is a weak basic and polar compound with a pK_a around 9.9 (Pichini, 2006). It is an oil at room temperature with a boiling point of 100-110ºC. It is invariable supplied as hydrochloride salt ($C_{11}H_{16}NClO_2$), a white crystalline material with a melting point of 148-149ºC. It has a chiral center giving rise to two enantiomers, each with different degrees of central nervous system (CNS) activity, and different affinities for the enzyme systems responsible for its breakdown. All currently popular syntheses produce a racemic mixture (Logan and Couper, 2003). These properties confer an easy diffusion across cells membranes and lipidic layer, and tissues or biological matrices with a more acidic pH than blood.

3,4-methylenedioxyamphetamine ($C_{10}H_{13}NO_2$) or MDA (Figure 1.1), a synthetic ring-substituted derivative of amphetamine (A; Figure 1.1), was first

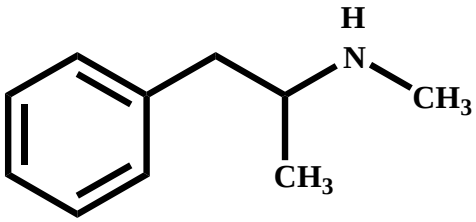
synthesized in 1910. It appears to produce similar pharmacological effects as MDMA. It is administered both orally and intravenously in doses of 50-250 mg as an illicit drug (Baselt, 1982). MDA is known popularly as love drug or love pill. MDA is a basic primary amine with a molecular weight of 179.22 a.m.u. MDA is a weak base drug with a pK_a around 10 (Garrett *et al.*, 1991). It is a liquid at room temperature, so is invariably supplied and used as the hydrochloride salt ($C_{10}H_{14}NClO_2$, 215.68 a.m.u.), which has a melting point of 187-188ºC. MDA also serves as a precursor in the synthesis of MDMA and MDEA. A number of studies have characterized the kinetics of MDA but there appears to be little information on MDA administered under controlled clinical settings. MDA has been reported to appear in oral fluid following the administration of MDMA in concentrations representing approximately 4-5% of MDMA (Cone and Huestis, 2007).

Methamphetamine ($C_{10}H_{15}N$) or MA (Figure 1.1), a central nervous system (CNS) stimulant with some legitimate therapeutic uses, has a tremendous potential for abuse. It has a history as a periodically popular drug of abuse, which at the time of writing is undergoing resurgence in popularity. MA is the common name for *N*,-dimethylphenethylamine, also referred to as desoxyephedrine, methylamphetamine, phenylisopropylmethylamine, and a variety of other similar systematic names.

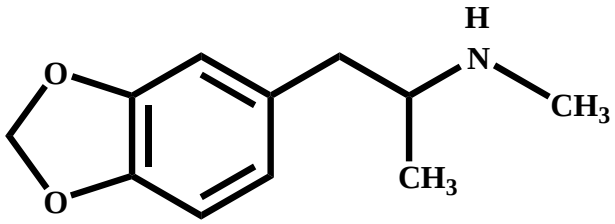
MA is an amphetamine (A) derivative and belongs to the class of amphetamines. The drug was first synthesized in Japan in 1919 by Ogata, patented in 1920, and later licensed to Burroughs Wellcome, who marketed it as the anorectic methedrine. MA is a secondary amine with a molecular weight of 149.24 a.m.u. MA is a weak base and polar compound with a pK_a around 9.5. It is a liquid at room temperature, so is invariably supplied and used as the hydrochloride salt ($C_{10}H_{16}ClN$, 185.74 a.m.u.), which has a melting point of 170-175 ºC, of note is the fact that this salt, unlike the hydrochloride salt of cocaine, volatilizes without pyrolysis at 300-305ºC, a temperature readily achieved in a butane lighter flame, meaning that it can be smoked in the salt form without the tedious conversion to the base required in order to smoke cocaine (Logan and Couper, 2002).



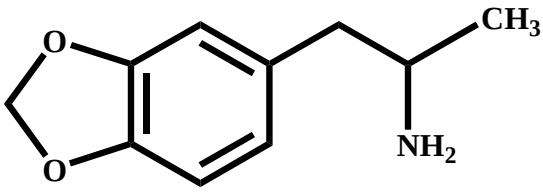
Amphetamine (A)



Methamphetamine (MA)



3,4-Methylenedioxymethamphetamine (MDMA)



3,4-Methylenedioxyamphetamine (MDA)

Figure 1.1 Chemical structures of A, MA, MDMA and MDA

1.2.3 Pharmacology of MDMA

1.2.3.1 Pharmacokinetics

1) Route of Administration

Ecstasy is almost uniformly sold in tablet form, and is usually taken orally, although there are reports of tablets being crushed and snorted. A popular variation on oral ingestion is parachuting, in which a tablets is crushed, wrapped in tissue paper, and swallowed for more rapid absorption. Sometimes, this is supplemented with uncrushed tablets to achieve both rapid onset and sustained effect (Logan and Couper, 2003).

2) Absorption

MDMA is readily absorbed from the intestinal tract and reaches its peak concentration in the plasma about 2 hours after oral administration. Dose of 50 mg, 75 mg and 125 mg to healthy human volunteers produced peak blood concentrations of 106 ng mL^{-1}, 131 ng mL^{-1} and 236 ng mL^{-1}, respectively (Kalant, 2001). These concentrations are quite low because the drug passes readily into the tissues constituents. De la Torre and coworkers (2000) reported the peak plasma concentrations at 1.5-4.0 h following ingestion of 50-150 mg of MDMA and the plasma MDA (metabolite) concentrations peaked later (4-6 h) and never exceeded 5% of the parent compound concentration. The author reports a combined mean peak plasma concentration (both enantiomers) of 0.06 mg L^{-1} at 2-4 h following the administration of 40 mg of MDMA, which is consistent with earlier references (Logan and Couper, 2003).

3) Distribution

MDMA shows low plasma protein binding. In practice, such low binding determines that almost the total amount of drug available in plasma may diffuse to the extravascular compartment. Information on the distribution parameter of MDMA in humans after controlled administration is very limited. With regard to MDMA enantiomers, Fallon and coworkers (1999) indicated that after the administration of 40 mg racemic MDMA, S-(+)MDMA, the more active form, had more extensive distribution than R-(-)MDMA enantiomer (595±204 L vs. 383±97 L). From the data

reported by De la Torre and coworkers (2000), a distribution volume of 452±137 L (6.4 L kg^{-1}) was reported following oral administration of 100 mg of MDMA.

4) Metabolism

MDMA metabolism in humans is complex and includes two main metabolic pathways (Figure 1.2). MDMA is primarily metabolized to 3,4-dihydroxymethamphetamine (DHMA; HHMA) by *O*-demethylenation via CYP2D6. A secondary pathway of MDMA metabolism is *N*-demethylation to MDA, a minor metabolite of MDMA. CYP1A2 is a major enzyme responsible for this pathway while CYP2D6 may also involve. Similarly to MDMA, MDA is *O*-demethylenated to 3,4-dihydroxyamphetamine (DHA; HHA). Both DHMA and DHA are metabolized by *O*-methylation to 4-Hydroxy-3-methoxymethamphetamine (HMMA) and 4-Hydroxy-3-methoxyamphetamine (HMA), respectively. HMMA and HMA are primarily excreted in urine as conjugated glucuronide or sulphate metabolites. There is a little data on the disposition of these metabolites in alternative matrices (Martinis *et al.*, 2007).

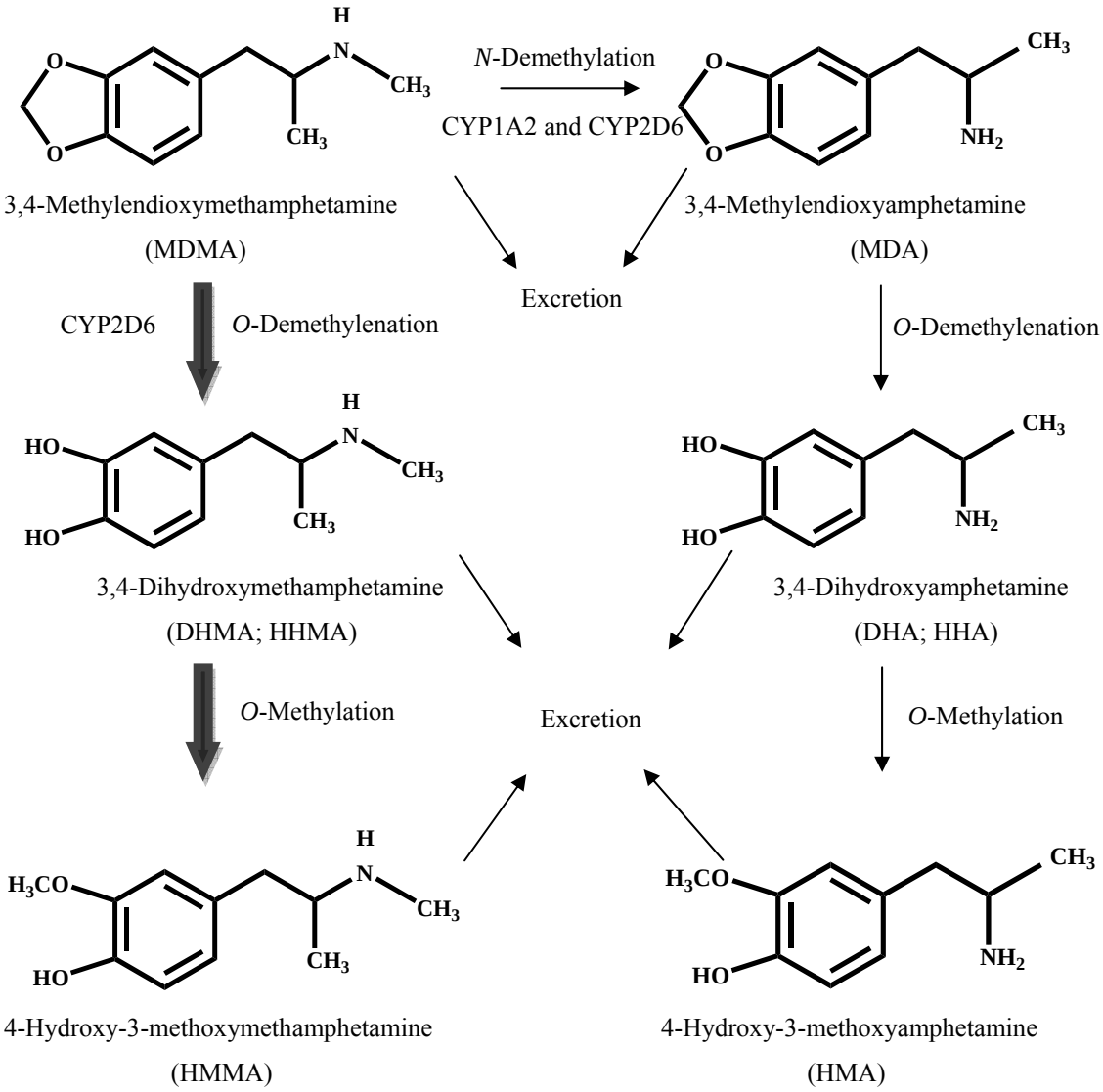


Figure 1.2 Metabolic pathway of MDMA in humans

5) Elimination

Elimination of MDMA from the body is moderately slow. The half-life for MDMA disappearance from blood is 8 h. Since it takes about 5 half-lives (*i.e.*, about 40 h) for over 95% of the drug to be cleared from the body, this may explain the persistence of troublesome after-effects for one or two days after use (Mas *et al.*, 1999). The apparent half-lives of the MDMA enantiomers are reported as 5.8±2.2 and 3.6±0.9 h for the R-(-) and S-(+)enantiomers, respectively (Fallon *et al.*, 1999).

In human urine, approximately 50-70% of the total MDMA dose is recovered as MDMA and other metabolites. Although MDMA is metabolized in the body, a large proportion is excreted unchanged in urine. The urinary recovery of HMMA, a metabolite of MDMA metabolism, remained unchanged. No significant changes in the urinary pH or creatinine clearance. Urinary clearance of different MDMA doses appeared to be rather constant. On the contrary, non-renal clearance was shown to be dose-dependent (De la Torre *et al.*, 2000). In a controlled clinical trial, the administration 125 mg oral MDMA resulted in 100% reduction of non-renal clearance (38.1±13.3 L h^{-1}) compared to a 75 mg dose (74.0±71.1 L h^{-1}), suggesting impairment in the MDMA hepatic clearance. After controlled administration of different MDMA dose, the urinary recovery is approximately 60%, independently from the dose given.

1.2.3.2 Pharmacodynamics

MDMA can be categorized as a stimulant as a result of its sympathomimetic effects, including peripheral vasoconstriction, brochodilation, and cardiorespiratory stimulation, papillary dilation, and appetite suppression. It can also be categorized as an empathogen, entactogen, and hallucinogen since it can produce these effects to varying degrees depending on dose. The hallucinations are generally mild, and frequently tactile in nature. While the drug is a sympathomimetic, it has significantly less central nervous system (CNS) stimulant properties than MA (Logan and Couper, 2003).

The reported effects of MDMA vary according to the dose and the frequency and duration of use. In general, the effects desired by most users are those produced by low doses on single occasions. It is, therefore, convenient to describe the effects separately for acute (single-occasion) and chronic (long-term) use and, within each category, to describe separately the mental and physical effects. A third category of effect, consisting of the serious or fatal toxicity seen at higher doses or in abnormally sensitive individuals, will be described separately (Kalant, 2001).

1) Acute effects

1.1) Desired effects

The desired effects for which MDMA is used are closely similar to those that account for the continuing popularity of the other amphetamines. Physically, it produces a marked increase in wakefulness, endurance and sense of energy, sexual arousal, and postponement of fatigue and sleepiness. The accompanying psychological effects are described as a sense of euphoria, well-being, sharpened sensory perception, greater sociability, extraversion, heightened sense of closeness to other people, and greater tolerance of their views and feelings (Peroutka *et al.*, 1988; Cohen, 1995).

1.2) Undesired effects

Like the amphetamines, MDMA also has adverse effects on many physical functions, even when taken in moderate doses for the recreational. Because the basic action of the amphetamines involves increased arousal and alertness, it is usually accompanied by an increase in tension, which is manifested as muscular tension, jaw clenching, tooth grinding (bruxism) and constant restless movement of the legs. The increased muscle activity, together with a direct action of the drug on the thermoregulatory system in the brain, leads to an increase in body temperature. Stiffness and pain in the lower-back and limb muscles are very common complaints during the first 2-3 days after the use of MDMA. Headache, nausea, loss of appetite, blurred vision, dry mouth and insomnia are other commonly reported physical symptoms during the drug experience and immediately afterwards. Heart rate and blood pressure, which are usually elevated during the drug experience, tend to fluctuate more widely than normal during the following days (Kalant, 2001).

Undesired psychological acute effects commonly reported during the drug experience similarly represent an exaggeration of the effects for which the drug is taken. The increased arousal, if carried to excess, is converted into hyperactivity, flight of ideas (with a resulting inability to focus one's thoughts in a sustained and useful manner) and insomnia. Related complaints often include mild hallucinations, depersonalization (a feeling of separation of the self from the body), anxiety, agitation, and bizarre or reckless behavior (Mørland, 2000). Occasionally these

symptoms lead to panic attacks, delirium or even brief psychotic episodes that usually resolve rapidly when the drug action wears off. The day or two after drug use, the most common mental or mood complaints are difficulty concentrating, depression, anxiety and fatigue. These symptoms strongly resemble, in miniature, the crash that is typically seen as a withdrawal reaction after the prolonged euphoria or manic state produced by heavy use of amphetamine, cocaine or other central nervous system stimulants. Despite these complaints, the majority of users find the overall balance of the experience positive rather than negative but, with frequent repetition of the experience, the negative effects tend to become more prominent and the beneficial or pleasurable ones less so.

2) Long-term effects

The ability of MDMA to increase the concentration of serotonin in the synapse probably underlies its production of improved mood and of sensory alterations. However, at higher doses the massive release of serotonin not only gives rise to acute psychotic symptoms (as described earlier) but also causes chemical damage to the cells that released it. This damage has been clearly demonstrated in animal experiments with MDMA and related drugs. Chemical and microscopic studies have shown reduced serotonin content of the brain, decreased numbers of identifiable serotonin-containing neurons and serotonin transporter molecules, and numerous degenerating serotonergic axons and axon terminals in the brains of animals treated with MDMA. Although there are conflicting theories concerning the mechanism of this neurotoxicity, it is clearly related to the excessive metabolic activity and neurotransmitter release in the serotonergic and, possibly, the dopaminergic neurons.

In humans, there has been only one postmortem study of changes in the levels of serotonin and its main metabolite in the brain of a single long-term MDMA user (McCann *et al.*, 2000). The levels were reduced by 50-80% in different regions of the brain, in comparison with those in the brains of controls who had not used MDMA, whereas the dopamine levels were unaltered (Kalant, 2001). In addition, psychiatric problems have been suggested that the neurotoxic effects of MDMA on the serotonin system may be the possible cause of a variety of mental and behavioural problems that outlast the actual drug experience by months or years.

3) Physical toxicity

3.1) Hepatic toxicity

A high proportion of the case reports of serious MDMA toxicity include the observation that the patients were jaundiced. Various explanations have been offered for this effect, including the possibility of an allergic drug reaction, a toxic contaminant in the individual batch of drug, or a secondary effect of hyperpyrexia (Andreu *et al.*, 1998; Jones and Simpson, 1999). However, the most probable explanation relates to the pathways of metabolism of the drug. MDMA and related drugs are largely metabolized in the liver by the CYP450 variety designated CYP2D6 (Wu *et al.*, 1997). The immediate product of this reaction is then processed further by other enzymes into a variety of secondary products, some of which are highly reactive with glutathione. A marked decrease in the level of free glutathione permits a series of biochemical changes (massive influx of calcium, oxidative change in the cell-membrane lipids, and so on) that result in cell death (Jones and Simpson, 1999).

3.2) Cardiovascular toxicity

MDMA and related drugs increase the net release not only of serotonin, but also of noradrenaline and dopamine. It is especially the noradrenaline that is responsible for most of the serious adverse effects on the cardiovascular system. These effects consist of two basic types: hypertension, with a consequent risk of ruptured blood vessels and internal hemorrhage, and tachycardia, with a consequently increased cardiac workload, and a resulting risk of heart failure (Kalant, 2001).

3.3) Cerebral toxicity

One of the consequences of the use of ecstasy at raves is profuse sweating as a result of both the vigorous physical activity and the pharmacological action of the drug on the thermoregulatory mechanism. Large amounts of sodium can be lost in sweat, and if the dancers drink large amounts of water in order to avoid overheating, the result is frequently hemodilution and resulting hyponatremia. An additional mechanism that can contribute to the same result is inappropriate secretion of the pituitary antidiuretic hormone, leading to retention of water by the kidneys, but in most cases it is probably caused by excessive water intake following profuse

sweating. This leads to passage of water from the blood into the tissues, including the brain. This has two serious consequences: initiation of epilepsy-like seizures and compression of the brain stem and cerebellum downward toward the foramen magnum, which can lead to fatal disruption of respiration or circulation (Kalant, 2001).

4) Fatalities due to ecstasy

All of the severe forms of toxicity described earlier have been capable of causing death. In addition, there have been deaths due to ecstasy induced depression that was severe enough to cause suicide, or to pre-existing depression in which the drug was used as the means of suicide (Iwersen and Schmoldt, 1996). There have also been several deaths due to accidents resulting from bizarre risk-taking behaviour while under the acute influence of the drug, or to motor vehicle accidents involving either drivers or pedestrians impaired by MDMA (Lora-Tamayo *et al.*, 1997). Conditions commonly contributing to death due to MDMA include dehydration, hyperthermia, disseminated intravascular coagulation, rhabdomyolysis, acute renal failure, tachycardia and other cardiac arrhythmias, and convulsions. In other fatal cases involving MDMA, necrosis of the liver and heart were found at autopsy as were various injuries to the brain such as focal hemorrhages and severe cerebral edema consistent with water intoxication.

1.2.4 Techniques for determination of MDMA and its analogues

There are various analytical methods available for initial screening, confirmation, and quantification of MDMA in forensic specimens (as shown in Table 1.1) such as thin-layer chromatography (TLC) (Kala and Madej, 1997; Zakrzewska *et al.*, 2007), capillary electrophoresis (CE) (Fanga *et al.*, 2002), high performance liquid chromatography (HPLC) (Costa *et al.*, 2004), gas chromatography (GC) (Mitrevski and Zdravkovski, 2005), liquid chromatography coupling mass spectrometry (LC-MS) (Costa *et al.*, 2004; Cheng *et al.*, 2006), and non-chromatographic techniques such as near infrared spectroscopy (NIR) (Schneider and Kovar, 2003), raman spectroscopy (Daeid and Waddell, 2005) and inductively coupled plasma (ICP) (Koper *et al.*, 2007).

Table 1.1 Methods of determination of MDMA and their analogues from 1991-present

Year	Technique	Drugs	Extraction method	Specimens	Derivatization reagents	Chromatographic column	%Recovery	Limit of detection (μg mL^{-1})	Reference
1991	HPLC-UV	MDMA, MA	LLE	plasma	-	-	-	-	Garrett *et al.*
1994	ICP-MS	MA	nitric acid	ecstasy tablets	-	-	-	-	Marumo *et al.*
1996	GC-MS	MDMA, MDEA, MA and A	SPME	urine	-	OV1 (25m×0.25mm i.d.)	-	-	Centini *et al.*
1997	TLC	A, opiates, cannabinoids	-	drug tablets	-	-	-	-	Kala and Madej
1998	CE-TOF/MS	A, MA, cocaine and heroin	dissolving in methanol	drug of abuse	-	fused silica (50mm i.d. and 190 mm o.d.)	-	-	Lazar *et al.*
1999	NIR	MDMA, MDE and A	-	ecstasy tablets	-	-	-	-	Sondermann and Kovar
1999	GC-NPD	MDMA, MDA, HMMA and HMA	SPE	plasma and urine	MBTFA	Ultra-2 (12m×0.20mm i.d.)	64-98	0.016-0.053	Ortuno *et al.*
2000	Raman spectroscopy	MDMA, MDA and MDEA	-	ecstasy tablets	-	-	-	-	Bell *et al.*

Table 1.1 Methods of determination of MDMA and their analogues from 1991-present (cont.)

Year	Technique	Drugs	Extraction method	Specimens	Derivatization reagents	Chromatographic column	%Recovery	Limit of detection (μg mL^{-1})	Reference
2001	ICP-MS	MDMA	nitric acid	ecstasy tablets	-	-	-	-	Comment *et al.*
2001	CZE-DAD	A	-	amphetamine tablets	-	-	-	-	Pietra *et al.*
2001	GC-PDHID	A, MA, MDA and MDMA	SME	urine	-	HP5 (30m×0.32mm i.d.)	-	0.015-0.500	Casari and Aadrews
2002	GC-MS	MDMA, MDEA, and MDA	SPE	hair	HFBA	RTX-5 (30m×0.25mm i.d.)	-	0.005-0.030	Piette *et al.*
2002	CZE-DAD	A	dilution	amphetamine tablets	-	-	-	-	Kala and Madej
2002	NACE/FL	MDMA	LLE	urine	-	-	-	-	Fanga *et al.*
2003	NIR	MDMA, MDE and A	-	ecstasy tablets	-	-	-	-	Sondermann and Kovar
2003	GC-FID	A, MA, MDA, MDMA and MDEA	SPME	urine	-	AT-5 (30m×0.25mm i.d.)	5.1-47.0	0.0034-0.040	Raikos *et al.*
2003	GC-MS	A, MDA and MDMA	SPE	urine	-	DB-35MS (30m×0.25mm i.d.)	73.0-104.6	0.002-0.004	Huang and Zhang *et al.*

Table 1.1 Methods of determination of MDMA and their analogues from 1991-present (cont.)

Year	Technique	Drugs	Extraction method	Specimens	Derivatization reagents	Chromatographic column	%Recovery	Limit of detection (μg mL^{-1})	Reference
2004	ICP-MS	MDMA	nitric acid	ecstasy tablets	-	-	-	-	Waddell *et al.*
2004	HPLC-FL	MDMA, MDEA and MDA	LLE	urine	-	ODS2 C18 (125mm×4mm i.d.)	85.5-105.1	0.015-0.030	Costa *et al.*
2005	HPLC-FL	MDMA, MDEA and MDA	LLE	oral fluid	-	Kromasil C8 (250mm×4.6mm i.d.)	-	0.002-0.010	Concheiro *et al.*
2005	LC-MS	A, MDA and MDMA	SPE	oral fluid	-	Xterra MS (150mm×2.1mm i.d.)	-	0.002-0.005	Wood *et al.*
2005	GC-FID	A, MA, MDA and MDMA	methanol	ecstasy tablets	-	DB-5MS (30m×0.25mm i.d.)	-	0.5-1.0	Mitrevskiet and Zdravkovski
2005	GC-MS	A, MA, MDA, MDMA and MDEA	SPME	urine	HFBA and HFBCl	Supelco SPB-50 (15m×0.32mm i.d.)	95.5-103.2	0.005-0.019	Chia and Huang
2006	LC-MS/MS	A, MA, MDA and MDMA	SPE	urine	-	Rocket EPS C18 (210mm×3.0mm i.d.)	92-100	0.02-0.06	Cheng *et al.*

Table 1.1 Methods of determination of MDMA and their analogues from 1991-present (cont.)

Year	Technique	Drugs	Extraction method	Specimens	Derivatization reagents	Chromatographic column	%Recovery	Limit of detection (μg mL^{-1})	Reference
2006	GC-MS	A, MDA and MDMA	LLE	ecstasy tablets	-	DB-5MS (30m×0.25mm i.d.)	-	-	Cheng *et al.*
2007	TLC	MDMA	-	ecstasy tablets	-	-	-	-	Zakrzewska *et al.*
2007	GC-MS	GHB, K, MA and MDMA	SPME	urine	pyridine and hexylchoroformate	VF-5ht (30m×0.25mm i.d.)	-	0.10	Brown *et al.*
2008	Raman spectroscopy	MDMA	-	human nail	-	-	-	-	Ali *et al.*
2008	Raman spectroscopy	MDMA	-	ecstasy tablets	-	-	-	-	Hargreaves *et al.*
2008	LC-APCI-MS	MDMA	SPE	urine	-	CPS C18 (150mm×4.6mm i.d.)	73.8-79.7	0.016	Pichini *et al.*
2008	LC-MS/MS	A, MA, MDA and MDMA	SPE	urine, blood	MBTFA	DB5-MS (30m×0.32mm i.d.)	86-97	0.05-0.10	Miki *et al.*

1.2.4.1 Near infrared spectroscopy (NIR)

Near infrared spectroscopy (NIR) is the measurement of the wavelength and intensity of the absorption of near-infrared light by a sample. Near-infrared light spans the 800 nm to 2500 nm range and is energetic enough to excite overtones and combinations of molecular vibrations to higher energy levels. NIR is typically used for quantitative measurement of organic functional groups, especially C-H, O-H, N-H, S-H and C=O. Detection limits are typically 0.1% and applications include pharmaceutical, agricultural, polymer and clinical analysis. NIR was employed to analyse and identify the illicit ecstasy samples. The analysis was rapid and simple, with the advantage of being non-destructive (Daeid and Waddell, 2005). Sondermann and Kovar (1999) developed a method using NIR to identify and quantify the commonly used substances of abuse being found in ecstasy tablets. This validated method was proved to be fast and precise without destroying the samples. So after determining the illegal substances, the pieces of evidence could be presented at court. To build a robust calibration, the investigators used self manufactured tablets. As these tablets were made with pharmaceutical knowledge by using chemical pure substances, this calibration can only reflect a part of the variety of the tablets existing on the illegal markets. In a later study, the same investigators developed a method determining the seized ecstasy tablets by NIR in diffuse reflectance and in transmission mode by applying seized ecstasy tablets for model building and validation (Schneider and Kovar, 2003). Quantification models for the mainly used actives have been developed using reference data from a high performance liquid chromatography with diode-array detection (HPLC-DAD) method. The authors evaluated the performance of each NIR measurement method with regard to its ability to predict the content of each tablet with a low root mean square error of prediction (RMSEP). Best calibration models could be generated by using NIR measurement in transmittance mode with wavelength selection and 1/x-transformation of the raw data. The authors can conclude that NIR analysis of ecstasy tablets in transmission mode is more suitable than measurement in diffuse reflectance to obtain quantification models for their active ingredients with regard to low errors of prediction.

1.2.4.2 Raman spectroscopy

Raman scattering is a powerful light scattering technique used to diagnose the internal structure of molecules and crystals. In a light scattering experiment, light of a known frequency and polarization is scattered from a sample. The scattered light is then analyzed for frequency and polarization. Raman scattered light is frequency-shifted with respect to the excitation frequency, but the magnitude of the shift is independent of the excitation frequency. This Raman shift is therefore an intrinsic property of the sample. Because Raman scattered light changes in frequency, the rule of conservation of energy dictates that some energy is deposited in the sample. A definite Raman shift corresponds to excitation energy of the sample (such as the energy of a free vibration of a molecule).

In general, only some excitations of a given sample are Raman active is only some may take part in the Raman scattering process. Hence the frequency spectrum of the Raman scattered light maps out part of the excitation spectrum. Additional information, related to the spatial form of the excitation, derives from the polarization dependence of the Raman scattered light. The shape of an excitation in a material, for example a vibration pattern of the atoms in a molecule, and the polarization dependence of the scattering, are determined by the equilibrium structure of the material through the rules of group theory. By this route one gleans valuable and unambiguous structural information from the Raman polarization dependence (Angela. R, 2002).

Raman spectroscopy is increasingly being used for the identification of drugs of abuse. This technique is a non-destructive, non-contact technique that requires minimal or no sample preparation (Hargreaves *et al.*, 2008). It has previously been applied for the identification of ecstasy, cocaine, barbiturates, and benzodiazepines. It has also been used for the quantitative analysis of drugs of abuse (Ali *et al.*, 2008).

Bell and coworkers (2000) concentrated on the analysis of ecstasy and related compounds using Raman spectroscopy with far-red excitation (783 nm). It was possible to differentiate the spectra of the illicit substances based on differences in the position of vibrational bands, even in the presences of adulterants and diluents. Semi-quantitative analysis was possible base on relative intensity of the band. In addition, the spectra could be used to link seizures to a common batch. In a later study, the

authors applied Raman spectroscopy to profile the composition of illicit ecstasy tablets. A sample population of 400 tablets was selected from seizure. It was concluded that a physical description of seizures and quantification of the illicit substance were not sufficient for definitive characterization due to significant variation observed within each sample population.

In another work, Ali and coworkers (2008) applied the Raman spectroscopy to the detection of drugs of abuse and non controlled substances used in the adulteration of drugs of abuse on human nail. Contamination of the nail may result from handling or abusing these drugs. An added difficulty in the analytical procedure is afforded by the presence of nail varnish coating the nail fragment; the authors demonstrated the discrimination of confocal Raman spectroscopy for the detection of drugs on uncoated nail and also under a coating of nail varnish.

In addition, Raman spectroscopy was demonstrated for the rapid identification of illicit substances in their containers in an airport environment (Hargreaves *et al.*, 2008). Raman spectra of drugs of abuse *in situ* were collected using portable Raman spectrometers; this technique offers distinct advantages to government agencies, first responders and forensic scientists working in the security field. They have demonstrated that the spectrometers are able to collect the spectra of suspect powders, including cocaine hydrochloride and amphetamine sulphate with unknown constituents rapidly and with a high degree of discrimination.

1.2.4.3 Inductively coupled plasma (ICP)

Inductively Coupled Plasma (ICP) is one of the most common techniques for elemental analysis. These techniques are the so-called "wet" sampling methods whereby samples are introduced in liquid form for analysis. In plasma emission spectroscopy (AES), a sample solution is introduced into the core of inductively coupled argon plasma (ICP), which generates temperature of approximately 8000ºC. At this temperature all elements become thermally excited and emit light at their characteristic wavelengths. This light is collected by the spectrometer and passes through a diffraction grating that serves to resolve the light into a spectrum of its constituent wavelengths. Within the spectrometer, this diffracted light is then collected by wavelength and amplified to yield an intensity measurement that can be

converted to an elemental concentration by comparison with calibration standards. This technique has high specificity, multi-element capability and good detection limits. Often, ICP is used in conjunction with other analytical instruments, such as the Atomic Emission Spectroscopy (AES) and the Mass Spectroscopy (MS). This is an advantageous practice, as both the AES and MS require that sample to be in an aerosol or gaseous form prior to injection into the instrument. Thus, using an ICP in conjunction with either of these instruments eliminates any sample preparation time which would be required in the absence of an ICP. The largest advantage of employing an ICP when performing quantitative analysis is multi-elemental analysis can be accomplished, and quite rapidly (Evans, *et al*. 2008).

Several publications describe the application of various techniques for trace element determinations in materials of concern to forensic science. ICP-MS have been used to identify inorganic impurities present in MA samples. Seventeen of the seized samples were classified into five groups based on the concentration of Sodium (Na) and Barium (Ba) present. This technique was shown to be a reliable and sensitive method for the determination of trace elements in illicit substance. Rapid and simultaneous multi-element analysis was possible although the technique is limited by expensive instrumentations and high maintenance costs (Marumo *et al.*, 1994).

Another works presented the method were analysed two different ecstasy seizures by ICP-MS and ICP-AES. The authors reported that heating solutions of the tablets in nitric acid in a microwave oven was a more effective method of sample preparation than vortexing and sonicating the solutions. ICP-MS was shown to be more sensitive than ICP-AES for all the elements studied and was used to generate inorganic impurity profiles for a number of ecstasy seizures. Elements, such as Silicon (Si), Potassium (K) and Iron (Fe), were determined in both the seizures and also in samples, which contained no illicit substance. These elements are abundant in the environment and will prove of little use in profiling illicit seizures and the organic impurity profiles, was essential to confirm the linkage (Comment *et al.*, 2001). In addition, ICP technique was identified the inorganic impurities in each tablet to create a chemical fingerprint. By comparing the fingerprints with each other, similar pills can be grouped together. Until now, the main obstacle to this kind of forensic analysis

has not been measuring a pill's fingerprint, but rather analyzing the data properly (Waddell *et al.*, 2004).

1.2.4.4 Thin-layer chromatography (TLC)

One type of chromatography that is relevant in forensic science is thin layer chromatography (TLC). TLC can be used to separate chemical compounds of differing structure based on the rate at which they move through a support under defined conditions. TLC is useful in detecting chemicals of forensic concern, including chemical weapons, explosives, and illicit drugs. Advances in TLC technique, largely driven by the efforts to quell terrorism, have benefited forensic science; the technique was an inexpensive, reliable, fast, and easy to perform means of distinguishing different compounds from each other (Lee and Wilmoth, 2006).

Fluorescent derivatives as well as certain color reactions have been reported to enable a more detailed differentiation of amphetamine and its derivatives. A characterization by formation of dansyl derivatives appears to be more effective on plates (Kala and Madej, 1997). A great variety of visualizing reagents for detection of amphetamine in TLC was reported. Apart from observation of spots under ultraviolet-visible (UV) light (254 nm), nihydrin and Marquis reagents are most often applied. Also fluorescamine spray, iodoplatinate solution were used. Zakrzewska and coworkers (2007) reported a new visualization and identification of amphetamines and its analogues in TLC, the derivatization reaction with phenyl isothiocyanate took place directly on the TLC plate before the developing step. Afterward, the plate was sprayed with a mixture of sodium azide and starch solution and then exposed to iodine vapors. The obtained limits of detection were compared with other commonly visualization techniques: ultraviolet-visible (UV) light, iodine vapor, Marquis and Simon's reagents, ninhydrin, Fast Black K. The results showed that visualization procedure based on iodine azide reaction belongs among the most sensitive methods of detection of amphetamines after their derivatization.

However, there are a number of disadvantages to TLC, one being a restricted resolving power. The relatively poor resolving power is due, not merely to relatively low plate efficiency, but more often to be due to the restricted physical size of the plate which limits the number of sample spots that can be accommodated in a given

sample. Thus, multi-component samples are not readily separated on a TLC plate due to the restricted plate capacity (this corresponds to the peak capacity obtainable from packed columns). The minimum detectable mass of solute in TLC (*i.e.* the sensitivity) is much larger than that in liquid chromatography and, consequently, the technique is not so suitable for trace analysis unless prior concentration is employed. Finally, the unique selectivity that is obtainable from reversed phases often cannot be comprehensively realized in TLC as the reversed phases are not wetted by solvent mixtures having high water contents and consequently, plate development becomes difficult if not impossible (Scott, 2008).

1.2.4.5 Capillary electrophoresis (CE)

Capillary electrophoresis (CE) is a family of related separation techniques that use narrow-bore fused-silica capillaries to separate a complex array of large and small molecules. High electric field strengths are used to separate molecules based on differences in charge, size and hydrophobicity. Sample introduction is accomplished by immersing the end of the capillary into a sample vial and applying pressure, vacuum or voltage. The separations are carried out in fused silica capillaries, typically 25-75 μm i.d. and 50-100 cm in length, filled with a background electrolyte. Electro-osmotic flow can ensure that both negatively and positively charged species migrate towards the same end of the capillary, where under typical conditions, is towards the cathode end, with neutral species not being separated and migrating with the electro-osmotic flow (Anastos *et al.*, 2005).

The CE technique is well suited for the analysis of illicit drug seizures. This technique can separate a wide variety of solutes, including compounds that are highly polar, thermally labile and nonvolatile, with high efficiency and selectivity. The CE can be classified into several separation techniques include Capillary zone electrophoresis (CZE), Micellar electrokinetic chromatography (MECC), Cyclodextrin-modified Capillary zone electrophoresis (CD-CZE), Capillary electrochromatography (CEC) and Capillary electrophoresis-mass spectrometry (CE-MS) (Lurie, 1998). The most widely used detection method in CE is on-column UV detection, which involves burning off a section of the polyimide coating of the capillary to form an optical window. This type of detector is standard on commercial

instruments, which can also be fitted with a diode array (DAD) detector for the simultaneous acquisition of spectra. However, the short internal diameter of the capillary (detection path length) limits the sensitivity of this detection system, which is 1-2 orders of magnitudes less than that found in HPLC. Other modes of detection include fluorescence and laser-induced fluorescence. On-column fluorescence detection encounters sensitivity limitations, due to the small path lengths provided by the capillaries. Thus, this detection mode is advantageous if selectivity is required, as only few molecules produce significant fluorescence. Capillary electrophoresis-mass spectrometry (CE-MS) provides an orthogonal approach to analysis in a single analytical run. CE-MS combines the advantage of both techniques, so that quantitative and migration time information, in combination with molecular masses and fragmentation patterns can be obtained in one analysis. In addition to the above detection techniques, a variety of other approaches have been taken, including chemiluminescence, conductivity, and a number of electrochemical methods, however, these can also be a disadvantage due to the lack of selectivity. The detection method requires modification to the basic capillary electrophoresis instrument, and is currently still research tools rather than routine detection techniques (Anastos *et al.*, 2005).

Several studies have reported using CE technique for analyze and identify amphetamine and their analogues from biological samples. CE-MS analysis of illicit drugs has the potential to become an indispensable tool for the forensic scientist. Lazar and coworkers (1998) have recently developed an electrospray time-of-flight mass spectrometry (ESI-TOF/MS) for use as a detector for fast and efficient liquid phase separations. The main features of this instrument are speed and sensitivity. This method describes the analysis of various mixtures of illicit drugs using CE-TOF/MS. Seized drug samples were analyzed either by continuous infusion or CE-TOF/MS. This resulted in relatively long migration times. Unfortunately, much shorter capillaries would be necessary to conduct separations in less than about 2 min. For small molecules, ESI produces primarily the protonated molecular ion (in the positive ion mode), and the resultant ESI mass spectra are very simple and easy to interpret. The identification of the main components of an unknown sample can be achieved simply by direct infusion without prior separation of the individual components. On

the other hand, for the investigation of complex mixtures, CE is an ideal technique to use prior to MS, since efficient separations can be achieved in a short time without extensive efforts at optimization.

Pietra and coworkers (2001) developed a CZE method for the identification of illicit amphetamines in 9 min, using an uncoated capillary with a low-pH run buffer. The relative standard deviation (RSD) (n=5), evaluated for the migration times of the analytes, ranged between 0.60 and 0.74%. A capillary electrophoresis method for the chiral separation of amphetamines was also described with the addition of hydroxypropyl-β-cyclodextrin to the previously described running buffer. Enantiomers of MA and 3-amino-1-phenylbutane were not resolved using the described chiral system. In this study, a CE procedure was provided greater peak symmetry and shorter run times.

Piette and Parmentier (2002) used CZE for the determination of amphetamine, MA, MDA, MDMA, *N*-methyl-1-(1,3-benzo-dioxol-5-yl)-2-butamine, and ephedrine, in illicit amphetamine seizures. A 50 μm × 47 cm capillary (40 cm effective length) was used with a running buffer consisting of 0.1 M phosphoric acid adjusted to pH 3.0 with triethanolamine. At this pH, triethanolamine is adsorbed to the wall, resulting in a reversal of the electro-osmotic flow. Separations were performed in less than 8 min, using an applied voltage of 25 kV at 25ºC with diode array (DAD) detection from 190-350 nm. No interferences from the adulterants, caffeine and paracetamol, were observed under these conditions. Excellent migration time RSD values (n=10) of 0.26% and 0.23% were obtained for amphetamine and MDMA, respectively. The MDMA content in one ecstasy tablet was determined using normalized peak areas with phenylephrine as an internal standard. The amount of MDMA present in the tablet was calculated to be 23.5%, yet, a secondary method to validate this result was not performed. A correlation coefficient of 0.9984 for MDMA using this method was obtained, as was an RSD of below 2.0% by means of duplicate preparation of the sample.

Fanga and coworkers (2002) demonstrated the non-aqueous capillary electrophoresis/fluorescence spectrometry (NACE/FS) was a sensitive analytical technique for the identification of MDMA in urine. This method provided results with in 5 min with a detection limit of 0.5 μg mL^{-1} with out sample pre-treatment. A

liquid-liquid extraction method was also developed with improved detection limits of 50 ng mL^{-1}. A 75 µm × 35 cm capillary (30 cm effective length) was used with a buffer composed of 100 mM sodium cholate and 20 mM ammonium acetate in a formamide-methanol solution (30:70, v/v). The separations were performed with an applied voltage of 15 kV at 77 K, with an excitation and emission wavelength of 285 and 320 nm, respectively. Poor intra- and inter-day peak area RSD values (n=6) of 7.47% and 9.88% for MDA were obtained, respectively. However, fair intra- and inter-day migration time RSD values of 1.48% and 3.86% were attained, respectively.

However, the small sample volume is also a major drawback of the CE system: optimal separation efficiency can be achieved only if the loading volume is below 2% of the total capillary volume, which means injection volumes of 1-100 nL. And this inevitably means poor sensitivity, especially when dilute solutions or low concentrations of metabolites in biological matrices are to be analysed (Keski-Hynnila *et al.*, 2000).

1.2.4.6 High performance liquid chromatography (HPLC) and liquid chromatography coupling mass spectrometry (LC-MS)

High performance liquid chromatography (HPLC) is the analytical chromatographic technique that is useful for separating ions or molecules that are dissolved in a solvent. If the sample solution is in contact with a second solid or liquid phase, the different solutes will interact with the other phase to differing degrees due to differences in adsorpting, ion-exchanging, partitioning, or size. These differences allow the mixture components to be separated from each other resulting to different transit times of the solutes. These instruments use a pump to force the mobile phase through and provide higher resolution and faster analysis time (Brian, 1996).

HPLC is widely used for MDMA analysis, and offers the advantage over GC in that MDMA and its polar metabolites can be quantified simultaneously without derivatization (Garrett *et al.*, 1991). The separation of MDMA and other amphetamine analogues are usually performed with silica-based reversed phase column, mainly C18 (Costa *et al.*, 2004; Cheng *et al.*, 2006) or C8 (Concheiro *et al.*, 2005; Schonberg *et al.*, 2006). Ability of HPLC and LC techniques to detect compounds depends on the type of detector used. The choice of the detection system is very important for

selectivity and sensitivity. Detection approaches include ultraviolet-visible (UV) detector, diode array (DAD) detector, fluorescence (FL) detector (e.g. excitation 285 nm and emission 320 nm), electrochemical (EC) detector and mass spectrometry (MS) detector (Nakashima, 2005).

For HPLC-UV and HPLC-FL techniques, a derivatization procedure is generally required to increase sensitivity which phenylisothiocyanate (PIT) derivatives and naphthaquinone-4-sulfonate (NQS) have been used to prepare UV absorbing species for HPLC (Logan and Couper, 2003). Costa and Coworkers (2004) described the development and validation of analytical methodology for the determination of the MDMA, MDEA and MDA in human urines by HPLC-FL detection. The chromatographic separation was achieved on a Spherisorb ODS2 C18 column (125 mm × 4 mm, 5 μm). The mobile phase, pumped at 1.0 mL min^{-1}, was composed of 50 mM solution of sodium dodecyl sulfate in HPLC-grade water, adjusted pH to 4.0 with 1 M HCl (60%) and acetonitrile (40%), in isocratic mode. The results showed high selectivity and enable quantification at concentration as low as 10 ng mL^{-1} instead of 130-200 ng mL^{-1} with the HPLC-UV absorbance detection. In another work, Concheiro and coworkers (2005) developed a simultaneous determination of MDMA, MDA, MDEA and *N*-methyl-1-(3,4-methylene dioxyphenyl)-2-butamine (MBDB) in oral fluid using HPLC-FL detection. The detector wavelength was fixed at 285 nm for excitation and 320 nm for emission. The mobile phase, a mixture of phosphate buffer (pH=5) and acetonitrile (75:25), and the column, Kromasil 100 C8 (5 μm 250 mm × 4.6 mm), allowed good separation of the compounds in an isocratic mode in only 10 min. The authors concluded that this method was a rapid, simple and specific method for the determination of MDMA, MDA, MDEA and MBDB in oral fluid and no interfering substances were detected. However, this method suffers from some drawbacks and limitations such as time-consuming cleanup procedures and co-extracted substances having native fluorescence interfering with the analysis.

Liquid chromatography coupling mass spectrometry (LC-MS) is a more versatile method for sensitive determination of many types of drugs including amphetamines and has generally been used in the forensic and toxicological fields. Atmospheric pressure sources such as electrospray ionization (ESI), present several

advantages as the sample are ionized directly in the liquid phase at a *quasi*-ambient temperature, is commercially available and have been employed for developing methods for determining MDMA and other amphetamine analogues (Pichini *et al.*, 2008). However, LC-MS is less sensitive than GC-MS as a result of the need to transfer the analytes from the liquid phase into a high-vacuum gas phase. Other limitation of LC-MS combination includes the inability to use nonvolatile buffers, the narrow optimum range for eluent flow rate influence of the proportion of organic modifier on the sensitivity, the narrow choice of ionization methods. LC-MS/MS have been developed for the detection of a single class or multiple classes of the analysis of illicit drugs simultaneously, since the small sample volume of forensic specimens. The high specificity and the increased signal-to-noise in combination with short chromatographic run times and a potential to reduce sample preparation because there is no need for derivatization, make LC-MS/MS the technique of choice for high-throughput confirmation of multiple illicit drugs in small sample such as oral fluid, saliva and hair samples (Wood *et al.*, 2005; Cheng *et al.*, 2006).

1.2.4.7 Gas chromatography (GC)

Gas chromatography (GC) is a chromatographic technique that can be used to separate volatile organic compounds. A gas chromatograph consists of a flowing mobile phase, an injection port, a separation column containing the stationary phase, and a detector. The organic compounds are separated due to differences in their partitioning behavior between the mobile phase and the stationary phase in the column. The GC technique is most frequently used to identify MDMA and related compounds in different matrices such as tablets, blood, urine, hair and saliva. MDMA and other amphetamine analogues determinations have been performed using GC coupled with different detection systems such as nitrogen-phosphorus detector (NPD) (Ortuno *et al.*, 1999), pulsed discharge helium ionization detector (PDHID) (Casari and Aadrews, 2001), flame ionization detector (FID) (Raikos *et al.*, 2003; Mitrevski and Zdravkovski, 2005) and mass spectrometry (MS) (Huang and Zhang, 2003; Cheng *et al.*, 2006).

In forensic laboratories, identification of amphetamine and its analogues, especially in forensic case, is performed by GC-FID as the most rational and universal

separation and identification technique (Lambrechts *et al.*, 1984; Centini *et al.*, 1996). Although it lacks superior sensitivity and specificity of GC-MS, the GC-FID technique has the advantage of simplicity, reproducibility and a wide range of linearity (Mitrevski and Zdravkovski, 2005). Amphetamine and its analogues are polar compounds, thus their direct determination is often followed by absorption in the injector system and column, or their degradation. These effects have resulted in peak tailing in the chromatograms, or even unsuccessful baseline separation. To avoid these undesirable effects the polar amino groups are being derivatized. The most common derivatization method is acylation, in which heptafluorobutyric anhydride (HFBA) (Skender *et al.*, 2002), chlorodifluoroacetic anhydride (CDFAA), trifluoroacetic anhydride (TFAA), or trifluoroacetamide (TFA) are commonly added to the evaporated analytes and incubated at 60-70ºC for 20-30 min. *N*-methyl-bis (trifluoroacetamide) (MBTFA) and acetic anhydride have also been used, but these are moisture sensitive, and leave an acidic residue that can cause chromatographic column deterioration over time (Logan and Couper, 2003). The derivatization prior to GC analysis is necessary for more stable, less polar and leads to their improved chromatographic behavior, such as better separation, more symmetrical peaks, greater sensitivity and determination of enantiomer composition. However, derivatization has several inherent disadvantages over direct analysis: it involves one more preparation; it requires more time; it carries the risk of introducing impurities and in some cases strong acid by-products are obtained which strongly affect the characteristics of the column (Mitrevski and Zdravkovski, 2005).

Recently, the mass spectrometry (MS) detector is favoring the detection system of choice for GC by virtue of its high selectivity. The GC coupled to MS is the most powerful technique for identification and confirmation of MDMA and other amphetamine analogues. The majority of published analytical papers use GC-MS with electron impact ionization followed by selected ion monitoring, using appropriate ions depending on the derivatizing reagent. The mass spectrum of MDMA is not highly characteristic, having a base peak of *m/z* 58; however, it has a recognizable spectrum, and is often identified underivatized. A more characteristic spectrum can be obtained following derivatization, which also increases sensitivity for polar drugs and metabolites.

Several studied have reported using GC-MS for determination MDMA and their analogues from human urine. Huang and Zhang (2003) have used the GC-MS for confirmation and quantification of amphetamine, MDA, MDMA in human urine samples after immunoassay screening. This method was found to be effective, reliable for identification of amphetamines and no derivatization is necessary.

In another work, Chia and Huang (2005) described a procedure for the determination of amphetamine-like drugs, amphetamine (A), MA, MDA, MDMA and MDEA in human urine samples were using SPME with on-fiber derivatization device. The analytes were derivatized with heptafluorobutyric anhydride (HFBA) and heptafluorobutyric chloride (HFBCl) and determined by GC-MS. The detection limits were obtained of 0.053, 0.016, 0.193 and 0.028 ng mL^{-1} for A, MA, MDA and MDMA respectively. This method proved to be a fast and economic method, with good sensitivity and reproducibility.

Brown and coworkers (2007) developed and validated for measuring four club drugs in human urine. These drugs include gamma-hydroxybutyrate (GHB), ketamine (K), MA and MDMA. The drugs were derivative using pyridine and hexylchloroformate to make them suitable for GC-MS analysis. This method has the advantage of an easy sample preparation with acceptable accuracy and precision for the simultaneous quantification of these four drugs of abuse and shows no interference from the urine matrix.

Although, the GC-MS technique is the most powerful for identification and confirmation of MDMA and other amphetamine analogues in biological samples, but there are laboratories around the world, especially in developing countries, which cannot afford such as an expensive instrument and which has forced researchers to use other methods (Mitrevski and Zdravkovski, 2005).

1.2.5 Sample preparation for determination of MDMA and its analogues

Sample preparation and clean-up procedures are important in most clinical, forensic and toxicological laboratories, both for assessing the reality and cause of an intoxication and for evaluating the level of impairment caused by drugs (Kintz and Samyn, 1999). The major goal of sample preparation is to isolate and concentrate an analyte from matrix components. The extent of isolation, purification, and concentration of the analyte is determined by the complexity and composition of the matrix itself, the concentration of the analyte in the matrix, the selectivity and sensitivity required for the subsequent analysis (Diana, 1997). The various different methods have commonly been used to isolate MDMA and other amphetamine analogues in ecstasy tablets and biological samples, including solvent microextraction (SME) (Casari and Aadrews, 2001), liquid-liquid extraction (LLE) (Luaa *et al.*, 2003; Makino *et al.*, 2003), solid phase microextraction (SPME) (Chia and Huang, 2005), protein precipitation/dilution (Mueller *et al.*, 2007) and solid phase extraction (SPE) (Alabdalla, 2005; Pichini *et al.*, 2008).

1.2.5.1 Liquid-liquid extraction (LLE)

Traditional liquid-liquid extraction (LLE) is one of the most useful techniques that are being used for selective extraction of MDMA and other amphetamine analogues from aqueous solutions and it is largely applied in the purification processes in numerous ecstasy tablets and biological samples (Chung, 2004).

LLE is based on the partition of organic compounds between the aqueous sample and an immiscible organic solvent. LLE can effectively remove protein components from biological samples. Extraction of MDMA and other amphetamine analogues in ecstasy tablets and biological matrices by LLE has been reported by several approaches which wide variety of solvents have been used, including *tert.*-butyl methyl ether (Pellegrini *et al.*, 2002), chloroform (Makino *et al.*, 2003), ethyl acetate (Luaa *et al.*, 2003), *n*-hexane/ethyl acetate (70:30, v/v) (Costa *et al.*, 2004), diethyl ether (Cheng *et al.*, 2006) and dichloromethane (Teng *et al.*, 2006). The more polar solvents are more effective for recovering the polar hydrolyzed metabolites. Extraction is performed under basic conditions using such as borate, sodium hydroxide, and sodium hydroxide/ammonium sulfate buffers (Logan and Couper,

2003). Several studies have reported using LLE for isolating MDMA and their analogues from biological samples and ecstasy tablets. In a previous study, Pellegrini and coworkers (2002) used the *tert*.-butyl methyl ether as organic solvent to extracting of amphetamine and related compounds from human urine samples where recovery of 62-85% was obtained from twenty volunteers that were taken drug abuse. In addition (Luaa *et al.*, 2003), urine samples were adjusted to pH 9.5 with bicarbonate buffer and extracted with ethyl acetate for isolating MDMA and its metabolites form human urine samples. The organic layer was evaporated to dryness under a stream of nitrogen gas. The dried extracts were derivatized with trichloroacetic anhydride and analyzed with gas chromatography-mass spectrometry (GC-MS). In another work, *n*-hexane/ethyl acetate (70:30, v/v) was used as organic solvent to extract MDMA, MDA and MDEA from human urine samples. The percentage recoveries were obtained in the range of 85.5-105.1% (Costa *et al.*, 2004). Sometime ecstasy tablet samples were dissolved directly in water, chloroform and methanol (Mitrevski and Zdravkovski, 2005). These methods are simple and suitable for most laboratories with a heavy workload.

The major disadvantages of the LLE methods are the need of a large volumes of organic solvents, some of these solvent are hazardous and toxic. The operators are laborious and automation is difficult. Emulsion formation and waste problems are also associated with these methods, as well as difficulties in handling small sample volumes (Soriano *et al.*, 2001). LLE methods require expensive glassware and distillation or evaporation apparatus. It also has limited selectivity, particularly for trace level analysis; there is a need for clean-up or analyte enrichment/concentration steps prior to instrumental analysis (Krystyna, 2007).

1.2.5.2 Solvent microextraction (SME)

Solvent microextraction (SME) is a LLE type in which the analytes distributed between the bulk aqueous phase and a microdrop of organic solvent, suspended directly at the tip of a microsyringe needle that is immersed in a stirred aqueous sample solution (Ebrahimzadeh *et al.*, 2007). After a certain time, when sufficient amounts of analytes are transferred into the organic extractor, the microdrop is retracted into the microsyringe, and subsequently part or all of the organic solvent is

injected into the chromatographic system. An important additional feature of SME is the integration of extraction and injection in a microsyringe, making it possible to employ this miniaturized medium for extraction as well as an injection device for the GC (Bagheri *et al.*, 2004). SME is very inexpensive, because it requires only simple laboratory equipments and 1-3 µl of solvent.

Since 1995, a solvent-minimized sample pretreatment procedure, known as SME, has been developed. Jeannot and Cantwell (1996) first proposed a method of microextraction using a one microliter organic drop suspended on the tip of a microsyringe. He and Lee (1997) further developed this technique by introducing the concepts of static and dynamic microextraction. Recently a new microextraction device was presented: a small piece of a porous hollow fiber, containing and organic solvent, immersed in a sample vial. SME can be applied to many different fields. Jager and Adrews (1999) developed a method to extract and detect organochlorine pesticides in river water.

Several studies have reported using SME for isolating MDMA and their analogues from human urine samples. Casari and Andrews (2001) developed a method to extract and detect some commonly abused illicit drugs, amphetamine, MA, MDA, MDMA, MDEA and phencyclidine (PCP) in urine. In these work, the authors have proposed a new method of detecting drugs in urine using SME. The analytes are extracted by chloroform as the extraction solvent and detected in urine at concentrations lower than the Substrance Abuse and Mental Health Services Administration (SAMHSA) guideline for drugs cut-off levels. SME is fast, inexpensive and could be easily automated, thus, improving detection limits and precision.

In another work, Jager and Andrews (2001) developed a screening method for determination cocaine and its metabolites. Drug extraction was achieved using the relatively new technique of SME. Chloroform was used as organic solvent to extract cocaine and its metabolites from urine samples. The authors have described that SME was capable of extensive sample clean up and provided sufficient preconcentration of the drugs of interest required for a screening method. The advantages of the SME methods are fast, simple, requiring a small amount of solvent and producing little

waste. This makes SME as an attractive alternative to conventional screening method (Jager and Andrews, 2001a).

Moreover, SME method has several drawbacks. As result of drop in stability at high stir rates, stir rate speeds are limited. Samples with particulate matter must also undergo some form of filtration in order to prevent the particles from colliding with the drop and dislodging it (Jager and Andrews, 2001a).

1.2.5.3 Protein precipitation

Protein samples commonly contain substances that interfere with downstream applications. Several methods exist for eliminating these substances from samples. Small soluble substances may be removed and the samples exchanged into appropriate buffers by dialysis or gel filtration. Another method for removing undesirable substances is to add a compound that causes protein to precipitate. Once proteins have been precipitate, separation of aqueous and solid protein must occur by filtering or centrifugation. The proposal of protein precipitation is to obtain a cleaner preparation. Some drugs are occluded in the precipitate, but can be at least partially recovered by washing the precipitated with hot water or hot dilute hydrochloric acid (HCl). Protein precipitation reagents include acetone, acetonitrile, methanol, 5-sulfosalicylic acid, perchloric acid and ammonium sulfate (Levine, 2003).

Mueller and coworkers (2007) validated the liquid chromatographic-electrospray ionization mass spectrometric (LC-ESI-MS) for determination of MDMA and its metabolites in squirrel monkey plasma. A simple sample preparation involving protein precipitation with perchloric acid was performed to measure the analytes in a small sample (100 µL) of plasma. After centrifugation (16000 × g, 5 min), the supernatants were transferred to autosampler vials and 5 µL were injected for LC-ESI-MS system. MDMA and its metabolites MDA, HHMA, and HMMA were extracted form squirrel monkey plasma.

The protein precipitation of whole blood or dilution of urine, e.g. with acetonitrile or methanol, are rapid and simple procedures, but large amounts of matrix material are co-extracted and may interfere with the analysis. Additionally, the analytes may stick into the surface of particles during precipitation, which lowers the recovery (Gergov, 2004).

1.2.5.4 Solid phase microextraction (SPME)

During the past few years, solid-phase microextraction (SPME) has emerged a valuable alternative for sample preparation in the analysis of a wide variety of compounds. SPME typically uses a polymeric-coated fused-silica fiber to extract the analytes from the samples. The extracted compounds are then desorbed and transferred to an analytical instrument for separation and detection. Desorption is normally attained by placing the fiber into the hot injector of a gas chromatograph or into the interface of a liquid chromatograph (Cháfer-Pericás *et al.*, 2004). This technique is based on an equilibrium forming between the fiber and sample which there are three types of extraction that can be utilised with an SPME fiber; direct extraction SPME (DI-SPME), headspace SPME (HS-SPME) and membrane protected SPME. SPME method has been extensively applied in a broad field of analysis including food, biological and pharmaceutical and forensic samples.

Centini and coworkers (1996) developed methods for isolating amphetamine and their analogues from urine sample by HS-SPME with polydimethylsiloxane (PDMS) fiber. The analytical method was employed gas chromatography-mass spectrometry (GC-MS). Raikos and coworkers (2003) studied a simple procedure for the analysis of amphetamine, MA, MDA, MDMA and MDEA in human urine using HS-SPME and analysed by gas chromatography equipped with flame ionization detector (GC-FID). The percentage recovery for amphetamine and their analogues ranged from 19.5 to 47.0%. The use of SPME as a sample preparation technique was once thought to be limited to volatile analytes. It is necessary to derivatize the amphetamine and related compounds to increase chromatographic efficiency. For the analysis of amphetamine and related compounds, several different derivatizing agents have been used, including alkyl chloroformates, heptafluoro-*n*-butyryl chloride, pentafluorobenzoyl chloride, propionic anhydride, hetafluorobutyric anhydride and *N*-methyl-bis (trifluoroacetylamide) (Brown *et al.*, 2007). Different derivatization techniques in SPME have been developed, and reviews on the development and application of derivatization with SPME have been published. Derivatization of the analytes in the solution followed by the SPME of the derivatives formed is a good option for the analysis of amphetamine, MA and MDMA both in aqueous sample and in urine. This method has been applied to measure amphetamine, MA and MDMA in

urine by liquid chromatography (LC), using the fluorogenic reagent 9-fluorenylmethyl chloroformate (FMOC) and carbowax-templated resin (CW-TR)-coated fibers as derivatizing agents. The mean recoveries of the analytes in the urine samples, the values were obtained of 106% for MA, 93% for MDMA and 84% for MA. This approach combines the simplicity of SPME for sampling and analyte isolation, and the advantages of chemical derivatization. This approach can be considered a simple and rapid altenative for determinating of amphetamine and their analogues at low concentration levels, which are the concentrations typically found in real samples, for example, in urine of drug abusers (Cháfer-Pericás *et al.*, 2004).

In another work, Chia and Huang (2005) developed the simultaneous derivatization and extraction of amphetamine-like drugs in urine with HS-SPME followed by GC-MS. In other study, amphetamine-like drugs were derivatized with heptafluorobutyric and hetafluorobutyric chloride. The influence of several parameters on the efficiency of microextraction (type of fiber coating, extraction time, extraction temperature, etc.) was investigated. The SPME method developed provides good sensitivity and high relative recovery between 95.5-103.2%. The authors improved design may also be used in the derivatization step of other systems using HS-SPME method of choice for the analysis of illicit formulation containing amphetamine and their analogues.

In addition, SPME fiber has many drawbacks, such as fragility of the fiber, nonresistance to high temperature and organic solvents. Its fibers are expensive and short life usability (Jager and Andrews, 2001b). Moreover, the partial loss of SPME fiber stationary phase can result in peaks that may co-elute with the analytes (Ebrahimzadeh *et al.*, 2007).

1.2.5.5 Solid phase extraction (SPE)

SPE is a widely used sample preparation technique for the isolation of selected analytes which based on the partition equilibrium between sorbent and eluting solvent. The analytes are transferred to the solid phase where they are retained. The principle goals of SPE are to preconcentrate, clean-up sample and transfer from the sample matrix to a different solvent. The SPE designs frequently used are in the form of a cartridge, syringe or disk (Simpson, 2000). Initially, silica (SiOH) was used as a

sorbent but the development of HPLC column coatings in the 1970's revolutionized the solid phase extraction sorbents. Bonded silica created from the reaction of organosilanes with activated silica yield a product with the functional group of the organosilane attached to a silica backbone by a silyl ether linkage. Silica having different properties is produced by altering the length and structure of the organosilane. Sorbents can be classified into four categories depending on their primary retention mechanism; reversed phase, normal phase, ion exchange, and adsorption (Simpson, 2000). Reversed phases, such as C18, C8 and C2 interact mainly via Van der Waals forces, thereby extracting relatively non-polar compounds. Normal phases, such as silica, alumina and florisil also extracting relatively polar called polar phases, can interact by dipole-dipole interactions compounds. Ion-exchange phases bind compounds through ionic interactions. These sorbents can be classified as either anionic or cationic; anionic sorbents retain negatively charged compounds and cationic resins retain positively charged compounds. Examples of ion exchange sorbents include strong cation exchange (SCX) and weak anion exchange (WAX). Adsorption phases are not considered bonded phases as they do not have hydrocarbon chains attached to the backbone. These sorbents interact mainly via polar interactions and can therefore extract relatively polar analytes (Yawney, 2000).

However, SPE technique has four main limitations. First, the sorbent must remain wet prior to sample loading. If the sorbent runs dry anytime before the sample loading step in cartridges or wells in 96-well extraction plates, the consequence are low and variable recoveries. Second, for polar drugs and metabolites, retention is weak and often resulting in breakthrough during the loading step, yielding unpredictably low recoveries and poor reproducibility. Third, basic analytes interact strongly with residual silanols, which, in turn, lead to low recovery with simple elution solvents. Fourth, the SPE method development is time consuming and tedious. Often, it takes a long time to develop a method, and this process can take up to two-thirds of the entire analysis (Cheng *et al.*, 1998).

Recently, hydrophilic-lipophilic balanced (HLB) sorbent and mixed-mode cation exchanges (MCX) were developed by Waters Corporation. Both of these columns are made of poly (divinylbenzene-co-N-vinylpyrrolidone), a reversed phase sorbent. HLB refers to the hydrophilic-lipophilic balance which describes that the

sorbent binds both polar and nonpolar compounds through the use of Van der Waals forces and dipole-dipole interactions. The MCX columns contain a mixed-mode cation exchange sorbent based on the polymer backbone with the addition of sulfonic acid groups to enable it to retain cations. It is considered a mixed-mode sorbent because it retains compounds by two different mechanisms; hydrophobic and ionic interactions. These sorbents are promoted for their unique ability to stay wet with water despite drying, retain a wide spectrum of both polar and non-polar compounds and remain stable from pH 1-14. Better recovery and increased reproducibility with the Oasis columns compared to silica based columns have also been reported (Yawney, 2000).

SPE method is becoming increasingly popular to isolate MDMA and its metabolites from simple biological matrices such as urine, plasma, serum, hair and saliva (Kintz and Samyn, 1999). Many reports evaluated the different types of SPE procedure and sorbent for isolation of MDMA and its metabolites were investigated. Cheng and coworkers (1998) have demonstrated the SPE clean-up of a wide range of compounds using a hydrophilic-lipophilic balanced (HLB) sorbent for extraction of verapamil and its metabolites in plasma. This method showed that the HLB sorbent is unique and universal for reversed-phase SPE application. This copolymer has no residual to complicate its interaction with basic analytes, such as verapamil and its metabolite. It is stable across a wide pH range (pH 0-14) advantageously in SPE method development to provide cleaner extracts for these basic compounds.

In another work, Ortuno and coworkers (1999) developed method for extraction of MDMA from plasma or hydrolyzed urine. The sample is first mixed with a pH 6.0 buffers. The biological samples are then added to Bond Elut Certify® cartridges preconditioned with methanol and pH 6.0 buffers. The cartridges are then rinsed with acetic acid and methanol. For plasma, MDMA and its polar metabolites are eluted using chloroform containing 2% ammonium hydroxide, while for urine, MDMA and its polar metabolites are eluted with ethyl acetate containing 2% ammonium hydroxide. In general, the more polar solvent, ethyl acetate, extracts the polar metabolites more efficiently than chloroform. SPE combined with gas chromatography-nitrogen-phosphorus detector (GC-NPD) of underivatized MDMA and MDA provided a very fast and sensitive method. The recoveries obtained from

plasma were ranged of 85.1-98.3% and from urine the recoveries were ranged of 64-90%. Therefore, Yawney (2000) developed a general screening method for acidic, neutral, and basic drugs from whole blood using the Oasis HLB cartridges and the Oasis MCX cartridges. He has proposed that the Oasis HLB cartridges were not suitable for the differential elution of acidic, neutral and basic drugs. Basic drugs eluted into the acidic elution fraction. The procedure may work for the extraction of basic/neutral drugs but more work is needed to validate the methodology. The Oasis MCX procedure was capable of differential elution of acidic, neutral drugs. Many drugs were extractable from whole blood and detectable at therapeutic concentrations. Good recoveries and clean extracts were achieved.

Wood and coworkers (2005) developed method for the simultaneous analysis of basic drugs which comprises a sample clean-up step, using mixed-mode SPE, and follow by LC-MS/MS analysis. After conditioning with 1 mL of methanol and 1 mL of 0.1 N hydrochloric acid (HCl), the dilute oral fluid samples were applied onto SPE cartridges. Clean-up procedure was accomplished with successive 1 mL wash of 0.1 N HCl, tetrahydrofuran and a mixture of methanol and water (50:50, v/v). The cartridges were eluted with 0.5 mL of 5% ammonia in methanol. The percentage recoveries obtained using this method were valued of 90.7% for amphetamine, 83.7% for MA, 82.2% for MDA and 76.6% for MDMA. After that, Cheng and coworkers (2006) reported using of C18 column for extraction of amphetamine and their analogues from urine. This method provides the percentage recovery of drugs of abuse in urine were ranged of 87.2-115.7% for different analytes.

In addition, Pichini and coworkers (2008) have developed a method for identification and quantification of the most common misused hallucinogenic designer drugs in urine of consumers enrolled in the survey. Sample preparation involved a solid phase extraction procedure at pH 6 of both non-hydrolyzed and enzymatically hydrolyzed urine samples. Urine samples added with 1 mL of 0.1 M phosphate buffer pH 6.0 underwent solid phase extraction procedure using Bond Elut Certify® cartridges. The cartridges were preconditioned with 2 mL methanol and 2 mL of 0.1 M phosphate buffer pH 6.0, washed with 1 mL of acetic acid and 4 mL of methanol. All analytes were eluted with 2 mL of ethyl acetate containing 2%

ammonium hydroxide. The recoveries of this method ranged between 55.4 to 95.6% for the different analytes.

Among the several advantages that SPE method offers over LLE are higher selectivity, more reproducibility, clean-up and concentration of analytes from various matrices and the avoidance of emulsion formation (Soriano *et al.*, 2001). The SPE methods are enable sample preparation in batches and automation of the extraction procedure, reduce analysis time, decrease solvent usage and disposal (Diana, 1997). This method improves and simplifies separations, increases chromatography column lifetime, and improves detection limits.

1.3 Objectives

The objectives of this work are to develop a simple GC-FID technique for the detection and determination of MDMA, MDA and MA in ecstasy tablets and urine samples. It is also aimed to determine the purity of MDMA in ecstasy tablets seized in Songkhla province and to perform the confirmation of MDMA, MDA and MA in urine samples of suspects. The methods of sample preparation for analysis of MDMA, MDA and MA in ecstasy tablets and human urine samples were studied.

CHAPTER 2

Methodology

2.1 Chemicals and materials

3,4-Methylenedioxymethamphetamine hydrochloride (MDMA), 3,4-methylenedioxyamphetamine hydrochloride (MDA) and methamphetamine hydrochloride (MA) (purity 99%) were supplied from the Regional Forensic Science Division 4, Office of the Police Forensic Science, Thailand and used as reference standards. Diphenylamine (DPA), an internal standard, was also provided by the Regional Forensic Science Division 4. Methanol HPLC grade (J.T. Baker) was obtained from Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA). Acetic acid (CH_3COOH), ammonium hydroxide (NH_4OH), formic acid (HCOOH) and ethyl acetate AR grade (J.T. Baker) were purchased from Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA). Hydrochloric acid (HCl, AR grade) was purchased from LAB-SCAN, Ireland. Potassium hydroxide (KOH, AR grade) was purchased from BDH Laboratory supplies, England. Sodium hydroxide (NaOH) was purchased from Merck, Darmstadt, Germany. Nitrogen, oxygen and hydrogen gases were 99.99% pure and supplied by TIG, Thailand. Oasis® HLB cartridge (1 mL/30 mg) was purchased from Waters Corporation (Waters, Massachusetts, Ireland).

2.2 Apparatus

A gas chromatograph system consisted of a gas chromatograph model GC 5890 A series II equipped with a flame ionization detector (Hewlett Packard, Minnesota, USA), a capillary column CP-Sil 24 CB; WCOT fused silica, 30 m × 0.32 mm i.d.; 0.25 μm film thickness of 50% phenyl/50% dimethylpolysiloxane (Varian, California, USA), a computer system model VL Vectra (Hewlett Packard, Minnesota, USA) and chemstation software (Agilent, California, USA).

Other instruments included a centrivap concentrator (LABCONCO, Bangkok, Thailand), ultrasonic bath model JAC-2010 (Kodo, Hwaseong, South Korea), refrigerated centrifuge (Sorvall RC-3B plus, California, USA), vortex Genie-2

(Sciencetific Industries, New York, USA) and automatic pipettes 100, 1000 and 5000 µL (Eppendorf, Hamburg, Germany).

2.3 Chromatographic condition

A gas chromatograph equipped with a flame ionization detector and a CP-Sil 24 CB WCOT fused silica capillary column (30 m × 0.32 mm i.d.; 0.25 µm film thickness of 50% phenyl/50% dimethylpolysiloxane stationary phase) were used. One microliter of sample was injected manually using a Hamilton micro-syringe in a splitless injection mode. Nitrogen gas was used as a carrier at a flow rate of 2.6 mL min^{-1}. The fuel gas (H_2) and make-up gas (N_2) flow rates were both 30 mL min^{-1}. The air zero or oxidant gas (O_2) flow rate was 300 mL min^{-1}. The temperatures of the injector and detector were 290ºC and 300ºC, respectively. The oven temperature was initially set at 80ºC (0 min) and then increased with a ramp rate of 20ºC min^{-1} to a final temperature of 270ºC where it was held for 1 min.

2.4 Preparation of standard solutions

2.4.1 Stock solutions

Stock solutions of 1000 µg mL^{-1} of MDMA, MDA, MA and DPA were prepared by separately dissolving a standard compound (1000 µg) with pure methanol (1 mL) in a 2 mL-microcentrifuge tube and stored at -20ºC.

2.4.2 Working standard solutions

MDMA, MDA and MA working standard solutions were prepared as a mixture by diluting a stock standard solution of each compound with pure methanol to a concentration of 500 µg mL^{-1}. This concentration was used for preparing calibration standard solution for the analysis of drugs in tablets and urine samples. For optimization of GC-FID condition, a mixture of MDMA, MDA and MA working standard solution with concentrations of 200, 100, 25 and 5 µg mL^{-1} were also prepared and a concentration of 5 µg mL^{-1} was used throughout the optimization process. The stock solution of the internal standard was also diluted with pure methanol to concentrations of 500 and 50 µg mL^{-1}.

2.4.3 Calibration standard solutions

For analysis of ecstasy tablets, the calibration standard solutions were prepared by adding an appropriate volume of a working standard mixture of MDMA, MDA and MA and a 20 µL-aliquot of 50 µg mL^{-1} DPA (final concentration of 10 µg mL^{-1}). Sample final volumes were made up to 100 µL with pure methanol to obtain the final concentrations of 3.125, 6.25, 25, 100 and 200 µg mL^{-1}.

For urine analysis, the calibration standard solutions were prepared by adding an appropriate volume of a working standard mixture of MDMA, MDA and MA solutions and a 100 µL-aliquot of 50 µg mL^{-1} DPA (final concentration of 5 µg mL^{-1}) to human urine blank (final volume 1 mL) so that final concentrations were 0.5, 1, 5, 10 and 20 µg mL^{-1}.

2.5 Experiments

2.5.1 Study 1: Optimization of the GC-FID conditions

The optimal condition of GC-FID system for analyzing of MDMA, MDA and MA were investigated. The parameters included flow rates of carrier gas (nitrogen gas), fuel gas (hydrogen gas), oxidant gas (air) and make-up gas (nitrogen gas), column temperature program, injector temperature and detector temperature. In this study, one microliter of 5 µg mL^{-1} of MDMA, MDA, and MA mixture was injected into gas chromatograph. Optimization was carried out by varying one parameter at a time while the others were kept constant. Five replicates were done for each test. The optimum condition was obtained base on the best or acceptable resolution, response and analysis.

2.5.1.1 Carrier gas (nitrogen gas) flow rate

Optimization was carried out by varying the flow rate of nitrogen carrier gas at 2.3, 2.6, 2.9 and 3.2 mL min^{-1}. The flow rate of make-up gas (nitrogen gas), fuel gas (hydrogen gas) and oxidant gas (air) were maintained at 20, 30 and 300 mL min^{-1}, respectively. Column temperature was initially set at 100ºC and hold for 1 min and was increased at a rate of 20ºC min^{-1} to 270ºC and hold for 1 min. Injector temperature and detector temperature were 290ºC and 290ºC, respectively.

The optimum flow rate of carrier gas (nitrogen gas) was determined based on the height equivalent to a theoretical plate (*HETP*) calculated using the following expressions. $HETP = \frac{L}{N}$ where *L* is column length (cm) and *N* is plate number.

N was derived from the formula: $N = 2\pi\left(\frac{t_R h}{A}\right)^2$ (Grob and Barry, 2004), where t_R is peak retention time (min), h is peak height (cm), and A is peak area (count). The flow rate of nitrogen carrier gas was selected as optimal based on the acceptable peak response, resolution, and analysis time.

2.5.1.2 Fuel gas (hydrogen gas) flow rate

Optimum flow rate of hydrogen was studied by varying the flow rates at 20, 30, 40 and 50 mL min^{-1}. Peak areas obtained from all flow rates were compared. Flow rate that produced the highest peak area response was selected.

2.5.1.3 Oxidant gas (air zero) flow rate

Optimum oxidant gas flow rate was investigated by varying its flow rates at 200, 300, 400 and 500 mL min^{-1}. The peak areas obtained from all flow rates were compared and the optimum condition was considered from the highest response.

2.5.1.4 Make-up gas (nitrogen) flow rate

Optimum flow rate of make-up gas (nitrogen) was investigated at different flow rates of 10, 20, 30, 40 and 50 mL min^{-1}. The peak area responses obtained from all flow rates were compared and the optimum condition was determined based on the highest response.

2.5.1.5 Column temperature program

Column temperature program consists of the five parts: 1) initial temperature, 2) holding time of the initial temperature, 3) ramp rate, 4) final temperature and 5) holding time of the final temperature. Their study values are shown in Table 2.1. The optimum column temperature program was obtained based on acceptable resolution, response and analysis time.

Table 2.1 Optimization of column temperature program

Step	Parameters	Study values
1	Initial temperature	70, 80, 90, 100, 110 and 120ºC
2	Holding time at initial temperature	0, 1, 2 and 3 min
3	Temperature ramp rate	15, 20, 25 and 30ºC min^{-1}
4	Final temperature	270, 280, 290 and 300ºC
5	Holding time at final temperature	0, 1, 2 and 3 min

2.5.1.6 Injector temperature

The optimization of injector temperature was studied by varying the temperatures at 260, 270, 280, 290 and 300ºC. The temperature that gave the highest response was selected as the optimum injector temperature.

2.5.1.7 Detector temperature

The optimization of detector temperature was studied by varying the temperatures at 260, 270, 280, 290 and 300ºC. The temperature that provided the highest response was selected as the optimum detector temperature.

2.5.2 Study 2: Determination of MDMA, MDA and MA in ecstasy tablets

2.5.2.1 Sample collection

Ecstasy tablets seized in Songkhla province during 2007-2008 and kept at the Regional Forensic Science Division 4 in Songkhla province as an evidence in a trial were randomly selected. Eighteen different tablets were included in the present work. Physical characteristics of each tablet including logo, color, diameter, thickness and weight were recorded. Each tablet was also photographed with a digital camera.

2.5.2.2 Selection of methods of extraction

A trial of using solvent- and ultrasonication methods for extracting MDMA, MDA and MA from seized tablets was carried out. Each tablet was finely crushed. Three milligrams of powder were placed into the centrifuge tube (three replicates) and

then 1 mL of 50 µg mL^{-1} DPA (final concentration of 10 µg mL^{-1}) was added. A final volume of 5 mL of the mixture was made up with each of following solvents, *i.e.* pure methanol, ethyl acetate or deionized water. For solvent extraction method, the mixture was mixed thoroughly by vortex for 1 min and then passed through a filter paper no.1 (Whatman). For ultrasonication method, the mixture was sonicated using ultrasonic bath at 25ºC for 2 min before passing through a filter paper no.1 (Whatman). The filtrates (1 µL) obtained from either solvent extraction or ultrasonication was injected into GC-FID system and their chromatographic responses were compared.

2.5.2.3 Preparation and analysis of seized tablets

According to section 2.5.2.2, method of extraction with pure methanol was the most efficient (see results in section 3.2.2) and therefore, was chosen for analysis of seized tablets. Prior to analysis, each tablet was finely crushed. Three milligrams of powder were dissolved in 5 mL of methanol and mixed thoroughly with a vortex mixer for 1 min. The solution was filtered using filter paper no.1 (Whatman). A 500 µL aliquot of the filtrate was mixed with 200 µL of 50 µg mL^{-1} DPA (final concentration of 10 µg mL^{-1}) and 300 µL of methanol. One microliter of this mixture was injected into the GC-FID (five replicates for each tablet). The analysis was performed using an optimum condition of GC-FID derived from this work (section 2.3). Sample preparation and analysis of seized tablets procedure were performed as follows (Figure 2.1)

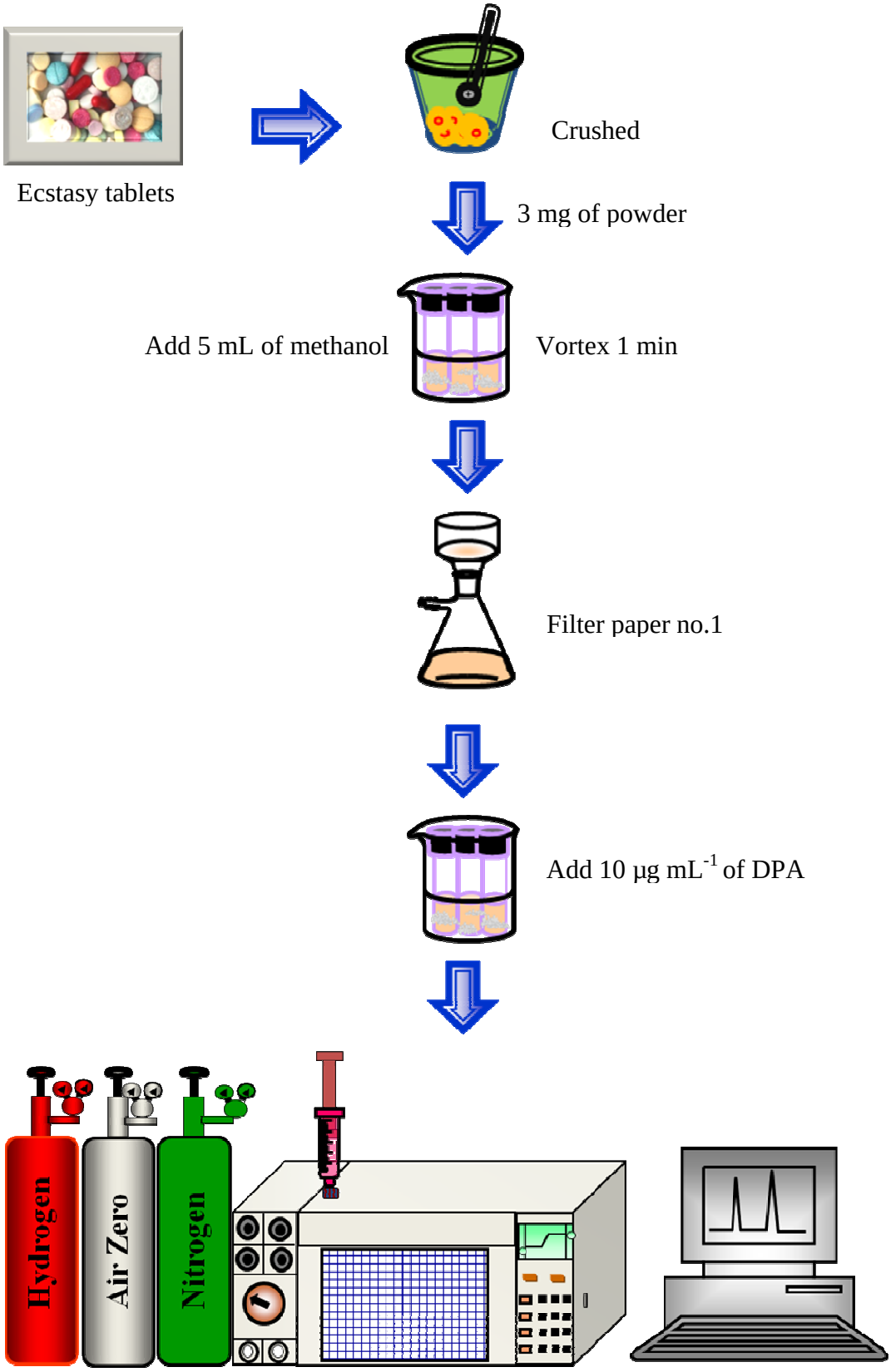


Figure 2.1 Sample preparation for determination of MDMA, MDA and MA in ecstasy tablets

2.5.2.4 Method validation for ecstasy tablets analysis

Method of analysis was generally validated according to the ICH guideline recommendation (Swartz and Krull, 1997), Bioanalytical method validation and its implication for forensic and clinical toxicology-A review (Peters and Maurer, 2002) and US Food and Drug Administration (FDA, 2001). Parameters included linearity and range, intra-day and inter-day precisions, accuracy, recovery, lower limit of quantification (LLOQ) and stability.

1) Linearity and range

Linearity was determined by preparing seven standard solutions as a mixture of MDMA, MDA and MA (3.125, 6.25, 12.5, 25, 50, 100 and 200 μg mL^{-1}; five replicates of each concentration), and 10 μg mL^{-1} of DPA in methanol. The calibration curve was constructed by plotting peak area ratios (y) of MDMA, MDA and MA to those of the internal standard versus the concentrations of MDMA, MDA and MA (x). Regression analysis was performed to obtain the calibration equation and correlation coefficient (r).

2) Precision

Both intra-day (repeatability) and inter-day (reproducibility) precisions were determined using four quality control (QC) samples which were a mixture of MDMA (3.125, 25, 100 and 200 μg mL^{-1}), MDA and MA (6.25, 25, 100 and 200 μg mL^{-1}), containing 10 μg mL^{-1} of DPA in methanol. Intra-day precision was determined by analyzing five samples of each concentration during the same day under the same instrumental operating condition. Inter-day precision was determined by analyzing of the samples for five consecutive days. The level of acceptance for precision is within 15% RSD except at the LLOQ where 20% RSD is accepted (FDA, 2001; Peters and Maurer, 2002). Precision was expressed as relative standard deviation (RSD) was calculated as follows.

$$\text{Relative standard deviation: RSD (\%)} = \frac{\text{standard deviation}}{\text{mean value}} \times 100$$

3) Accuracy

The accuracy was determined by using four QC samples which were a mixture of standards MDMA (3.125, 25, 100 and 200 µg mL^{-1}), MDA and MA (6.25, 25, 100 and 200 µg mL^{-1}) and contained 10 µg mL^{-1} of DPA in methanol (five replicates for each concentration). The accuracy was expressed as the deviation (DEV) and is acceptable when the DEV is within ±15% (FDA, 2001). The deviation (DEV) was derived as follows.

$$\text{Deviation: DEV (\%)} = \left[\frac{\left(\text{measured concentration - nominal concentration}\right)}{\text{nominal concentration}} \right] \times 100$$

4) Lower limit of quantification (LLOQ)

The lower limit of quantification (LLOQ) were determined as lowest concentration on the calibration curve that could be determined with a precision of 20% and accuracy of 80-120 %, *i.e.* %DEV is within ± 20% (FDA, 2001).

5) Stability

5.1) Short-term stability

The standard mixture of MDMA, MDA and MA in methanol at low (10 µg mL^{-1}) and high (100 µg mL^{-1}) concentrations and 10 µg mL^{-1} of DPA (three replicates of each concentration) were left at room temperature (21ºC) and analyzed at 0, 6, 12, 18 and 24 h. The measured concentrations as percentage at varying time and that at the baseline (t=0) were compared.

5.2) Long-term stability

Stock solution (200 µg mL^{-1}) of a mixture of MDMA, MDA and MA was prepared. Aliquots of 100 µL and 10 µL were made to produce different amounts (100 µg and 10 µg) of each compound. The aliquots were dried by using centrivap concentrator (65ºC). The residues were kept at -20ºC in a freezer for 2 months. On the day of analysis, the residues were dissolved in pure methanol. The measured amounts as percentage at 1 and 2 month (s) storage were compared (three replicates) with those at the baseline (0 month).

2.5.3 Study 3: Determination of MDMA, MDA and MA in human urine

2.5.3.1 Sample collection

Twenty human urine samples were obtained from the Regional Medical Science Center Songkhla. These samples were collected from individuals suspected of drug abuse in public in Songkhla province during 2007-2008. Urine samples were measured the pH and centrifuged at 1500 × g for 15 min. The supernatant was collected and kept at -20ºC until analysis.

2.5.3.2 Sample preparation studies

Conditions affecting the efficiency of sample preparation using solid phase extraction (SPE) were studied. Those included 1) pH of sample and a reagent used to adjust pH of sample and 2) a solvent used to wash and elute sample. Three replicates of samples were done and each sample was injected three times. The optimum condition was considered based on the highest peak area response.

1) pH of sample study

Effect of urine pH on recovery of extraction was investigated. Various acidic and basic reagents including 1 N HCl, 1 M CH_3COOH, 1 M HCOOH, 1 N NaOH, 5 M NH_4OH and 1 M KOH were used. Urine samples containing 5 μg mL^{-1} of DPA were spiked with a mixture of MDMA, MDA and MA to obtain a concentration of 20 μg mL^{-1} and then were either acidified to pH 3 with acidic reagents or alkalinized to pH 12 by basic reagents. The samples were mixed by vortex for 30 s and loaded into the Oasis® HLB cartridges that were preconditioned by passing sequentially 1 mL of methanol and equilibrated by 1 mL of deionized water. The fluids obtained after loading were collected and then evaporated to dryness under speed vacuum at 65ºC. The residue was reconstituted with 50 μL of pure methanol and one microliter was injected into the GC-FID system using an optimum condition. The pH value and reagent making the analytes retained the most in the cartridges was selected.

2) Wash-elute study

Urine blanks (1 mL) containing 20 µg mL^{-1} of MDMA, MDA and MA and 5 µg mL^{-1} of DPA were prepared. The mixture was alkalinized with 30 µL of 1 M KOH before loading into the Oasis® HLB cartridges that were preconditioned by passing sequentially 1 mL of methanol and equilibrated by 1 mL of deionized water. For washing step, the cartridges were passed with different compositions, *i.e.* 0, 10, 20, 30, 40, 50, 60, 70 and 80% (v/v), of methanol-water mixtures containing 2% ammonium hydroxide. For eluting step, the cartridges were passed with different compositions, *i.e.* 0, 10, 20, 30, 40, 50, 60, 70 and 80% (v/v), of methanol-water mixtures containing 2% acetic acid. The fluids obtained after washing and eluting steps were collected separately and then evaporated to dryness under speed vacuum at 65ºC. The residues were reconstituted with 50 µL of methanol prior to GC-FID analysis. The solvent mixture making the analytes retained the most in the cartridges was selected for washing step while the one making them eluted the most from the cartridges was selected for eluting step.

2.5.3.3 Preparation and analysis of urine samples

Regarding to section 2.5.3.2, an appropriate reagent and pH of samples including solvent mixtures using in SPE process were selected (see results in section 3.3.2). For urine analysis, sample extraction procedure was performed as follows (Figure 2.2). One milliliter of urine sample was placed in 2 mL-microcentrifuge tubes and then combined with 100 µL of DPA (final concentration of 5 µg mL^{-1}). The mixtures were adjusted to pH 12 by adding 30 µL of 1 M KOH and then mixed by vortex for 30 s. The samples were passed through the Oasis HLB® cartridges that were preconditioned by passing sequentially 1 mL of methanol and equilibrated by 1 mL of deionized water. The cartridges were washed with 1 mL of 5% methanol-water mixtures containing 2% ammonium hydroxide. Analytes were eluted from cartridges with 1 mL of 70 % methanol-water mixtures containing 2% acetic acid. The eluents were evaporated to dryness under speed vacuum at 65ºC and reconstituted with 50 µL of methanol. One microliter was injected into the GC-FID system under an optimum condition.

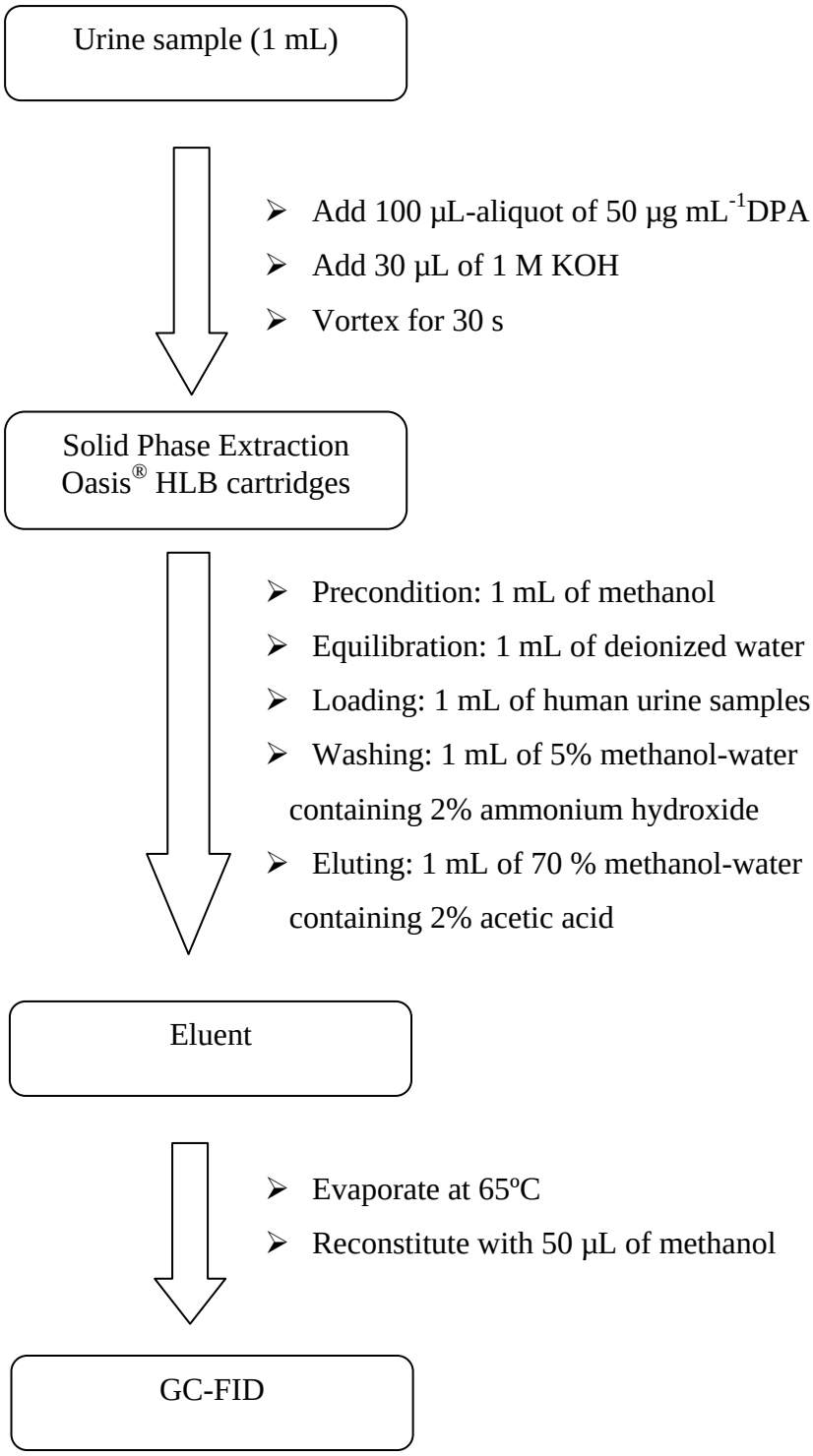


Figure 2.2 Urine sample preparation for determination of MDMA, MDA and MA

2.5.3.4 Method validation for urine analysis

Method of analysis was validated according to the same guideline as used for tablets analysis (section 2.5.2.4). Parameters included linearity and range, intra-day and inter-day precisions, accuracy, recovery and lower limit of quantification (LLOQ). Human urine samples, donated from volunteers who were students of Faculty of Science, Prince of Songkla University, were used throughout the validation process.

1) Linearity and range

Linearity was determined by preparing five standard solutions as a mixture of MDMA, MDA and MA (0.5, 1, 5, 10 and 20 μg mL^{-1}; five replicates of each concentration), and 5 μg mL^{-1} of DPA in urine blanks. The calibration curve was constructed by plotting peak area ratios (y) of MDMA, MDA and MA to those of the internal standard versus the concentrations of MDMA, MDA and MA (x). Regression analysis was performed to obtain the calibration equation and correlation coefficient (r).

2) Precision

Four QC samples obtained by preparing of MDMA, MDA and MA (1, 5, 10 and 20 μg mL^{-1}), and 5 μg mL^{-1} of DPA in urine blank were used. Intra-day precision was determined by analyzing five samples of each concentration during the same day under the same instrumental operating condition. Inter-day precision were determined by analyzing of the samples for five consecutive days. The level of acceptance for precision is within 15% RSD except at the LLOQ where 20% RSD is accepted (FDA, 2001; Peters and Maurer, 2002). The relative standard deviation (RSD) was calculated as follows.

$$\text{Relative standard deviation: RSD (\%)} = \frac{\text{standard deviation}}{\text{mean value}} \times 100$$

3) Accuracy

Four QC samples of standards MDMA, MDA and MA mixed in urine blank samples at concentrations of 1, 5, 10 and 20 μg mL^{-1} and 5 μg mL^{-1} of DPA were used (three replicates for each concentration). The accuracy was expressed as the deviation (DEV) and is acceptable when the DEV is within ±15% (FDA, 2001). The deviation (DEV) was derived as follows.

$$\text{Deviation: DEV (\%)} = \left[\frac{(\text{measured concentration - nominal concentration})}{\text{nominal concentration}} \right] \times 100$$

4) Recovery

The recovery of MDMA, MDA and MA after extracting from urine samples was determined at LLOQ (1 μg mL^{-1}), low (5 μg mL^{-1}), medium (10 μg mL^{-1}) and high (20 μg mL^{-1}) concentrations and 5 μg mL^{-1} of DPA (three replicates of each concentration) by comparing with the response obtained after direct injection of standard MDMA, MDA, MA and DPA mixtures prepared in methanol. The percentage recovery was calculated as follows.

$$\text{Recovery (\%)} = \frac{\text{response after extraction}}{\text{response after direct injection}} \times 100$$

5) Lower limit of quantification (LLOQ)

The lower limit of quantification (LLOQ) were determined as the lowest concentration on the calibration curve that could be determined with a precision of 20% and accuracy of 80-120 %, *i.e.* %DEV is ± 20% (FDA, 2001).

2.6 Statistical analysis

The results are presented as the mean value ± SD. Data were analyzed by one-way ANOVA. A difference was considered significant at $p<0.05$. The software used was the SPSS Version 11.50 statistical software program.

CHAPTER 3

Results and discussion

3.1 Study 1: Optimization of the GC-FID conditions

3.1.1 Carrier gas (nitrogen gas) flow rate

Flow rate carrier gas was optimized by considering Van Deemter plot (HETP v.s. carrier gas flow rate). The flow rate producing the lowest HETP for separation of MDMA, MDA and MA was 2.6 mL min^{-1} (Figure 3.1). Therefore, this flow rate was considered optimal in providing the highest column efficiency.

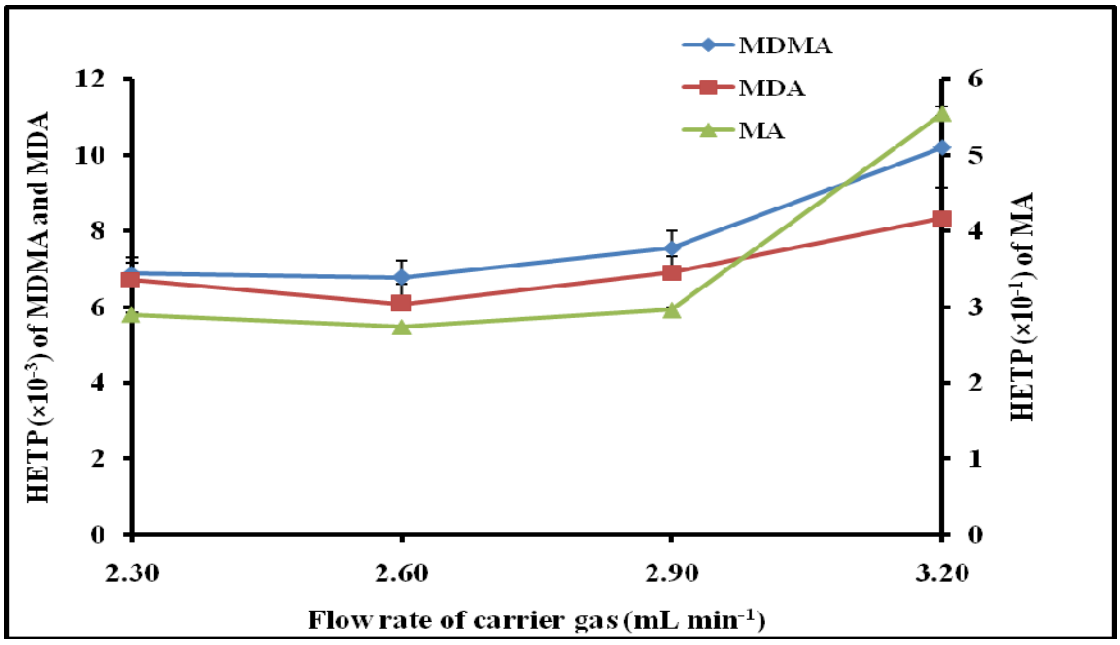


Figure 3.1 Van Deemter plots of MDMA, MDA and MA (n=5)

3.1.2 Fuel gas (hydrogen gas) flow rate

The FID detector was operated in a hydrogen-rich mode and used oxidant gas (air zero) for combustion. Therefore, flow rates of hydrogen gas and air may influence the detector sensitivity and level of noise. The different fuel gas flow rates were varied (20, 30, 40 and 50 mL min^{-1}). Detection responses of all analytes except MDA and MA at 50 mL min^{-1} were significantly higher than those a flow rate of 20 mL min^{-1} (p<0.05) (Figure 3.2). All analytes were detected with the highest response at a rate of 30 mL min^{-1}. Therefore, this flow rate was chosen.

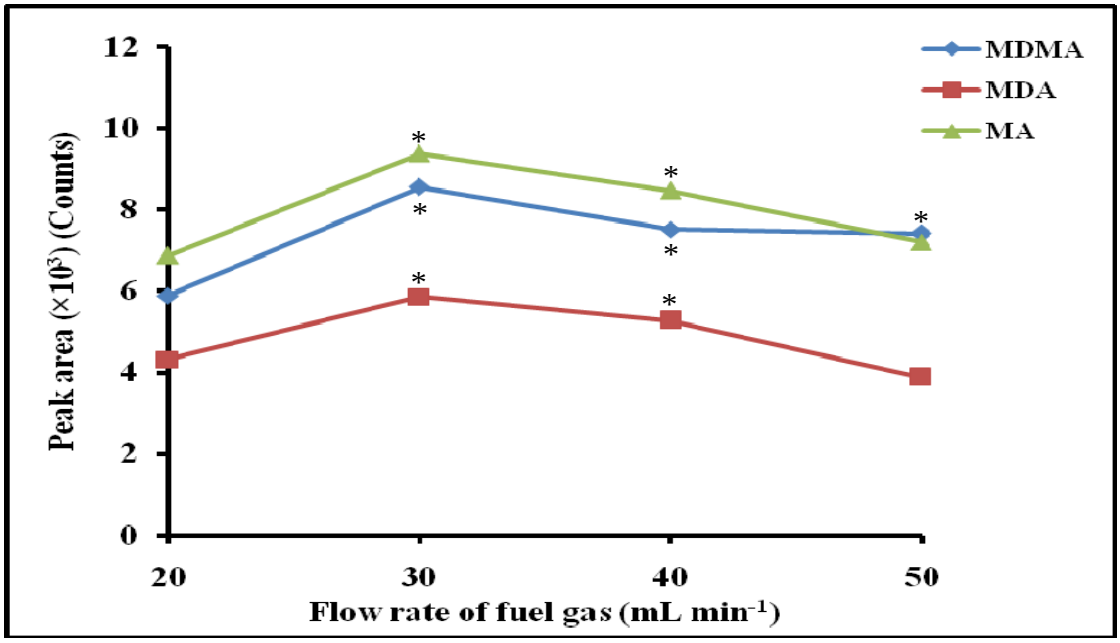


Figure 3.2 Peak areas of MDMA, MDA and MA (5 µg mL^{-1}) in a mixture of standard solutions at various fuel gas flow rates (n=5)

* Indicated that the results are significantly different from fuel gas flow rates of 20 mL min^{-1} at p<0.05

3.1.3 Oxidant gas (air zero) flow rate

In this work, air zero (O_2) used as an oxidant gas was varied from 200, 300, 400 and 500 mL min^{-1}. The effect of air flow rate on the responses is shown in Figure 3.3. Peak responses of all analytes were significantly higher than those obtained at a rate of 200 mL min^{-1} (p<0.05). Although peak responses were the highest at gas flow rates of 400 and 500 mL min^{-1}, the results showed that peak responses of MDMA and

MA were not significantly difference. For cost saving purpose, a flow rate of 300 mL $min^{-1}$was selected.

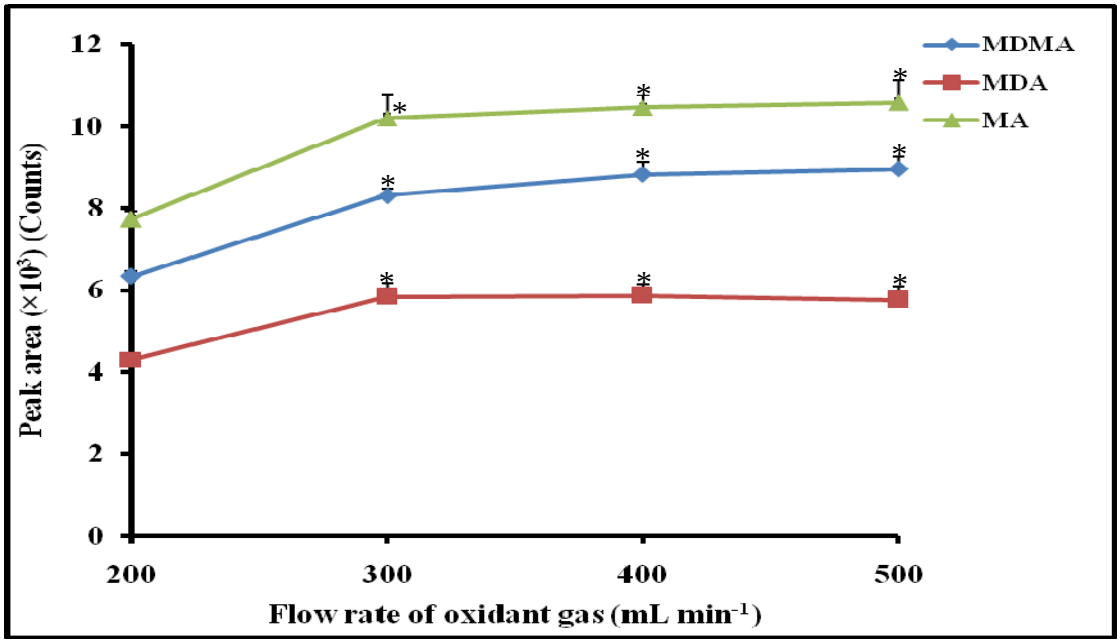


Figure 3.3 Peak areas of MDMA, MDA and MA (5 µg mL^{-1}) in a mixture of standard solutions at various oxidant gas flow rates (n=5)

* Indicated that the results are significantly different from oxidant gas flow rates of 200 mL min^{-1} at $p<0.05$

3.1.4 Make-up gas (nitrogen) flow rate

Make-up gas was used to carry the analyte from the column to the detector zone. In this study, the flow rate of the make-up gas was varied at 10, 20, 30, 40 and 50 mL min^{-1}. The effect of make-up gas flow rate on the responses is shown in Figure 3.4. At a rate of 30 mL min^{-1}, detection responses of all analytes except MDA were significantly higher than those at 10 mL min^{-1} ($p<0.05$). Those responses detected at 40 and 50 mL min^{-1} were significantly decreased. Therefore, the rate of 30 mL min^{-1} was considered to be the optimum rate.

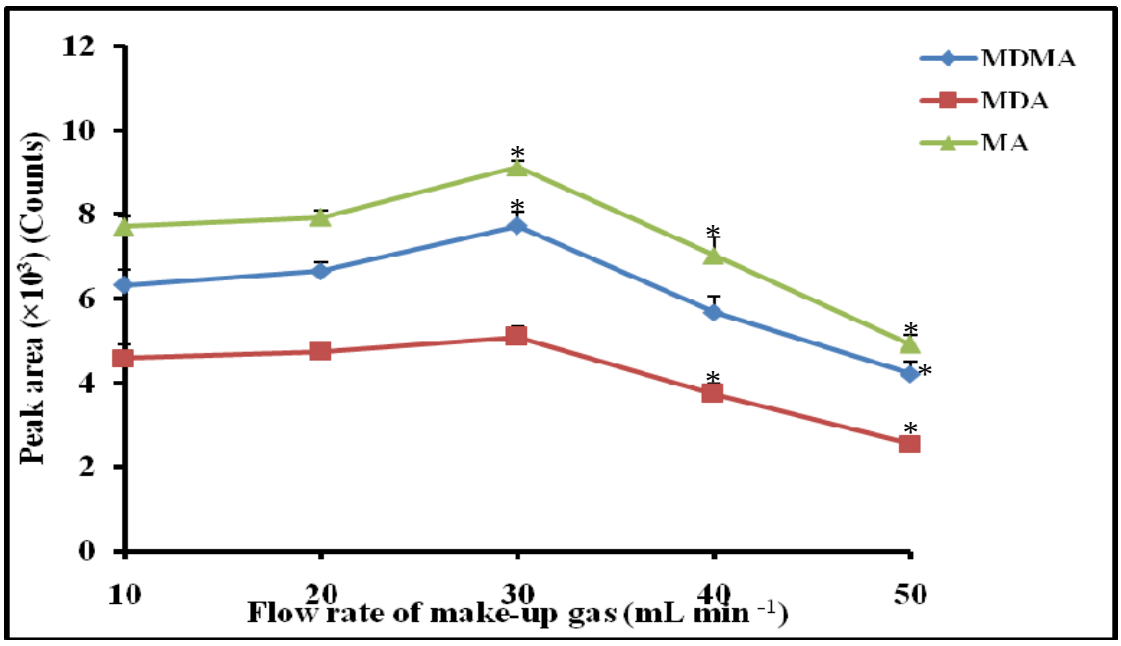


Figure 3.4 Peak areas of MDMA, MDA and MA (5 µg mL^{-1}) in a mixture of standard solutions at various make-up gas flow rates (n=5)

* Indicated that the results are significantly different from make-up gas flow rates of 10 mL min^{-1} at $p<0.05$

3.1.5 Column temperature program

Column temperature is an important parameters in gas chromatographic technique since it leads to the good peak resolution. For simultaneous separation of several compounds, a temperature program was set for analysis as it was more effective than an isothermal system. The temperature program was used to minimize the time for eluting or separating the components of interest while narrowing peak widths, increasing integrating detectability and sample throughout and reducing analysis time (Grob and Barry, 2004). The optimal column temperature programming was investigated by varying the initial temperature, initial holding time, ramp rate, final temperature and final holding time.

Varying initial column temperature affected peak responses of MDMA, MDA and MA and analysis time (Figure 3.5). As the temperature increased, the peak responses of MDA and MA were significantly decreased while those of MDMA were significantly increased ($p<0.05$). However, the analysis time was significantly decreased compared with those at temperature of 70ºC ($p<0.05$). An initial

temperature of 80ºC was selected due to acceptable good responses and analysis time (11.50 min). Although at temperature of 90ºC and 100ºC, the analysis times were shorter (11.00 and 10.50 min) and MDMA response was better (p<0.05) compared with at temperature of 80ºC, but MDA response was poorer.

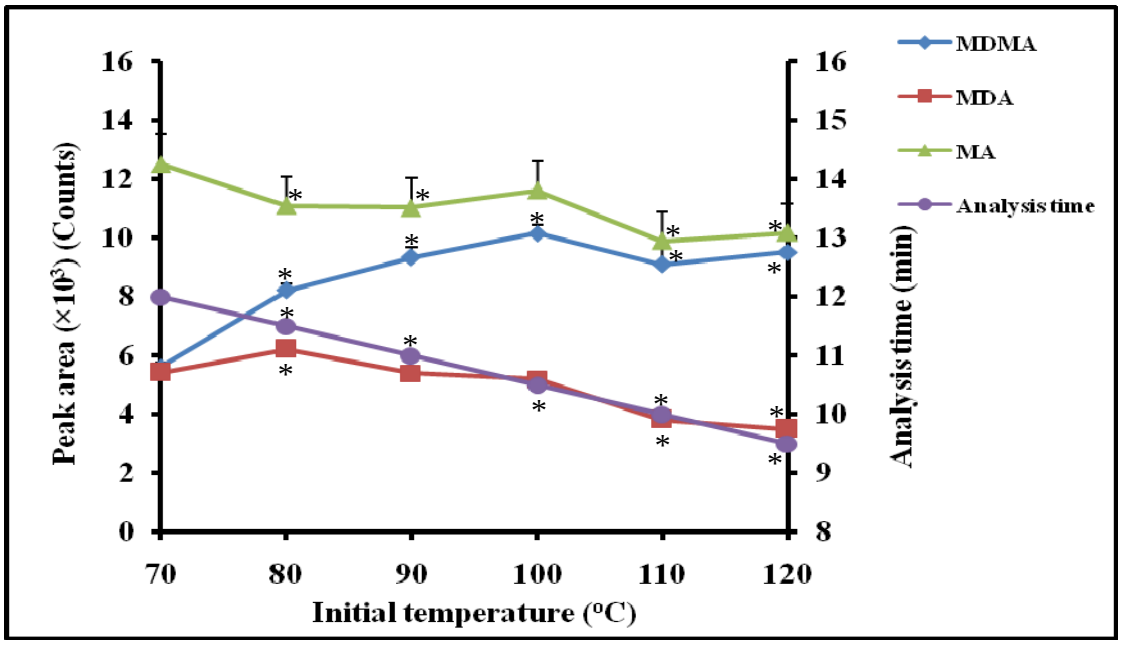


Figure 3.5 Peak areas of MDMA, MDA and MA (5 µg mL^{-1}) in a mixture of standard solutions at various initial temperatures (n=5)

* Indicated that the results are significantly different from initial temperature of 70ºC at p<0.05

Holding time at the initial temperature of 80ºC was investigated and the results are shown in Figure 3.6. As the holding time increased from 0 to 3 min, the analysis time significantly increased. Peak response of all analytes tended to decrease (significantly for MDMA only; p<0.05). Holding time of 0 min was chosen as optimal since it provided the shortest analysis time (10.50 min) with the best separation.

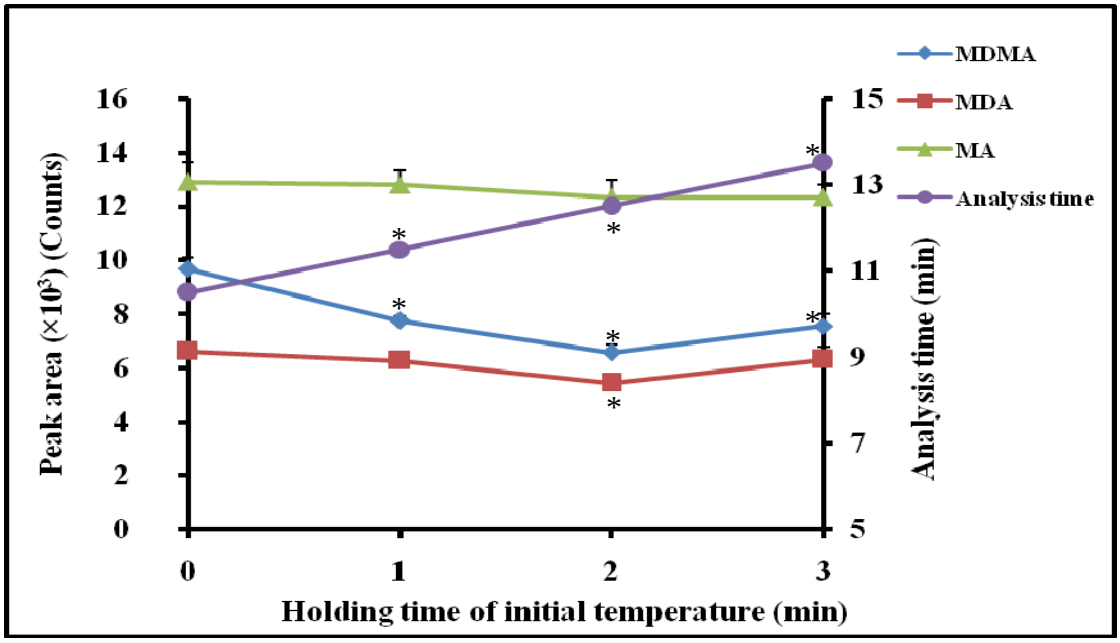


Figure 3.6 Peak areas of MDMA, MDA and MA (5 µg mL^{-1}) in a mixture of standard solutions at various holding time of initial temperature (n=5)

* Indicated that the results are significantly different from initial holding time of 0 min at $p<0.05$

Ramp rates of column temperature (15, 20, 25 and 30ºC min^{-1}) were investigated. The analysis time was significantly decreased ($p<0.05$) while the responses of all analytes were the same when the ramp rates of temperature increased (Figure 3.7). At the rate of 20ºC min^{-1}, the responses were slightly lower but the resolutions were as good as at the rate of 15ºC min^{-1}. Since the analysis time was shorter, *i.e.* 10.50 min, therefore the ramp rate of 20ºC $min^{-1}$was chosen as optimal.

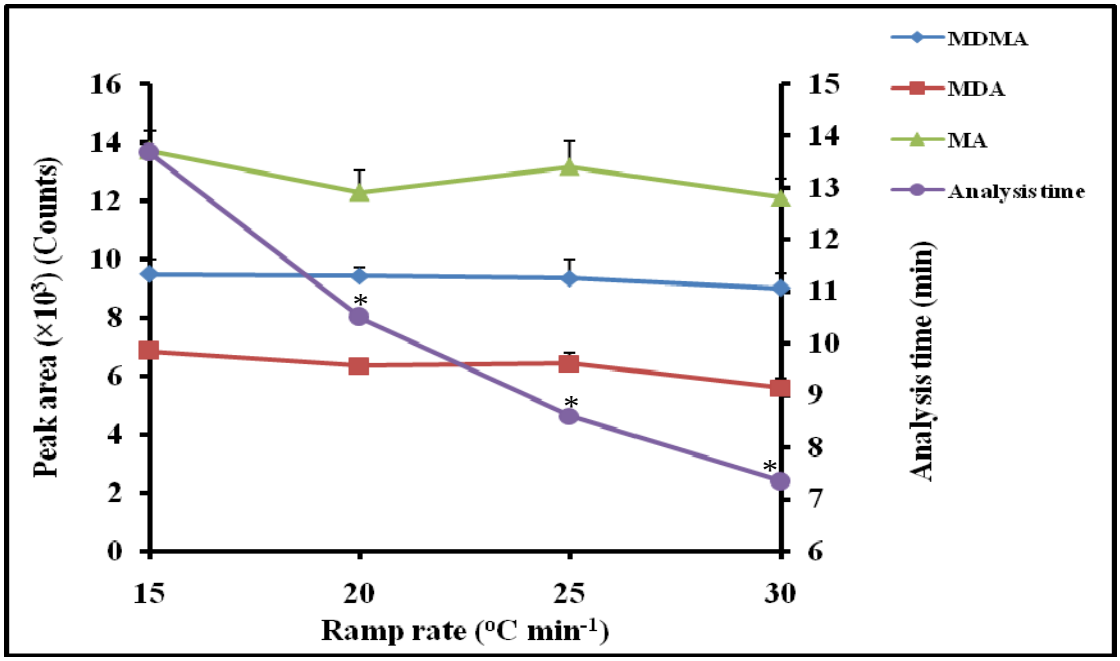


Figure 3.7 Peak areas of MDMA, MDA and MA (5 µg mL^{-1}) in a mixture of standard solutions at various ramp rates (n=5)

* Indicated that the results are significantly different from ramp rates of 15°C min^{-1} at $p<0.05$

The optimum final temperature was investigated by varying the temperatures at 270, 280, 290 and 300°C. Since the temperature of 300°C is the maximum acceptable temperature for CP-SIL 24 CB WCOT column, therefore, the temperature above 300°C was not tested. The analysis time was significantly decreased ($p<0.05$) when the final temperature increased (Figure 3.8). Peak response of MDMA and MDA also significantly decreased. At the final temperature of 270°C the detection responses of MDMA and MDA were the highest. Thus, the temperature of 270°C was selected as the optimum final temperature.

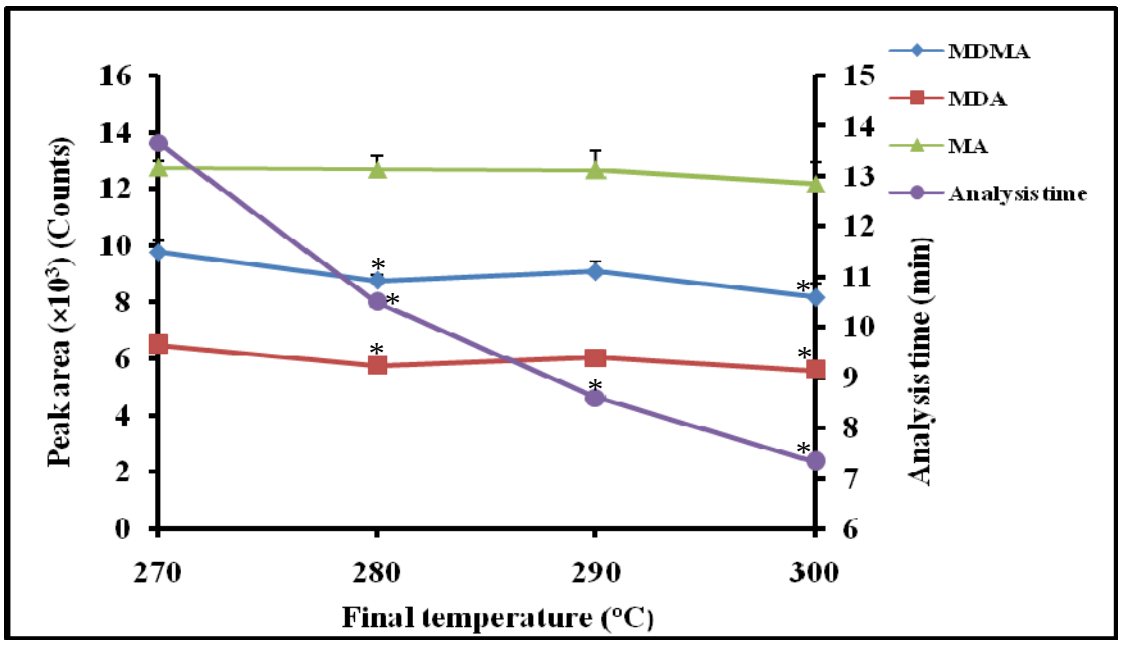


Figure 3.8 Peak areas of MDMA, MDA and MA (5 µg mL^{-1}) in a mixture of standard solutions at various final temperatures (n=5)

* Indicated that the results are significantly different from final temperature of 270ºC at $p<0.05$

The holding time of the selected final temperature was obtained by considering the time that MDMA, MDA and MA could be eluted completely from the column with the optimum condition. The purpose of holding the final temperature was to allow the signal returns to baseline. The analysis time was significantly increased when the holding time of the final temperature increased ($p<0.05$) (Figure 3.9). Peak responses of MDMA and MDA significantly increased while those of MA significantly decreased. Holding time of the final temperature for 1 min was sufficient to produce the best responses with a short analysis time (10.50 min).

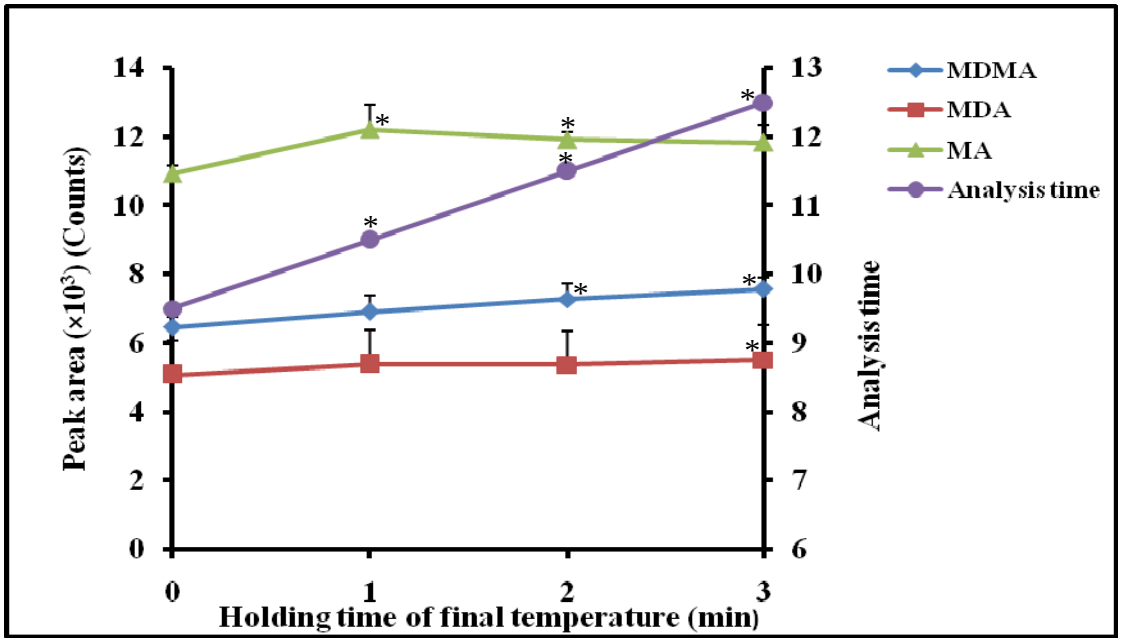


Figure 3.9 Peak areas of MDMA, MDA and MA (5 µg mL^{-1}) in a mixture of standard solutions at various holding time of final temperature (n=5)

* Indicated that the results are significantly different from holding time of final temperature of 0 min at $p<0.05$

3.1.6 Injector temperature

Injector is a part of the system that performs the physical task of transferring the sample from a syringe into gas chromatograph. The injector temperature should be set high enough so that there is enough thermal mass and energy in the inlet to vaporize the injected sample without causing the inlet to cool significantly, but not so high that the sample components are decomposed. The temperatures of the injector were varied at 260, 270, 280, 290 and 300ºC. The detection responses of MDMA, MDA and MA are shown in Figure 3.10. As the injector temperature was increasing, the peak response of MDA was also significantly increasing ($p<0.05$). Peak responses of MDMA and MA were unchanged except at the injector temperature of 300ºC. Since at 290ºC, the best MDA detection was produced, such temperature level was selected.

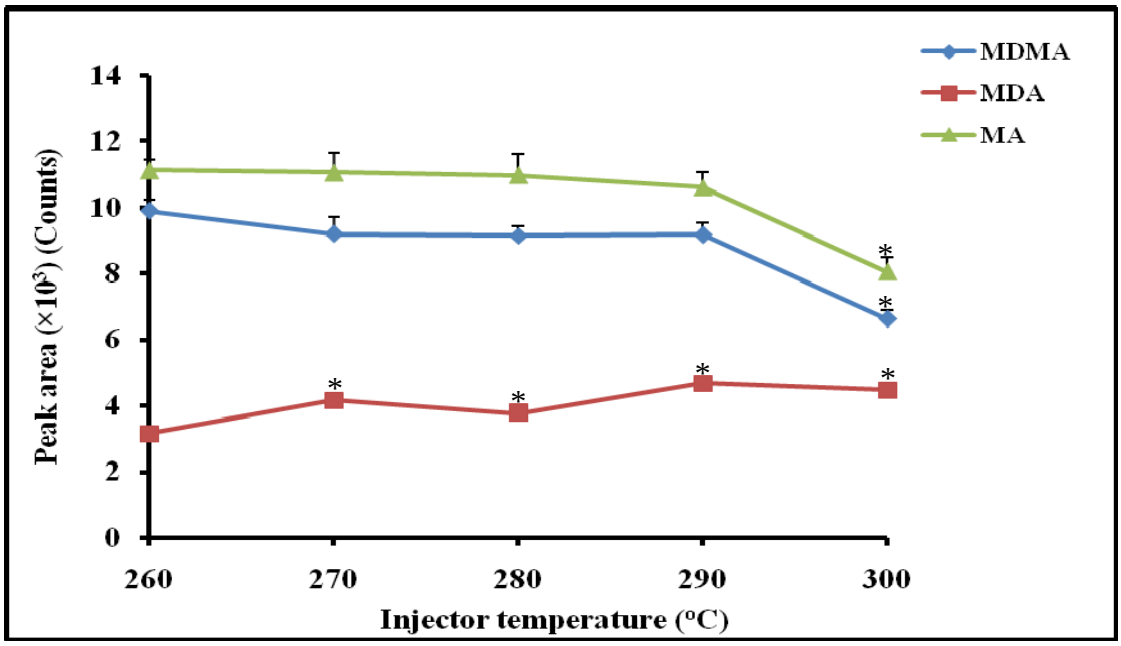


Figure 3.10 Peak areas of MDMA, MDA and MA (5 µg mL^{-1}) in a mixture of standard solutions at various injector temperatures (n=5)

* Indicated that the results are significantly different from injector temperature of 260ºC at $p<0.05$

3.1.7 Detector temperature

The responses derived from various detector temperatures are shown in Figure 3.11. The relationship between detector temperature and the detection response indicated that the response increased with temperatures. When the detector temperature was set at 300ºC, peak responses of all analytes were significantly increased to the highest compared to the temperatures of 260, 270 and 280ºC. The detector temperature of 300ºC was therefore chosen.

In this system, the analytes eluted from the column were detected by a flame ionization detector (FID). The FID consists of a small hydrogen-air diffusion flame burning at end of a jet, to which the eluted components from the column are directed with carrier gas and make-up gas flow. The basic of the FID is that the effluent from the column is mixed with hydrogen and burned in air to produce a flame which should have sufficient high energy to ionize solute molecules having low ionization potentials. The resulting current is amplified by an electrometer (Grob and Barry,

2004). Detector temperature and the detection response indicated that the response increased with temperatures.

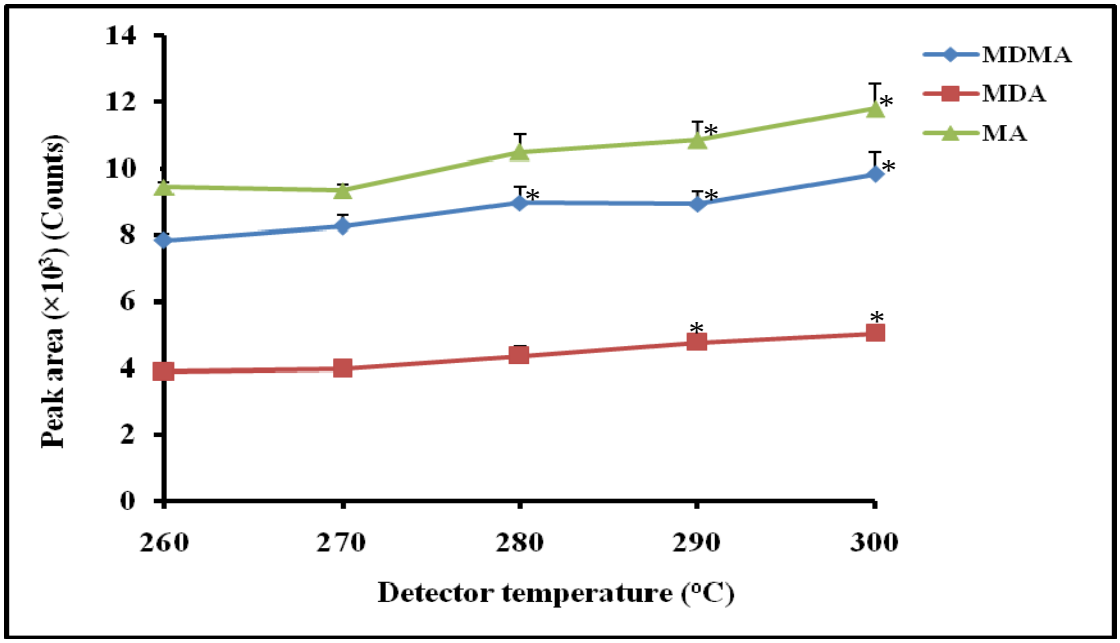


Figure 3.11 Peak areas of MDMA, MDA and MA (5 µg mL^{-1}) in a mixture of standard solutions at various detector temperatures (n=5)

* Indicated that the results are significantly different from detector temperature of 260°C at $p<0.05$

To sum up, the optimum condition for simultaneous MDMA, MDA and MA analysis using a capillary column of 0.25 µm film thickness (30 m × 0.32 mm i.d.) with flame ionization detector is described in Table 3.1

Table 3.1 The optimum condition of GC-FID for simultaneous MDMA, MDA and MA analysis

Parameter		**Optimum condition**
Gas flow rate	Carrier gas (N_2)	2.60 mL min^{-1}
	Fuel gas (H_2)	30 mL min^{-1}
	Oxidant gas (O_2)	300 mL min^{-1}
	Make-up gas (N_2)	30 mL min^{-1}
Column Temperature	Initial (holding time)	80 ºC (0 min)
	Final (holding time)	270 ºC (1 min)
	Ramp rate	20ºC min^{-1}
Injector temperature		290ºC
Detector temperature		300ºC

3.2 Study 2: Determination of MDMA, MDA and MA in ecstasy tablets

3.2.1 Sample collection

Eighteen ecstasy tablets randomly selected from those seized in Songkhla province during 2007-2008 were included in the present work. Their physical characteristics are described in Table 3.2. The tablets of ecstasy weighed between 195 and 460 mg. The diameter ranged from 0.75 to 1.40 cm and the thickness ranged from 0.40 to 0.80 cm. The colors were beige, pink, green, white, blue, orange and yellow. The logos printed on some of these tablets included Mitsubishi, Crown, Diamond, Xtriangle, Tree, SKY, cK, Bird, 88 and DMT. The rest was unidentified. The ecstasy tablets Mitsubishi, Crown, Diamond and SKY were also appeared in Japan and Hong Kong (Teng *et al.*, 2006). In 1995 to 2002, the Bureau of Drugs reported the physical characteristics of ecstasy tablets seized in Thailand (179 samples). The tablets of ecstasy weighed between 47 and 453 mg. The diameter ranged from 0.50 to 1.00 cm and the thickness ranged from 0.20 to 0.70 cm. The logos printed on some of these tablets included CU, Butterfly, B-29 and Rolex (Kosin and Qawnaom., 2006).

However, the tablets seized in Chonburi province during 1998 to 2004 were different from those seized in Songkhla province. The most frequently seen logos were CU, OX, B-29, Rolex and 2000 and imprinted a butterfly (Ratanavaraporn, 2006).

Table 3.2 Physical characteristics of ecstasy tablets samples

Sample no.	Tablet characters					
	Color	**Weight (mg)**	**Diameter (cm)**	**Thickness (cm)**	**Logo**	**Picture**
1	Beige	270	0.90	0.50	Mitsubishi	
2	Pink	291	0.90	0.60	Mitsubishi	
3	Beige	204	0.80	0.45	Crown	
4	Green	312	0.90	0.50	Diamond	
5	White	308	0.95	0.50	Crown	
6	Pink	460	1.40	0.80	Xtriangle	
7	Pink	310	0.90	0.50	Tree	
8	Green	310	0.85	0.45	SKY	
9	Blue	270	0.85	0.45	Bird	
10	Green	317	0.85	0.45	88	
11	Green	301	0.90	0.50	DMT	
12	Green	278	0.80	0.40	cK	

Table 3.2 Physical characteristics of ecstasy tablets samples (cont.)

Sample no.	Tablet characters					
	Color	**Weight (mg)**	**Diameter (cm)**	**Thickness (cm)**	**Logo**	**Picture**
13	Beige	328	0.90	0.45	-	
14	Orange	283	0.85	0.40	-	
15	Green	269	0.85	0.40	-	
16	Pink	284	0.80	0.45	-	
17	Beige	195	0.75	0.40	-	
18	Yellow	299	0.80	0.50	-	

3.2.2 Selection of method of extraction

Selection of an appropriate extracting solvent is important for the optimization of the extraction process. The purpose of this experiment were to evaluate the capability of extraction using different solvents, including methanol, ethyl acetate and deionized water, combined with ultrasonication.

The responses of MDMA after extraction of ecstasy tablets with various solvents are shown in Figure 3.12. The values of mean±SD of peak area ratio of MDMA are as follows: 27.94±0.92 for extraction with deionized water combined with ultrasonication, 25.19±0.22 for using methanol combined with ultrasonication, 19.24±1.13 for using ethyl acetate combined with ultrasonication and 24.86±0.46 for using methanol alone. MDMA responses after extraction with either methanol or ethyl acetate combined with ultrasonication were significantly lower than those after extraction with deionized water combined with ultrasonication (p<0.05). This indicated that extraction with deionized water followed by ultrasonication was the

most efficient. This is due to high polarity index when compared with methanol and ethyl acetate (Table 3.3). Extraction efficiency of solvent depends on polarity index, dipole moment and solubility in water. All analytes are polar compound. Therefore, the solvents with similar properties would provide good extraction efficiency. However, water is not suitable for GC-FID system because, it can cause the formation of iron oxide of collector resulting to the reduction of the detector performance. Since ethyl acetate extraction was inefficient because it has low polarity index, using methanol was reasonable. MDMA responses after extraction with methanol followed by ultrasonication were not significantly different from those of methanol alone (without ultrasonication) (Figure 3.12). Therefore, in order to simplify sample preparation procedure and save time, extraction with methanol without ultrasonication is appropriate for ecstasy tablet sample preparation in this study.

Table 3.3 Polarity index and solubility in water of studied solvents

Solvents	**Polarity index**	**Solubility in water (%w/w)**
Ethyl acetate	4.4	8.7
Methanol	5.1	100
Deionized water	10.2	100

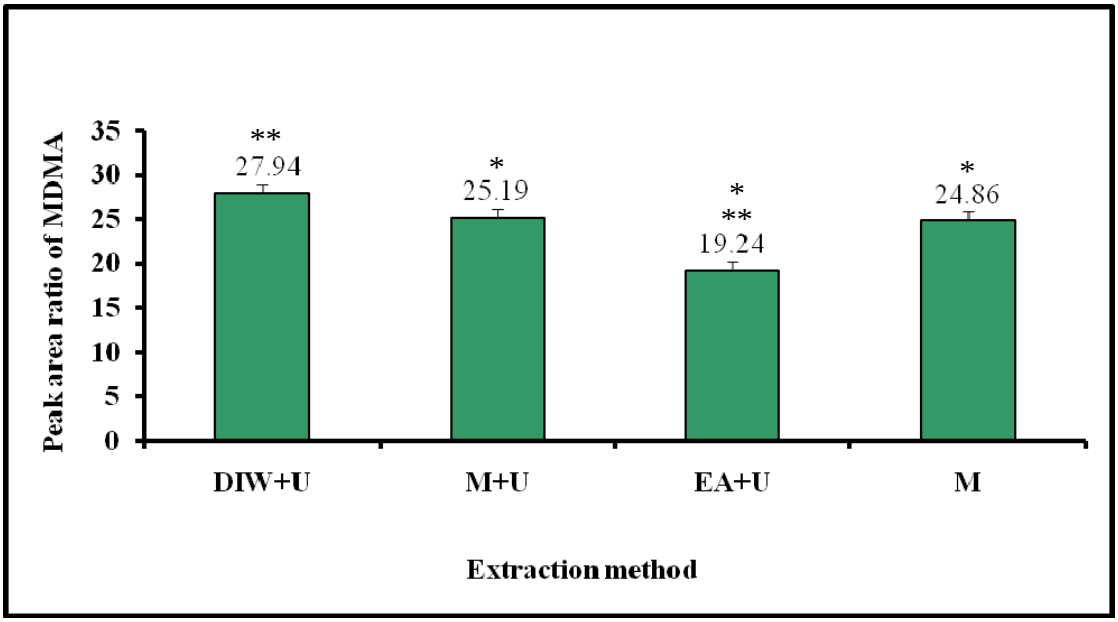


Figure 3.12 Responses (mean±SD) of MDMA after extraction of ecstasy tablets with different solvents (n=3): DIW, deionized water; M, methanol; EA, ethyl acetate; U, ultrasonication

*Indicated that the results are significantly different from deionized water combined with ultrasonication at $p<0.05$

** Indicated that the results are significantly different from methanol at $p<0.05$

3.2.3 Chromatographic profile of a standard mixture of MDMA, MDA and MA

Chromatographic separation of a standard mixture of MDMA, MDA, and MA (10 μg mL^{-1}) is shown in Figure 3.13. All analytes and the internal standard were well separated with no interference within 10.0 min. The retention times were 4.31, 6.93, 7.21 and 9.33 min for MA, MDA, MDMA and DPA, respectively. DPA was a good internal standard because it caused no chromatographic interference with the analytes.

The separation times of MDMA, MDA, MA and DPA obtained from the present study were longer than those reported by Mitrevski and Zdravkovski (2005), *i.e* 9 vs. 6 min. This is due to the difference in the column length (*i.e.* 30 vs. 15 m). However, the time required for each run or each sample injection of this study was shorter, *i.e*. 10.5 vs. 15 min.

Jimenez and coworkers (2006) used a derivatizing agent to produce a more stable and less polar form leading to an improvement of chromatographic profile, such as better separation, more symmetrical peaks and greater sensitivity. However, derivatization has several inherent disadvantages over direct analysis: it involves more complicate preparation step (s); it requires more time and carries the risk of introducing impurities and, in some cases, strong acid by-products are obtained which strongly affect the characteristics of the column (Mitrevski and Zdravkovski, 2005). However, derivatization is not necessary in the present study.

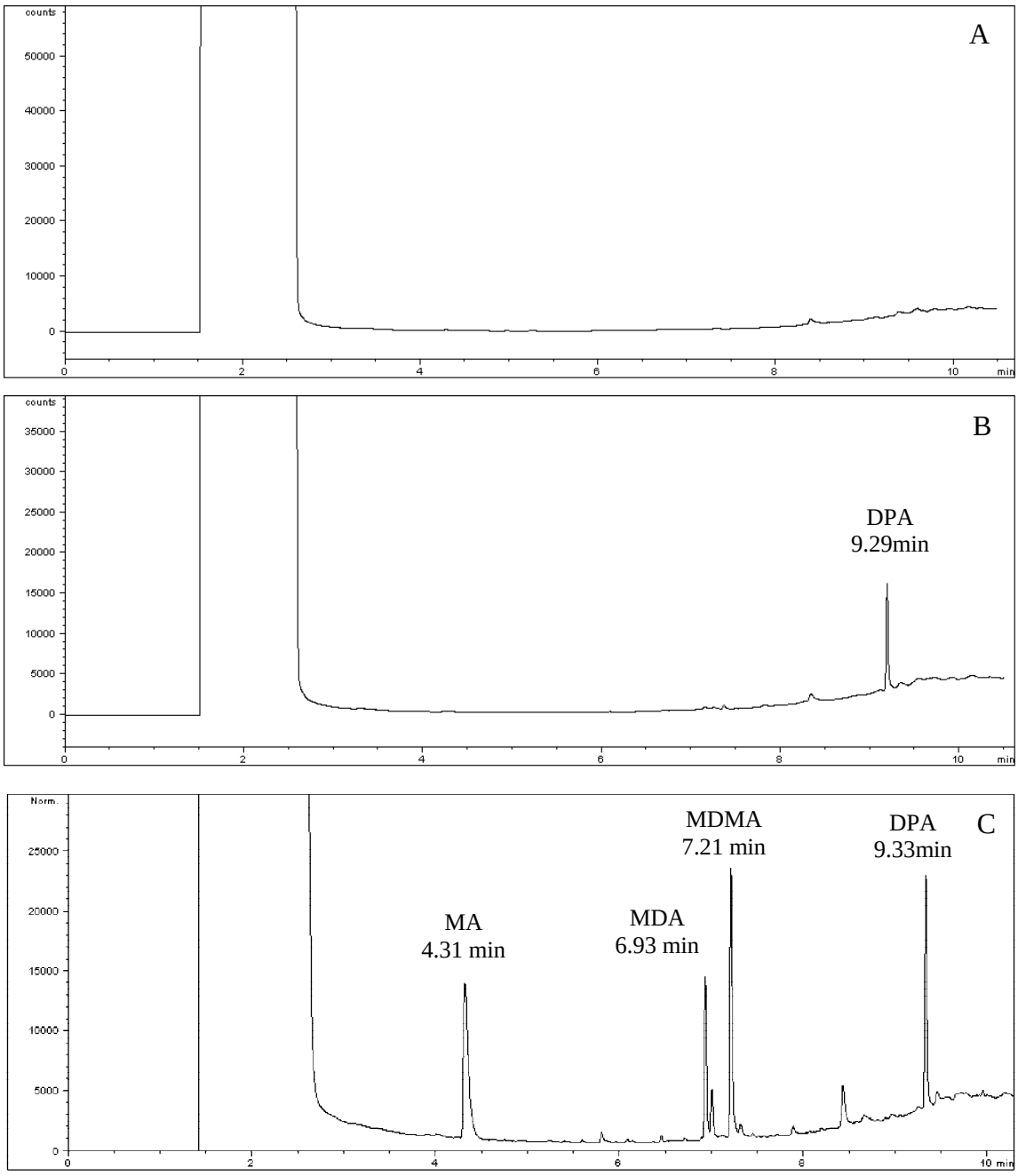


Figure 3.13 Representative chromatograms of standard MDMA, MDA and MA in methanol; (A) methanol blank; (B) internal standard (DPA) blank (10 μg mL^{-1}); (C) standard mixture of MDMA, MDA and MA (10 μg mL^{-1}) and DPA (10 μg mL^{-1}) in methanol

3.2.4 Preparation and analysis of ecstasy tablet samples

The optimum condition of GC-FID was used to determine purity of MDMA in ecstasy tablets seized in Songkhla province during 2007-2008. For qualitative analysis, retention times of the MDMA, MDA and MA in ecstasy tablet samples were compared with those of the standards to identify their peaks. According to a standard mixture, the average retention times were 4.26±0.08 min for MA, and 6.85±0.05 min for MDA, and 7.09±0.05 min for MDMA (n=5). The average retention times of MDMA and MA in ecstasy tablets samples were 7.06±0.05 min (n=17) and 4.24±0.02 min (n=2), respectively. The representative chromatograms of seized tablets are shown in Figure 3.14.

In this work, the method used to determine the analytes in ecstasy tablets, resulting in good separation and symmetrical peaks. The capillary column coated with 50% phenyl/50% dimethylpolysiloxane is a good choice for separation of these amphetamines and their analogues, as well as for the chosen internal standard, since they were eluted as symmetrical peaks at a wide range of concentrations. The method has a wider linear range compared with the previous GC-MS methods (Mitrevski and Zdravkovski, 2005). It is especially useful for analysis of seized tablets with a wide range of possible concentrations of MDMA, MDA and MA. Our method, in conjunction with preliminary methods such as color reaction and thin layer chromatography, provide identification test and simultaneous determination of MDMA, MDA and MA in ecstasy tablets.

For quantitative analysis, peak responses (area ratio) of MDMA, MDA and MA in ecstasy tablets samples were used. Quantities of these compounds are shown in Table 3.4. Among eighteen different tablets, almost all of them contained only MDMA as an active ingredient with purity varying considerably ranging from 1.69 to 80.90% w/w or 4.57 to 243.51 mg/tablet. One tablet with a ‘Tree’ logo also contained a detectable amount of caffeine. HPLC analysis of ecstasy tablets seized in Japan between 2000 and 2001 revealed that these tablets contained MDMA, MDA, MA and other components such as ephedrine, caffeine and ketamine (Raikos *et al.*, 2003). Thirty-five tablets contained MDMA as the sole active ingredient. The content range (calculated as purity MDMA) was 37-160 mg/tablet. One tablet contained MDA alone calculated as a purity of at 75 mg/tablet. Ecstasy tablets seized in Taiwan between

2002 and 2005 contained only MDMA. Analysis with GC-MS showed that the MDMA contents varied from 16 to 193 mg/tablet (Teng *et al.*, 2006). In addition, the ecstasy tablets seized in Chonburi province during 1998 to 2004 (173) contained only MDMA as an active ingredient with purity varying considerably ranging from 60 to 180 mg/tablet. Other tablet contained MA and other components such as caffeine, ketamine, ephedrine, nimetazepam and nitrazepam. The Bureau of Drugs reported that the ecstasy tablets seized in Thailand during 1995 to 2002 (179 sample) contained only MDMA as an active ingredient with purity varying considerably ranging from 52 to 200 mg/tablet. Other tablet contained MA, ketamine, ephedrine and other components such as phenethylamine (2C-B), MDA, MBDB, amphetamine, pemoline and paracetamol. A data of this study can be used as a guideline for inspection of suspicious drugs in the future (Kosin and Qawnaom., 2006).

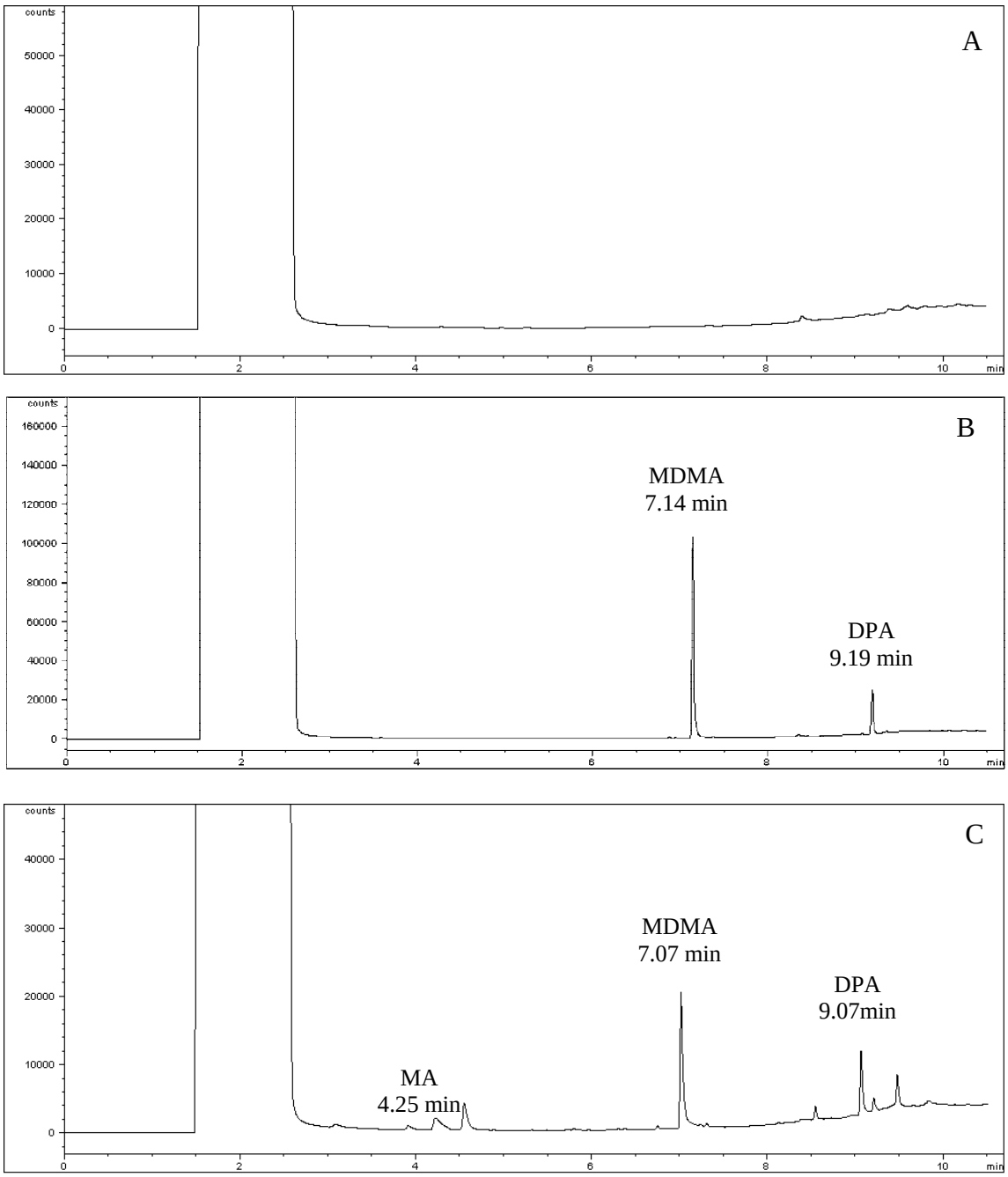


Figure 3.14 Representative chromatograms of ecstasy tablet samples; (A) methanol blank; (B) the ecstasy tablet sample no.1; (C) the ecstasy tablet sample no.14

Table 3.4 Purity of MDMA, MDA and MA in samples of ecstasy tablets seized in Songkhla province from 2007-2008

Sample no.	Amount (mg) [Purity (%, w/w)]		
	MDMA	**MDA**	**MA**
1	43.85 [16.24]	ND	ND
2	80.28 [27.59]	ND	ND
3	57.24 [28.06]	ND	ND
4	73.92 [23.69]	ND	ND
5	123.61 [40.13]	ND	ND
6	202.25 [43.97]	ND	ND
7	8.27 [2.67]	ND	30.07 [9.70]
8	ND	ND	ND
9	39.6 [14.67]	ND	ND
10	202.03 [63.73]	ND	ND
11	243.51 [80.90]	ND	ND
12	216.09 [77.73]	ND	ND
13	113.82 [34.70]	ND	ND
14	79.24 [28.00]	ND	12.26 [4.33]

Table 3.4 Purity of MDMA in samples of ecstasy tablets seized in Songkhla province from 2007-2008 (cont.)

Sample no.	Amount (mg) [Purity (%, w/w)]		
	MDMA	**MDA**	**MA**
15	4.57 [1.69]	ND	ND
16	182.71 [64.33]	ND	ND
17	6.17 [3.16]	ND	ND
18	221.26 [74.00]	ND	ND

ND; not detectable

3.2.5 Method validation for ecstasy tablets analysis

1) Linearity and range

For the analysis of ecstasy tablet samples, regression analysis results showed that the calibration curves were linear over the concentration ranges of 3.125-200 μg mL^{-1} for MDMA and 6.25-200 μg mL^{-1} for MDA and MA (Figure 3.15) (five replicates of each concentration). The regression equation was y = (0.06±0.00) *x*- (0.14±0.09) (r = 0.9993) for MDMA, y = (0.02±0.00) *x*- (0.17±0.06) (r = 0.9980) for MDA and y = (0.10±0.00) *x*- (0.07±0.22) (r = 0.9989) for MA (Table 3.5). When *x* is the concentrations of MDMA, MDA and MA and y is the peak area ratio.

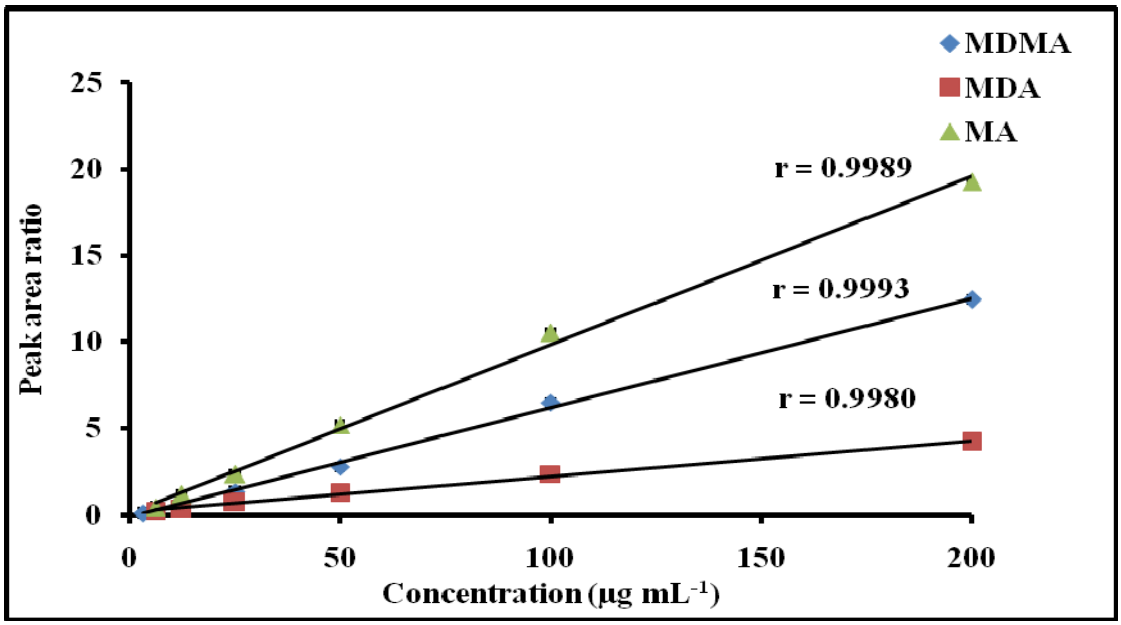


Figure 3.15 Linearity plots of mean peak area ratio±SD against different concentrations of MDMA, MDA and MA spiked in methanol; correlation coefficient (r) = 0.9993 for MDMA, 0.9980 for MDA and 0.9989 for MA (n=5)

Table 3.5 Linear regression analysis of MDMA, MDA and MA (n=5)

Analyte	**Range of linearity (µg mL^{-1})**	**Calibration equation** [a]	**Correlation coefficient (r)**
MDMA	3.125-200	(0.06±0.00) *x* – (0.14±0.09)	0.9993
MDA	6.25-200	(0.02±0.00) *x* + (0.17±0.06)	0.9980
MA	6.25-200	(0.10±0.00) *x* + (0.07±0.22)	0.9989

[a] Mean ± SEM

2) Precision

Both intra-day (repeatability) and inter-day (reproducibility) precisions were determined using four quality control (QC) samples prepared by dissolving MDMA (3.125, 25, 100 and 200 µg mL^{-1}), MDA and MA (6.25, 25, 100 and 200 µg mL^{-1}) in pure methanol. Intra-day precision was determined by analyzing five samples of each concentration during the same day under the same instrumental operating condition. Inter-day precision were determined by analyzing of the samples for five consecutive days. Intra- and inter-day precisions for determining MDMA, MDA and MA are shown in Table 3.6. The relative standard deviation (%RSD) values ranged from 1.56 to 10.38% for MDMA, 1.15 to 9.15% for MDA and 2.51 to 9.77% for MA. Both values for all analytes were found to be within the acceptable value (15%RSD) (FDA, 2001; Peters and Maurer, 2002). The results indicated that the method of analysis was precise.

3) Accuracy

The accuracy of an analytical method is defined as the degree to which the determined value of analyte in a sample corresponds to the true value. In this work, the accuracy was determined by using four QC samples of standard MDMA (3.125, 25, 100 and 200 µg mL^{-1}), MDA and MA (6.25, 25, 100 and 200 µg mL^{-1}) in methanol (five replicates for each concentration). The accuracy was expressed using the deviation (%DEV) and the results are shown in Table 3.6. The accuracy (%DEV) ranged from (-) 7.11 to (+) 17.51% for MDMA, (-) 7.18 to (+) 11.72% for MDA and (-) 19.17 to (+) 6.91 for MA. The results for determination of all analytes ranged between ±15% and ±20% for the concentration at LLOQ (FDA, 2001).

Table 3.6 Precision and accuracy of the method for determination MDMA, MDA and MA in ecstasy tablets (n=5)

Analytes	Concentration ($\mu g\ mL^{-1}$)	Precision (%RSD)		Accuracy (%DEV)	
		Intra-day	Inter-day	Intra-day	Inter-day
MDMA	200	2.92	1.95	-0.57	-0.23
	100	2.92	1.56	+4.59	+1.12
	25	3.02	6.03	-7.11	-2.92
	3.125	10.38	7.08	+17.51	+3.34
MDA	200	3.59	4.84	-2.87	+0.34
	100	2.32	1.15	+8.51	-1.34
	25	3.22	4.17	+11.72	+5.20
	6.25	3.55	9.15	-7.18	-6.24
MA	200	3.70	3.78	-1.34	-0.68
	100	3.06	4.10	+6.91	+3.08
	25	6.58	2.51	-2.56	-6.84
	6.25	6.50	9.77	-19.17	-0.71

4) Lower limit of quantification (LLOQ)

For ecstasy tablet samples, the LLOQ was determined by using five calibration curves of standard MDMA in methanol at concentrations of 3.125, 25, 100 and 200 $\mu g\ mL^{-1}$ and MDA and MA in methanol at concentrations of 6.25, 25, 100 and 200 $\mu g\ mL^{-1}$. It was found that the LLOQ of MDMA was 3.125 $\mu g\ mL^{-1}$ while those of MDA and MA were 6.25 $\mu g\ mL^{-1}$. The sensitivity of the method was in the range of micrograms per component on-column, being quite enough for analysis MDMA and their analogues in ecstasy tablets. The LLOQs obtained with this method were higher than those shown by Mitrevski and Zdravkovski (2005) detecting amphetamine, MA, MDA, MDMA and MDEA with GC-FID. This may be due to the age of the instrument or capillary column resulting to a decrease in the sensitivity of the analysis.

5) Stability

5.1) Short-term stability

The standard mixture of MDMA, MDA and MA in methanol at low (10 µg mL^{-1}) and high (100 µg mL^{-1}) concentrations (three replicates of each concentration) were left at room temperature for 24 h. They were analyzed at 0, 6, 12, 18 and 24 h. The measured concentrations as percentage at varying time and that at the baseline (t=0) were compared. Although the percentage of concentrations tended to decrease during 24 h, but there was no significant difference (Table 3.7). The results indicated that the standard solutions of MDMA, MDA and MA were stable during 24 h storage at room temperature (21ºC).

5.2) Long-term stability

Stock solution (200 µg mL^{-1}) of a mixture of MDMA, MDA and MA was prepared. An aliquot of 100 µL and 10 µL was made to produce different amounts (10 µg and 100 µg) of each compound. The aliquots were dried using centrivap concentrator. The residues were kept at -20ºC in a freezer for 2 months. On the day of analysis, the residues were dissolved in pure methanol. The measured amounts as percentage at 1 and 2 month (s) storage were compared (three replicates) with those at the baseline (0 month). Table 3.7 shows that after 1 month storage, the percentage amounts of all analytes both at low and high amounts were not significantly different from those at the baseline, except at high amount of MDA. However, the percentage amounts after 2 month storage significantly decreased compared with those at baseline both at low and high amounts ($p<0.05$).

The results indicated that keeping the stock solution at -20ºC for 1 month caused no change in the stability (Table 3.8). However, an approximate 35-67% loss occurred after 2 months storage. This is a significant degradation of the compounds. These findings correspond with those of Paul and coworkers (1993), who investigated the effect of freezing (at -16ºC to -18ºC) on the concentrations of amphetamine and methamphetamine in spiked urine samples stored for 45 days. Our observations are also in accordance with those obtained by Jimenez and coworkers (2006) reported that non relevant changes in concentrations were also observed for MDMA and MA after

storage at 4ºC and -20ºC for 6 months, comparing with initial concentration in non-sterile samples.

Table 3.7 The percentage response of short-term stability of MDMA, MDA and MA at high and low concentrations (n=3)

Stability	**Concentration (μg mL^{-1})**					
	MDMA		**MDA**		**MA**	
	100	**10**	**100**	**10**	**100**	**10**
Short-term						
0 h	100	100	100	100	100	100
6 h	97.8±1.5	94.3±9.1	94.6±10.8	100.0±4.4	99.5±7.9	99.7±5.0
12 h	97.8±5.9	105.7±5.9	89.8±5.5	99.0±7.2	99.1±9.1	99.7±3.4
18 h	93.9±3.6	102.8±9.1	87.7±16.2	92.3±8.7	98.4±14.5	93.2±6.6
24 h	92.3±5.0	100.0±12.8	82.2±7.0	98.1±7.6	94.3±5.4	94.3±11.5

The data (mean±SD) represent measured concentrations as percentage compared with baseline (t=0 h)

*Indicated that the results are significantly different from baseline (t= 0 h) at $p<0.05$

Table 3.8 The percentage response of long-term stability of MDMA, MDA and MA at high and low amounts (n=3)

Stability	Amount (μg)					
	MDMA		MDA		MA	
	100	10	100	10	100	10
Long-term						
0 m	100	100	100	100	100	100
1 m	98.7±3.2	93.9±2.6	78.5±3.3*	93.9±3.0	99.7±0.9	100.4±6.8
2 m	65.3±4.4*	33.3±1.4*	57.1±0.6*	33.3±9.1*	38.0±1.6*	55.7±2.9*

The data (mean±SD) represent measured concentrations as percentage compared with baseline (t=0 month)

*Indicated that the results are significantly different from baseline (t= 0 month) at $p<0.05$

The biggest problem encountered during stability testing for bioanalytical method in forensic and clinical toxicology is the fact that there is a great number of different sampling vessels. Stability must be included in the method validation. Knowledge of the stability of a drug is of importance for toxicologists in several situations, limitation of a logistic nature often introduces variable time intervals between sampling of the matrices and analysis. Even in these kinds of situations, the toxicologist should be able to determine if interpretation of the obtained quantitative data can be performed reliably. It is very important to know the optimal storage condition to keep drugs stable at least during the storage time. Sample and standard should be tested over at least a 24 h period. Stability procedures should evaluate the stability of analytes during sample collection and handling, after long-term and short-term storage.

3.3 Study 3: Determination of MDMA, MDA and MA in human urine

3.3.1 Sample preparation studies

1) pH of sample

Effect of changing of urine pH on the extraction of MDMA, MDA and MA was investigated. Various acidic and basic reagents including 1 N HCl, 1 M CH_3COOH, 1 M HCOOH, 1 N NaOH, 5 M NH_4OH and 1 M KOH were used. Figure 3.16 shows that peak areas of MDMA, MDA and MA, after passing through Oasis® HLB cartridges, in spiked urine adjusted to pH 3 varied among different acidic reagents used. Adjusting with acetic acid resulted in the lowest responses of all analytes compared with hydrochloric- and formic acids adjustment (significantly for MA only; $p<0.05$) while the response of DPA was not detected. This means that most of all analytes were retained when the sample was pretreated with acetic acid. However peak responses after sample alkalinizaion were apparently lower than those obtained from acidification (Figure 3.17). That means higher proportions of analytes were retained in the cartridge. Using sodium hydroxide seems to be in appropriate since it provided significantly higher than peak responses of MA compared with those of ammonium hydroxide and potassium hydroxide. When comparisons ammonium hydroxide and potassium hydroxide were used the basic reagents. The results showed that peak responses of MA were not significantly difference.

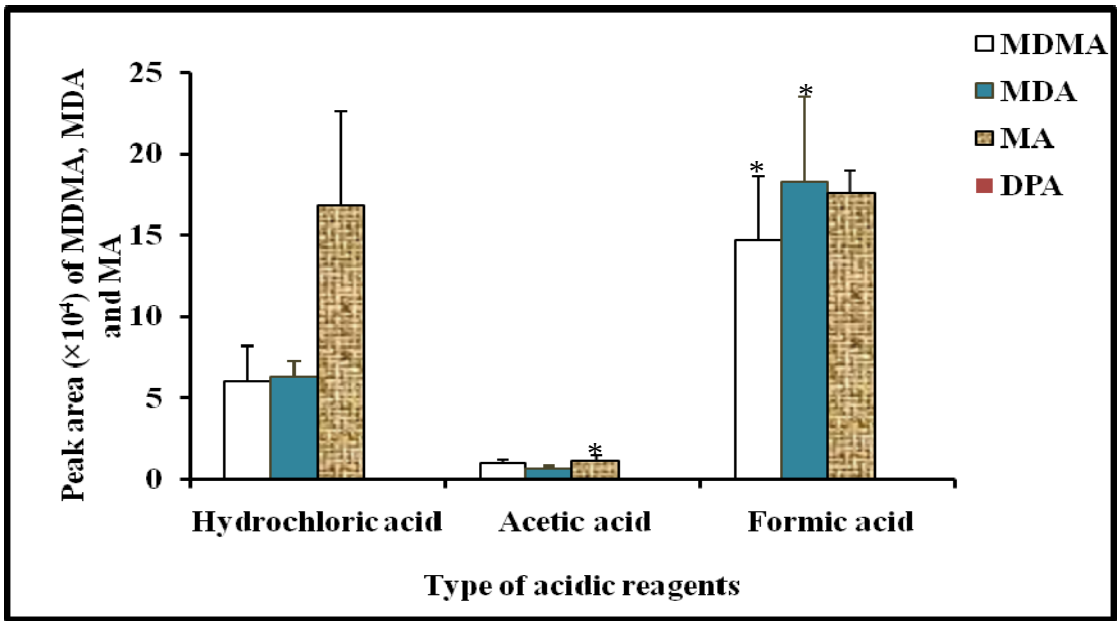


Figure 3.16 Peak responses (mean±SD), after passing through Oasis® HLB cartridge, of MDMA, MDA and MA in spiked human urine samples adjusted to pH 3 using various acidic reagents (n=5)

*Indicated that the results are significantly different from hydrochloric acid at $p<0.05$

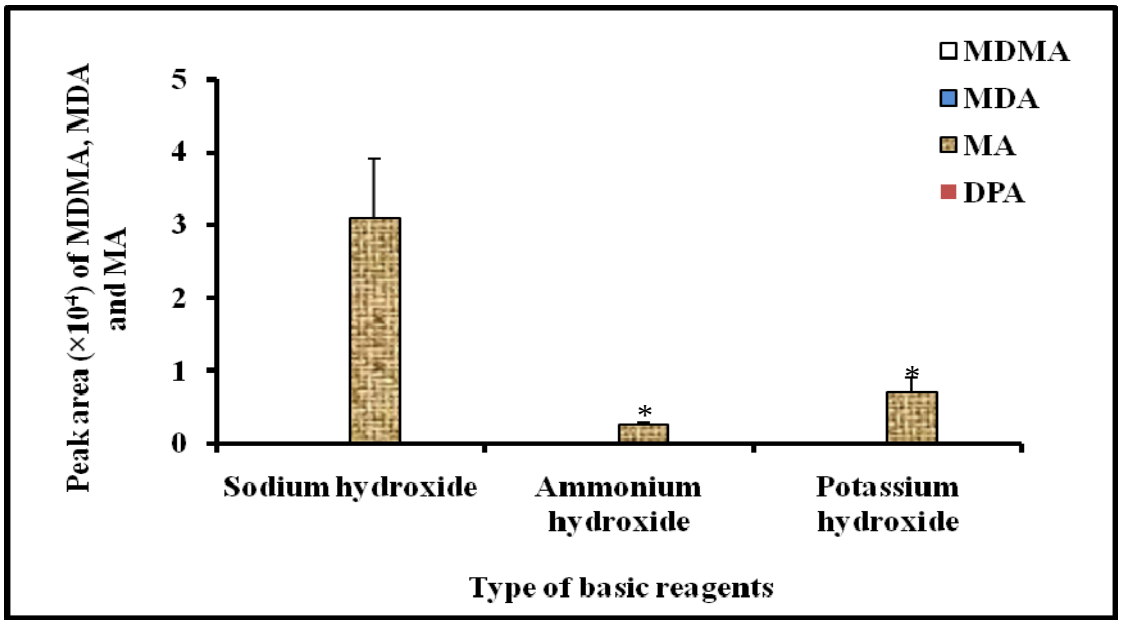


Figure 3.17 Peak responses (mean±SD), after passing through Oasis® HLB cartridge, of MDMA, MDA and MA in spiked human urine samples adjusted to pH 12 using various basic reagents (n=5)

*Indicated that the results are significantly different from sodium hydroxide at $p<0.05$

For the best recovery of extraction, theoretically, the drugs have to be strongly retained on the cartridge during loading and washing steps. The Oasis® HLB cartridges, divinylbenzene-based polymers provide excellent pH stability ranging between pH 0 and 14. This broad pH range allows for increased flexibility in SPE methods development. Varying of pH has an influence on degree of the ionization of the compounds, based on the Henderson-Hasselbach equations _1 and _2.

Acid: $pH = pK_a + \log [I] / [UI]$ equation _1

Base: $pH = pK_a + \log [UI] / [I]$ equation _2

The calculated percentage of unionized form of MDMA, MDA, MA and DPA were expressed in Table 3.9. MDMA, MDA and MA were completely ionized (99%) when urine was acidified to pH 3 while approximately 99% of all analytes were unionized at pH 12. This indicated that at low pH, MDMA, MDA and MA could be lost during loading step because the analytes are mostly in an ionized form, which are less retained in the cartridges. At high pH, MDMA and MDA were highly retained in cartridges even though MA was slightly lost. The analytes which mostly are in an unionized form had higher retention at the high pH than at low pH. Although adjustment with ammonium hydroxide resulted in no loss of MDMA and MDA, potassium hydroxide was considered to be used to adjust pH of human urine samples because it is a strong base, therefore, a small volume is used for adjust pH (*i.e.* 30 μL KOH, 30 μL NaOH and 800 μL NH_4OH).

From this study, it appeared that Oasis® HLB cartridge interacted with unionized compounds stronger than ionized compounds (*i.e.* all analytes were lost at pH 3 and retained in the cartridges at pH 12). The Oasis® HLB cartridges are able to retain both polar and nonpolar compounds through Van der Waals and dipole-dipole interactions (Yawney, 2000).

Table 3.9 Percentage of unionized form of MDMA, MDA, MA and DPA in spiked urine adjusted to pH 3 using various acidic reagents and adjusted to pH 12 using different basic reagents

Reagent type	% Unionized form of analytes			
	MDMA (pK_a 9.5)	**MDA (pK_a 10.0)**	**MA (pK_a 9.9)**	**DPA (pK_a 1.0)**
Acidic reagents				
1 N HCl	0.01	0.01	0.01	98.94
1 M CH_3COOH	0.01	0.01	0.01	98.94
1 M HCOOH	0.01	0.01	0.01	98.94
Basic reagents				
1 N NaOH	99.21	99.01	99.68	100
5 M NH_4OH	99.21	99.01	99.68	100
1 M KOH	99.21	99.01	99.68	100

2) Wash-elute study

Aim of wash-elute study is to determine the percentage of methanol mixture required in the washing and eluting steps of SPE. It was found that when the cartridge was passed with a methanol-water mixture (0-80%, v/v) containing 2% ammonium hydroxide, MDMA, MDA and DPA were not eluted throughout the whole range, while some amount of MA was found in the eluent (Figure 3.18). The results suggested that a methanol-water mixture, 0-80%, could be used as washing solvent. In this work, 5% methanol-water mixture containing 2% ammonium hydroxide was chosen because it could remove most neutral and polar acidic interferences.

After passing the cartridge with a mixture of methanol and water (0-80%, v/v) containing 2% acetic acid, MDMA, MDA and MA were eluted (Figure 3.19). DPA was eluted when percentage of methanol was 30% and over. The peak area of DPA was significantly increased compared with those at 0% of methanol. At 70% of methanol all analytes were mostly eluted because it provided the highest response of all analytes. Peak area of MDMA, MDA and MA were not statistically different from those at 0% of methanol. Therefore, the 70% methanol-water mixture containing 2% acetic acid was selected as the eluting solvent.

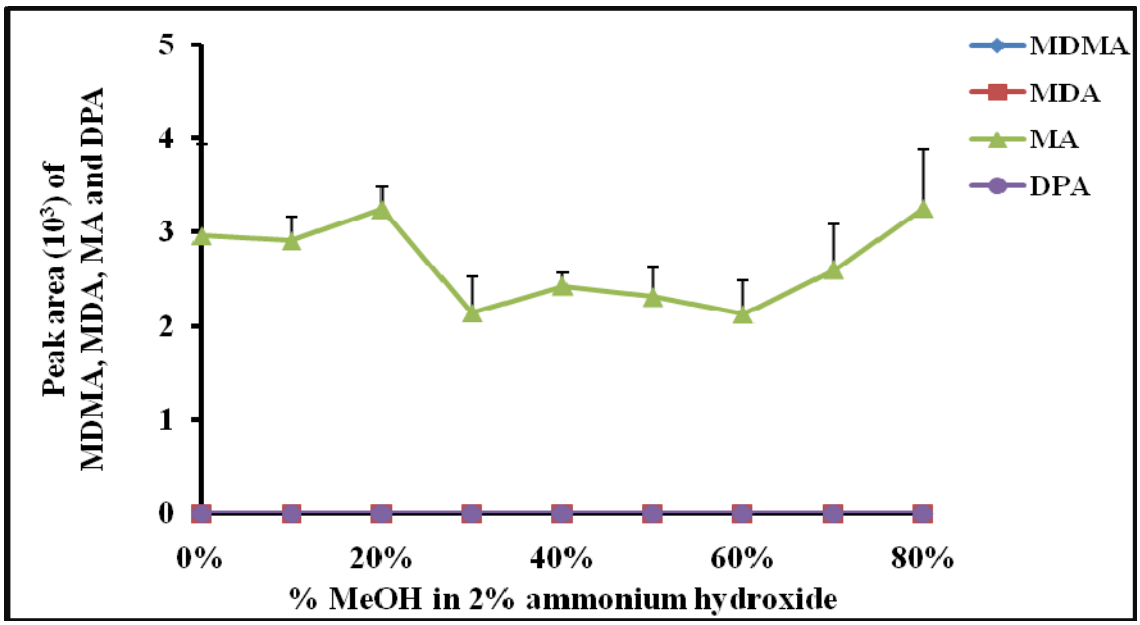


Figure 3.18 Peak responses of MDMA, MDA, MA and DPA in spiked urine passing through Oasis® HLB cartridge and eluted with varying percentage of a methanol-water mixture containing 2% ammonium hydroxide

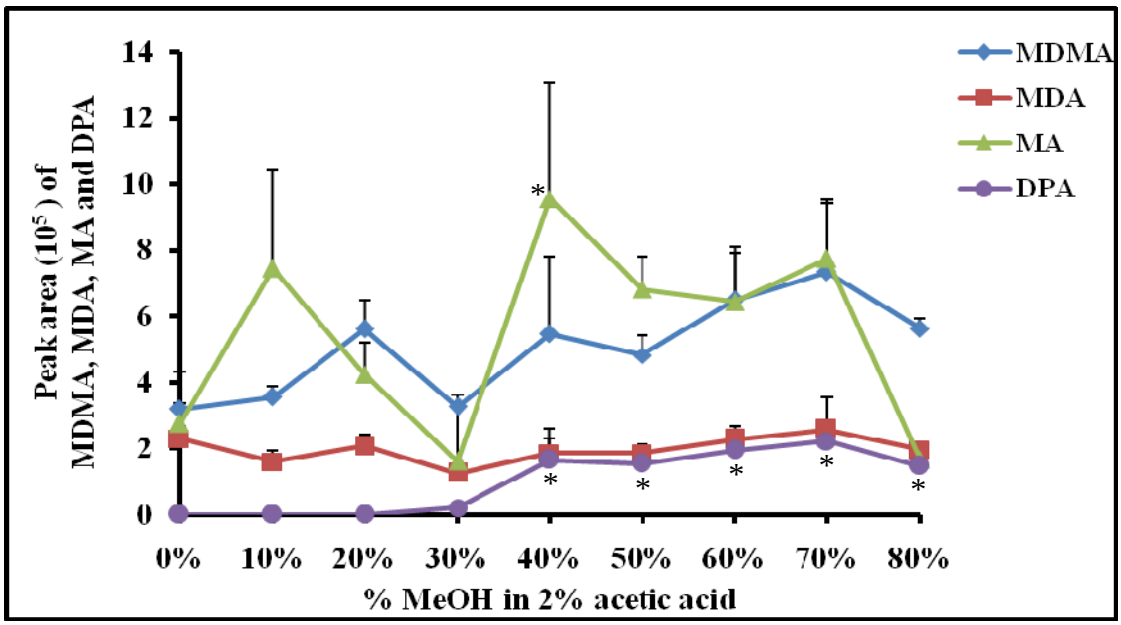


Figure 3.19 Peak responses of MDMA, MDA, MA and DPA in spiked urine passing through Oasis® HLB cartridge and eluted with varying percentage of a methanol-water mixture containing 2% acetic acid

*Indicated that the results are significantly different from 0% methanol at $p<0.05$

3.3.3 Comparison of 2D-SPE methods with one- and two washing step(s)

In this study, extraction of MDMA, MDA, MA and DPA in human urine using 2D-SPE method with one washing and two washing step(s) were compared. After one washing step with 1 mL of 5% methanol-water containing 2% ammonium hydroxide prior to elution with 1 mL of 70% methanol-water mixture containing 2% acetic acid, chromatogram showed good separation of all analytes but some interference were seen (Figure 3.20A). In contrast, two washing steps with 1 mL of 5% methanol-water mixture containing 2% ammonium hydroxide followed by 1 mL of 60% methanol-water mixture containing 2% ammonium hydroxide before elution with 1 mL of 70% methanol-water mixture containing 2% acetic acid resulted in more effective elimination of urine interferences (Figure 3.20B). However, this method decreased sensitivity of MA. Therefore, in this work, the 2D-SPE method with one wash was chosen for extraction of MDMA, MDA and MA in human urine samples. This method was simple and provided high recovery of extraction, better sensitivity and a clean background.

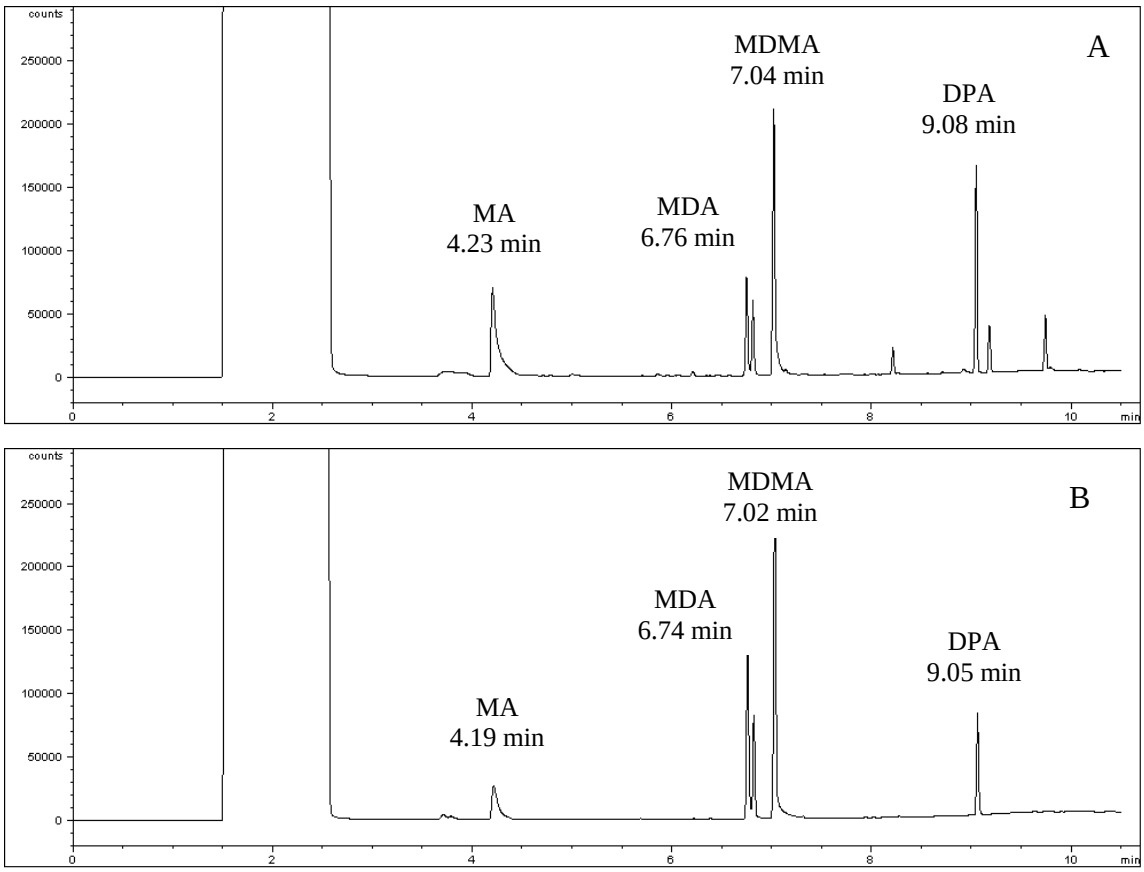


Figure 3.20 Representative chromatograms of extraction of MDMA, MDA and MA in spiked human urine using 2D-SPE method; (A) 2D-SPE method with one wash using 5% methanol-water mixture containing 2% ammonium hydroxide; (B) 2D-SPE method with two washes using 5% methanol-water mixture containing 2% ammonium hydroxide followed by 60% methanol-water mixture containing 2% ammonium hydroxide.

3.3.4 Chromatographic profile of standards MDMA, MDA and MA in human urine samples

The chromatographic separation of standard mixture of MDMA, MDA, and MA in human urine using SPE and GC-FID technique is shown in Figure 3.21. Some unknown peaks were seen in the blank urine (Figure 3.21A). Peaks of MDMA, MDA, MA and DPA in spiked human urine samples were good in shape and well separated from unknown peaks (Figure 3.21B). The retention times of MA, MDA, MDMA and DPA were 4.20, 6.79, 7.06 and 9.06 min, respectively, within a run time of 10.0 min.

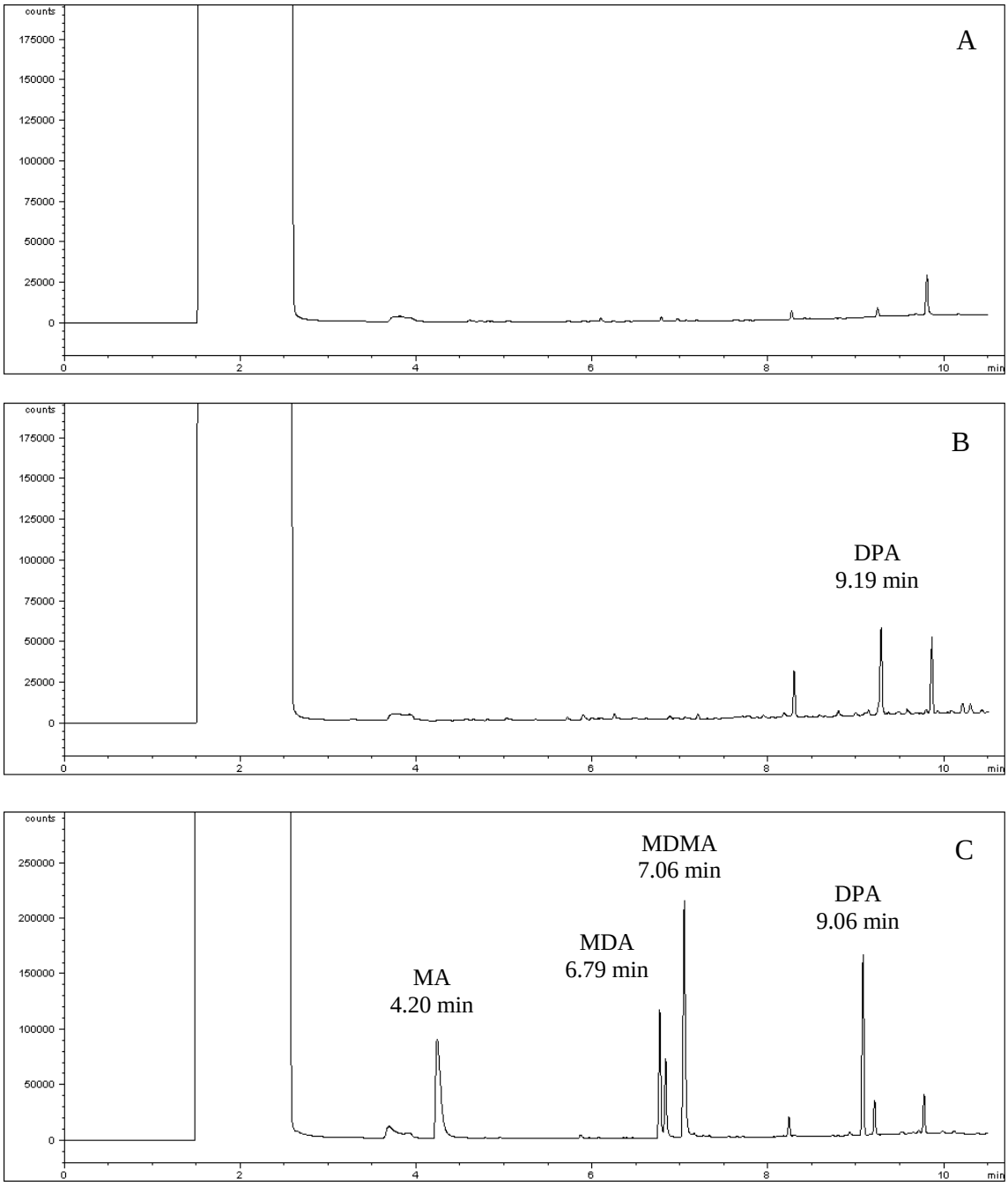


Figure 3.21 Representative chromatograms for MDMA, MDA and MA in human urine; (A) blank urine; (B) blank urine spiked with internal standard (DPA) (5 μg mL^{-1}); (C) blank urine spiked with standard mixture of MDMA, MDA, MA (10 μg mL^{-1}) and DPA (5 μg mL^{-1})

The most undesirable effect in direct separation (*i.e.* without derivatization) of MDMA and its analogues separation is peak tailing in the chromatogram. This makes the acquisition of chromatographic signal information more difficult, affecting retention time, peak area, peak width at half peak height, peak overlapping. etc. (Mitrevski and Zdravkovski, 2005). The method in this work provided good separation, symmetrical peaks, and short analysis time. The capillary column coated with 50% phenyl/50% dimethylpolysiloxane has been shown to be a good choice for separation of MDMA, MDA and MA from human urine, as well as for the chosen internal standard, since the eluted peaks were symmetrical at a wide range of concentrations.

Previous studies used either liquid-liquid extraction (Luaa *et al.*, 2003) or protein precipitation techniques for extracting of drugs of abuse from biological matrixes (McDowall, 1989; Levine, 2003). However, the major disadvantages of both techniques are the need of a large volume of organic solvents, some of which are hazardous and toxic. The methods are expensive and the operators are laborious and automation is difficult. Emulsion formation and waste problems are also associated with these methods, as well as difficulties in handling small sample volumes. Recently, solid phase microextraction (SPME) has been used for extracting of amphetamines in biological fluids. Although, this new method is simple, solvent-free and easily automated, it has not been widely used due to its high cost, worse precision and more critical optimization procedure (Huang and Zhang, 2003). In the present method, solid phase extraction (SPE) was used for extracting MDMA, MDA and MA from human urine samples. The alkalinized (pH 12) human urine samples were loaded onto Oasis® HLB cartridges. The reason for using a high pH value was to increase a fraction of unionized form of basic analytes to be retained in cartridges. The cartridges were washed with 5% methanol-water mixture containing 2% ammonium hydroxide to remove proteins in the human urine matrix while components were still in the cartridges. All analytes were eluted with 70% methanol-water mixture containing 2% acetic acid. At this low pH condition, basic analytes are ionized and therefore, easily removed from the cartridges. The extraction method proposed in this study was simple and the samples were cleaned effectively with high

recovery (> 80.35%) for all analytes. Furthermore, the method was highly specific to MDMA, MDA and MA in urine samples and no back-extraction step is needed.

3.3.5 Preparation and analysis of urine samples

For qualitative analysis, MDMA, MDA and MA detected from human urine samples were identified using their retention times, compared with those of the standards. The average retention time of standard mixture spiked in human urine was 4.21±0.01 min for MA and 6.74±0.01 min for MDA, and 7.02±0.01 min for MDMA (n=5). The representative chromatogram of human urine sample no.11 containing MDMA, MDA and MA is shown in Figure 3.22.

Quantitative analysis of urinary concentrations of MDMA, MDA and MA are shown in Table 3.10. The concentrations ranged from 1.36 to 20.59 μg mL^{-1} for MDMA, 1.65 to 4.12 μg mL^{-1} for MDA, and 1.80 to 19.98 μg mL^{-1} for MA. This method was successful in the verification of recreational drug use since its sensitivity was concordant with the cut-off value recommended by the Narcotics Act of B.E.2543 (2000) (No.3) of Thailand *i.e.* 1 μg mL^{-1} for MDMA, MDA and MA confirmation in human urine. MDMA, MDA and MA were found together in human urine sample no. 14-18 and 20. It is likely that the suspects took both ecstasy and methamphetamine (YaBa) tablets. For other four urine samples (no. 1, 5, 7 and 8), MDMA and MDA but not MA, were detected. These suspects probably took only ecstasy tablets.

The presence of MDMA in urine after drug ingestion indicates that urine, which is an easily accessible human biological fluid, is an appropriate collecting matrix for confirmation of MDMA abuse. Luaa and coworkers (2003) reported that the urinary concentrations of MDA and MDMA in human urine samples were 5.60 and 1.90 μg mL^{-1}, respectively. In cases of death caused by ecstasy overdose, the urinary concentration of MDMA reached upto 170 μg mL^{-1} and 4 μg mL^{-1} for MDA (Letter *et al.*, 2002). Weinmann and Bohnert (1998) reported, respectively, 201, 7.1 and 0.135 μg mL^{-1} of MDEA, MDA and MDMA urinary concentrations in over dose case.

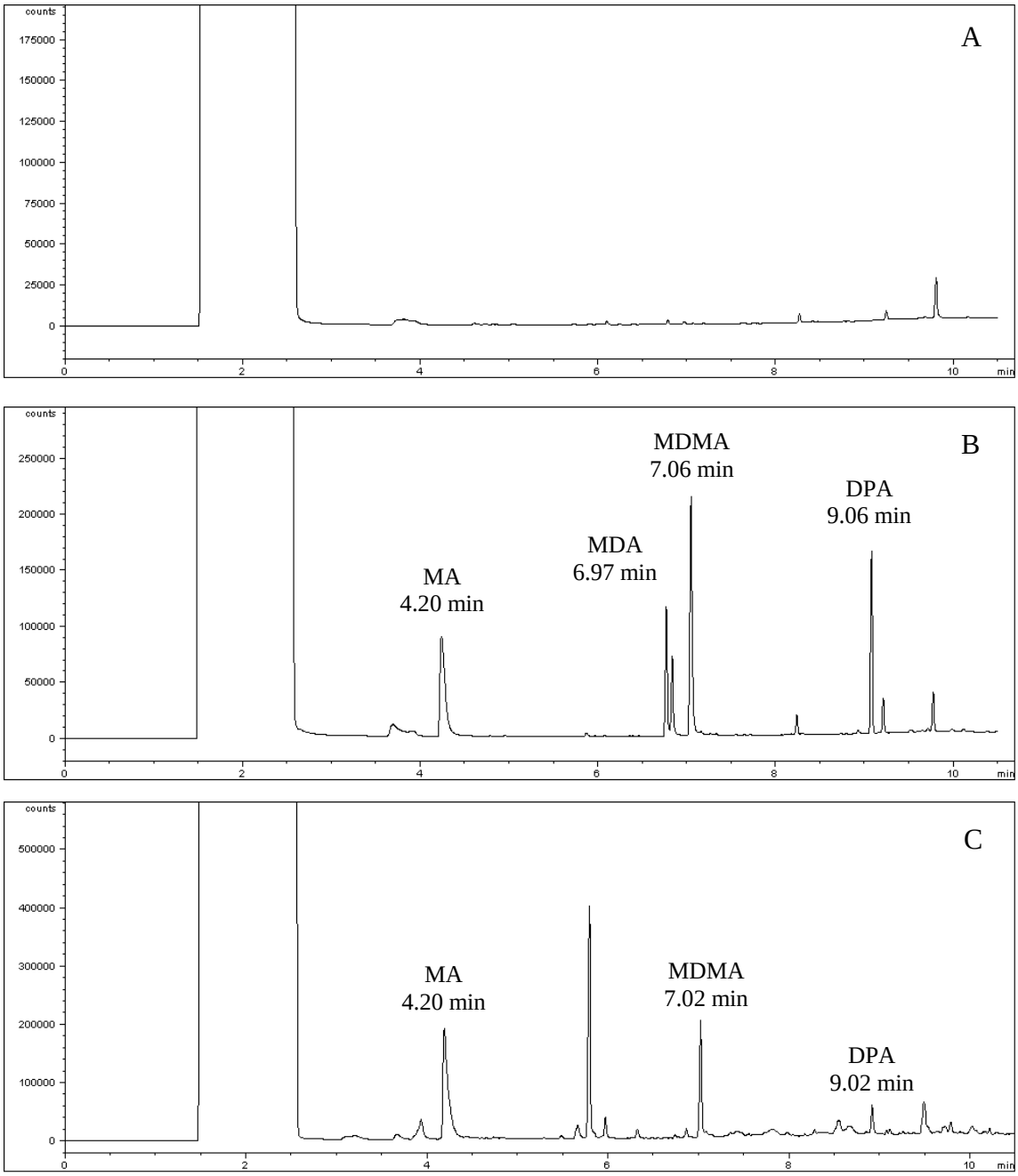


Figure 3.22 Representative chromatograms of human urine sample no.11 containing MDMA and MA; (A) blank urine; (B) blank urine spiked with standard mixture of MDMA, MDA, MA (10 μg mL^{-1}) and DPA (5 μg mL^{-1}); (C) human urine sample no.11

Table 3.10 Concentrations of MDMA, MDA and MA in urine of drug abuse suspects

Sample no.	Concentration (μg mL^{-1})		
	MDMA	**MDA**	**MA**
1	16.99	1.65	ND
2	2.34	ND	5.80
3	6.65	ND	18.50
4	5.98	ND	ND
5	2.93	< LLOQ	6.01
6	8.05	ND	18.46
7	< LLOQ	< LLOQ	1.80
8	6.19	< LLOQ	8.12
9	14.23	ND	ND
10	16.54	ND	ND
11	19.34	ND	19.98
12	ND	ND	12.52
13	20.59	ND	ND
14	3.42	3.68	2.39
15	16.77	3.57	12.42
16	3.26	1.83	12.85
17	2.43	1.96	7.41
18	1.36	4.12	7.45
19	12.01	ND	ND
20	2.17	1.76	5.67

ND; not detectable

3.3.6 Method validation for urine analysis

1) Linearity and range

Regression analysis results showed that the calibration curves were linear over the concentration ranges of 1-20 µg mL^{-1} for MDMA, MDA and MA (Figure 3.23) (five replicates of each concentration). The regression equation was y = (0.17±0.01) *x*- (0.04±0.03) (r = 1.0000) for MDMA, y = (0.05±0.01) *x*- (0.02±0.02) (r = 0.9971) for MDA and y = (0.14±0.01) *x*- (0.11±0.05) (r = 0.9998) for MA (Table 3.11). When *x* is the MDMA, MDA and MA concentrations and y is the peak area ratio.

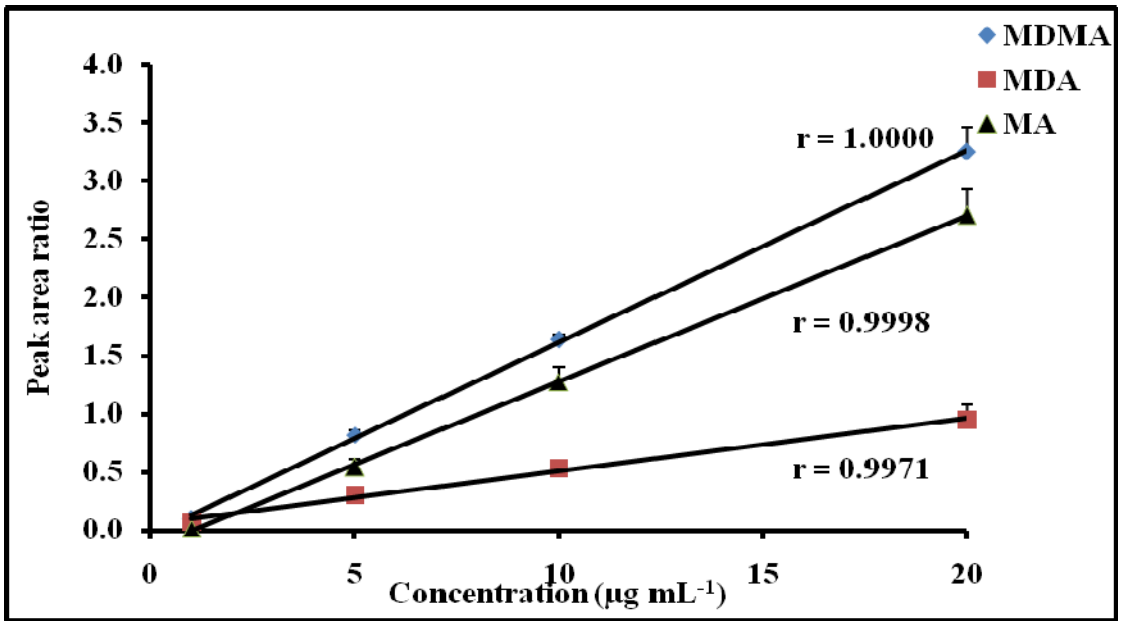


Figure 3.23 Linearity plot of mean peak area ratio±SD against different concentrations of MDMA, MDA and MA spiked in human urine; correlation coefficient (r) = 1.0000 for MDMA, 0.9971 for MDA and 0.9998 for MA, respectively (n=5)

Table 3.11 Linear regression analysis of MDMA, MDA and MA (n=5)

Analyte	Range of linearity (μg mL^{-1})	Calibration equation [a]	Correlation coefficient (r)
MDMA	1-20	(0.17±0.01) *x* - (0.04±0.03)	1.0000
MDA	1-20	(0.05±0.01) *x* + (0.02±0.02)	0.9971
MA	1-20	(0.14±0.01) *x* - (0.11±0.05)	0.9998

[a] Mean ± SEM

2) Precision

Both intra-day (repeatability) and inter-day (reproducibility) precisions were determined using four QC samples obtained by spiking of MDMA, MDA and MA in blank urine (1, 5, 10 and 20 μg mL^{-1}). Intra-day precision was determined by analyzing five samples of each concentration during the same day under the same instrumental operating condition. Inter-day precision were determined by analyzing of the samples for five consecutive days. Intra- and inter-day precisions for determining MDMA, MDA and MA are shown in Table 3.12. The relative standard deviation (% RSD) values ranged from 2.97 to 17.31% for MDMA, 6.28 to 16.54% for MDA and 6.86 to 17.18% for MA, respectively. Both intra- and inter-day precisions for all analytes were found to be within the acceptable value, *i.e.* 15%RSD for the concentrations above LLOQ and 20%RSD for the concentration at LLOQ (FDA, 2001; Peters and Maurer, 2002).

3) Accuracy

The accuracy was determined by using four QC samples of standards MDMA, MDA and MA in urine blanks at the concentrations of 1, 5, 10 and 20 μg mL^{-1} (three replicates for each concentration). The results are shown in Table 3.12. The accuracy (%DEV) ranged from (-) 16.68 to (+) 4.28% for MDMA, (-) 18.10 to (+) 14.94% for MDA and (-) 18.47 to (+) 0.60 for MA, respectively. The accuracy (%DEV) for determination of all analytes were ±15% and ±20% for the concentration at LLOQ (FDA, 2001).

Table 3.12 Precision and accuracy of the method for determination MDMA, MDA and MA in blank urine samples (n=5)

Analyte	Concentration ($\mu g\ mL^{-1}$)	Precision (%RSD)		Accuracy (%DEV)	
		Intra-day	Inter-day	Intra-day	Inter-day
MDMA	20	6.43	10.87	-0.54	-0.11
	10	2.97	10.45	+1.49	-0.89
	5	4.53	14.20	+4.28	+5.30
	1	17.31	12.18	-16.68	-16.55
MDA	20	13.23	12.98	-2.61	+0.08
	10	6.99	6.68	+6.75	-2.50
	5	11.63	6.28	+14.94	+7.75
	1	16.54	11.93	-17.62	-18.10
MA	20	8.43	8.17	+0.60	+0.36
	10	10.05	7.43	-0.89	-0.91
	5	13.39	6.86	-6.92	-3.58
	1	17.18	11.64	-8.72	-18.47

4) Recovery

The recovery of an analyte in an assay is the comparison of the detector response obtained from an amount of the analyte added to and extracted from the biological matrix with that obtained for the true concentration of the pure authentic standard. Recovery pertains to the extraction efficiency of an analytical method within the limit of variability (FDA, 2001).

The results of extraction recovery for determining of MDMA, MDA and MA in human urine are shown in Table 3.13. The mean percentages of recovery were 84.17 to 95.01% for MDMA, and 92.56 to 107.60% for MDA and 80.35-90.74% for MA. The percentage of recovery in this study was higher than that obtained from other studies. For examples, Pellegrini and coworkers (2002), who used LLE method to extract amphetamine and their analogues in urine samples prior to detecting with GC-MS, reported that the percentages of recovery were ranged from 62 to 66% for

amphetamine and 73 to 85% for MDMA. Raikos and coworkers (2003) determined levels of amphetamine in human urine by headspace solid-phase microextraction (HS-SPME) and gas chromatography and reported that the percentages of recovery were in the range of 19.50% to 47.00% for amphetamine and 20.00% to 38.10 for MA. Another work determined designer drugs in human urine of consumer using the Bond Elut Certify® SPE (Pichini *et al.*, 2008). The authors reported that the percentages of recovery were ranged from 73.80 to 79.70% for MDMA. The results of the present study indicate that the 2D-SPE method using Oasis® HLB cartridges with one wash was highly efficient for the extracting of MDMA, MDA and MA from human urine samples.

Table 3.13 Extraction recovery for determination of MDMA, MDA and MA in human urine samples (n=3)

Compound	**Concentration ($\mu g\ mL^{-1}$)**	**Mean peak area ratio**		**%Recovery[a]**
		Direct injection	**After extraction**	
MDMA	20	3.311	3.112	93.98 (5.71)
	10	1.905	1.753	92.02 (3.68)
	5	1.061	1.008	95.01 (7.02)
	1	0.139	0.117	84.17 (2.48)
MDA	20	2.327	2.227	95.70 (9.62)
	10	1.238	1.160	93.70 (9.04)
	5	0.667	0.718	107.60 (10.21)
	1	0.103	0.095	92.56 (8.26)
MA	20	3.033	2.752	90.74 (6.93)
	10	1.604	1.390	86.64 (9.80)
	5	0.945	0.759	80.35 (4.78)
	1	0.163	0.145	88.75 (9.44)

[a]Mean (SD)

5) Lower limit of quantification (LLOQ)

For human urine samples, LLOQ was determined by using five calibration curves of standard MDMA, MDA and MA spiked in urine samples at concentrations of 0.5, 1, 10 and 20 µg mL^{-1}. The results showed that LLOQ of all analytes were 1 µg mL^{-1}. The LLOQ obtained with this method was higher than those obtained from Raikos and coworkers (2003) who also used GC-FID technique (LOQ for MDA, MDMA and MDEA; 172.5, 177.0 and 181.0 ng mL^{-1}, respectively). When compared with a selective detector such as nitrogen-phosphorus detector (NPD). The nitrogen-phosphorus detector (NPD) was provided lower LOQ value than the present work (Ortuno *et al.*, 1999; Soriano *et al.*, 2001). This may be due to the age of the instrument or capillary column resulting in a decrease in the sensitivity of the analysis. However, the sensitivity of the present method was sufficiently high for determining MDMA, MDA and MA in human urine samples.

CHAPTER 4

Conclusions

The present study described the method for direct separation and determination of MDMA, MDA and MA in ecstasy tablets and human urine samples of suspects seized in Songkhla province by GC-FID. Chromatographic separation was carried out by CP-SIL 24 CB WCOT fused silica capillary column of 0.25 μm film thickness (30 m × 0.32 mm i.d.). Condition for GC-FID was optimized and the results are shown in Table 4.1.

Table 4.1 The optimum condition of GC-FID for simultaneous MDMA, MDA and MA analysis

Parameter		**Optimum condition**
Gas flow rate	Carrier gas (N_2)	2.60 mL min^{-1}
	Fuel gas (H_2)	30 mL min^{-1}
	Oxidant gas (O_2)	300 mL min^{-1}
	Make-up gas (N_2)	30 mL min^{-1}
Column Temperature	Initial (holding time)	80 ºC (0 min)
	Final (holding time)	270 ºC (1 min)
	Ramp rate	20ºC min^{-1}
Injector temperature		290ºC
Detector temperature		300ºC

This method was simple, sensitive, precise and accurate to simultaneously determine MDMA, MDA and MA in ecstasy tablets and human urines. This GC-FID method was successfully applied to determine the purity of MDMA in ecstasy tablets and quantified the MDMA, MDA and MA levels in human urine of drug abuse suspects. This technique is universal and cost-saving and facilitates routine analytical work. It would be useful for the laboratories of the Forensic Science Division or any departments responsible for identifying narcotic substances. The information about purity of MDMA and other substances in ecstasy tablets and human urine samples is often necessary to verify drug ingestion of suspects. This can be used as an evidence to support the trial case, narcotic offence, punishment and narcotic seizure reward

Preparation of ecstasy tablet samples involved simple steps of solvent extraction resulting in sufficiently high recovery of extraction. Urine samples were prepared by using solid-phase extraction with Oasis® HLB cartridges, containing divinylbenzene-based polymers stationary phase. The samples were prepared by alkalinizing with 30 µL of 1 M KOH before loading into Oasis® HLB cartridges. The cartridges was washed with 5% methanol-water mixture containing 2% ammonium hydroxide and eluted with 70% methanol-water mixture containing 2% acetic acid. This method was simple and provided high recovery of extraction, better sensitivity, a clean background and no derivatization.

The drawback of this method is only the sensitivity. Generally, the flame ionization detection is less sensitive than the mass spectrometry detection. This method is therefore impossible for quantifying the analyte existed in the nanogram level. Nevertheless, this method is less time consuming. Since this method has short run time, a great number of samples can be run within a given time. This method is, therefore, appropriate for the work which the sample are routinely sent for analysis.

Future works are to modify this method to simultaneously determine MDMA, MDA, MA and other drugs such as amphetamine, MDEA, caffeine, ketamine and ephedrine in seized tablets and blood, urine or other biological fluids. The method involving with isolation of MDMA and its metabolites from fluids biological matrices such as, plasma, serum, tissues and saliva should also be developed. In Forensic Science, the physical characteristic of ecstasy tablets such as diameter, thickness, weigh, color and logo is the database. The chemical characteristic of ecstasy tablets

have to be investigated in term of organic components and traces is generally created as drug profile. This drug profile is useful for investigation. This information could help law enforcement agencies to trace about source, batch and manufacturer of the seized tablets.

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Appendix-1

Chemical properties of diphenylamine (DPA)

Diphenylamine ($C_{12}H_{11}N$) or DPA (Figure 1) is a secondary amine with a molecular weight of 169.22 a.m.u. DPA is a base drug with a pKa around 1.03. It is white, crystalline solid with a melting point range of 52-54ºC which has a boiling point of 302ºC. It is soluble in water, acetonitrile, methanol, octanol and hexane. DPA is widely used as a rubber antioxidant and accelerator, an insecticide (directly and by fusion with sulfur), solid fuel rocket propellant, stabilizer for explosives, preparation of azo dyes, pharmaceuticals, veterinary medicine, storage preservation of apples, and as a reagent in analytical chemistry.

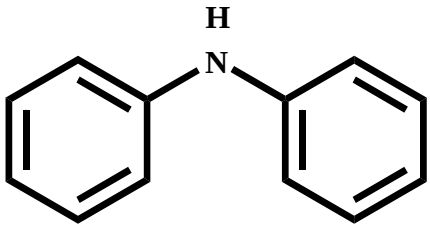

Figure 1 Chemical structure of DPA

Appendix-2

Table 1 The height equivalent to a theoretical plate (HETP) of MDMA, MDA and MA (5 µg mL^{-1}) standard solution at various carrier gas flow rates (n=5)

Flow rate (mL min^{-1})	**Mean HETP [x10^{-3} cm (%RSD)]**		
	MDMA [x10^{-3} cm]	**MDA [x10^{-3} cm]**	**MA [x10^{-1} cm]**
2.30	6.88 (6.19)	6.71 (6.80)	2.90 (8.61)
2.60	6.78 (6.31)	6.08 (8.65)	2.74 (7.29)
2.90	7.55 (6.08)	6.91 (6.22)	2.96 (9.86)
3.20	10.20 (8.16)	8.34 (9.45)	5.55 (16.91)
3.50	8.15 (3.77)	7.58 (3.55)	3.27 (13.66)

Table 2 Peak areas of MDMA, MDA and MA (5 µg mL^{-1}) in a mixture of standard solutions at various fuel gas flow rates (n=5)

Fuel gas flow rate (mL min^{-1})	**Peak area (Count); (%RSD)**		
	MDMA×(10^3)	**MDA×(10^3)**	**MA×(10^3)**
20	5.89 (3.28)	4.31 (5.14)	6.86 (3.42)
30	8.55 (3.28)*	5.85 (6.37)*	9.38 (1.74)*
40	7.51 (2.88)*	5.28 (7.30)*	8.47 (3.73)*
50	7.41 (4.87)*	3.87 (5.04)	7.19 (5.42)

* Indicated that the results are significantly different from fuel gas flow rates of 20 mL min^{-1} at $p<0.05$

Table 3 Peak areas of MDMA, MDA and MA (5 μg mL^{-1}) in a mixture of standard solutions at various oxidant gas flow rates (n=5)

Oxidant gas flow rate (mL min^{-1})	**Peak area (Count); (%RSD)**		
	MDMA×(10^3)	**MDA×(10^3)**	**MA×(10^3)**
200	6.32 (2.17)	4.30 (2.97)	7.72 (2.40)
300	8.30 (1.99)*	5.83 (5.59)*	10.19 (5.62)*
400	8.82 (3.46)*	5.85 (4.82)*	10.45 (2.78)*
500	8.94 (3.33)*	5.75 (5.73)*	10.57 (5.19)*

* Indicated that the results are significantly different from oxidant gas flow rates of 200 mL min^{-1} at $p<0.05$

Table 4 Peak areas of MDMA, MDA and MA (5 μg mL^{-1}) in a mixture of standard solutions at various make-up gas flow rates (n=5)

Make-up gas flow rate (mL min^{-1})	**Peak area (Count); (%RSD)**		
	MDMA×(10^3)	**MDA×(10^3)**	**MA×(10^3)**
10	6.32 (5.70)	4.58 (7.33)	7.71 (3.05)
20	6.65 (3.96)	4.74 (4.00)	7.93 (2.20)
30	7.72 (4.36)*	5.09 (4.95)	9.12 (1.56)*
40	5.68 (6.39)	3.73 (6.55)*	7.03 (5.97)*
50	4.20 (6.96)*	2.54 (6.98)*	4.91 (4.35)*

* Indicated that the results are significantly different from make-up gas flow rates of 10 mL min^{-1} at $p<0.05$

Table 5 Peak areas of MDMA, MDA and MA (5 μg mL^{-1}) in a mixture of standard solutions at various initial temperatures (n=5)

Initial temperature (°C)	**Peak area (Count); (%RSD)**			**Analysis time (min)**
	MDMA×(10^3)	**MDA×(10^3)**	**MA×(10^3)**	
70	5.58 (3.14)	5.42 (5.32)	12.51 (2.94)	12.00
80	8.20 (3.02)*	6.21 (2.64)*	11.10 (3.81)*	11.50*
90	9.33 (3.70)*	5.40 (3.12)	11.02 (4.45)*	11.00*
100	10.15 (2.79)*	5.19 (4.72)	11.62 (3.80)	10.50*
110	9.09 (5.92)*	3.80 (6.00)*	9.89 (5.93)*	10.00*
120	9.52 (14.32)*	3.49 (5.16)*	10.16 (6.12)*	9.50*

* Indicated that the results are significantly different from initial temperature of 70ºC at $p<0.05$

Table 6 Peak areas of MDMA, MDA and MA (5 μg mL^{-1}) in a mixture of standard solutions at various holding time of initial temperature (n=5)

Holding time of initial temperature (min)	**Peak area (Count); (%RSD)**			**Analysis time (min)**
	MDMA×(10^3)	**MDA×(10^3)**	**MA×(10^3)**	
0	9.66 (4.56)	6.60 (4.92)	12.91 (5.46)	10.50
1	7.75 (2.17)*	6.26 (2.69)	12.79 (4.02)	11.50*
2	6.56 (4.33)*	5.43 (5.41)*	12.34 (5.25)	12.50*
3	7.56(5.81)*	6.30 (7.47)	12.33 (3.78)	13.50*

* Indicated that the results are significantly different from initial holding time of 0 min at $p<0.05$

Table 7 Peak areas of MDMA, MDA and MA (5 μg mL^{-1}) in a mixture of standard solutions at various ramp rates (n=5)

Ramp rate	**Peak area (Count); (%RSD)**			**Analysis time**
(°C min^{-1})	**MDMA×(10^3)**	**MDA×(10^3)**	**MA×(10^3)**	**(min)**
15	9.48 (5.10)	6.83 (4.13)	13.72 (5.00)	13.67
20	9.41 (3.05)	6.34 (2.81)	12.27 (6.27)	10.50*
25	9.34 (7.00)	6.41 (5.67)	13.15 (6.74)	8.60*
30	9.00 (5.54)	5.57 (5.79)	12.09 (5.49)	7.33*

* Indicated that the results are significantly different from ramp rates of 15ºC min^{-1} at $p<0.05$

Table 8 Peak areas of MDMA, MDA and MA (5 μg mL^{-1}) in a mixture of standard solutions at various final temperatures (n=5)

Final temperature	**Peak area (Count); (%RSD)**			**Analysis time**
(°C)	**MDMA×(10^3)**	**MDA×(10^3)**	**MA×(10^3)**	**(min)**
270	9.78 (4.11)	6.48 (3.78)	12.75 (2.07)	13.67
280	8.77 (2.34)*	5.76 (5.13)*	12.69 (3.73)	10.50*
290	9.07 (3.83)	6.04 (3.30)	12.66 (5.36)	8.60*
300	8.17 (6.25)*	5.59 (4.13)*	12.17 (6.34)	7.33*

* Indicated that the results are significantly different from final temperature of 270ºC at $p<0.05$

Table 9 Peak areas of MDMA, MDA and MA (5 μg mL^{-1}) in a mixture of standard solutions at various holding time of final temperature (n=5)

Holding time of final temperature (min)	**Peak area (Count); (%RSD)**			**Analysis time (min)**
	MDMA×(10^3)	**MDA×(10^3)**	**MA×(10^3)**	
0	6.45 (4.64)	5.08 (3.04)	10.94 (2.03)	9.50
1	6.90 (6.90)	5.38 (5.23)	12.21 (5.86)*	10.50*
2	7.28 (6.44)*	5.36 (1.15)	11.91 (1.90)*	11.50*
3	7.56 (4.46)*	5.51 (2.34)*	11.82 (4.44)	12.50*

* Indicated that the results are significantly different from holding time of final temperature of 0 min at $p<0.05$

Table 10 Peak areas of MDMA, MDA and MA (5 μg mL^{-1}) in a mixture of standard solutions at various injector temperatures (n=5)

Injector temperature (ºC)	**Peak area (Count); (%RSD)**		
	MDMA×(10^3)	**MDA×(10^3)**	**MA×(10^3)**
260	9.89 (3.45)	3.15 (3.93)	10.94 (2.03)
270	9.19 (5.49)	4.16 (4.36)*	12.21 (5.86)
280	9.14 (3.21)	3.76 (4.36)*	11.91 (1.90)
290	9.16 (3.98)	4.68 (4.92)*	10.61 (4.18)
300	6.61 (4.21)*	4.47 (3.85)*	8.05 (5.49)*

* Indicated that the results are significantly different from injector temperature of 260ºC at $p<0.05$

Table 11 Peak areas of MDMA, MDA and MA (5 μg mL^{-1}) in a mixture of standard solutions at various detector temperatures (n=5)

Detector temperature (ºC)	**Peak area (Count); (%RSD)**		
	MDMA×(10^3)	**MDA×(10^3)**	**MA×(10^3)**
260	7.83 (2.63)	3.87 (1.49)	9.43 (1.53)
270	8.24 (3.90)	3.96 (5.32)	9.32 (2.05)
280	8.95 (5.42)*	4.35 (6.69)	10.47 (5.30)
290	8.92 (4.41)*	4.76 (5.53)*	10.82 (5.19)*
300	9.83 (6.61)*	5.02 (4.67)*	11.78 (6.21)*

* Indicated that the results are significantly different from detector temperature of 260ºC at $p<0.05$

Appendix-3

Table 12 Peak responses (mean±SD), after passing through Oasis® HLB cartridge, of MDMA, MDA and MA in spiked human urine samples adjusted to pH 3 using various acidic reagents (n=5)

Type of acidic reagent	Peak area (Count); (SD)			
	MDMA×(10^4)	MDA×(10^4)	MA×(10^4)	DPA×(10^4)
Hydrochloric acid	5.99 (22.23)	6.27 (0.97)	16.83 (5.84)	ND
Acetic acid	0.93 (0.24)	0.64 (0.19)	1.13 (0.32)*	ND
Formic acid	14.71 (3.91)*	18.29 (5.25)*	17.65 (1.39)	ND

*Indicated that the results are significantly different from hydrochloric acid at $p<0.05$

ND; not detectable

Table 13 Peak responses (mean±SD), after passing through Oasis® HLB cartridge, of MDMA, MDA and MA in spiked human urine samples adjusted to pH 12 using various basic reagents (n=5)

Type of basic reagent	Peak area (Count); (SD)			
	MDMA×(10^4)	MDA×(10^4)	MA×(10^4)	DPA×(10^4)
Sodium hydroxide	ND	ND	3.10 (0.81)	ND
Ammonium hydroxide	ND	ND	0.30 (0.03)*	ND
Potassium hydroxide	ND	ND	0.70 (0.21)*	ND

*Indicated that the results are significantly different from sodium hydroxide at $p<0.05$

ND; not detectable

Appendix-4

Table 14 Peak responses of MDMA, MDA, MA and DPA in spiked urine passing through Oasis® HLB cartridge and eluted with varying percentage of a methanol-water mixture containing 2% ammonium hydroxide

% MeOH	Peak area (Count); (SD)			
	MDMA×(10^3)	MDA×(10^3)	MA×(10^3)	DPA×(10^3)
0	ND	ND	2.97 (0.97)	ND
10	ND	ND	2.91(0.25)	ND
20	ND	ND	3.24 (0.24)	ND
30	ND	ND	2.14 (0.39)	ND
40	ND	ND	2.43 (0.14)	ND
50	ND	ND	2.31 (0.32)	ND
60	ND	ND	2.13 (0.37)	ND
70	ND	ND	2.60 (0.49)	ND
80	ND	ND	3.25 (0.63)	ND

ND; not detectable

Table 15 Peak responses of MDMA, MDA, MA and DPA in spiked urine passing through Oasis® HLB cartridge and eluted with varying percentage of a methanol-water mixture containing 2% acetic acid

% MeOH	Peak area (Count); (SD)			
	MDMA×(10^5)	MDA×(10^5)	MA×(10^5)	DPA×(10^5)
0	3.19 (0.17)	2.29 (0.39)	2.76 (1.54)	ND
10	3.56 (0.32)	1.60 (0.34)	7.45 (2.99)	ND
20	5.61 (0.86)	2.07 (0.33)	4.24 (0.97)	ND
30	3.26 (0.36)	1.25 (0.25)	1.63 (1.75)	0.20 (0.03)*
40	5.47 (2.35)	1.85 (0.75)	9.55 (3.54)*	1.66 (0.65)*
50	4.82 (0.62)	1.86 (0.28)	6.83 (0.97)	1.54 (0.17)*
60	6.48 (1.42)	2.29 (0.38)	6.43 (1.65)	1.95 (0.31)*
70	7.33 (2.09)	2.58 (0.96)	7.73 (1.78)	2.22 (0.65)*
80	5.63 (0.31)	1.96 (0.22)	1.67 (0.15)	1.48 (0.10)*

*Indicated that the results are significantly different from 0% methanol at $p<0.05$

ND; not detectable

Table 16 Responses (mean±SD) of MDMA after extraction of ecstasy tablets with different solvents (n=3)

Type of solvent extraction	Peak area ratio (%RSD)
Deionized water + ultrasonication	27.94 (3.29)**
Methanol + ultrasonication	25.19 (0.88)*
Ethyl acetate + ultrasonication	19.24 (5.85)*,**
Methanol	24.86 (1.85)*

*Indicated that the results are significantly different from deionized water combined with ultrasonication at $p<0.05$

** Indicated that the results are significantly different from methanol at $p<0.05$

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Poster Presentation

Phonchai, A., Janchawee, B., Prutipanlai, S. and Thainchaiwattana, S. Purity Determination of 3,4-Methylenedioxymethamphetamine (MDMA) in Ecstasy Tablets Seized in Songkhla Province Using GC-FID Technique. 34th Congress on Science and Technology of Thailand, Bangkok, Thailand, 2007.