การดัดแปรวิธี QuEChERS เพื่อการตรวจวัดมอร์ฟีนและโคเดอีนในปัสสาวะ

นาง ชานิดา แสงสุริย์

MODIFICATION OF QuEChERS METHOD FOR DETERMINATION OF MORPHINE AND CODEINE IN URINE

Mrs. Chanida Sangsuri

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By   Mrs. Chanida Sangsuri

Field of Study   Chemistry

Thesis Advisor   Assistant Professor Natchanun Leepipatpiboon, Dr.,rer.nat.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

………………………………………Dean of the Faculty of Science

(Professor Supot Hannongbua, Dr.,rer.nat.)

THESIS COMMITTEE

…………………………………………. Chairman

(Associate Professor Sirirat Kokpol, Ph.D.)

…………………………………………. Thesis Advisor

(Assistant Professor Natchanun Leepipatpiboon, Dr.,rer.nat.)

……………………………………… Examiner

(Assistant Professor Pakorn Varanusupakul, Ph.D.)

……………………………………… External Examiner

(Assistant Professor Panthira Ketkaew, Ph.D.)

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วิธีการหนึ่งในการพิสูจน์ผู้เสพเฮโรอีน คือการตรวจหามอร์ฟีนและโคเดอีนในปัสสาวะ
การเตรียมตัวอย่างค่อนข้างยุ่งยาก ใช้เวลาและปริมาณตัวอย่างมาก งานวิจัยนี้ศึกษาการเตรียม
ตัวอย่างโดยใช้เทคนิค QuEChERS (Quick Easy Cheap Effective Rugged and Safe) อาศัยหลักการ
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แผ่นบางสมรรถนะสูง และเทคนิคแก๊สโครมาโทกราฟี แมสสเปกโทรเมตรี โดยเทคนิคโคร
มาโทกราฟีแบบแผ่นบางสมรรถนะสูง ใช้ตัวอย่างปัสสาวะ 5 มิลลิลิตร สกัดในตัวทำละลาย
เอทิลอะซิเตต แยกชั้นสารละลายสกัดด้วยแมกนีเซียมซัลเฟต และกำจัดสารรบกวนในขั้นตอน
สุดท้ายด้วยตัวดูดซับอะลูมินา สำหรับเทคนิคแก๊สโครมาโทกราฟี แมสสเปกโทรเมตรีใช้ตัวอย่าง
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สาขาวิชา	เคมี	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก
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CHANIDA SANGSURI: MODIFICATION OF QuEChERS METHOD FOR DETERMINATION OF MORPHINE AND CODEINE IN URINE. ADVISOR: ASST. PROF. NATCHANUN LEEPIPATPIBOON, Dr.,rer.nat, 68 pp.

One way of proving persons taking heroin is the detection of trace amounts of morphine and codeine in the defendant's urine. The test may be complicated, by being time consuming and requiring a large amount of samples. The samples must be enriched enough for the test. A QuEChERS (Quick Easy Cheap Effective Rugged and Safe) method is literally introduced for use in the test process. It has been successfully modified for the urine tested samples by using an alumina-N sorbent as the dispersive solid-phase extraction to reduce matrix interferences and to achieve the enrichment. The treated sample was detected by a High Performance Thin Layer Chromatography (HPTLC) and Gas chromatography–mass spectrometry (GC–MS). The proposed procedure required 5 mL and 1 mL of a urine sample, respectively. It was found that the limit of morphine and codeine detection by HPTLC were 100 ng/mL and 300 ng/mL, respectively, and by GC–MS were 39.1 ng/mL and 40.6 ng/mL, respectively. This method is quick, easy, cheap, effective, rugged, safe, and applicable in a routine work. Especially for the HPTLC method, with the QuEChERS, is cheaper than other methods. This proposed method solves the problem of a limited amount of samples. The remaining sample can be used for further confirmation procedures as required by Thai legislation.

Department : _____Chemistry_____ Student's Signature _____

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LIST OF ABBREVIATIONS

Alumina-N	Alumina neutral
AOAC	Association of Official Analytical Chemist
BSTFA	N, O-bis(Trimethylsilyl)trifluoroacetamide
CI	Chemical Ionization
cm	centimeter
COD	Codine
C_{18}	Octadecyl
°C	degree celsius
DAD	Diode array detector
d-SPE	dispersive solid phase extraction
EI	Electron Impact Ionization
EtOAc	ethyl acetate
FAB	Fast atom bombardment
GC	gas chromatography
GC-MS	gas chromatography mass spectrometry
GC-NPD	gas chromatography-nitrogen-phosphorous detection
GCB	graphitized carbon black
g	gram
HCl	Hydrochloric acid
He	Helium
HPLC	High performance liquid chromatography
HPTLC	High performance Thin Layer chromatography
H_2	gas hydrogen
ICP-MS	Inductively coupled plasma mass spectrometry
Kpa	kilopascal
LC	liquid chromatography
LC-ESI	liquid chromatography-electrospray ionisation
LC-MS	liquid chromatography tandem mass spectrometry
LLE	liquid-liquid extraction
LOD	limits of detection
LOQ	limits of quantification

MeCN	acetonitrile
MeOH	Methanol
MOR	Morphine
MW	Molecular weight
M3G	morphine-3-glucuronide
M6G	morphine-6-glucuronide
6-MAM	6-acetylmorphine
min	minute
mg	milligram
mL	milliliter
N_2	gas nitrogen
NH_2	Aminopropyl
ng/mL	nanogram per milliliter
PSA	primary secondary amine
QuEChERS	Quick Easy Cheap Effective Rugged and Safe
RF	radio-frequency
RIA	Radio Immune Assay
R.S.D.	relative standard deviation
R^2	correlation of determination
S.D.	standard deviation
SIM	selected ion monitoring
S/N	signal to noise ratio
SPE	solid-phase extraction
TMCS	trimethylchlorosilane
TOF	Time-of-Flight Mass Analyzer
TLC	thin-layer chromatography
t_R	retention times
UV	ultraviolet
v/v	volume by volume
µg/mL	microgram per milliliter
µL	microliter
µm	micrometer
%	percentage

CHAPTER I

INTRODUCTION

1.1 Problem Definition

Detection of morphine and codeine in the urine of Heroin addicts was the purpose of this study to prove drug abuse within national legislation *(1)*. From urine samples which were sent to the Regional Medical Science Center 4th Samut Songkhram from 2007 to 2010 it was found that in drug addicts, who take more than one type of narcotic, have increased year on year. Morphine was found in drug abusers who take more than one type of narcotic. This can be found along with Methamphetamine and Cannabinoid. Even in small numbers of drug abusers who take more than one type of narcotic, it is also of importance for data preparing policies in surveillance and the suppression of narcotics in Thailand. Volumes of sample which are used for narcotic identification vary by the type of narcotic, for some tests consume high volumes of sample. For morphine confirmation the method currently used in routine is Thin layer Chromatogarphy (TLC). Two systems for developing a solvent are required, requiring a volume of 25 mL for one system, and need to use a sample of 50 mL per test. Many samples did not contain enough volume to test for levels of morphine because they consume in high volume and in some cases need to tested for more than one type of narcotic. Preliminary testing for morphine in urine uses a test kit which has a cut-off level 300 ng/mL because it is specified under legislation that we can identify morphine found in the body, when the results of morphine in urine are more than 300 ng/mL *(1)*. Within legal process, positive samples must be confirmed, but in many cases the volume of samples remaining were not enough to confirm. Sample preparation for testing of morphine and codeine is difficult, it consumes time and volume of the sample and had high noise in analytical process. Liquid - Liquid extraction (LLE) is the sample preparation method which is used at present. This method has many steps causing use of time and samples and solvents were consumed. The solid phase extraction (SPE) was used to prepare urine samples too, but expensive instruments such as SPE-cartridge cause high cost of analysis.

The QuEChERS method (Quick Easy Cheap Effective Rugged and Safe) is easier and faster when compared with the cartridge method. With no need of insoluble organic solvents and the efficiency of interference removal were advantages in this method. The QuEChERS technique was recognized officially by AOAC in 2007 *(2)* as the standard method to determine pesticide residues in vegetables and fruits. Later studies have extended this technique to widely cover more substances. QuEChERS should be used instead of LLE because it is cheaper, faster and consumes a low amount of sample that is important in confirmation of the method.

1.2 Literature Review

Morphine is an opioid analgesic that is used to treat severe pain. The World Health Organization recommended that it be used for the relief of moderate cancer-related pain. Morphine is considered to be the gold standard, or benchmark, of analgesics used to relieve severe pain or suffering *(3)*. It is the choice opioid prescribed in palliative and terminal care. Morphine is metabolized in humans primarily through conjugation with uridine diphosphoglucuronic acid in the 3-position and cleared from the body by metabolism to morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) (Figure 1.1).
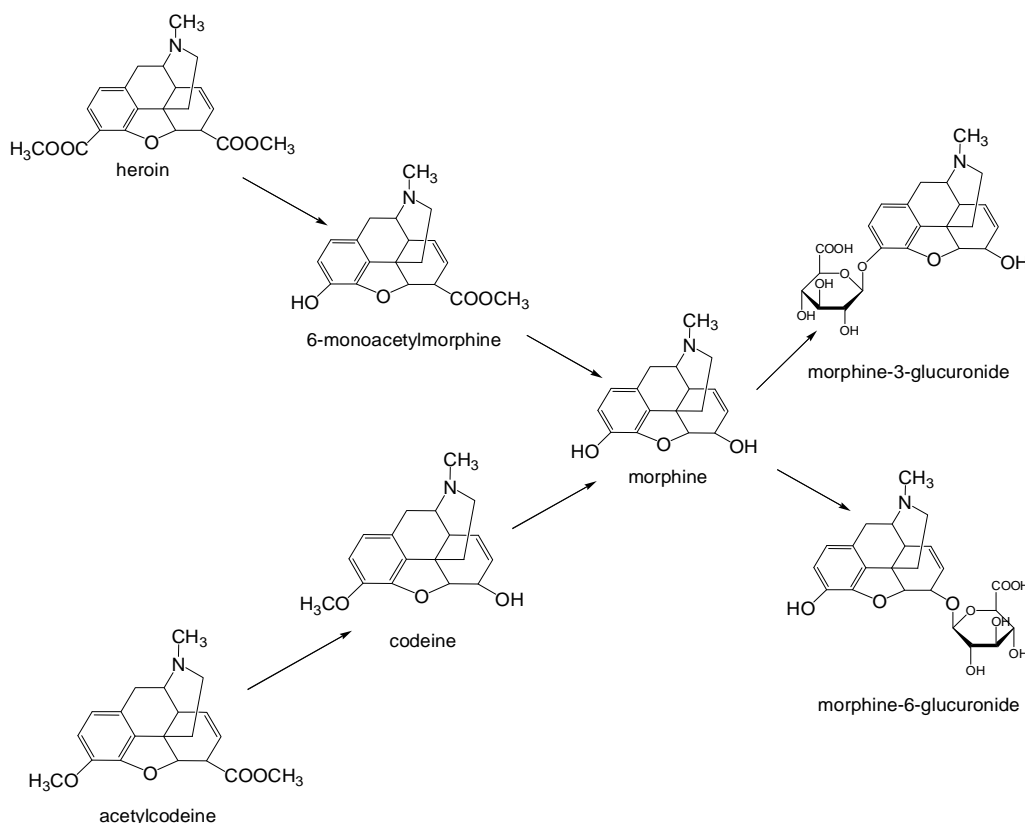
Figure 1.1 Metabolic pathway of heroine and acetylcodeine.

1.2.1. Immunoassay

The assay of drugs in biological fluids is presented by many difficult problems of analysis. Often, the drugs are strongly bound to proteins and are present in complex matrices. The analytical techniques employed to overcome the above difficulties includes immunoassays, enzyme-multiplied immunoassay and chromatographic methods, such as thin-layer chromatography, gas chromatography and high-performance liquid chromatography, coupled with both liquid–liquid and solid–liquid extraction. Immunoassay is a fast and simple method which is used for initial testing of specimens. For the detection of opiates in whole blood, plasma or serum, non-chromatographic methods are rare. In some papers, the use of immunoassay as a screening step has been reported *(4-10)*. However, positive results must continue with determination by the confirmed method which is at least as sensitive as the screening test. On the other hand, hair analysis studies in abused drugs by RIA (Radio Immune Assay) and history of drug use is estimated by sectional hair analysis*(10, 11)* .

1.2.2 Chromatographic method

Small amounts of drug / metabolite in a sample due to the drugs potency, is difficult to assay by conventional sample preparation. In addition, high rates of metabolism, distribution and excretion diminish analyze concentration, thus demanding greater assay sensitivity. Chromatographic assay has high reliability and versatility to identify and quantitatively determine organic compounds in a wide range of absolute and relative concentrations.

1.2.2.1 Thin layer chromatography (TLC)

TLC is a widely used technique for the separation and identification of narcotics *(12-13)*. It offers a screening method prior to examination by Gas chromatography (GC) and High performance liquid chromatography (HPLC). Though HPLC has been used extensively for the accurate quantitative analysis of complex mixture of alkaloids, silica gel and alumina are the sorbents of choice. Thin layers impregnated with the metallic salts have been tested for realizing mutual separations of alkaloids using mixed organic eluents. The practical utility of metal salt impregnated in thin layers is restricted due to the formation of occasional tailing spots, their lesser stability in acidic solvents, and their tendency to absorb moisture. A TLC procedure for rapidly determining morphine in urine has been utilized as a versatile, economic and relatively rapid tool for analyzing large numbers of specimens. These methods and techniques were relatively simple to perform and could detect the psychoactive drugs in the range of 1 to 5 µg/mL of urine. High-performance thin layer chromatography (HPTLC) is the method which was developed for complex and dirty samples where impurities remain absorbed at the stationary phase. HPTLC is well suited for the separation of drugs over a large polarity range. Therefore, planar chromatography is an important separation technique in the fields of forensic blood and urine analysis *(14-18)*. The use of thin layer chromatography in combination with colour reactions to visualise chromatographic spots is wide spread.

1.2.2.2 High-performance liquid chromatography (HPLC)

The use of liquid chromatography (LC) for analysis both of morphine and its metabolites are used with a sensitive detector such as ultraviolet-visible (UV), diode array detection (DAD), fluorescence, electrochemical and mass spectrometry (MS). Moreover, HPLC method was developed for the determination of morphine in plasma *(19).* Samples were extracted using Zeolite Y column followed by reversed phase with fluorescence detection. This method was based on an ex-calibration procedure and was linear between 20 and 200 ng/mL of morphine. Furthermore; mass spectrometric detection is more specific than most of the LC detectors but it is still expensive and not widely used. Nevertheless, many reports had shown that liquid chromatography mass spectrometry (LC–MS) is the method of choice, if the glucuronides of morphine are to be covered. In all other cases, Gas chromatography mass spectrometry (GC–MS) is preferable *(20-22)* . Polettini et al. *(23-24)* determined heroin, morphine, M3G, M6G, 6-acetylmorphine, codeine and acetylcodeine in blood and urine by LC–TSP-MS–MS. The urine samples were extracted with Sep Pak C_{18} cartridges and subjected to analysis of M3G and morphine in SIM and full scan mode. LC-MS with electrospray ionization for the detection of M3G and M6G was reported by Tyrefors et al. in 1996 *(25)* . Separation was achieved on a reversed phase column using a gradient from 4 to 70% acetonitrile with formic acid; the flow rate was at 1.0 mL/min. The eluant was diluted 1:50 prior to mass spectral analysis. Compounds were detected by selected-ion monitoring. The linear range spanned 5-500 ng/mL (M3G) and 2-100 ng/mL (M6G). Short analysis times of less than 5 minutes were the advantage of this technique compared with previous methods involving 45 minute run times. In 1997, Bourquin et.al. *(26).* developed a reversed-phase HPLC method with a diode-array detector to detect metabolites of morphine including M3G and M6G in plasma. Ethylmorphine served as the internal standard and sample cleanup involved SPE with C_{18} cartridges yielding recoveries >80%. The LOQ was 25 ng/mL for each compound. The concentrations of M3G and M6G in serum, urine, and cerebrospinal fluid (CSF) of patients or volunteers receiving morphine during a clinical study were determined using HPLC-electrospray-MS with a C_{18} column and a mobile phase of 1% acetonitrile, 1% tetrahydrofuran and 0.1% formic acid in water as a reversed-phase. Their retention times were 1.7 and 3.2 minutes. The LOQ was reported as 0.5 ng/mL for M6G and 2 ng/mL for M3G. Analytes were extracted from serum and urine with C_2 SPE. Reversed

phase separation on an Atlantis dC_{18} column was achieved in 10 minutes, under gradient conditions. The method was fully validated, including linearity. This procedure was shown to be sensitive and specific, and was applied to 156 real cases from road fatalities *(27)*.

1.2.2.3 Gas chromatography (GC)

Codeine can be changed to morphine by conjugation and demethylation; however, the reverse pathway of codeine from morphine does not occur *(28)*. Both morphine and codeine are generally found in biological fluids and these results show the need for simultaneous assay in both morphine and codeine. Amphoteric nature of morphine became a problem in co-extraction of morphine and codeine, cation exchange SPE columns were developed to solve this problem *(29)*. The poor chromatographic characteristics of underivatized morphine analogues necessitate production of stable derivatives. LLE for the isolation of morphine and codeine from urine and other biological fluids was performed for simultaneous assays of morphine and codeine by GC-MS with selected ion monitoring (SIM) *(30)*. Many methods have been developed for assay of morphine in biological specimens such as a GC–MS SIM method with CI, ammonia–methane (1:5), for the determination of free and hydrolysed morphine in serum and cerebrospinal fluid *(31)*. This method was also used for determination of the pharmacokinetics of morphine in human beings *(32)*. On-line dialysis is purification step by automate for determine opiates in plasma and whole blood by using gas chromatography-nitrogen-phosphorous detection (GC-NPD) and GC-MS *(33)*.

1.2.3 Sample preparation

For chromatographic methods, which are combined with mass spectral detection, it needs a complex sample preparation procedure. The sample preparation is a very important step prior to analyzing. Extraction methods, which were used for the last few years, are the classic LLE, SPE. The SPE often shows better extraction efficiencies than the LLE, especially for polar components or metabolized drugs. Due to limitations in the type of sorbents available, no real fast and easy multi - residue sample preparation methods were possible. Nowadays polymeric or mixed-moded

sorbents are able to separate acidic, neutral and basic drugs from different biological samples by hydrophobic or ion-exchange interactions. Through this, a multi - residual determination of pharmaceuticals in body specimens is possible. The disadvantage of SPE is the high consumption and many types of solvents. SPE has made several steps, emulsion formation in step SPE extraction of some samples and toxicity of chloroform, which can be used as a solvent. Therefore it was necessary to develop a method for preparing a new model to replace the original. The Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS), was recognized officially by Association of Official Analytical Chemists (AOAC) in 2007 *(2)* as the standard method to determine pesticide residues in vegetables and fruit*s (34).* The advantages of this method were no need for insoluble organic solvent and efficiency of interference removal. Later studies have extended this technique to widely cover more substances *(35-37).* QuEChERS is comprised of easy and simple steps *(38).* Firstly the sample was extracted with a solvent into the water species (Water-miscible). Next, salt was added to absorb large amounts of water in the sample, the salting-out effect used high-speed centrifuge with a 2-5 minute rotation. After the removal the of matrix by dispersive-SPE, which was added in the collecting tube and then centrifuge, the supernatant was analyzed directly or adjusted into the appropriate concentration. This method is convenient for the simultaneous determination of more than 40 pharmaceuticals belonging to various therapeutic categories in whole blood *(39).*

1.3 Purpose of The Study

From the review, many researches have paid attention to simple sample preparation. However, all of these extraction methods still used higher amounts of organic solvent consumptions, higher analysis time and higher sample interferences. Thus; this research aimed to develop a new sample preparation method for the extraction of morphine and codeine in urine by the QuEChERS method, which based on dispersion of solid phase substance in sample for noise reduction. This technique is widely used for the detection of pesticide residue in fruit and vegetables. QuEChERS can be applied to urine samples, which can lead to reductions in time and cost. This method is safer to technicians than LLE method and the sample will remain enough to confirm.

CHAPTER II

THEORY

2.1 Physical and Chemical Properties of Morphine and Codeine

2.1.1 Morphine (and Heroin)

Morphine (Astramorph®, Duramorph®, Infumorph®, Kadian®, Morphine Sulfate®, MSIR®, MS-Contin®, Oramorph SR®, Roxanol®) and heroin (Or Mexican brown, Mexican black tar heroin, bags, blue-steel, China white, H, horse, junk, no-name, silk, skag, smack, scramble, bone, chippers), white crystalline powders which may vary in color from white to dark brown due to impurities, or may appear as a black tar-like material.

Morphine is a natural substance which is extracted from seeds of the poppy plant (*Papavar somniferum)*. There are many numbers of alkaloids in opium and about 4-21% of it is morphine. Morphine can be also made from industrial process by extracting alkaloids from mature dried poppy straw.

Morphine is used medicinally for the relief of moderate to severe pain in both acute and chronic pain management by oral, intramuscular, intravenous, rectal, epidural, or intrathecal administration. Heroin has no currently accepted medical uses in the U.S., however, it is an analgesic and antitussive. Morphine is a schedule II controlled substance and is available in a variety of prescription forms: injectables (0.5-25 mg/mL strength); oral solutions (2-20 mg/mL); immediate and controlled release tablets and capsules (15-200 mg); and suppositories (5-30 mg). Heroin is a schedule I controlled substance and is produced from morphine by acetylation at the 3 and 6 positions. The majority of heroin sold in the U.S. originates from Southeast Asia, South America (Columbia) and Mexico. Low purity Mexican black tar heroin is most common on the West coast, while high purity Columbian heroine dominates in the East and most mid-western states.

Morphine has 20-40% of oral bioavailability, and 35% is bound in plasma. It has a short half-life about 1.5 - 7 hours and is primarily glucuroconjugated at positions 3 and 6, to morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G), respectively. About 5% is demethylated to normorphine. M6G is an active metabolite with a higher potency than morphine, and can accumulate following chronic administration or in renally impaired individuals. From a single morphine dose 95% of it will eliminated in the kidney. There was found 75% of M3G and less than 10% as unchanged morphine after 72 hours in urine.

2.1.2 Codeine
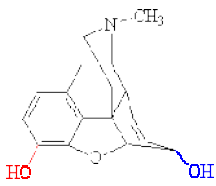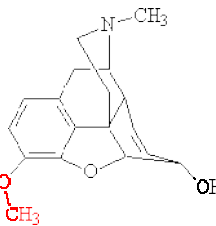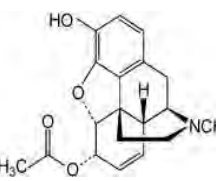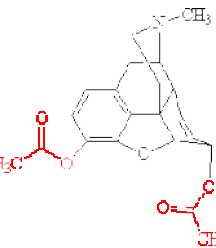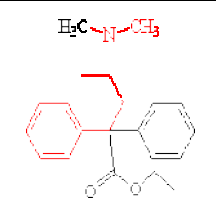
Codeine (codeine, methylmorphine, morphine 3-methylether, morphine monomethyl ether) is obtained either naturally from opium (extracted from *Papaver somnifera*) or by methylation of morphine. It is a phenanthrenic alkaloid and constitutes 0.5% of raw opium.

Toxic doses of codeine produce unconsciousness, pinpoint pupils, slow and shallow respiration, cyanosis, weak pulse, hypotension and in some cases pulmonary oedema, spasticity and twitching of the muscles. The main and most dangerous effect is respiratory depression. Death from respiratory failure may occur within 2 to 4 hours after an oral dose. Convulsions may occur, especially in children. Hallucinations, trembling, uncontrolled muscle movements, mental depression and skin rash may be observed. Chronic ingestion or injection leads to addiction. In this case pinpoint pupils and changes in mood may be observed (or no evident signs of use). The withdrawal syndrome is characterized by yawning, lacrimation, pilomotor reactions, severe gastrointestinal disturbances with cramps, vomiting, diarrhea or constipation, sweating, fever, chills, increase respiratory rate, insomnia, tremor, mydriasis and myalgia.

Codeine is absorbed from the gastrointestinal tract then it is rapidly distributed from the intravascular spaces to the various body tissues, with preferential uptake by the liver, spleen, and kidneys. 90% of an oral dose is eliminated via the kidneys within 24 hours, urinary secretion products consist of 70% free and glucuronide conjugated

codeine, the other substances are free and conjugated norcodeine (about 10%), free
and conjugated morphine (about 10%), normorphine (4%), and hydrocodone (1%)

Table 2.1 Structure and property of opioid substances

Opioid substances	Chemical structure	Chemical formula	Molecular mass (g/mol)	pKa	Half-Life
Morphine		$C_{17}H_{19}NO_3$	285.34	7.9	2-3 Hours
Codeine		$C_{18}H_{21}NO_3$	299.36	8.2	2.5-3 hours
6-Monoacetyl morphine (6-MAM)		$C_{19}H_{21}NO_4$	327.37	11.05	6-25 minutes
Heroine		$C_{21}H_{23}NO_5$	369.41	7.9	<10 minutes
Methadone		$C_{21}H_{27}NO$	309.44	8.2	24-36 hours

2.2 Sample Preparation Techniques (QuEChERS)

Dispersive - SPE (d-SPE), often referred to as the "QuEChERS" method (Quick, Easy, Cheap, Effective, Rugged, and Safe)**,** is a sample preparation approach entailing solvent extraction of high moisture samples with acetonitrile, ethyl acetate, or acetone and partitioning with magnesium sulfate alone or in combination with other salts followed by cleanup using d-SPE *(38, 40)*. It is very flexible and since its inception, there have been several modifications of the technique depending on analytes, matrices, instrumentation, and analyst preferences. In d-SPE, food or agricultural samples are first extracted with a water-miscible solvent (for example, acetonitrile) in the presence of high amounts of salts (for example, sodium chloride and magnesium sulfate) and buffering agents (for example, citrate) to induce liquid separation and stabilize acidic and basic labile pesticides, respectively. Upon shaking and centrifugation, an aliquot of the organic phase is subjected to further cleanup using dispersive SPE (adding small amounts of bulk SPE packing sorbents to the extract). After sample clean up, the mixture is centrifuged and the resulting supernatant can be analyzed directly or can be subjected to a concentration and solvent exchange step if necessary. This method is most convenient when the SPE sorbents acts as a "chemical filter" to remove matrix components and analytes are unretained. The choice of sorbents, various types of characteristic and effective in cleanup matrix components, as follows in Table 2.2

Table 2.2 Effective in cleanup matrix components of sorbent

Sorbent	Effective
Primary Secondary Amine (PSA)	Removes polar organic acid, some sugars and lipids
Octadecylsilane (C_{18})	Removes polar organic acid, sterols, some sugars and lipids
Alumina- N (Al_2O_3)	Removes fatty acids, other organic acids, sugars and pigments
Graphitized Carbon Black (GBC)	Removes pigments, polar organic acid, some sugars and lipids
Aminopropyl ($-NH_2$)	Removes sugar and other polar substances through hydrogen bonding and acidic compounds like free fatty acids through anion exchange

2.3 High Performance Thin Layer Chromatography (HPTLC)

High-performance thin-layer chromatography (HPTLC) is a form of thin-layer chromatography (TLC) that provides superior separation power using optimized coating material, novel procedures for mobile-phase feeding, layer conditioning and improved sample application*(41)*. It promotes higher separation efficiencies, shorter analysis time, lower amounts of mobile phase and efficient data acquisition and processing. The main difference between HPTLC and TLC is the particle and pore size of sorbents. The other differences are shown in Table 2.3

Table 2.3 Difference of HPTLC and TLC *(42)*

	HPTLC	TLC
Layer of Sorbent	100µm	250µm
Efficiency	High due to smaller particle size generated	Less
Separations	3 - 5 cm	10 - 15 cm
Analysis Time	Shorter migration distance and the analysis time is greatly reduced	Slower
Solid support	Wide choice of stationary phases like silica gel for normal phase and C_8 , C_{18} for reversed phase modes	Silica gel , Alumina & Kiesulguhr
Development chamber	New type that require less amount of mobile phase	More amount
Sample spotting	Auto sampler	Manual spotting
Scanning	Use of UV/ Visible/ Fluorescence scanner scans the entire chromatogram qualitatively and quantitatively and the scanner is an advanced type of densitometer	Not possible

In thin layer chromatography, a solid phase, the adsorbent is coated onto a solid support such as glass, plastic or aluminum as a thin layer. A solvent, or mixture of solvents, called the eluant, is allowed to flow up the plate by capillary action. The mixture of sample is dissolved in a solvent and the resulting solution is spotted onto the thin layer plate near the bottom of the plate.

The solid will adsorb a certain fraction of each component of the mixture and the remainder will be in solution. A substance that is strongly adsorbed will spend more time sitting still and less time moving, while a weakly adsorbed substance will spend

less time sitting and more time moving. Thus, the more weakly a substance is adsorbed, the farther up the plate it will move and the more strongly a substance is adsorbed, the closer it will stays near the origin.

The sample is applied to the layer of adsorbent, near one edge, as a small spot of solution.   After the solvent has evaporated, the adsorbent-coated sheet is propped more or less vertically in a closed container, with the edge to which the spot was applied down. The spot on the thin layer plate must be positioned above the level of the solvent in the container (Figure 2.1a). If it is below the level of the solvent, the spot will be washed off the plate into the developing solvent. The solvent, which is at the bottom of the container, creeps up the layer of adsorbent, passes over the spot, and as it continues up, effects the separation of the materials in the spot (developing the chromatogram). When the solvent front has nearly reached the top of the adsorbent, the thin layer plate is removed from the container (Figure 2.1b).

a)   b)
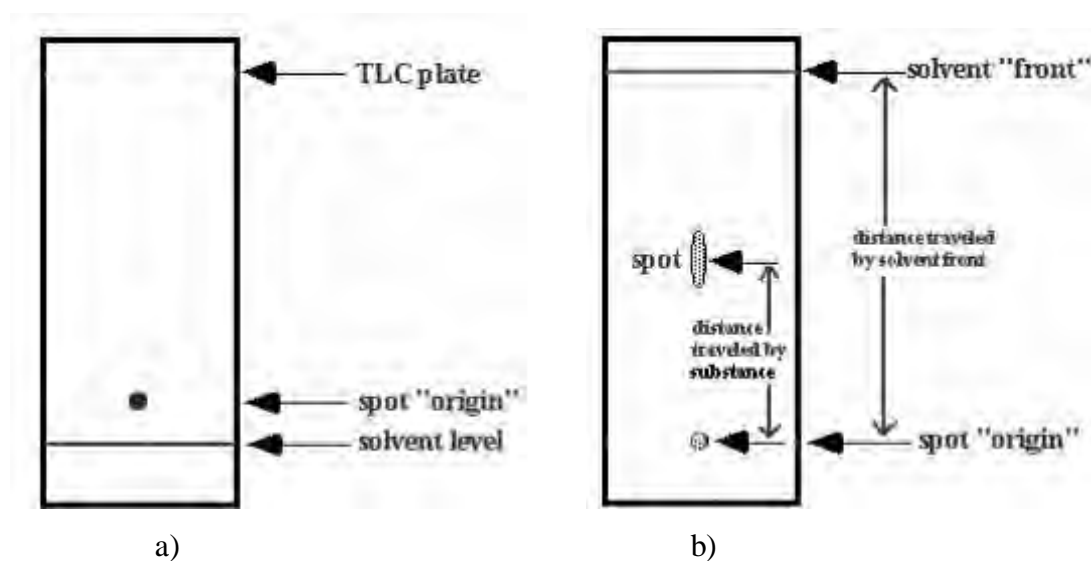
Figure 2.1 a) Position of the spot on a thin layer plate.

   b) TLC plate showing distances traveled by the spot and the solvent after

   solvent front nearly reaches the top of the adsorbent

Since the amount of adsorbent involved is relatively small, and the ratio of adsorbent to sample must be high, the amount of sample must be very small, usually much less than a milligram. For this reason, thin-layer chromatography (TLC) is usually used as an analytical technique rather than a preparative method. With thicker layers (about 2 mm) and larger plates with a number of spots can be used as a preparative method. The separated substances are recovered by scraping the adsorbent off the plate (or cutting out the spots if the supporting material can be cut) and extracting the substance from the adsorbent.

Because the distance travelled by a substance relative to the distance travelled by the solvent front depends upon the molecular structure of the substance, TLC can be used to identify substances as well as to separate them. The relationship between the distance travelled by the solvent front and the substance is usually expressed as the R_f value:

$$R_f \text{ value } = \text{ [distance traveled by substance] / [distance traveled by solvent front]}$$

The R_f values are strongly dependent upon the nature of the adsorbent and solvent. Therefore, experimental R_f values and literature values do not often agree very well. In order to determine whether an unknown substance is the same as a substance of known structure, it is necessary to run the two substances side by side in the same chromatogram, preferably at the same concentration.

The major factors which affect chromatographic separation are: chromatographic adsorbents, eluting solvent and adsorbability of organic compounds. The adsorbent should show a maximum selectivity toward the substances being separated so that the differences in the rate of elution will be larger. For the separation of any given mixture, some adsorbents may be too strongly adsorbing or too weakly adsorbing. Table 2.4 lists a number of adsorbents in order of adsorptive power.

Table 2.4 Adsorbents in order of adsorptive power

Most Strongly Adsorbent	Alumina	Al_2O_3
	Charcoal	C
	Florisil	MgO/SiO_2 (anhydrous)
Least Strongly Adsorbent	Silica gel	SiO_2

When one substance is relatively soluble in a solvent, this can result in its being eluted faster than another substance. However, a more important property of the solvent is its ability to be itself adsorbed on the adsorbent. If the solvent is more strongly adsorbed than the substances being separated, it can take their place on the adsorbent and all the substances will flow together. If the solvent is less strongly adsorbed than any of the components of the mixture, its contribution to different rates of elution will be only through its difference in solvent power toward them. If, however, it is more, strongly adsorbed than some components of the mixture and less strongly than others, it will greatly speed the elution of those substances that it can replace on the absorbent, without speeding the elution of the others. Table 2.5 lists a number of common solvents in approximate order of increasing adsorbability.

Table 2.5 Eluting solvents for chromatography

Least Eluting Power	Petroleum ether (hexane; pentane)
	Cyclohexane
	Carbon tetrachloride
	Benzene
	Dichloromethane
	Chloroform
	Ether (anhydrous)
	Ethyl acetate (anhydrous)
	Acetone (anhydrous)
	Ethanol
	Methanol
	Water
	Pyridine
Greatest Eluting Power	Organic acids

If the substances in the mixture differ greatly in adsorbability, it will be much easier to separate them. Often, when this is so, a succession of solvents of increasing eluting power is used. One substance may be eluted easily while the other stays at the top of the column, and then the other can be eluted with a solvent of greater eluting power. Table 2.6 indicates an approximate order of adsorbability by functional group.

Table 2.6 Adsorbability of organic compounds by functional group

Least Strongly Adsorbed	Saturated hydrocarbons; alkyl halides
	Unsaturated hydrocarbons; aIkenyl halides
	Aromatic hydrocarbons; aryl halides
	Polyhalogenated hydrocarbons
	Ethers
	Esters
	Aldehydes and ketones
	Alcohols
Most Strongly Adsorbed	Acids and bases (amines)

2.4 Gas Chromatography Mass Spectrometry (GC-MS)

Gas Chromatography-Mass Spectrometry (GC-MS) is the combining of 2 analytical techniques between Gas Chromatography (GC) and a mass spectrometer (MS). A sample mixture passes through a chromatographic column so that it can emerge sequentially then MS is used for examining the compound, also in the gas phase, so that its structure or identity can be deduced from its mass spectrum. The combined GC-MS allows for more information to be gained than is obvious from the simple sum of the two separate instruments.

2.4.1 Gas Chromatography (GC)

A chemical analysis instrument for separating chemicals in a complex sample. A gas chromatograph uses a flow-through narrow tube known as the column, which different chemical constituents of a sample pass through a gas stream (carrier gas, mobile phase) at different rates depending on their various chemical and physical

properties. Their interaction with a specific column filling, called the stationary phase. As the chemicals exit the end of the column, they are detected and identified electronically. The function of the stationary phase in the column is to separate different components, causing each one to exit the column at a different time (retention time). Other parameters that can be used to alter the order or time of retention are the carrier gas flow rate, and the temperature.

Gas chromatography provides both qualitative and quantitative information for individual compounds present in a sample. Compounds move through a GC column as gases, either because the compounds are normally gases or they can be heated and vaporized into a gaseous state. The compounds partition between a stationary phase, which can be either solid or liquid, and a mobile phase (gas). The differential partitioning into the stationary phase allows the compounds to be separated in time and space*(43-45)*.
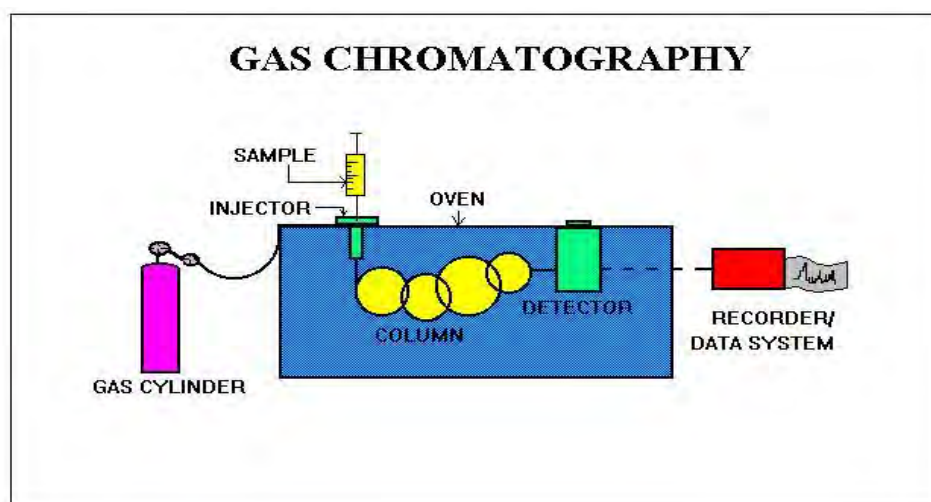
Figure 2.2 Schematic of a Gas Chromatographic System

2.4.1.1 Carrier Gas

The carrier gas (He, H_2 or N_2) serves as the mobile phase that moves the sample through the column. The carrier gas flow can be quantified by either linear velocity, expressed in cm/sec, or volumetric flow rate, expressed in mL/min. The linear

velocity is independent on the column diameter while the flow rate is dependent on the column diameter.

2.4.1.2 Injector

The injector is a hollow, heated, glass-lined cylinder where the sample is introduced into the GC. The sample will be vaporized in the injector, where the temperature is controlled.

2.4.1.3 Column

The GC column is most important part of the system. It is coated with stationary phases which have large molecular weight polysiloxane, polyethylene glycol, or polyester polymers film thickness (0.1 to 2.5 micrometer). The structure of the stationary phase plays an important role in the separation of the compounds because it affects the amount of time the compounds take to move through the column. A typical capillary column is 15 to 60 meters in length and 0.25 to 0.32 mm ID. A typical packed column is 6 to 12 feet long and 2.2 mm ID.

2.4.1.4 Oven

The column is placed in an oven where the temperature can be controlled very accurately over a wide range of temperatures. Typically, GC oven temperatures range from room temperature to $300^{o}C$, but cryogenic conditions can be used to operate at temperatures from about $-20^{o}C$ to $20^{o}C$.

2.4.1.5 Detector

Signals are generated by an interaction between compounds and detector, the size of the signal depends on the amount of compound that is present in the sample. Detectors vary by compounds that are analyzed (e.g., FID : compounds with C-H bonds, a poor response for some non-hydrogen containing organics, NPD : nitrogen and phosphorous containing compounds, ECD : halogens, nitrates and conjugated carbonyls) and can measured from 10^{-15} to 10^{-6} g. of a single component.

2.4.1.6 Data Recorder System

Chromatogram is the plot which is generated by the data recorder over analytical time. The retention time, which is when the component elutes from the GC system, is qualitatively indicative of the type of compound. The data recorder also has an integrator component to calculate the area under the peaks or the height of the peak. The area or height is indicative of the amount of each component.

2.4.2 Mass Spectrometry (MS)

Mass Spectrometry is a powerful technique for identification of compounds by their mass-to-charge ratios. An advantage of Mass Spectrometry is its ability to identify the very small amounts of unknown compounds*(46-47)*. The sample, which may be a solid, liquid or vapor, is introduced into a high vacuum system through an inlet; which is then ionized by an ionization source. The ions are separated according to their mass-to-charge ratios in the mass analyzer. The ions are then collected by a detector which is connected to a data system for data processing that produces a Mass Spectrum. Mass Spectrum is a plot of the m/z ratios of the ionic species present versus abundance. The molecular weight and the structure of the compounds can be obtained from the information of the ion fragmentations and their abundances.
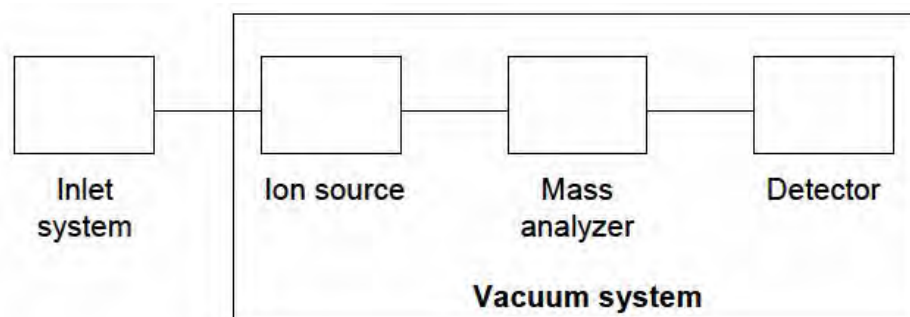
Figure 2.3 Schematic diagram of MS system.

2.4.2.1 Ion source

2.4.2.1.1 Electron Impact Ionization

Electron Impact Ionization (EI) is the bombardment of the sample by very fast moving electrons. The sample is chemically influenced by the fast moving electrons, causing an ejection of its own electron, forming a radical cation. This process takes about 10E-14 seconds. These unstable parent ions fragment to form stable daughter and granddaughter ions
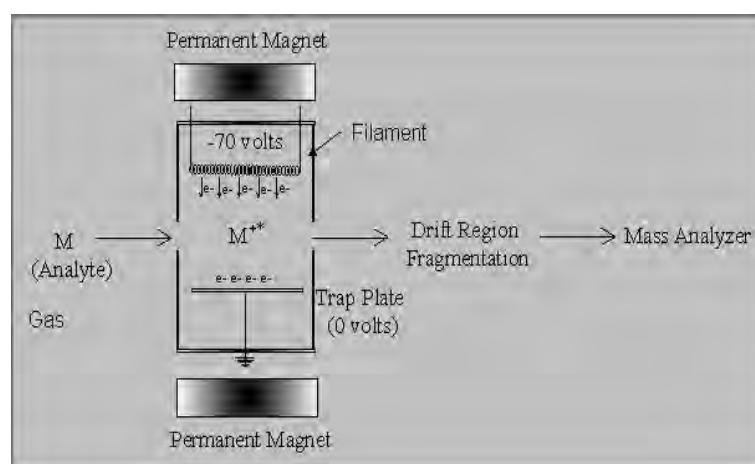
Figure 2.4 Schematic of Electron Impact Ionization

2.4.2.1.2 Chemical Ionization

Chemical Ionization (CI) is a relatively gentle process of proton transfer from pre-ionized reagent gas, such as methane. Ionization is made possible by collision of sample molecules with the reagent ions. Chemical ionization produces quasimolecular ions which do not fragment as readily as the molecular ions formed by electron ionization. Therefore, CI spectra are normally simpler than EI spectra in containing abundant quasimolecular ions and few fragment ions. It is advantageous to run both CI and EI spectra with the same compound to obtain complementary information
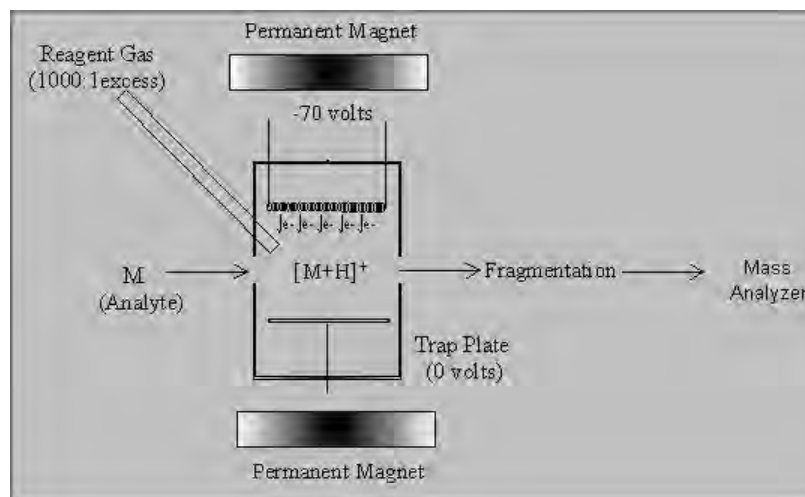
Figure 2.5 Schematic of Chemical Ionization

2.4.2.1.3 FAB Ion Source

Fast atom bombardment (FAB) is an ionization method of mass spectrometry. In this method ions are produced in a mass spectrometer from non volatile organic molecule by bombarding the compound in the condensed phase with energy rich neutral particles. It is used to analyze non-volatile and high-polarity compounds. The material to be analyzed is mixed with a non-volatile chemical protection environment known as a 'matrix'. It is bombarded under a vacuum with a high energy (4000 to 10000 electron volts) beam of atoms. The atoms are usually from an inert gas such as argon or xenon. The bombarding ions are usually rare gases either Xe or Ar, these atoms are first ionized and then passed through an electric field to increase their kinetic energy. After acceleration, the fast moving ions pass into a chamber containing further gas atoms, in which bombardment of the ions and atoms leads to charge exchange;

$$Ar^{+\cdot} + Ar(thermal) \longrightarrow Ar(fast) + Ar^{+\cdot}(thermal)$$

The fast atom that is formed retains most of the original kinetic energy of the fast ions and continues in the same direction. The negatively charged deflection plate can be used to direct the ions away. Positive or negative ions may be formed in the bombardment process with extraction of ions from the sample plate being dependent

on whether the extraction slits are held at a positive or negative voltage. The rapid sample heating from the noble- gases reduces sample fragmentation.
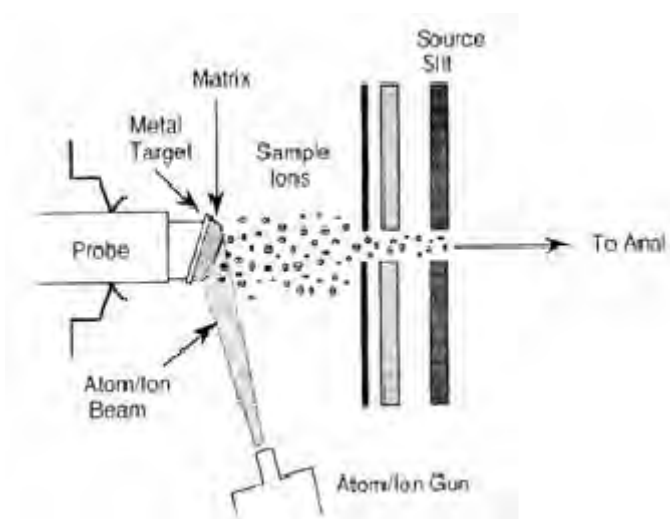
Figure 2.6 Schematic of Fast atom bombardment

2.4.2.1.4 ICP Ion Source

The inductively coupled plasma (ICP) source converts the atoms of the elements in the sample to ions. These ions are then separated and detected by the mass spectrometer. Argon gas flows inside the concentric channels of the ICP torch. The RF load coil is connected to a radio-frequency (RF) generator. As power is supplied to the load coil from the generator, oscillating electric and magnetic fields are established at the end of the torch. When a spark is applied to the argon flowing through the ICP torch, electrons are stripped off of the argon atoms, forming argon ions. These ions are caught in the oscillating fields and collide with other argon atoms, forming an argon discharge or plasma. The sample is typically introduced into the ICP plasma as an aerosol, either by aspirating a liquid or dissolved solid sample into a nebulizer or by using a laser to directly convert solid samples into an aerosol. Once the sample aerosol is introduced into the ICP torch, it is completely desolvated and the elements in the aerosol are converted first into gaseous atoms and then ionized towards the end of the plasma.
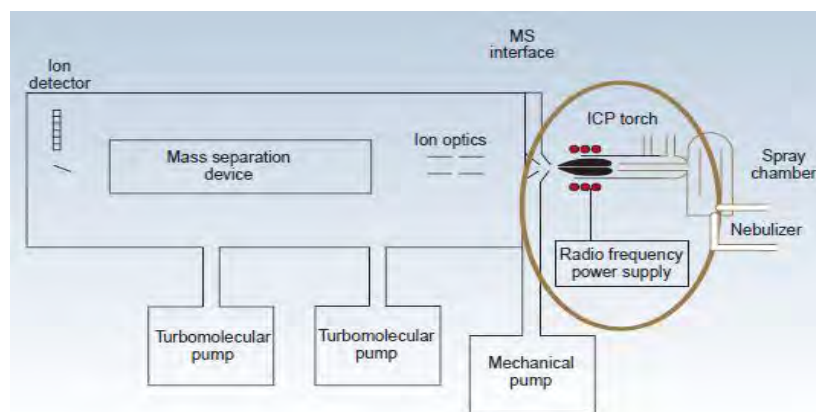
Figure 2.7 Schematic of an ICP-MS system

2.4.2.2 Mass Analyzer

2.4.2.2.1 Quadrupole Mass Analyzer

A quadrupole analyzer is a mass filter that creates a quadrupole field with a DC component and an RF component in such a manner as to allow transmission only of ions having a selected mass-to-charge ratio. A Quadrupole analyzer consists of four parallel rods arranged symmetrically. The opposite two are connected electrically to the RF generator and DC potential. By varying the ratio of RF/DC, the ions selected at m/z ratio can avoid collision with the rods and go through the central space between the rods to reach the detector.
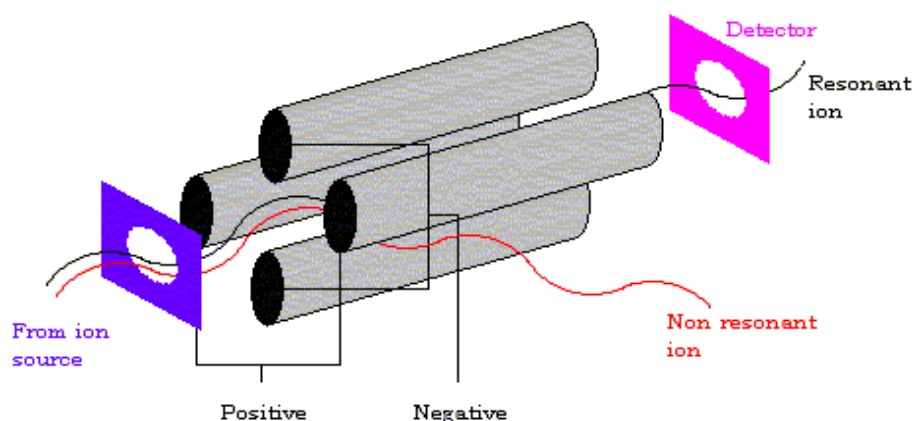
Figure 2.8 Schematic of a Quadrupole Mass Analyzer

2.4.2.2.2. Ion Trap Mass Analyzer

The ion-trap mass analyzer uses three electrodes to trap ions in a small volume. The mass analyzer consists of a ring electrode separating two hemispherical endcap electrodes. Ions are trapped by applying specific AC voltages to the electrodes. A mass spectrum is obtained by changing the electrode voltages to eject the ions from low mass to high mass (in fact, m/z) from the trap. The advantages of the ion-trap mass spectrometer include compact size, and the ability to trap and accumulate ions in time to increase the signal-to-noise ratio of a measurement
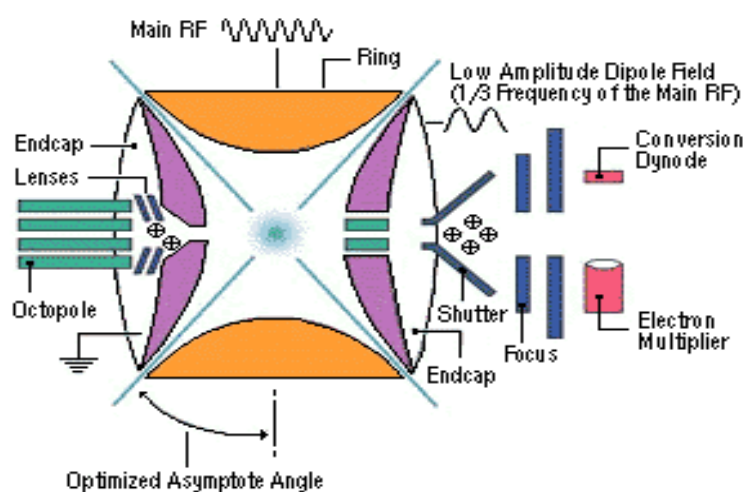
Figure 2.9 Schematic of an ion-trap mass analyzer

2.4.2.2.3 Time-of-Flight Mass Analyzer (TOF)

Time-of-Flight Mass Analyzer (TOF) is based on the fact that ions with the same energy but different masses travel with different velocities. Basically, ions formed by a short ionisation event are accelerated by an electrostatic field to a common energy level and travel over a drift path to the detector. The lighter ones arrive before the heavier ones and a mass spectrum is recorded, measuring the flight time for each ion allowing the determination of its mass. This cycle is repeated with a repetition rate that depends on the flight time of the highest mass to be recorded.
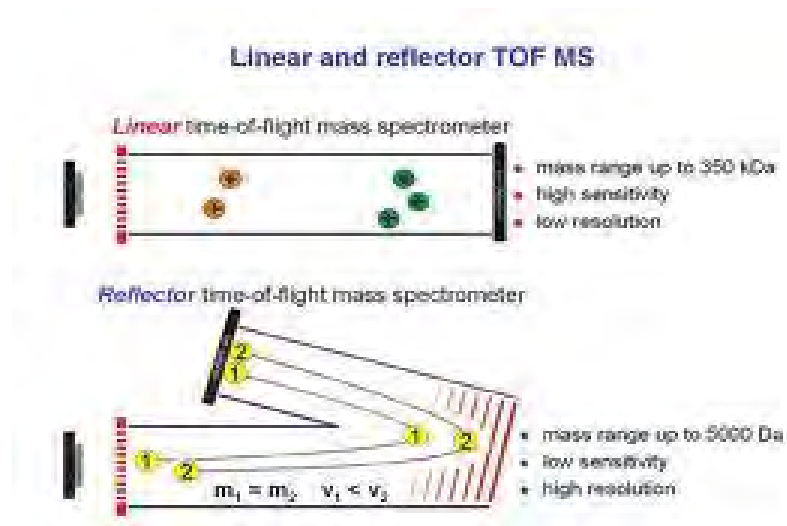
Figure 2.10 Schematic of the Time-of-Flight mass analyzer

2.4.2.3 Detector

Mass spectrum is generated by measurement of the ions leaving the mass analyzer. A detector is selected by speed, dynamic range, gain and geometry. Most of detectors currently used to amplify the ion signal are electron multiplier tube (Figure 2.11) and photo multiplier tube (Figure 2.12). Electron multiplier tube offers electron from surface of the tube for analyte ions. The entrance of the tube is held with potential charge opposite from the analyte ions. Analyte ions are attracted to the entrance of the tube and collide with the tubes surface, where the inner surface coated with electron-emissive material releases electrons. These electrons are accelerated to hit another portion of the tube by electrostatic force and surface loses more electrons in every collision. Amplified electrons are counted by an electrical circuit and displayed as signal intensity. The photo multiplier tube comprises a photocathode and a series of dynodes. In the high voltage tube, incident photon strikes the photo cathode and emits electrons due to the photoelectric effect. These electrons are accelerated towards a series of additional electrodes called dynodes, the amount of electrons is increased at every collision. This creates an amplified signal that is finally collected and measured at the anode.
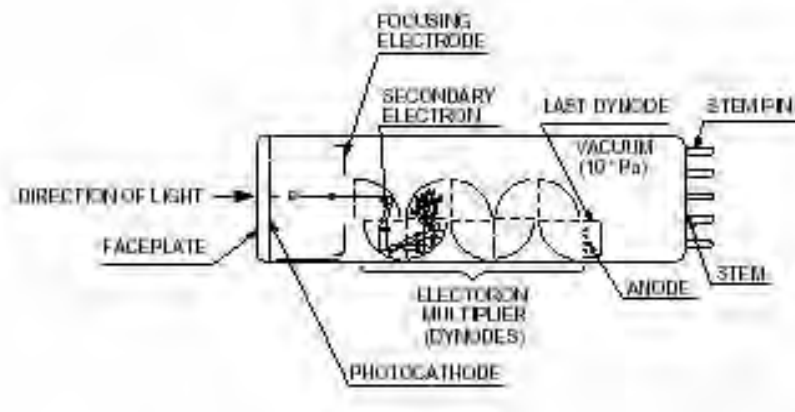
Figure 2.11 Schematic of electron multiplier tube
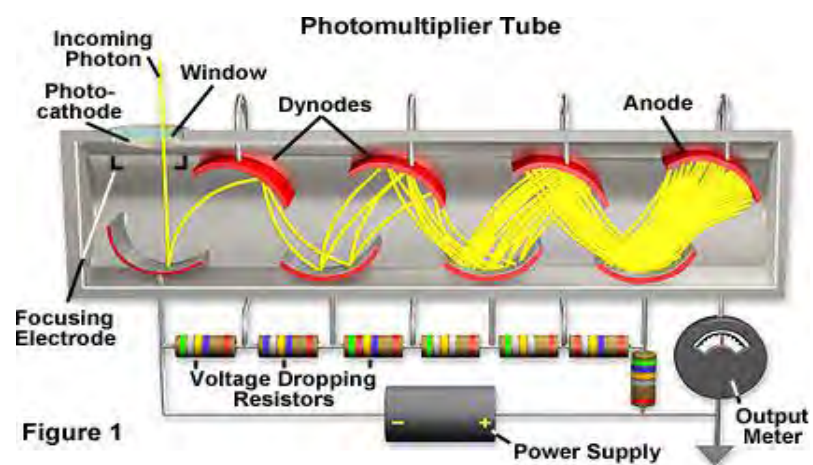
Figure 2.12 Schematic of photo multiplier tube

CHAPTER III

EXPERIMENTAL

3.1 Instrument and Apparatus

3.1.1 Gas chromatography mass spectrometer (GC-MS) was performed on an HP 6890N GC coupled to HP 5973N mass selective detectors (Santa Clara, CA, USA). The GC system was equipped with an electronic pressure control (EPC), split-splitless injector, and an HP 7683 autosampler. Chemstation software was used for instrument control and data acquisition/processing.

3.1.2 GC column: 30 m × 0.25 mm i.d. × 0.25 µm film thickness HP-5MS capillary column (Agilent).

3.1.3 High purity helium (He) (Chatakorn lab center, Bangkok, Thailand)

3.1.4 Analytical balance (5 digits), Model BP 210 D, Max 210 g, Sartorius AG Goettingen, Germany.

3.1.5 Analytical balance (4 digits), Model LA 230 S, Max 230 g, Sartorius AG Goettingen, Germany.

3.1.6 Vortex mixer, IKA, Type MS1, IKA-works Industries, Willmington, U.S.A.

3.1.7 Centrifuge, KOKUSAN, Type H-701FR, KOKUSAN Coperation, Co., Ltd., Tokyo, Japan

3.1.8 Hot air oven, Model UNB 500, MEMMERT, Germany

3.1.9 Micro-pipettes 10-100 µL, 100-1000 µL, 500 µL, 1000 µL, 5 mL, 10mL and tips, Eppendorf, Hamburg, Germany.

3.1.10 Developing tank 10 x 10 cm., CAMAG, Germany

3.1.11 Saturation Pads, Vertical chromatography, Thailand

3.1.12 HPTLC silica gel 60 F 254 Aluminium sheets 20x20 cm., Merck, Germany

3.1.13 Beakers 10 mL, 50 mL, 150 mL, 250 mL.

3.1.14 Volumetric flasks 10.00 mL, 25.00 mL, 50.00 mL,100.00 mL.

3.1.15 Centifuge tube screw cap 2 mL, 10 mL, 15 mL, 50 mL, Hycon, Plastics Inc.

3.1.16 Syringe filters, 0.45µm nylon, Vertical Chromatography Co., Ltd.

3.1.17 GC vials 2 mL with PTFE caps, Agilent technologies, Pola Alto, U.S.A.

All experimental glassware was cleaned with detergents and rinsed with deionized water before use.

3.2 Chemicals, materials and samples

3.2.1 Standard Compounds

Morphine, codeine, 6-monoacetylmorphine(6-MAM), heroine and methadone (>97% purity) were Sigma-Aldrich (St. Louis, MO, USA) donated from the Bureau of Drug and Narcotic, Department of Medical Sciences, Ministry of Public Health, Thailand.

3.2.2 Organic Solvents and other chemicals

Methanol (MeOH), Acetonitrile (MeCN), ethyl acetate (EtOAc) and acetone were pesticide residue analysis grade sourced from Kanto Chemical (Kanto, Japan). Dichloromethane and chloroform were purchased from Merck (Darmstadt, Germany). Hydrochloric acid (HCl) was analytical grade obtained from J.T. Baker (Philipsburg, NJ, USA). Pyridine was analytical grade purchased from Merck (Darmstadt, Germany). A mixture of N, O-bis(Trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (99:1, v/v) was supplied by Supelco (Bellefonte, PA, USA). Anhydrous $MgSO_4$ fine powder was obtained from Panreac (Barcelona, Spain). NaCl was supplied by Ajax Finechem (Taren Point, Australia). PSA sorbent purchased from Varian (Harbor City, CA, USA). Alumina neutral (alumina-N) was obtained from Macherey-Nagel (Düren, FRG). Octadecyl (C_{18}), Aminopropyl (NH_2), graphitized carbon black (GCB) sorbent was sourced from Merck.

3.2.3 Positive and blank Sample

Positive-urine samples of heroine abusers and blank urine samples (which were obtained from healthy volunteers with no previous history of drug abuse) were provided by Bureau of Drug and Narcotic, Department of Medical Sciences, Ministry of Public Health, Thailand. All urine samples were kept in polypropylene bottles at 4°C prior to analysis.

3.3   Preparation of Standard Solution

3.3.1   Stock Standard Solutions

Stock standard solution of 1,000 µg/mL of each compound were prepared by dissolving an accurate weight of approximately 0.0250 g of each standard in methanol and made up to 25.00 mL in volumetric flask with methanol. These stock standard solutions were kept in amber glass bottles with screw caps and stored in the refrigerator at 4 ℃, prior to testing.

3.3.2   Intermediate Standard Solutions

The intermediate standard solutions of 100 µg/mL of each compound were prepared with diluted stock standard solution in 50.00 mL volumetric flasks with methanol. This intermediate standard solution was kept in amber glass bottles with screw caps and stored at 4 ℃, prior to testing.

3.3.3   Working Standard Mixture Solutions

A working standard mixture of morphine and codeine at 10 µg/mL in methanol was prepared from the stock solutions and refrigerated at 4°C.

3.4   The Optimum Sample Preparation

The major problem of conventional sample preparation method (appendices x) for TLC is high volume of sample and reagent consume. This work will solve this problem by using QuEChERS for the sample preparation in the urine sample.

3.4.1 QuEChERS Optimization

QuEChERS is a sample preparation approach entailing solvent extraction of high moisture samples with acetonitrile, ethyl acetate, or acetone and partitioning with magnesium sulfate alone or in combination with other salts followed by cleanup using dispersive-SPE.

Basically, the sample is first extracted with a water-miscible solvent (for example, acetonitrile) in the presence of high amounts of salts (for example, magnesium sulfate and sodium chloride) to induce phase separation. The salting-out effect also influences analyte partition, which is dependent upon the solvent used for extraction. The concentration of salt can influence the percentage of water in the organic phase and can adjust its "polarity". Upon shaking and centrifugation, an aliquot of the organic phase is subjected to further cleanup using dispersive SPE. The SPE sorbent is chosen to retain matrix components and not the analytes of interest.

This study focuses on the optimization of extraction solvent and dispersive-SPE cleanup conditions to achieve most practical benefits and good analytical results. (Table 3.1) The optimum condition was selected for further experiment.

Table 3.1 The parameters of optimum condition for HPTLC and GC-MS

Parameter	Optimum condition for HPTLC	Optimum condition for GC-MS
Type of solvent	ethyl acetate, acetonitrile, acetone, dichloromethane, chloroform	ethyl acetate, acetonitrile, acetone
Amount of $MgSO_4$	500 mg, 1,000 mg, 1,500 mg, 2,000 mg and 2,500 mg	-
Type of sorbent	PSA, C_{18}, Alumina –N, GCB and NH_2	PSA, C_{18}, Alumina –N
Amount $MgSO_4$ and sorbent	same amount of each $MgSO_4$ and sorbent were 25 mg, 50 mg, 75 mg, 100 mg, 125 mg, 150 mg, 175 mg, 200 mg and 250 mg	-
Clean-up	clean-up, no clean-up	clean-up, no clean-up

3.4.1.1 QuEChERS Optimization for HPTLC

1. Pipette 5.00 mL urine sample and 5.00 mL 0.1 M HCl into test tube-I

2. Vortex the tube for 1 min and incubate at 100°C for 30 min

3. Cool down to room temperature

4. Adjust to pH 9 with ammonia solution

5. Decant the extract into a 50 mL centrifuge tube-II containing 2.0 g anh. $MgSO_4$

6. Rinse tube-I with 5.00 mL ethyl acetate, vortex brifely, and decant the solution into tube-II

7. Vortex tube-II vigorously for 1 min to prevent crystalline agglomerations due to dehydration of anh. $MgSO_4$

8. Centrifuge the tube at 4000 rpm for 5 min

9. Transfer 3.5 mL of the upper layer into a 15 mL centrifuge tube containing 50 mg anh. $MgSO_4$ + 50 mg Alumina-N

10. Vortex the tube for 1 min

11. Centrifuge the tube at 4000 rpm for 5 min

12. Pipette 3.00 mL of the supernatant into cup

13. Evaporate to dryness for analyze with HPTLC

3.4.1.2 QuEChERS Optimization for GC-MS

1. Pipette 1.00 mL urine sample into test tube-I

2. Add 1 mL of 0.1 M HCl and vortex tube-I for 1 min

3. Place tube-I in a heating block and incubate at 80°C for 30 min

4. Cool down to room temperature

5. Adjust to pH 9 with ammonia solution

6. Decant the extract into a 10 mL centrifuge tube-II containing 0.5 g anh. $MgSO_4$ + 0.25 g NaCl;

7. Rinse tube-I with 2.00 mL MeCN, vortex briefly, and decant the solution into tube-II

8. Vortex tube-II vigorously for 1 min to prevent crystalline agglomerations due to dehydration of anh. $MgSO_4$

9. Centrifuge the tube at 4000 rpm for 5 min

10. Transfer 1.00 mL of the upper layer into a 2 mL minicentrifuge tube containing 25 mg. anh. $MgSO_4$ + 25 mg PSA

11. Vortex the tube for 1 min

12. Centrifuge the tube at 4000 rpm for 5 min

13. Pipette 0.2 mL of the supernatant into 5 mL glass centrifuge tube and evaporate to dryness under vacuum with N_2

14. Add 0.2 mL of pyridine and vortex briefly

15. Add 0.2 mL mixture of BSTFA:1% TMCS (v/v) and vortex for 1 min

16. Incubate the tube at 70°C for 30 min and cool down to room temperature

17. Filter the final extract through a 0.45µm nylon syringe filter into autosampler vial for GC–MS analysis.

3.5 Method of Determination
3.5.1   HPTLC

1. The residue was reconstituted in 50 µL chloroform : methanol (1 : 1, v/v)

2. All the samples, 50 µL, and standards were spotted onto the HPTLC plate

3. Developing chamber (10 cm x 10 cm) with two developing systems ; ethyl acetate–methanol–ammonia (85:10:5, v/v/v, 10 mL) and toluene–acetone–methanol–ammonium hydroxide (45:45:7:3, v/v/v, 10 mL) as mobile phase, and dip the saturated pads (10 cm x 10 cm) for saturated with solvent vapor

4. The HPTLC plate was developed in a saturated developing chamber

5. The separation distance 7 cm, removed plate and the solvent was allowed to evaporate

6. The plate was sprayed with an acidified iodoplatinate reagent

7. Interpretation by comparison of R_f and color of spot between the sample and the standard

3.5.2 GC-MS

1. GC–MS analysis was performed on an HP 6890N GC coupled to HP 5973N mass selective detectors

2. The GC system was equipped with an electronic pressure control (EPC), split- splitless injector, and an HP 7683 autosampler. Chemstation software was used for instrument control and data acquisition/processing.

3. High purity helium (He) was used as carrier gas at 1 mL/min constant flow rate, which was controlled by EPC.

4. Inject 1 µL into double-tapered liner using splitless (1 min) injection at 260°C.

5. The analyte separation was carried out on a 30 m × 0.25 mm i.d. × 0.25 µm film thickness HP-5MS capillary column

6. The GC oven was set at initial temperature 150°C for 1 min, ramped to 260°C at 35°C/min, and then ramped to 280°C at 5°C/min and held for 7 min (to remove less volatile compounds remaining in the column). The total run time was 14.9 min (last eluter was 7.87 min).

7. The MS transfer line, ion source, and quadrupole temperature were default instrument setting at 280°C, 230°C, and 150°C, respectively.

8. The electron ionization energy was -70 eV.

9. The MS detection was operated in selected ion monitoring (SIM) mode.

3.6 Method validation

3.6.1 Method validation of HPTLC

3.6.1.1 Specificity

Standard solution and spiked blank urine extracts were performed on a HPTLC plate. The method was demonstrated by the presence with the Rf of morphine, codeine, heroine, 6-MAM and methadone.

3.6.1.2 LOD

LOD refers to the method lowest concentration of analyte detected. LOD of this method was performed for HPTLC method (3.4.1.1, 3.5.1), with the 3 replicates of each 5 concentrations of spiked blank urine extracts, 50 ng/mL, 100 ng/mL, 200 ng/mL, 300 ng/mL and 400 ng/mL of morphine and Codeine.

3.6.2 Method validation of GC-MS

3.6.2.1 Specificity

To verify the absence of interfering substance around the retention time of the analytes, by analyzing reagent blank, blank urine extracts from different volunteers, and spiked blank urine extracts, and checking for the presence of all selected ions and interference peaks eluted at the retention time of target analytes.

3.6.2.2 Linearity

The linearity of a test procedure is its ability (within a given range) to obtain test results proportional to the concentration of analyte in the sample. Linearity of this method was obtained from the standard calibration curve of morphine and codeine. Correlation coefficient (R^2) represents the linearity of the proposed method. The linearity was performed by spiking standard morphine and codeine in blank urine extracts in 3 replicates of each 10 concentration levels ranging from 10-450 ng/mL (10, 50, 100, 150, 200, 250, 300, 350, 400, and 450 ng/mL). The calibration curves were plotted as concentration over peak area of each analyte.

3.6.2.3 Recovery

Recovery and repartition were carried out by analyzing spiked blank urine samples for 10 replicates of each 3 spiked levels: 50, 150, and 300 ng/mL.

% recovery = (volume of compound discovered in spiked sample – amount of

compound in sample) / amount of compound extra x 100

3.6.2.4 Precision

The precision is the closeness of agreement between independent test results obtained under same condition. The two categories of precision are intra-assay precision and intermediate precision. The intra-assay precision is the precision derived from repeated tests on the same method with single analytical run, while the intermediate precision is the precision acquired from the repeated test on the same method with different operators or different times. In this work, the precision in terms of intermediate-precision was conducted for 12 replicates of spiked blank samples at 100 ng/mL for 2 consecutive days (n=24 overall). The replicate sample determinations were made together with a simple statistical assessment of the results including the percent of relative standard deviations (% RSD).

3.6.2.5 LOD and LOQ

LOD refers to the method lowest concentration of analyte detected, while LOQ is the lowest concentration of analyte which can be quatitatively determined. The LOD and LOQ were calculated at a signal to noise (S/N) ratio of 3 and 10, respectively.

3.7   Analysis of real urine samples

The feasibility of the proposed method was evaluated with urine samples obtained from different suspected heroine abusers. The tested urine samples followed the method as described in Section 3.4.1.1, 3.4.1.2, 3.5.1 and 3.5.2

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 The Optimization of sample preparation by QuEChERS technique

4.1.1 Optimization of QuEChERS for HPTLC

4.1.1.1 Extraction Solvents

Ethylacetate, acetonitrile, acetone, dichloromethane and chloroform were tested for finding the appropriated solvent for this study. After salt addition to induce phase separation, the result from Table 4.1 shows that, Ethylacetate is the most suitable due to the fast evaporation and its ability to provide well defined spots as shown in Figure 4.1

Table 4.1 Comparison of extraction between the five solvents

Solvent	Separation	Evaporation	Spot appear
Ethyl acetate	well separated	Quick dry	distinctive spots
Acetone	not well separated	Dry slowly	not well spots
Acetonitrile	not well separated	Dry slowly	not well spots
Dichloromethane	well separated	Quick dry	blur spots
Chloroform	well separated	Quick dry	blur spots
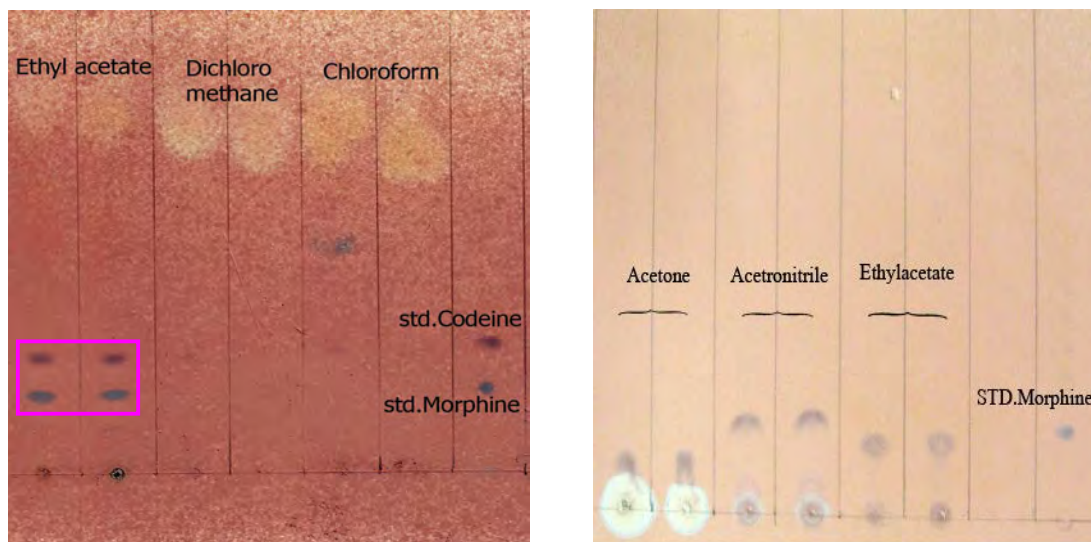
Figure 4.1 Comparison of extraction solvents for extract morphine and codeine in spiked blank urine samples (300 ng/mL each)

4.1.1.2 Amount of MgSO$_4$

Anh. MgSO$_4$, used as salt were employed as phase separation agents and induced target analyses to partition in the ethyl acetate phase. The amount of 2 g and 2.5 g of anh. MgSO$_4$ provide similar clear spot of morphine and codeine, in the Table 4.2. Therefore, selected amount 2 g anh. MgSO$_4$ was used for the experiment to remove water and polar matrix co-extractives in the extract, and improved spot analyze as shown in Figure 4.2

Table 4.2 Various amount of anh. MgSO$_4$

Amount of anh. MgSO$_4$	Spot appear
500 mg	+
1,000 mg	+
1,500 mg	++
2,000 mg	+++
2,500 mg	+++

Note : + Blur spot, ++ Clear spot, +++ Distinctive spot
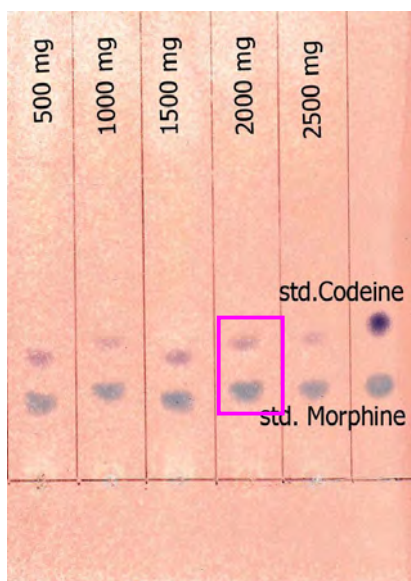
Figure 4.2 HPTLC obtained for morphine and codeine from varied amounts of $MgSO_4$ in the extract of spiked blank urine samples of morphine and codeine (300 ng/mL each)

4.1.1.3 Sorbent

The spot of morphine and codeine were obtained from using different sorbents in dispersive-SPE step. The result from Table 4.3 shows that. PSA, NH_2 and GCB can remove polar organic acid, some sugars and lipids; in addition GCB can also remove pigments from sample too. C_{18} is suitable for removing non-polar compounds, moderately polar compounds, sterols, some sugars and lipids. C_{18} gave poor spot of morphine and codeine because C_{18} itself could not retain polar matrix interference, the same trend was also observed with no-cleanup step. Alumina-N is neutral and high polar. It can retain polar co-extractives components, such as fatty acids, other organic acids, sugars and pigments. From Figure 4.3 Alumina-N obtained similar results to the PSA, but spot of codeine in Alumina-N more clearly than PSA. Therefore Alumina –N was selected for this experiment. The results of clean up in the extracts better than no clean up as show in Figure 4.4

Table 4.3 The comparison of sorbent type for cleanup

Type of sorbent	Spot appear	
	MOR	**COD**
PSA	++++	++++
C_{18}	+	+
Alumina – N	+++++	+++++
GCB	+++	+++
NH_2	++	++

Note : The most number of symbol refer to the most obvious spot.
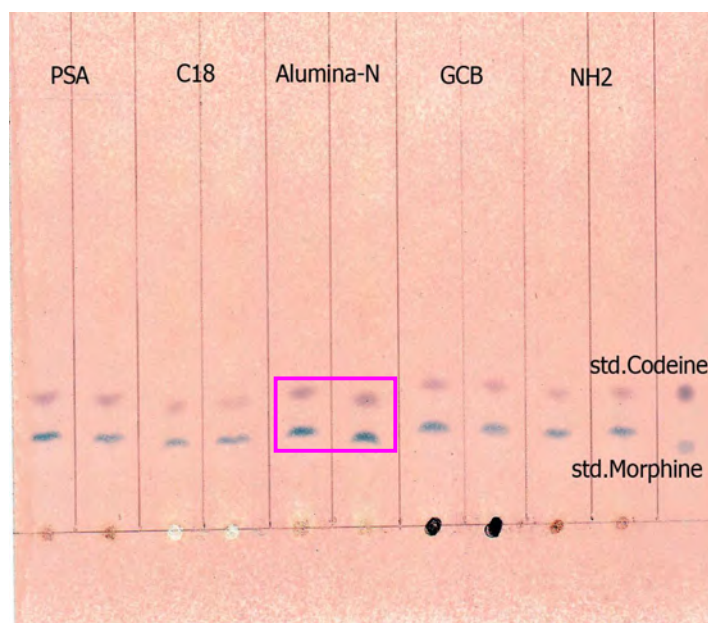
Figure 4.3   HPTLC obtained for morphine and codeine from varied sorbents in the extract of spiked blank urine samples of morphine and codeine (300 ng/mL each).
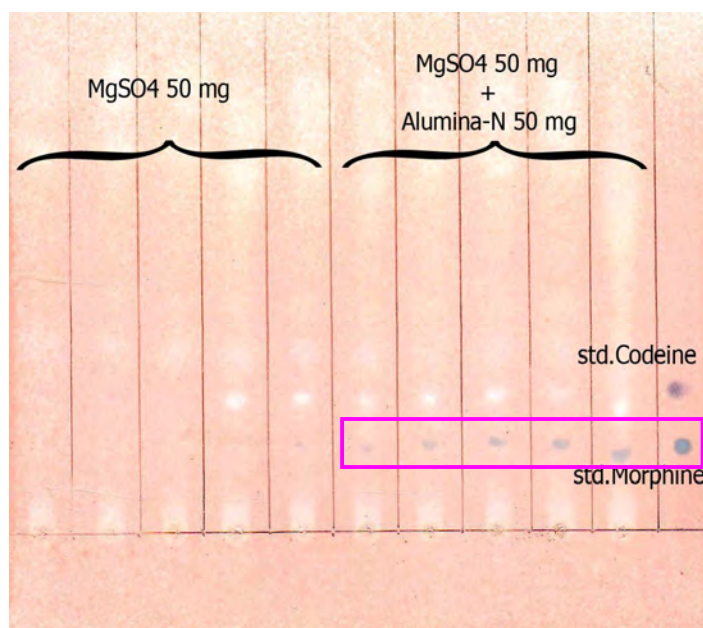
Figure 4.4 Comparison between clean-up and no clean up from in the extract of spiked blank urine samples of morphine and codeine (300 ng/mL each)

4.1.1.4 Amount of MgSO$_4$ and sorbent

With regards to dispersive-SPE cleanup, the sorbents combination and amount of sorbents were optimized to obtain good analytical results. In QuEChERS methods PSA is used for effective removal of fatty acids and some polar co-extracted components in foods. The addition, the anh. MgSO$_4$ helps to remove water residual in the extract. In this study, we designed experiments by mixing anhydrous MgSO$_4$ and Alumina-N in 1:1 ratio, varying amounts from 25 to 200 mg per 3 mL of extract. Increasing amount of alumina-N provided cleaner extracts, but it also significantly decreased amounts of morphine and codeine due to their adsorptions on alumina-N sorbents. Thus, 50 mg anh. MgSO$_4$ + 50 mg alumina-N was found to be the most suitable sorbents in this method, providing sufficient clean up of matrices in urine extracts as shown in Table 4.4 and Figure 4.5

Table 4.4 The different amounts of anh. MgSO$_4$ and alumina-N for cleanup

Amount of anh. MgSO$_4$ and alumina-N	Spot appear	
	MOR	**COD**
25 mg	+	+
50 mg	+++++++	+++
75 mg	++++++	++
100 mg	+++++	+
125 mg	++++	–
150 mg	+++	–
175 mg	++	–
200 mg	+	–

Note

+   The most number of symbol refer to the most obvious spot

-   Not detectable
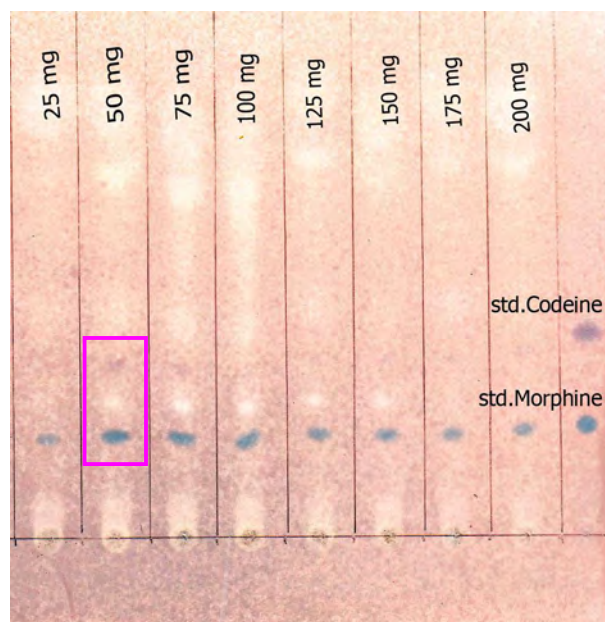
Figure 4.5 HPTLC obtained for morphine and codeine from varied amounts of anh. MgSO$_4$ and alumina-N in the extract of spiked blank urine samples of morphine and codeine (300 ng/mL each).

4.1.2 Optimization of QuEChERS for GC-MS

4.1.2.1 Extraction Solvent

MeCN, EtOAc, acetone, and MeOH were tested for finding suitable solvent for urine extraction by QuEChERS. MeOH did not separate well from the aqueous layer (the major composition of urine) after adding salts to induce phase separation (salting-out mechanism). Acetone gave low recoveries for both analytes and also showed high co-extractive interferences in the front part of total ion chromatogram. Figure 4.6 shows the results in a comparison of extraction solvents. MeCN and EtOAc provided no difference in term of recovery >75% for codeine and ≈100% for morphine. However, EtOAc has more solvent strength than MeCN gaving dirtier extracts, interferences were not much affected due to the analyte recoveries but they can cause contamination in MS ion source, deteriorate the performance of the instrument, and require frequent cleaning. Therefore, MeCN was chosen as the extraction solvent and used the 0.5 g anh. $MgSO_4$ + 0.25 g NaCl for removal of water and polar matrix co-extractives in the extract, and improved analyte recoveries.
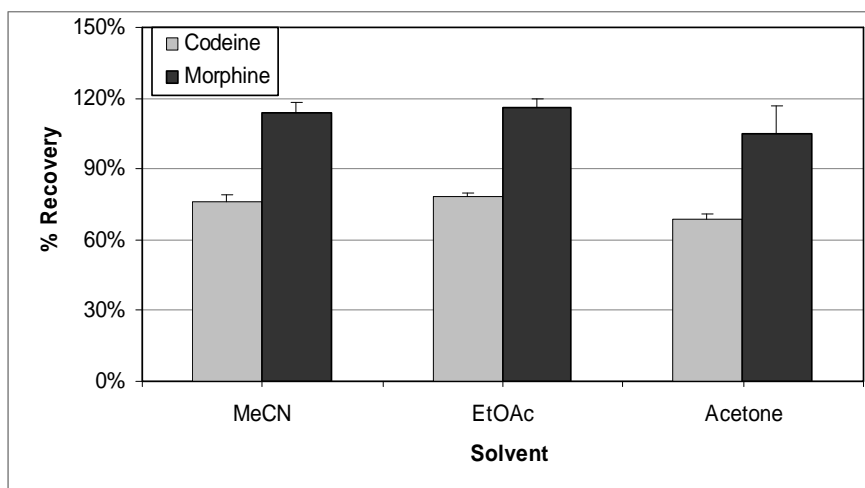
Figure 4.6 Recoveries of morphine (1.2 µg/mL) and codeine (0.6 µg/mL) spiked urine sample obtained from various extraction solvents.

4.1.2.2 Sorbent

Figure 4.7 shows the recoveries of morphine and codeine obtained from using different sorbents in dispersive-SPE step. Although differences in recoveries were not obtained for PSA, C_{18}, alumina-N, and no-clean up in spiked blank urine samples (Figure 4.7a), significant differences were obviously observed in spiked positive-urine samples (Figure 4.7b). This is probably due to the difference of urine compositions. Urine normally consists of water as a major constituent, and endogenous substances (such as albumin, urea, uric acid, creatinine, enzymes, fatty acid, glucose, carbohydrates, pigments, and dissolved ions). C_{18} gave suitable recovery for codeine but negatively impacted on morphine (<60% recovery) because C_{18} itself could not retain polar matrix interference, which tend to bound with morphine molecules. The same trend was also observed with no-cleanup step. Alumina-N, is a very polar sorbent, not only clean up matrix co-extractives but it also retained polar analytes, resulting in low recoveries. PSA gave recoveries better than other sorbent. Therefore, PSA was chosen as the sorbent for clean up, with using 25 25 mg PSA + mg anh. $MgSO_4$ in this method.
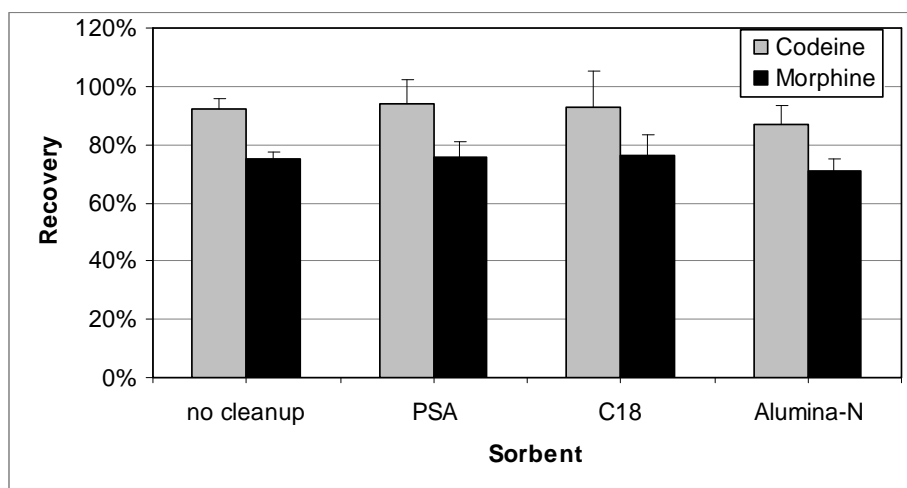
Figure 4.7 a)   spiked blank urine samples
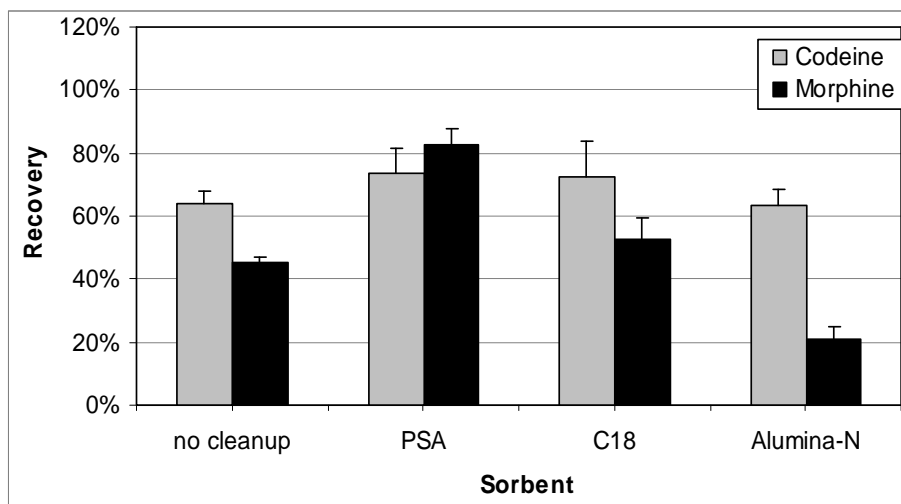
Figure 4.7 b)   spiked positive-urine samples

Figure 4.7 Comparison of different sorbents for dispersive-SPE cleanup of morphine (480 ng/mL) and codeine (1200 ng/mL) in urine extracts, a) spiked blank urine samples, b) spiked positive-urine samples.

4.2 The Optimization of the Detection Method

4.2.1   HPTLC method

QuEChERS is sample preparation method for determination of morphine and codeine in urine by HPTLC.   This proposed method solves the problem of the limited amount of samples. The remaining sample can be used for further confirmation procedures. The results of morphine and codeine were clear and easy to interpret. This method uses less than five times the sample volume, takes less analysis time from the original analysis, approximately from one day to only 4 hours for 10 samples and the cost is reduced from 300 baht to 130 baht per sample as shown in Table 4.5

Table 4.5 Advantage of HPTLC method development for determination of morphine and codeine in urine.

Parameter	Traditional Method (TLC)	Optimization of HPTLC method	Advantage of method development
Volume of urine sample	25 mL	5 mL	Less amount samples
Plate	TLC plate 20 cm x 20 cm	HPTLC plate 10 cm x 10 cm	Cheaper
Developing Tank	20 cm x 20 cm	10 cm x 10 cm	Convenient
Developing Solvent	100 mL	10 mL	Less amount solvent
Solvent consumption	large	Low	Less hazardous waste
Cost per test	300 baht	130 baht	Low cost
Time consumption	1 day / 10 samples	4 hours / 10 samples	Less time consumption

4.2.2 GC-MS method

Under optimum conditions, full-scan mass spectrum of TMS-morphine and TMS-codeine showed not only the number of fragmentation ions in EI but an abundance of high mass ions were also increased compared to underivatized forms (low mass ions). These high mass ions provided selective mass characteristics and showed less

amounts of matrix interferences, increasing detectability of the method. In this study, we operated MS in SIM mode, a base peak ion (highest abundance) at high m/z was chosen for quantitation and other 4 most abundant ions were monitored for identification purposes (Table 4.6)

Table 4.6 Parameters of GC–MS in SIM mode for analysis of codeine and morphine

Analyte	MW	t_R (min)	Acceptable t_R window (min)	Selected *m/z* (ion ratio)		dwell time (min)
				quantifier	qualifier	
codeine	299.36	7.57	7.27 – 7.86	371 (100)	196 (42), 234 (40), 313 (23), 343 (22)	20
morphine	285.34	7.86	7.57 – 8.14	429 (100)	236 (70), 324 (29), 401 (36), 414 (51)	20

The matters of forensic investigation had the chemical residues rather than in foods and environments. High efficient chromatographic and selective MS (/MS) techniques have become important issues for identification and quantitation of the presence of illicit drugs and their metabolites and doping substances in various specimens. Even if MS can provide unequivocal information which is unique to the chemical structure of each analyte, MS identification criteria is a basic requirement and should be included in the qualitative method in order to confirm false-positive, false-negative and meet all analytical needs. The factors of identification criteria for making qualitative decision in this study included: (1) Absence of positive findings in blank sample. (2) Expected t_R within ± 5sd of average t_R, chromatographic peak shape of quantifier ion matches that of reference standard. (3) Expected t_R within ± 5sd of average t_R, chromatographic peak shape of qualifier ion are similar as quantifier ion. (4). % relative intensity of the base ion is ≤10% of standard analyte in electron impact mode (5). Signal-to-noise ratio ≥3 for quantifier ion and qualifier ion.

Table 4.6 summarizes the selected ions, ion ratios, and tolerance window of retention time and ion ratio for morphine and codeine. The data obtained from calibration standard results was used for setting the acceptable range of retention times and ion ratios. These windows were taken into consideration for identifying the presence of analytes in validation experiments and real samples to enhance confidence in quantitative results and meet the performance of identification.

4.3 Method validation

4.3.1 Method validation for HPTLC

4.3.1.1 Specificity

The testing of 5 standard substances of opioid compounds are shown in Table 4.7 and the substances can be separated significantly by R_f. When compared between two systems, it gave the same results in both systems. This result showed that this method could be applied to identify opioid compounds from samples as shown in Figure 4.8

Table 4.7 R_f and color of spot of morphine, codeine, 6-MAM, heroine and methadone

Substances	Color of Spot	$R_f \pm SD$	
		System 1	System 2
100 µg/mL Morphine (3 µL)	Blue (light)	0.20 ± 0.01	0.18 ± 0.00
1000 µg/mL Codeine (1 µL)	Purple	0.32 ± 0.02	0.31 ± 0.01
1000 µg/mL 6-MAM (1 µL)	Purple-Brown	0.48 ± 0.02	0.44 ± 0.01
1000 µg/mL Heroine (1 µL)	Blue (deep)	0.53 ± 0.03	0.56 ± 0.03
1000 µg/mL Methadone (1 µL)	Red-Purple	0.74 ± 0.06	0.77 ± 0.01
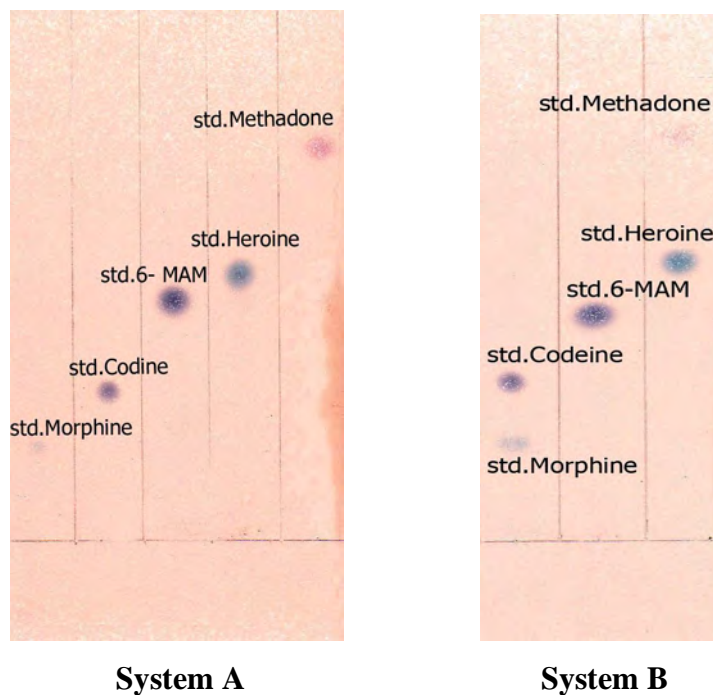
System A   **System B**

Figure 4.8 R$_f$ of substances from standard solution at 100 µg/mL Morphine, 1,000 µg/mL Codeine, 1,000 µg/mL 6-MAM, 1,000 µg/mL Heroine and 1,000 µg/mL Methadone.

System A: ethylacetate : methanol :ammonium hydroxide (8.5 : 1.0 : 0.5)

System B: toluene : acetone : methanol : ammonium hydroxide (4.5 : 4.5 : 0.7 : 0.3)

4.3.1.2 LOD of Morphine and Codeine

The standard solutions of morphine and codeine were 50 ng/ml - 400 ng/ml (Table 4.8) spiked into urine samples for determination of the limit of detection. After extraction and clean up following the proposed method, the limit of detection (LOD) of morphine and codeine are found to be 100 ng/mL and 300 ng/mL respectively (Figure 4.9). The LOD of this technique conforms to the Thai legislation for the detection of such drugs in a defendants' urine.

Table 4.8 Concentration of Morphine and Codeine in experiment of LOD

Concentration	Spot appear	
(n = 3)	MOR	COD
50 ng/ml	-	-
100 ng/ml	+	-
200 ng/ml	+	-
300 ng/ml	+	+
400 ng/ml	+	+

Note

- Not Detectable

+ Detected
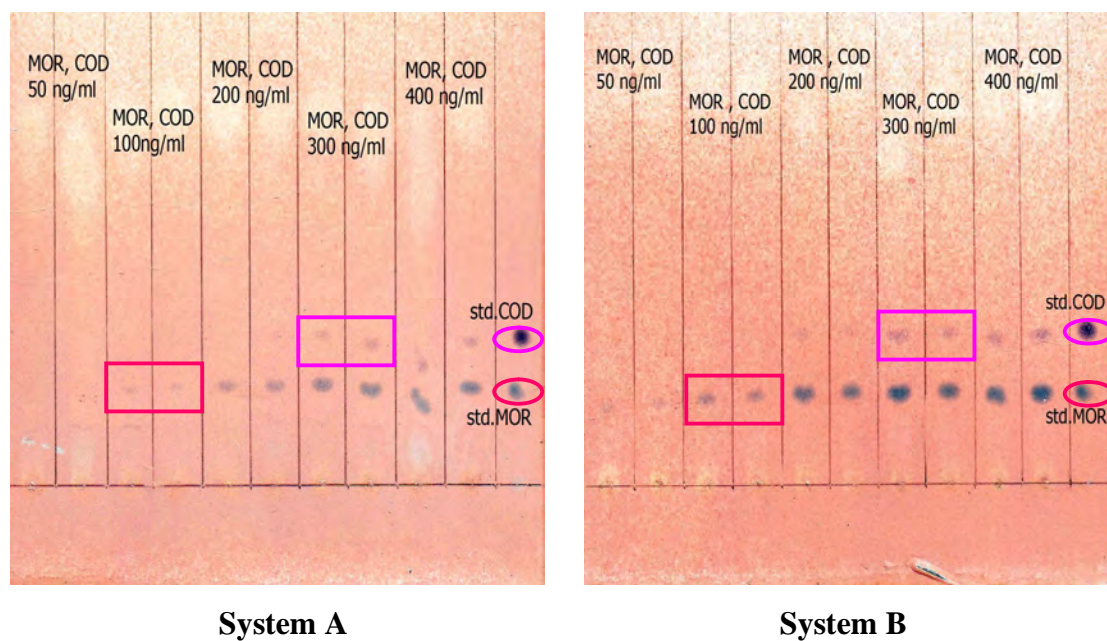
System A   System B

Figure 4.9 HPTLC for determination of LOD of morphine and codeine at
50 ng/mL - 400 ng/mL in spiked urine samples
system A : ethylacetate : methanol :ammonium hydroxide (8.5 :1.0 : 0.5)
system B : toluene : acetone : methanol : ammonium hydroxide (4.5 : 4.5 : 0.7 : 0.3)

4.3.2   Method validation for GC-MS

4.3.2.1 Specificity

This experiment was performed by separate injection of reagent blank, 10 of blank urine extracts, spiked blank urine extracts, and positive-urine extracts. Selectivity of the method was demonstrated by the presence or absence of interfering peaks at the elution time of codeine (7.57 min) and morphine (7.86 min). None of interfering peaks co-eluted with the analyte peaks or eluted close to the t_R of analytes in any sample extracts.

4.3.2.2 Linearity

Linearity of the method was performed using matrix-matched standard calibrations. The 10-point calibration curves (not included 0-point) for quantifier ion of each analyte were linear with regression coefficient (R^2) 0.997 for both morphine and codeine concentration range of 10-450 ng/mL. (Figure 4.10)
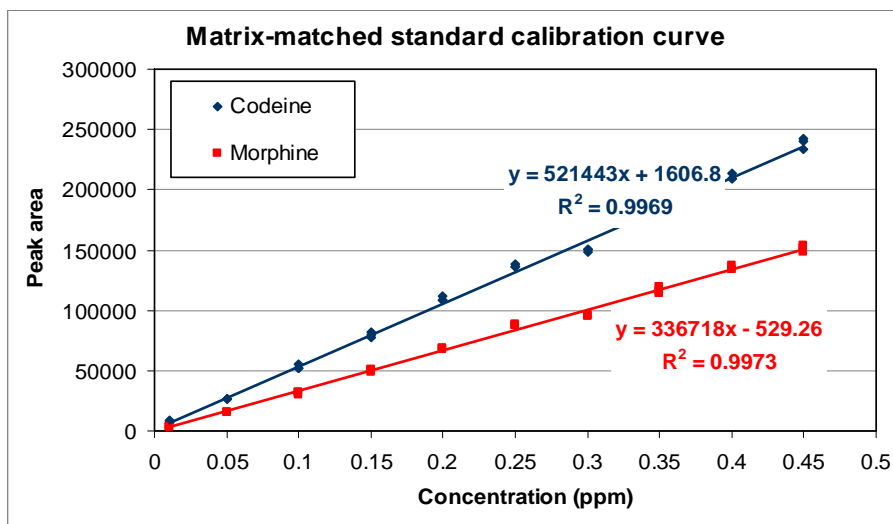
Figure 4.10 Matrix matched standard calibration curve of morphine and codeine

4.3.2.3 Recovery

The accuracy of the method was conducted using spiked blank urine samples at 50, 150, and 300 ng/mL (cut-off levels). The results of the recovery study are given in Table 4.9. The average overall recoveries of morphine and codeine were >70% and >90% (n=15), respectively in all cases. Morphine showed slightly lower recoveries, which were due to its polarity and partial retention on PSA sorbents in dispersive-SPE and/or incomplete hydrolysis of morphine-conjugates.

4.3.2.4 Precision

The precision in terms of repeatability and intermediate-precision (interday-precision) is reported in Table 4.9. Within-sequence repeatability (same spiking level) gave RSD <6% (n=15) in all cases, which were lower than the acceptable limit RSD of 13-17% calculated from Horwitz equation. Also, repeatability of all results (n=45) gave <6% RSD. Intermediate-precision of the method was ≈ 4 % RSD (n=24, 2 days), which was within the acceptance limit of 15% RSD. These results demonstrate well-suited proposed method for quantitation of morphine and codeine in urine sample.

Table 4.9 Average recoveries, repeatability, and intermediate-precision results for codeine and morphine.

| analyte | recovery (n=15) | | | | | | repeatability (n=45) | intermediate-precision (100 ng/mL) | | | |
| | 50 ng/mL | | 150 ng/mL | | 300 ng/mL | | | day1 (n=12) | day2 (n=12) | overall (n=24) | accepted %RSD* |
	%recovery (%RSD)	accepted %RSD*	%recovery (%RSD)	accepted %RSD*	%recovery (%RSD)	accepted %RSD*					
codeine	92 (1.6)	16.6	98 (3.6)	14.0	94 (5.6)	12.7	95 (4.9)	97 (3.4)	94 (4.8)	96 (4.3)	14.9
morphine	79 (3.7)		80 (2.8)		73 (5.6)		77 (5.4)	77 (3.3)	76 (5.4)	77 (4.4)	

*accepted %RSD values calculated from Horwitz equation *(48)*

4.3.2.5 LOD and LOQ

LODs and LOQs were estimated by analyzing spiked blank urine extract at lowest calibrated level and determining the concentration of each analyte which provided signal-to-noise ratio equal to 3 and 10, respectively. The LOD values were 39.1 ng/mL for morphine and 40.6 ng/mL for codeine. The LOQs were 130.4 ng/mL for morphine and 135.3 ng/mL for codeine. These values were lower than the established cut-off concentrations, indicating the performance of the proposed method for the determination of morphine and codeine at trace levels. However, these values can be lowered by using selective tandem MS (MS/MS), which provides higher sensitivity and more specificity.

4.3.2.6 Ruggedness

Ruggedness refers not only the consistency of the instrument, but it also means the entire sample preparation procedure. For the QuEChERS method, the efficiency has been demonstrated in many studies. However, for the derivatization method, selectivity of chemical reaction (including completion of reaction, amount of reactants, temperature, time, etc.) becomes a crucial factor in part of the method. Thus, this can lower the selectivity and affects to long term-ruggedness of the method. In this study, method ruggedness was evaluated from the t_R of analytes and sensitivity of the method. Automated MS tuning was performed before starting each injection sequence. From multiple day analyses without performing any maintenance to instrumentation, the t_R of morphine and codeine were consistent with overall RSD $\approx 1\%$ (n>100). The matrix-matches calibration curves (6 days) gave good linearities with average R^2 of 0.998±0.002 and did not show the significant difference of slopes at 95% confidential Student's t-test. These demonstrated good ruggedness in terms of sensitivity and high reproducibility of the derivatization method and in the performance of the instrument.

4.4 Analysis of real urine samples

4.4.1 Analysis of real urine samples for HPTLC

The feasibility of the proposed method was evaluated with urine samples obtained from positive urine of morphine. Thirty urine samples were tested for morphine and codeine by using SPE as the sample preparation method then analysed with GC-MS, compared with QuEChERS which is the successful method developed for HPTLC. From GC-MS with SPE, seven samples were found only morphine and twenty three samples found both morphine and codeine. For QuEChERS of HPTLC method, five samples were found only morphine and nineteen samples found both morphine and codeine.   As a result, twenty four samples have been shown to contain a high accuracy of HPTLC (QuEChERS) techniques which was equally to eighty percentages of GC-MS with SPE. (Table 4.10)

Three samples found 6-MAM from both the HPTLC and GC-MS method as shown in Figure 4.11**.** Thus, the successful method of QuEChERS was developed for the determination of morphine and codeine in urine sample with a high efficiency using small amount of sample. Moreover, this technique can also detect 6-MAM.

Table 4.10 Comparison of results of morphine and codeine in real urine samples for HPTLC and target value with GC-MS.

Result	Number of sample	
	GC-MS (SPE)	**Develop method (QuEChERS) for HPTLC**
MOR + COD	23	19
MOR	7	5
COD	0	3
Not found	0	3
Total	30	30
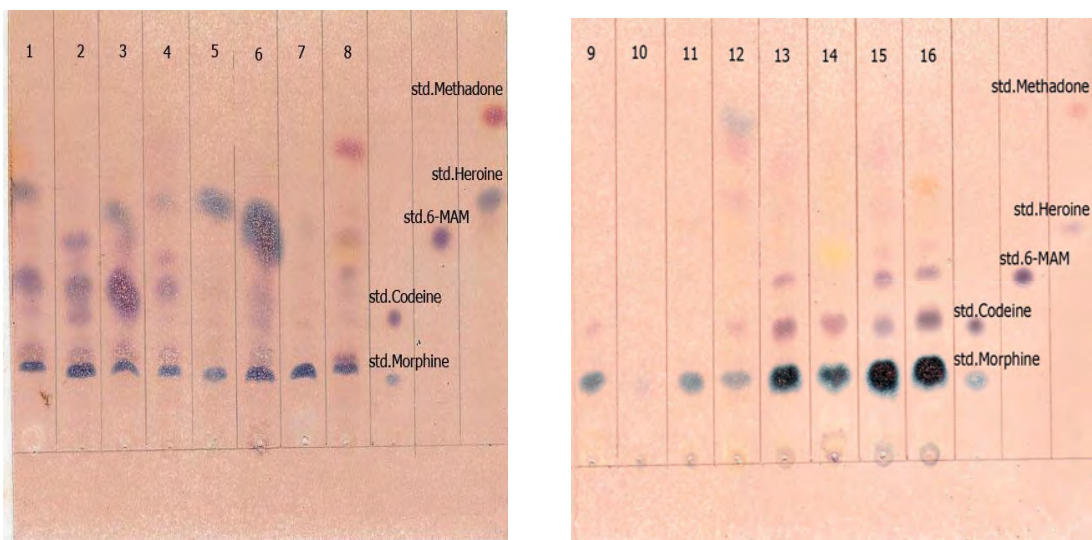
Figure 4.11 HPTLC obtained the results of morphine, codeine and 6-MAM (6-MAM found in no.13,15,16) from real urine samples compared with standard Morphine Codeine, 6-MAM, Heroine and Methadone.

4.4.2 Analysis of real urine samples for GC-MS

The feasibility of the proposed method was evaluated with urine samples obtained from different suspected heroin users. The tested urine samples were prepared as described in procedure. Matrix-matched standards were used for quantification of morphine and codeine in these samples, whereas retention times ($\pm$3sd), chromatographic factors, and ion ratios ($\pm$3sd) were used for identification. From 20 urine samples, 8 samples showed positive results (Table 4.11) with the presence of morphine and codeine exceeded the cut-off levels of 300 ng/mL and these positive-results met all identification criteria of the study.

Table 4.11 Results of real urine samples for GC-MS

Result (GC-MS)	Number of detected sample
MOR + COD	8
Not found	12
Total	20

CHAPTER V

CONCLUSION AND SUGGESTION FOR FURTHER STUDY

This work was aimed at developing sample preparation methods for determination of morphine and codeine in a defendant's urine. The traditional method, LLE, is complicated, time consuming and requires a large amount of the samples; the samples must be enriched enough for the test. A QuEChERS (Quick Easy Cheap Effective Rugged and Safe) is a sample preparation method for this work.

QuEChERS is used for detection of morphine and codeine in urine by HPTLC. LOD of this method is 100 ng/mL and 300 ng/mL, respectively. This method can reduce volume of tested sample from 25 ml to 5 ml. Solvents for QuEChERS are safer and cheaper than the conventional method. This method can be used in samples that need to consider more than 1 type of narcotic by TLC technique.

It also found 6-MAM in this method at level 1,000 ng/mL, and it was confirmed by GC-MS. Because of 6-MAM has very short half-life, positive results depend on many factors, such as time and volume of sample. HPTLC method can detect of 6-MAM in the urine while conventional TLC could not be detected. However, HPTLC method is the sensitive method that could be further developed for detection of 6-MAM in the urine. Therefore, this method is efficient, rapid, simple, selective, cost effective, time saving, and no special equipment required. Moreover, it can be used for routine work.

GC-MS method is successfully for single-laboratory validates and provides good results (linearity, recovery, precision, and ruggedness). This proposed method shows good sensitivity with LOD and LOQ of both morphine and codeine. Sensitivity of both methods was lower than cut-off levels established by SAMHSA and Thailand Narcotics Control Act. A rapid and efficient QuEChERS method has been developed for the identification and quantitation of morphine and codeine in urine of heroin users. This method also provides several practical advantages, such as quick, easy, low cost, high sample throughput, less time consumption, less hazardous waste, and

less labor usage, compared to the other traditional methods. The method can overcome the amount of repetitions due to the small amount of urine sample needed for extraction, allowing the re-analysis to confirm the presence of illicit drugs in the urine.

The procedure of QuEChERS for detection morphine and codeine in urine by HPTLC and GC-MS as shown in Figure 5.1 and Figure 5.2, respectively.

| Pipette 5 mL of urine and 5 mL 0.1 M HCl into test tube-I |

↓

| Vortex 1 min and incrubate at 100 $^{\circ}$C 30 min |

↓

| Cool down to room temperature and adjust to pH 9 by ammonia solution |

↓

| Decant the extract into a 50 mL centrifuge tube-II containing 2.0 g anh. MgSO$_4$ |

↓

| Rinse the tube-I with 5 mL ethyl acetate, vortex briefly, and decant the solution into the tube-II |

↓

| Vortex vigorously for 1 min, and centrifuge at 4000 rpm for 5 min |

↓

| Transfer 3.5 mL of the upper layer into a 15 mL centrifuge tube containing 50 mg anh. MgSO$_4$ + 50 mg Alumina-N |

↓

| Vortex for 1 min, and centrifuge at 4000 rpm for 5 min |

↓

| Pipette 3 mL of the supernatant into cup |

↓

| Evaporate to dryness then analyze with HPTLC |

Figure 5.1 Schematic diagram of optimized sample preparation for HPTLC

Pipette 1 mL of urine and 1 mL 0.1 M HCl into test tube-I

↓

Vortex 1 min and incubate at 80 $^{\circ}$C 30 min

↓

Cool down to room temperature and adjust to pH 9 by ammonia solution

↓

Decant the extract into a 10 mL centrifuge tube-II

containing 0.5 g anh. $MgSO_4$ + 0.25 g NaCl

↓

Rinse the tube-I with 2 mL MeCN, vortex briefly,

and decant the solution into the tube-II

↓

Vortex vigorously for 1 min, and centrifuge at 4000 rpm for 5 min

↓

Transfer 1 mL of the upper layer into a 2 mL minicentrifuge tube

containing 25 mg. anh. $MgSO_4$ + 25 mg PSA

↓

Vortex for 1 min, and centrifuge at 4000 rpm for 5 min

↓

Pipette 0.2 mL of the supernatant into 5 mL glass centrifuge tube,

and evaporate to dryness under vacuum with N_2

↓

Add 0.2 mL of pyridine and vortex briefly,

Add 0.2 mL mixture of BSTFA:1% TMCS (v/v) and vortex for 1 min

↓

Incubate the tube at 70°C for 30 min and cool down at room temperature

↓

Filter through a 0.45μm nylon syringe filter into vial for GC–MS analysis

figure 5.2 Schematic diagram of optimized sample preparation for GC-MS

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APPENDIX

APPENDIX

Determination of morphine and codeine in urine by TLC

1. Add 25 ml. of urine sample into flask

2. Add 10 ml of conc. HCl , boil on hot plate for 5 min. (start from boiling)

3. Cool down to room temperature

4. Adjust to Neutralize pH with 50% Sodium hydroxide and filter through filter paper

5. Decant the extract into separation funnel

6. Adjust to pH 2-3 with 6 N HCl

7. Add diethylether equal volume to remove impurities, then discard ether

8. Adjust to pH 8.5 – 10 with ammonia solution

9. Add ethyl acetate or chloroform: isopropanol (3 : 1) equal volume for extraction

10. Take organic solvent phase and remove water by Sodium sulfate anhydrous

11. Evaporate to dryness then analyze with TLC

12. The residue was reconstituted with chloroform : methanol   (1 : 1, v/v)

13. The samples and standards were spotted onto the TLC plates

14. The Developing chamber (20 cm x 20 cm) with two developing systems ;
    as mobile phase. The saturated pads were dipped (20 cm x 20 cm)
    saturated with solvent vapor.

15. The TLC plate was developed in a saturated developing chamber

16. The separation distance 10 cm, removed plate and the solvent was allowed to
    evaporate

17. The plate was sprayed with an acidified iodoplatinate reagent

18. Interpretation by comparison of R_f and color of spot between the sample and the
    standard

Developing solvent

systems A: ethylacetate–methanol–ammonia (85 : 10 : 5)

systems B: toluene–acetone–methanol–ammonium hydroxide (45 : 45 : 7 : 3)

Acidified iodoplatinate reagent

0.25 g of   platinic chloride and 5 g of   potassium iodide in 100 ml of water,
then add 5 mL of conc. HCl

VITA

Mrs Chanida Sangsuri was born on July 16, 1971, in Samutsongkhram, Thailand. She had graduated a Bachelor's degree of Science in Chemistry from Rajabhat Ubon Ratchathani in 1994. Afterwards, she continued her academic education for Master degree at Department of Chemistry, Faculty of Science, Chulalongkorn University. She had completed the program in April 2010 and received her Master's degree of Science in Analytical Chemistry in July 2011. At present, she has been working in Regional Medical Sciences Center 4th Samut Songkhram, Department of Medical Sciences Ministry of Public Health, since 1997.