Analytical Method Development and Validation for Forensic Investigation of Psychoactive Compounds and Gastrointestinal Fluids Characterization

"For the attention, this thesis includes standalone chapters; therefore, the literature review and the introduction will reflect this. And all include topics that are not related to each other."

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Declaration of Authenticity and Author's Rights

'This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.'

Signed: Abdullah Aldasem

Date: July,2024

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By the name of Allah

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Abdullah Aldasem

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List of Abbreviations

Abbreviation	Explanation
Δ^8-THC	Δ^8-tetrahydrocannabinol
AAIs	Aminoalkylindoles
AC	alternating current
ACMD	British Advisory Council on the Misuse of Drugs
ADB-PINACA-COOH	ADB-PINACA 5-Pentanoic Acid
ADHD	attention-deficit/hyperactivity disorder
AMP	Amphetamine
APCI	atmospheric pressure chemical ionization
AUCs	Areas under the curve
BCS	biopharmaceutical classification system
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
cAMP	cyclic adenosine monophosphate
CB1	cannabinoid receptor 1
CB2	cannabinoid receptor 2
CBD	Cannabidiol
CHL	cholesterol
CI	chemical ionization
CID	collision-induced dissociation
CNS	central nervous system
COD	codeine

Abbreviation	Explanation
CVS	cardiovascular system
DAG	diacylglycerides
DC	direct current
DEA	Drug Enforcement Administration
DoA	Drugs of abuse
DTE	dithioerythritol
DZP	Diazepam
ECS	endocannabinoid system
EI	electron impact
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
ESI	electrospray ionization
FAB	fast ion bombardment
FFA	free fatty acids
GABA	gamma-aminobutyric acid
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GIT	gastrointestinal tract
GMO	glyceryl monooleate
HER	heroin
HGF	human gastric fluids
HIF	human intestinal fluids
HPLC	high-performance liquid chromatography
IUPAC	The International Union of Pure and Applied Chemistry

Abbreviation	Explanation
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LLE	liquid–liquid extraction
LOD	limit of detection
MAG	monoacylglycerides
MALDI	matrix assisted laser desorption/ionization
MAMP	methamphetamine
MENA	the Middle East and North Africa
MOR	morphine
MRM	multiple reaction monitoring
MS	mass spectrometry
MS-MS	Tandem mass spectrometry
MSTFA	N-methyl-N-(trimethylsilyl)trifluoroacetamie
MTBE	Methyl tert-butyl ether
NPS	Novel Psychoactive Substances
ODS	octadecyl silane
O-TMS	Oleate-TMS
PGB	pregabalin
PPT	protein precipitation
PTR	proton transfer reaction ionization
Q	quadrupole mass analyzer
QqQ	triple quadrupole mass spectrometer
RSD%	relative standard deviation

Abbreviation	Explanation
SCs	Synthetic cannabinoids
SIM	selected ion monitoring
SO	sodium oleate
SPE	solid phase extraction
SRM	selected reaction monitoring
TAG	triacylglycerides
THC	Δ9-tetrahydrocannabinol
TMCS	trimethylchlorosilane
TMS	trimethylsilyl
TMSDM	Trimethylsilyldiazomethane
TMSI	N-trimethylsilylimidazole
TOF	time-of-flight mass analyzer
TQ-ESI	triple quadrupole electrospray ionization
UGT	UDP-glucuronosyltransferase
UHPLC	ultra-high-performance liquid chromatography
UNODC	United Nations Office on Drugs and Crime

Abstract

Drugs of abuse (DoA) involve a wide range of substances, including illicit drugs (Heroin), prescription medications when used non-medically (benzodiazepines), and even legal substances when misused (alcohol). DoA pose significant public health challenges and social problems, therefore, effective strategies are essential to address these concerns. The aim of this thesis was to screen common DoA in Kuwait and draw a picture on the common trends in illicit DoA. First, ten synthetic cannabinoids (SCs) were investigated, and a LC-MS/MS method was developed for their separation and quantitation. The method was used for screening of these SCs in street samples in Kuwait (**Chapter 2**). Another three SCs were screened in urine samples collected in Kuwait. A solid phase extraction (SPE) procedure was developed for extraction of the three SCs from urine, followed with a sensitive and specific LC-MS/MS method for their quantitation. (**Chapter 3**). Next, a sensitive and specific LC-MS/MS method was developed for the detection of six DoA common in Kuwaiti market, namely pregabalin, morphine, amphetamine, methamphetamine, codeine, and diazepam. The six drugs were screened in 150 urine samples collected in Kuwait (**Chapter 4**).

Nowadays, gastrointestinal tract (GIT) media are commonly used to determine drug solubility and bioavailability in vitro during drug development phase. GIT media are susceptible to large variability between individuals and to inter-day fluctuations, in addition to meal intake and biliary and pancreatic secretions. These facts show the importance of characterization of GIT fluid components

for accurate determination and prediction of drug bioavailability. The aim of work in this chapter was to characterize common GIT fluid components such as sodium oleate (SO), glyceryl monooleate (GMO), and cholesterol (CHL). An accurate and specific GC-MS method was developed for the determination of these compounds. A derivatization procedure was optimized for the three compounds, where silylation reagent mixture was used to produce their silyl derivatives (**Chapter 5**).

Chapter 1 General Introduction and Literature Review

1.1. Synthetic Cannabinoids

1.1.1. Introduction

Synthetic cannabinoids (SCs) represent the most common group of Novel Psychoactive Substances (NPS) that is consumed by young people nowadays. NPS are new compounds that are designed to mimic the psychoactive effects of some known controlled drugs. The United Nations Office on Drugs and Crime (UNODC) was the first organization to use the term NPS and defined it as “substances of abuse, either in a pure form or a preparation, that are not controlled by the 1961 Single Convention on Narcotic Drugs or the 1961 Convention on Psychotropic Substances, but which may pose a public health threat” [1]. NPS are new chemicals that are developed to replace drugs of abuse that are banned, and their chemical structures are continually changing to stay ahead of the law [2].

During the 2000s, the quality of some well-known illicit drugs such as cocaine was low. This fact together with drug users being open for new experiences transformed the consumption behavior of illicit drugs significantly. NPS appeared at this time, as a first generation, synthetic cathinones [3]. These compounds are usually synthesized in massive amounts in underground labs mainly in China prior to their shipping to other countries. NPS are usually mislabeled, to circumvent the law, such as “designer drugs”, “legal highs”, “bath salts”, “internet drugs”, “research chemicals” and even “potpourri” [2].

NPS are grouped into several classes such as synthetic cannabinoids, synthetic cathinones, synthetic opioids, ring substituted amphetamines, novel benzodiazepines, psychedelic phenethylamines, tryptamine derivatives, aminoindanes, and piperazines [2, 4]. However, NPS are classified by European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) into four groups: synthetic cannabinoids (MDMB-CHMICA),

synthetic stimulants (synthetic cathinones), new opioids (furanylfentanyl) and new antidepressants [5]. Several reasons have made NPS very common currently among drug users; such as legality, easy availability and affordability, curiosity, social media users' experiences, and the potent psychoactive effects [6].

UNODC have emphasized the risks posed by NPS usage among youths and many studies have also addressed the use of NPSs by teenagers and young adults [7]. UNODC identify NPS as one of the major health issues worldwide. According to UNODC, NPS have been emerging at an average rate of one substance per week. The extent of drug use among people in age range 18–25 remains higher than that among old people [8]. Many studies have also addressed the use of NPS by teenagers and young adults this is observed in countries in most regions and for most drug types [7]. Synthetic cannabinoids and cathinones represent the most common groups of NPS being monitored globally [9].

1.1.2. Cannabinoids

Cannabinoids are terpeno-phenolic compounds found in *Cannabis sativa* plant which are psychoactive in nature. *Cannabis sativa,* also known as marijuana, is the source of a large number of pharmacologically active compounds, the most famous one of these is cannabinoids [10]. According to their source, these compounds are classified into three groups namely: phytocannabinoids from cannabis plant, endocannabinoids formed in animals and humans, and synthetic cannabinoids synthesized in laboratories [11]. Pharmacologically, the term "Cannabinoids" indicates compounds that can activate cannabinoid receptor 1 (CB1) or cannabinoid receptor 2 (CB2) or both. Other molecules included under this class of drugs are structurally similar to cannabinoids, but do not activate CB1 and CB2 receptors. Also, many compounds

found in *Cannabis sativa* that do not activate these receptors are referred to as cannabinoids [10].

Phytocannabinoids are lipid-soluble phytochemicals found in *Cannabis sativa L.* Δ^9-tetrahydrocannabinol (THC) and Δ^8-tetrahydrocannabinol (Δ^8-THC) are phytocannabinoids that have affinity for the cannabinoid receptors and elicit a psychotropic effect, while some others do not exhibit such effects such as Cannabidiol (CBD) [12], **Figure 1.1**. Phytocannabinoids are also found in non-cannabis plants such as β-caryophyllene which is widespread in several plant essential oils [13], **Figure 1.1**.

Endocannabinoids (endogenous cannabinoids) are a group of naturally occurring members of eicosanoid super-family, which activate cannabinoid receptors [10]. Anandamide, 2-arachidonoylglycerol and virodhamine (**Figure 1.2**) are examples for this class. They are involved in many regulatory functions in animals such as sleep wakefulness cycle, pain perception, regulation of egg implantation, control of sensorimotor and motivational aspects and memory function. Endocannabinoids induce these effects by interacting with either CB1 or CB2 receptors [14-16].

SCs are laboratory generated chemicals that can bind to CB1 and CB2 and produce psychoactive effects similar to THC e.g. JWH-200, WIN-55, HU-210, and AM-906 [17].

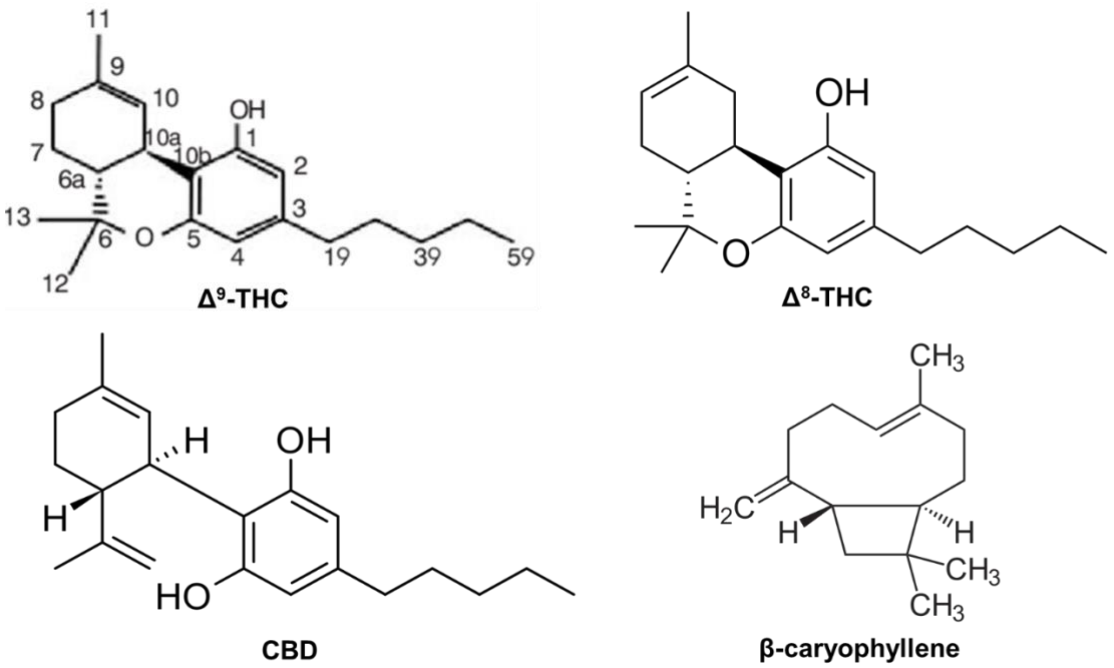


Figure 1.1 Chemical structure of phytocannabinoids (Δ^9-THC, Δ^8-THC, CBD, and β-caryophyllene), showing carbon numbering on Δ^9-THC

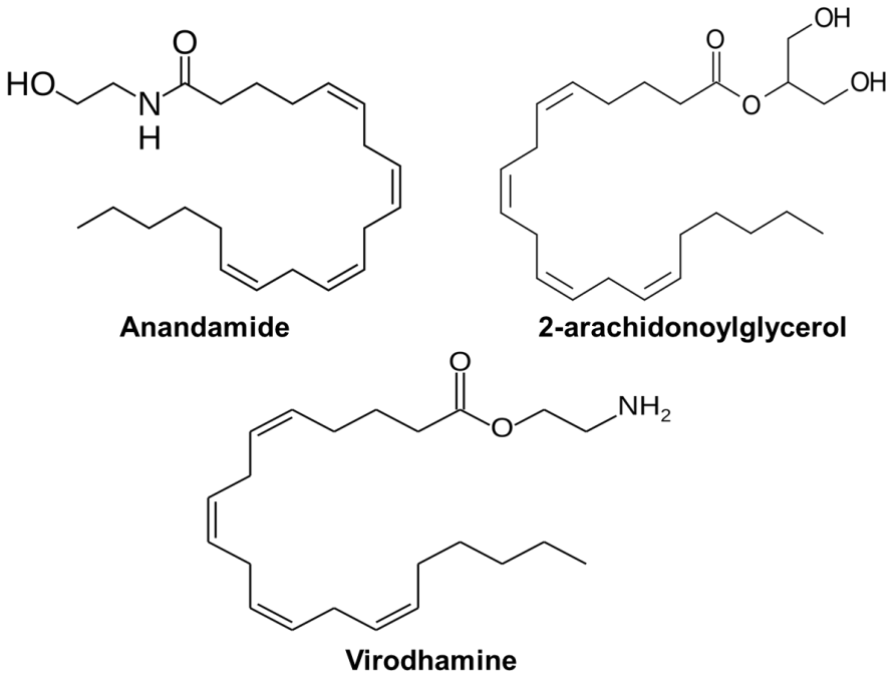


Figure 1.2 Chemical structure of endocannabinoids (anandamide, 2-arachidonoylglycerol, and virodhamine)

1.1.3. History

Since the discovery of natural cannabinoids, novel synthetic analogues with similar structures have been prepared by different research groups. In 1964, Raphael Mechoulam and his research group isolated one of the most active compounds from *Cannabis sativa* (THC) [18]. The same research group managed to synthesize this compound chemically, which allowed further biological studies and THC was confirmed as the major active compound in Cannabis. Subsequently, about 60 other phytocannabinoids and endogenous cannabinoids have been identified by Mechoulam's research group [19, 20]. Several research groups have significantly contributed to the field of synthetic cannabinoids such as Mechoulam et al. and Huffman et al., with the later group synthesizing 450 synthetic cannabinoids [21]. SCs were initially synthesized with the intention of exploring therapeutic effects and to study cannabinoid receptors and the endocannabinoid system (ECS). However, in the early 2000s, SCs started to be produced commercially and abused [17].

1.1.4. Synthetic cannabinoid and drug market

SCs emerged as common drugs of abuse when preparations branded as "K2" in USA or "Spice" in Europe became available online and in "head shops". These products were advertised as "harmless incense blends", which lead to the misleading guess that they were safe products. The SC drug formulations mainly contain plant materials such as "wild dagga" (*Leonotis leonurus*) and "Indian warrior" (*Pedicularis densiflora*) [22]. This herbal matrix gives the impression that these are natural products, but they have been intentionally fortified with SC. The SCs are first dissolved in a solvent such as ethanol, and then added to the herbal material. The solvent evaporates but leaves a highly variable concentrations of the SCs in the plant material [23].

Products of SCs are relatively cheaper and more available than marijuana, which make them very tempting for young people who want to try drugs [23]. Additionally, SCs are not detected in standard drug inspections. These facts caused a rapid increase in the use of K2 in college and high schools, making it the second most prevalent illicit drug after marijuana between 2008-2011 [23].

1.1.5. Chemistry of Synthetic Cannabinoids

SCs are either structurally related to THC (**Figure 1.1**) or have different skeletons that may or may not contain heterocyclic rings. Most SCs are lipid soluble and non-polar, consisting of 22–26 carbons. For optimal pharmacological activity, SCs should have 4-9 saturated carbon atoms [21].

Despite structural diversity, SCs share certain chemical moieties with THC, enabling them to interact with CB receptors [24]. An aromatic ring system is crucial for the binding affinity at the CB receptors. This aromatic ring interacts with hydrophobic regions within the receptor. THC contains a tricyclic structure with an aromatic phenolic ring, while SCs often include an aromatic or heteroaromatic ring system, such as a naphthoyl, cyclohexylphenol, or indole ring [25]. THC has a pentyl side chain (C_5) attached to its aromatic ring. This side chain is essential for binding to the CB1 receptor, as increasing its length has been shown to enhance affinity and activity at the receptor. Similarly, SCs typically possess an alkyl chain (C_5 or longer chains) attached to their core structures. Terminal fluorination of this alkyl chain generally enhances potency of CB1 receptor activation [26]. THC has a hydroxyl group attached to its phenolic ring, which is involved in hydrogen bond interactions with CB receptors, contributing to its binding affinity and partial agonism. SCs often include a functional group capable of hydrogen bonding, such as hydroxyl, carbonyl, or amide groups. These groups can interact with polar regions in the CB1 or CB2 receptors contributing

to their overall affinity and activity [27]. THC is lipophilic, which enables it to readily cross the blood-brain barrier and interact with the hydrophobic binding pocket of CB receptors. SCs are generally designed to be highly lipophilic, often surpassing THC in this regard [24].

The nomenclature of SCs is not fully systematized, it contains abbreviations and numbering. Each SC series is usually named with a two- or three-letter abbreviation for the place where the substance was synthesized, analyzed or tested or the scientist who synthesized it. This letter abbreviation is followed by a three- or four-figure number to identify them within that series. The most commonly reported series (their names' origin), are JWH (John W. Huffman), HU (Hebrew University), AM (Alexandros Makriyannis), CP (Pfizer), RCS (Research Chemical Supply), and WIN (Sterling Winthrop) [28]. Recently, with extensive SC synthesis, the names have more ambiguous origins to help promote the products. XLR-11, for example, is a type of rocket engine from the 1960s, and AKB-48 (APINACA) originate from a popular Japanese girl band [29].

Cannabinoids are now classified with a system that is adopted by UNODC [30] and the British Advisory Council on the Misuse of Drugs (ACMD) [31]. According to this classification system, cannabinoids are divided into the following classes [32]:

a) **Classical cannabinoids**

   Derivatives of dibenzopyran (e.g. THC, other constituents of cannabis; and their structurally related synthetic analogues e. g. HU-210, AM-906, AM-411, O-1184).

b) **Non-classical cannabinoids**

   Cyclohexylphenols or 3-arylcyclohexanols such as CP-47,497-C8, CP-55,940, CP-55,244.

c) **Hybrid cannabinoids**

Combinations of structural features of classical and non-classical cannabinoids, e. g. AM-4030.

d) **Aminoalkylindoles** (AAIs)

They can be further divided into naphthoylindoles (e. g. JWH-018, JWH-073, JWH-398, JWH-015, JWH-122, JWH-210, JWH-081, JWH-200, WIN-55,212); phenylacetylindoles (e. g. JWH-250, JWH-251); naphthylmethylindoles and benzoylindoles (e. g. pravadoline, AM-694, RSC-4).

e) **Eicosanoids**

Endocannabinoids such as anandamide, and their synthetic analogues e.g. methanandamide.

f) **Others**

Heterocyclic compounds that are not attributed to a specific class based on small number of compounds prepared or insufficient understanding of activity. Diarylpyrazoles (selective CB1 antagonist Rimonabant®), naphtoylpyrroles (JWH-307), naphthylmethylindenes or derivatives of naphthalene-1-yl-(4-pentyloxynaphthalen-1-yl)methanone (CRA-13).

Examples for the chemical structure for several SCs groups are shown in **Figure 1.3**.

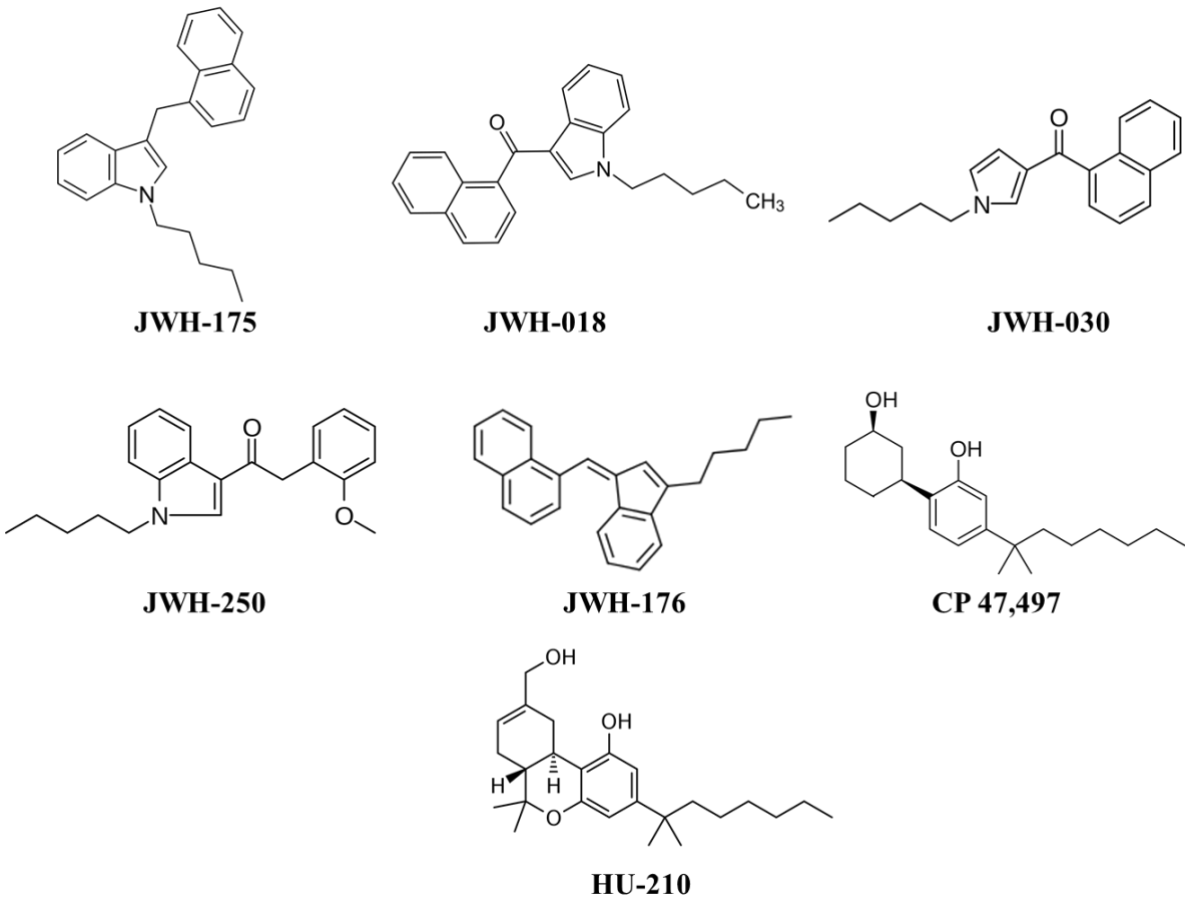


Figure 1.3 Chemical structures of some known synthetic cannabinoids

1.1.6. Pharmacology of Synthetic Cannabinoids

The ECS contains two receptors known as CB1 and CB2. CB1 receptors are found in the nervous system (central and peripheral), heart, liver, vascular endothelium, lung, bone and reproductive systems [33]. CB2 receptors are primarily in the immune system, and can be found in the central nervous system but at lower levels than CB1 [34]. SCs and THC bind to CB1 and CB2 and activate CB1 more than CB2. Activation of CB1 receptors decreases cellular cyclic adenosine monophosphate (cAMP) levels and provokes cannabimimetic responses., while CB2 stimulation has immunomodulatory effects [24].

SCs are strong CB1 receptor agonists, whereas THC is a moderate CB1 receptor agonist. Therefore, SC bind to the cannabinoid receptors with a higher affinity than

THC. In animals, CB1 stimulation produces the cannabinoid tetrad of hypothermia, analgesia, catalepsy and locomotor suppression [35]. SCs can bind to cellular membranes, opioid and benzodiazepine receptors, prostaglandin synthetic pathways and protein metabolism. These properties are the reason for complex interactions and may promote toxicity [36].

These compounds are metabolized via oxidation by cytochrome P450 and then conjugation with UDP-glucuronosyltransferase (UGT). The metabolites of some SC, such as JWH-018 and JWH-073, maintain high activity on CB1 receptors, whereas metabolites of THC have reduced affinity for CB1 receptors [35].

Pharmacologically, SCs can be classified as cannabinomimetics which have cannabis-like activity (agonists at CB1), antagonists which bind to the CB receptors and block the effects of other cannabinomimetics, and compounds which do not bind to the CB receptors significantly and do not have pharmacological effects mediated by these receptors [30].

1.1.7. Toxicity of Synthetic Cannabinoids

SCs are more potent, toxic and unpredictable than THC, which make them a substantial health concern. Clinical reports have shown that K2 use can lead to acute central nervous system (CNS) and cardiovascular system (CVS) toxicity, which makes SCs a major public health alarm. The reports suggested possible adverse effects of SC exposure such as tachycardia, anxiety, psychosis, and the addiction potential of these substances [37]. SCs have been reported to produce a wide range of harmful effects such as cardiotoxicity, kidney damage, seizure activity, and even death. Other toxicities related to SCs involve dizziness, irritability, delusions, hypertension, vertigo, chest pain, nausea, etc. [17]. **Figure 1.4** illustrates the harmful effects reported to occur upon ingestion of SCs.

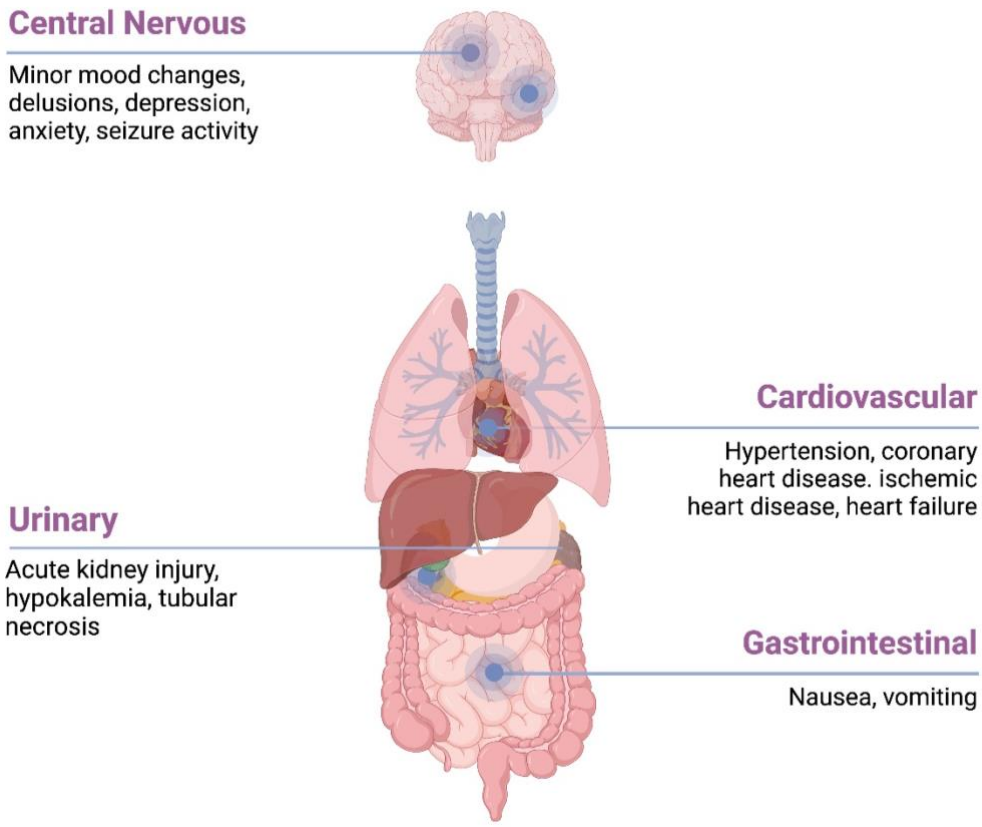


Figure 1.4 Harmful effects of SCs

The manufacture of SC products without quality control leads to huge variations in the SC concentrations in K2 or Spice products. Moreover, K2 or Spice products usually contain more than one chemical SC compound that can interact in unpredictable ways [23]. For example, the coadministration of JWH-018 and JWH-073 in mice can produce additive, synergistic or antagonistic interactions. Similar synergistic effects occurring among the multiple SCs present in K2 products may increase their relative potency and contribute to negative side effects commonly associated with these drugs [38].

Different types of SCs are synthesized regularly, and each type has a unique binding affinity for cannabinoid receptors. These different classes provoke variable responses and have unknown contaminants. The structures are different from THC and,

therefore, are not detected in routine drug screens [39]. These facts highlight the case for SCs as a very dangerous source for drugs of abuse.

1.1.8. Synthetic Cannabinoids in Kuwait

Despite the increasing interest regarding the use, spread and effects of SCs, there is little information regarding this in the Middle East countries, and especially Kuwait. SCs have only been categorized in Kuwait as schedule II substances by 2016, which may be the reason for deficiency of information about SCs in Kuwait.

The Middle East and North Africa (MENA) are major areas for illicit substance trafficking, in terms of production and use. Thanks to its central location and its widespread borders, the MENA area is a major transit area for illicit substances and links the major drug producers in Asia to markets in Europe and the Gulf area [40]. Flow through the MENA area include several illicit substances such as opiates (Golden Crescent to Europe), cocaine (Latin America to Europe), cannabis (Morocco to the Gulf Countries), and amphetamine-type stimulants (Western Africa to Europe) [40].

Trading, abuse and addiction in the MENA area are relatively high, being highly important in drugs trafficking. As a result, legislative authorities in the MENA countries have introduced harsh penalties to restrict and reduce the outbreak of recreational use of illicit drugs [41].

In 1960, the Kuwaiti government launched the Act for Trading and Using Illicit Drugs. In 1983, a list of abused drugs and their derivatives were added to the 1960 act. Products under the name of Spice/Bath Salt have been banned in 2015. Several new SC have been added to Schedule II of the 1960 act in 2016, such as CP-47,497, CP-55,940, CP-55,244, HU-331, HU-308 and AM-356. While AM-2201 and JWH-018 were added to Schedule I in the same year.

A survey conducted on Kuwaiti male students (n = 1587) concluded that the most usually used illicit substances were marijuana (11%), stimulants such as amphetamines (7.1%), cocaine (2.2%), and heroin (1.3%) [42]. Radovanovic et al. [43] studied the prevalence and trend of different psychoactive substances in Kuwait from 1992 to 1997 by examination of 3781 biological samples. From this report, the most commonly used materials were cannabinoids, benzodiazepines, opiates, and amphetamines. Some other substances were also recognized but at minor levels; which included phencyclidine, methadone and cocaine [43].

1.1.9. Synthetic Cannabinoids in United Kingdom

SCs were not legislated in UK before 2009, this where the name ‘legal highs’ was invented. Once the presence of SCs in herbal products was monitored and their harm was established, modifications were made to the legislation to include these compounds [31]. The main legislation controlling abused or harmful substances in UK is the Misuse of Drugs Act issued in 1971. Controlled drugs are defined in Schedule II of this act, and classified into three groups: A, B and C. The act also states the penalties for possessing and supplying these drugs [44]. Some of SC, including JWH-018, CP-47,497 and HU-210, were included as Class B substances under the 1971 act in 2009. In the following years more SCs were added.

The Misuse of Drugs Regulations issued in 2001 regulates the use of controlled drugs in legitimate scenarios, where the drugs are classified into five different schedules [45]. Having no accepted legitimate medical uses, SCs are controlled by Schedule 1 of this, and have the strongest controls surrounding their use. However, with the escalating synthesis of SCs and the introduction of new compounds onto the market each year, it became very difficult to keep the legislation up to date. As a result, the Psychoactive Substance Act was issued in 2016 [46]. The NPS, which are controlled by the act, are

defined as “any that is capable of producing a psychoactive effect in a person who consumes it and is not an exempted substance”. The 2016 act contains two schedules, the first one listing exempt substances (such as alcohol, caffeine, and nicotine) and the second is used for exempt activities (such as licensed research).

1.1.10. Analysis and determination of Synthetic Cannabinoids

The speed at which new SCs are emerging poses a significant challenge for forensic science. While authorities routinely review the legislation and add new substances to the schedules, manufacturers usually make only slight changes to SC structures to discriminate them from the listed ones. This challenge necessitates the development of new analytical methods for identifying of these new substances [47].

Conventional screening tests -such as enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), fluorescence polarization immunoassay (FPIA), enzyme multiplied immunoassay technique (EMIT) and kinetic interaction of microparticles in solution (KIMS)- are not suitable, as they are based on immunoassays which are not specific [48]. The use of highly specific and sensitive analytical techniques is now required to determine SCs and their metabolites [49].

A suitable selection of the biological samples to be analyzed is important. Urine is one of the most commonly used biological fluids in forensic analysis, with several studies reporting analysis of SCs in urine [50-54]. However, analysis of urine samples does not always provide the best detection of SC, as they are quickly metabolized to a large number of unknown compounds [55]. Oral fluid is another specimen that can be applied for the determination of SCs, due to the convenience and speed of collection and the difficulty of tampering [56, 57]. However, oral detection is only valid after fresh consumption, and the diffusion rate of SC from blood to the oral fluid is very low [58].

Several analytical methods were applied for SC detection in serum [58, 59]. This allows direct detection of SCs such as JWH family instead of their metabolites; which determines the composition of the SC in the sold herbal mixture [60]. Blood is a widely used biological sample for detection of SCs [61]. However, having a short half-life in this particular matrix, makes SC acute intoxication the only possible situation for using this specimen. Hair is another matrix that can be used for SCs detection [62, 63]. In this matrix, SCs are less susceptible to changes in metabolism which gives the advantage of evaluating long-term consumptions. Hair also presents a greater stability and a wide detection window [55]. However, the assay in hair may show limited sensitivity to detect some analytes and also some analytes can be incorporated into the hair by contamination [64].

1.1.11. Sample preparation for Synthetic Cannabinoids

The larger part of laboratory work in SCs determination is related to sample preparation. Pretreatment is important at initial stages of the analysis process, as it removes the matrix components that interferes with the analytes. This is especially noticed in more complex matrices, as whole blood, giving better results with reduced background noise. Sample preparation allows preconcentration of analytes, increasing sensitivity and giving a higher limit of detection (LOD) [55]. The sample pretreatment procedures commonly used in forensic analysis are protein precipitation (PPT), liquid–liquid extraction (LLE) and solid-phase extraction (SPE). A hydrolysis step is usually required when analyzing urine specimens.

PPT is usually used with complex matrices, such as whole blood, serum and plasma. It is a simple and rapid extraction technique, but may not remove all the matrix interferences [65]. For SCs, the most commonly used solvents for PPT are acetonitrile, perchloric and trichloroacetic acids. The most effective precipitant in terms of recovery

is acetonitrile (up to 80% recovery). In acid precipitations, the analyte recoveries show high variability, which may be due to possible co-precipitation of the analytes with the proteins [66].

The most commonly used sample pretreatment procedure for SC, regardless of the biological specimen, is LLE [29, 67]. LLE is usually used with SCs due to their high hydrophobicity. It works by adding an immiscible organic solvent to the biological specimen, where the analytes are moved from the sample layer (usually aqueous) to the organic solvent [55]. Single solvents such as chloroform, tert-butylmethyl and diethyl ethers have been used for extraction of SCs. However, the most common procedures involve solvent mixtures such as chlorobutane:isopropanol and hexane:ethylacetate to cover larger range of polarities of different SCs [68, 69]. For preparation of hair samples, a washing step should precede the extraction step, to eliminate possible contaminants [70]. LLE can be recognized as a more efficient sample pretreatment procedure compared to PPT regarding recovery. Nevertheless, the need for a concentration step (solvent evaporation) is considered a drawback [55].

Recently, SPE has become more popular as an extraction technique for illicit drugs. The extraction of SCs using SPE has been used in several biological specimens, with different types of cartridges [65]. The biological sample passes through the cartridge to waste, with the analytes being retained. Then, washing steps are applied to remove matrix interferences, and then the target analytes are eluted from the sorbent using a suitable solvent and collected in a clean vial [76]. SPE can be considered the only extraction type that will efficiently remove the endogenous compounds which may cause matrix effects. However, the high cost and time spent on the method development and extraction are the main disadvantages of SPE [55].

The metabolites of SCs are usually glucuronidated, so hydrolysis is often necessary in case of urine samples. The hydrolysis of these samples results in the cleavage of glucuronide conjugates, enabling the determination of the free metabolites [71]. The most widely used procedure for urine samples pretreatment involves hydrolysis with strong acids, bases or enzymes before extracting the compounds. β-glucuronidase is the most common enzyme for hydrolysis. Enzymatic hydrolysis is the most common procedure for the deconjugation, as it is gentler than chemical hydrolysis [72].

According to the literature survey, LC-MS is the most common technique used for detection of SCs. This can be attributed to the fact that LC-MS is now one of the most sensitive and specific techniques. By searching the literature for the work involved LC-MS for cannabinoids detection, more than 100 articles were retrieved. That's why in this thesis we will focus on the most recent work done in this field (2018-2024) for detection of SCs only. The methods were summarized in

Table 1.1.

Table 1.1 Summary of LC-MS methods used for determination of SCs.

Column	Mobile Phase	Detection	Application	Ref.
Acquity UPLC® HSS T3	Gradient 0.1% (v /v) formic acid in water (A) and acetonitrile (B).	MS/MS	Blood	[73]
Accucore Phenyl-Hexyl	Gradient 1 mM ammonium formate and 0.1% formic acid (A). 70% acetonitrile, 1 mM ammonium formate, and 0.1% formic acid (B).	MS/MS	Urine	[74]
Phenomenex Synergy Polar RP	Gradient 10mM ammonium formate buffer in water containing 0.1% (v/v) formic acid, pH 3.5 (A) Acetonitrile containing 0.1% (v/v) formic acid (B).	MS/MS	Urine	[53]
Hypersyl Gold PFP	Gradient Formate buffer 2 mM in 0.1% formic acid (A) and acetonitrile (B)	MS/MS	Hair	[75]
RP Poroshell 120 EC-C18	Isocratic Acetonitrile: 10 mM ammonium formate buffer solution (70:30, v/v)	MS	Herbal products/ blood	[76]
Zorbax Eclipse Plus C18 RRHD	Graient 2 mM ammonium formate: 0.2% formic acid in distilled water (A). 2 mM ammonium formate: 0.2% formic acid in methanol (B).	MS/MS	Hair	[77]
Force Biphenyl	Gradient Methanol (A) and 0.1% formic acid in water (B)	MS/MS	Wastewater	[78]

Table 1.1 Summary of LC-MS methods used for determination of SCs, continued.

Column	Mobile Phase	Detection	Application	Ref.
Kinetex C18	Gradient 5.0 mM ammonium formate in water with 0.05% formic acid (A) and acetonitrile with 0.05% formic acid (B).	MS/MS	Breath and whole blood	[79]
BEH Shield RP18	Gradient 0.1% formic acid in water (A) and acetonitrile (B)	MS/MS	Food	[80]
ZORBAX Eclipse-Plus C18	Gradient 5 mM ammonium formate buffer, adjusted to pH 4 with formic acid (A) and acetonitrile with 0.01% formic acid (B).	Q-TOF	Urine Hair	[81]
Kinetex C18	Gradient water (A) and methanol (B); both contained 0.1% formic acid and 2 mM ammonium formate.	MS/MS	Dietary supplements	[82]
Acquity UPLC® HSS T3	Gradient 0.1 % aqueous formic acid (A) and acetonitrile containing 0.1 % formic acid (B)	MS/MS	Hair	[83]
Acquity UPLC BEH C18	Gradient 1 mM ammonium formate (pH 3.1)-formic acid, 999:1, v/v (A) and methanol-formic acid, 999:1, v/v (B).	MS/MS	Plasma	[84]
ZORBAX Eclipse Plus C18	5 mM ammonium formate buffer, adjusted to pH 4 with formic acid (A), and acetonitrile with 0.01% formic acid (B).	MS/MS	Hair Urine	[85]

1.2. Drugs of Abuse

Drugs of abuse (DoA) are substances that alter mental and physical functions and are commonly misused, leading to negative consequences such as addiction, dependence, health problems, and social issues. Understanding these substances and their classifications is crucial for effective prevention, treatment, and regulation efforts [86]. DoA encompass a wide range of substances, including illicit drugs like cocaine and heroin (HER), prescription medications like opioids and benzodiazepines when used non-medically, and even legal substances like alcohol and nicotine when misused [86]. DoA pose significant public health challenges, contributing to substance use disorders, overdose deaths, infectious diseases, mental health issues, and social problems such as crime and family disruption. Effective prevention, treatment, and harm reduction strategies are essential to address these concerns. Understanding the classifications of drugs of abuse is vital for healthcare professionals, policymakers, law enforcement agencies, and the general public to implement evidence-based interventions and policies aimed at reducing substance misuse and its associated harms. According to Drug Enforcement Administration (DEA) of USA, drugs of abuse can be classified into [87]:

1. Narcotics: e.g. morphine, fentanyl, HER, and methadone.
2. Stimulants: e.g. amphetamines, methamphetamine, and cocaine.
3. Depressants: e.g. barbiturates, and benzodiazepines.
4. Hallucinogens: e.g. MDMA, ketamine, and LSD.
5. Steroids: e.g. testosterone, nandrolone, and oxandrolone.
6. Cannabis
7. Inhalants: e.g. glue, lighter fluid, and paint.
8. Designer Drugs: e.g. synthetic cannabinoids and synthetic cathinones.

In Kuwait, some drugs are known for being commonly abused, among these drugs are pregabalin (PGB), morphine (MOR), amphetamine (AMP), methamphetamine (MAMP), codeine (COD), and diazepam (DZP). These six compounds will be the focus of our study of drugs of abuse in Kuwait.

PGB is a structural analogue of gamma-aminobutyric acid (GABA), however it does not directly interact with GABA receptors. Instead, it exhibits selective binding to the α2-δ subunit of voltage-gated calcium channels in the CNS, specifically in the dorsal horn of the spinal cord. This binding reduces calcium influx and subsequently inhibits the release of excitatory neurotransmitters [88]. The modulation of calcium channel function by PGB ultimately results in its anticonvulsant, analgesic, and anxiolytic effects [89].

MOR is a natural opiate alkaloid derived from the opium poppy plant, *Papaver somniferum*. It is classified as a schedule II controlled substance due to its high potential for abuse and addiction [87]. MOR is a potent opioid analgesic that primarily acts as an agonist at the μ-opioid receptors in the CNS, resulting in analgesia, sedation, and euphoria. It also interacts with kappa and delta opioid receptors to a lesser extent, contributing to its overall pharmacological effects [90]. MOR's analgesic effects are mediated primarily by its actions in the spinal cord and brainstem, where it inhibits the transmission of pain signals [90]. COD is an opioid analgesic that acts as a prodrug, meaning it requires metabolism by the liver enzyme CYP2D6 to its active form, MOR, to exert its pharmacological effects [91]. Additionally, COD has antitussive properties, believed to be mediated by its effects on the cough center in the brainstem [91].

AMP and MAMP are highly potent psychostimulants that primarily function by increasing the release of monoamine neurotransmitters, such as dopamine,

norepinephrine, and serotonin, from presynaptic neurons [92]. This effect is achieved by reversing the direction of monoamine transporters, which includes the dopamine, norepinephrine, and serotonin transporters, causing the neurotransmitters to be expelled into the synaptic cleft. Consequently, there is a notable increase in the synaptic concentrations of dopamine and norepinephrine, particularly in various brain regions associated with the mesolimbic reward pathway [93]. AMP and its related compounds have clinical applications in the treatment of attention-deficit/hyperactivity disorder (ADHD) and narcolepsy [94].

DZP is a benzodiazepine with anxiolytic, sedative, muscle relaxant, anticonvulsant, and amnestic properties. It acts by enhancing the effect of GABA, the major inhibitory neurotransmitter in the CNS, by binding to the benzodiazepine binding site on GABA-A receptors. This results in increased chloride ion influx through the GABA-A receptor channel, leading to membrane hyperpolarization and inhibition of neuronal excitability [95]. DZP's CNS depressant effects contribute to its therapeutic actions in anxiety disorders, insomnia, muscle spasms, and seizure disorders [96].

1.2.1. Analysis of drugs of abuse

The six drugs under investigation were determined by several analytical techniques as single compounds or in their mixtures with other drugs. Due to the large number of reported methods on these drugs, we will focus on the LC methods reported for analysis of their mixtures only in our literature survey. Also, with large number of methods reported for the analysis of the binary mixtures of MOR/COD and AMP/MAMP, our survey will be limited to the last five years (2020-2024).

The liquid chromatographic methods utilized for determination of mixtures of the six compounds were summarized in

Table 1.2. No single method is reported for the analysis of these compounds simultaneously. The six drugs share the fact they are mainly excreted via the kidney, so their determination in urine presents a potential technique for polydrug abuse detection.

Table 1.2. Summary of LC methods used for determination of the drugs of abuse under study.

Analytes	Column	Mobile Phase	Detection	Application	Ref
MOR, AMP	Supelcosil LC-18 DB	KH_2PO_4 (0.077 M) in water: methanol: acetonitrile: tetrahydrofuran: triethylamine, 600: 100: 25: 7: 1.5, by volume	DAD (204 nm)	Meconium	[97]
MOR, DZP, pethidine	C18	Methanol: 25 mmol/L KH_2PO_4, 90:10	UV (254 nm)	Blood	[98]
MOR, COD, their metabolites	Gemini C18	Gradient elution Acetonitrile and phosphate buffer (0.0125 M, pH 7.5)	UV (210 nm)	Urine	[99]
MOR, COD	C18	Acetonitrile: sodium acetate (pH 4, 10 mM), 10: 90, v/v.	UV (285 nm)	Urine	[100]
MOR, COD	C18	Gradient elution 10 mM ammonium formate in water with 0.1% formic acid. Methanol with 0.1% formic acid.	MS/MS	Urine	[100]
AMP, MAMP, and their metabolites	Kinetex Biphenyl	Gradient elution 0.1% formic acid in water. 0.1% formic acid in methanol.	MS/MS	Postmortem Canine Tissues and Fluids	[101]
AMP, MAMP, MDMA, and their enantiomers	Chiral Lux	Gradient elution 5 mM ammonium bicarbonate (pH 11). Methanol (for AMP) methanol/acetonitrile mixture (for MDMA)	MS/MS	Street samples	[102]
AMP, MAMP, famprofazone	Accucore C18	Gradient elution Formic acid in water. 0.015% formic acid in acetonitrile	MS	Liver	[103]

Table 1.2 Summary of LC methods used for determination of the drugs of abuse under study, continued.

Analytes	Column	Mobile Phase	Detection	Application	Ref
MOR, COD, methadone, their metabolites	Kinetex Biphenyl	Gradient elution Water and 0.1% formic acid. Methanol with 0.1% formic acid.	MS	*Lucilia sericata* species	[104]
MOR, COD, thebaine, papaverine, noscapine	BEH C18	Gradient elution Acetonitrile 10 mM ammonium acetate (0.05% aqueous ammonia)	MS/MS	Rat plasma	[105]
MOR, COD, thebaine, narcotine, papaverine	Kinetex Biphenyl	Gradient elution Water and 0.1% formic acid. Methanol with 0.1% formic acid.	MS/MS	Seasonings	[106]

1.3. Gastrointestinal fluids and drug solubility

Oral administration of drugs is the most popular method for drug therapy, where tablets and capsules account for about 70% of the marketed medicinal products. It is well-known that solid dosage forms must dissolve from the medicinal product in the fluids of the gastrointestinal tract (GIT), remain in solution and then permeate through the intestinal wall to reach portal and systemic circulation. Inconsistency in drug bioavailability is often attributed to variations in hepatic first-pass effect, body distribution, and kidney excretion [107]. FDA's biopharmaceutical classification system (BCS) demonstrates that drug solubility and GIT permeability are the main factors controlling oral bioavailability [108].

A range of factors affect the solubility and dissolution of drug substances, such as the drug's pKa, crystal form and particle size, formulation, dissolution medium pH and presence of surfactants [109]. It was evident that the composition of dissolution media is a very important factor and influences the solubility, dissolution and therefore bioavailability of poorly soluble drugs. Also, the physicochemical relationships between media components and their effect on drug solubility are critical factors [110, 111]. Therefore, it's challenging to predict drug's biopharmaceutical performance and relate in vitro drug dissolution to in vivo drug profiles. This challenge is an important one, since about 40% of new chemicals are rejected in the early drug development stages because of low aqueous solubility [112]. However, some low water soluble drugs show higher GIT solubility and hence increased bioavailability [113]. Therefore, in vitro solubility and dissolution testing of solid oral dosage forms is a key step in drug development, but it inadequately predicts in vivo performance [114].

In these drugs, variability in bioavailability may be increased by GIT factors, including motility, gastric emptying rate, and intestinal fluid composition [115]. The main function

of GIT is to supply nutrition from different food products, together with the elimination of metabolic waste products. This function is achieved by the secretion of fluids, and muscular activity to mix food with the residues being pushed forward. Therefore, it is understood that the complex physiology of GIT influences drug absorption [116].

FDA classify drug substances based on their aqueous solubility and intestinal permeability in what is known as biopharmaceutical classification system (BCS) [108]. BCS takes into account three major factors that govern the rate and extent of drug absorption from solid oral dosage forms which are dissolution, solubility and intestinal permeability. According to the BCS, drug substances are classified into four classes as shown in **Figure 1.5** [108].

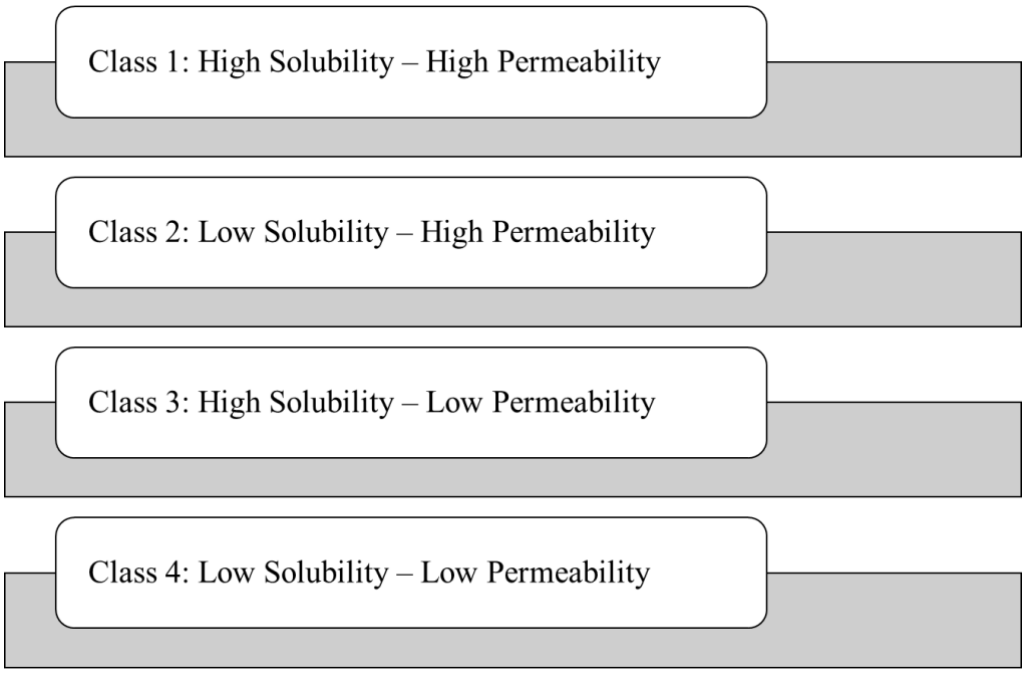


Figure 1.5 Biopharmaceutical Classification System (BCS) for drug solubility and permeability

GIT media composition is highly important for its effect on the dissolution and permeation of lipophilic drugs with a limited aqueous solubility, i.e. class II and IV compounds [117]. Expecting the oral absorption potential of these drugs requires in vitro tools for dissolution, solubility, and permeability, and pharmacokinetic simulation models that account for GIT media composition and its variability [118].

Bile salts, pH, phospholipids, lipid degradation products, cholesterol, and enzymatic secretions are important factors for the absorption of drugs. pH affects drug ionization behavior [114]. Bile salts and phospholipids have surfactant properties that contribute to the solubilization of lipophilic drugs [119]. Lipolytic hydrolysis products formed after enzymatic digestion of meals, form colloidal structures and mixed micelles, which influence the solubility and permeability of lipophilic drugs. These hydrolytic products include triacylglycerides (TAG), diacylglycerides (DAG), monoacylglycerides (MAG), and free fatty acids (FFA) [120]. Recently, cholesterol has been confirmed to affect solubility of BCS class II drugs, to improve bilayer stability and to form mixed micelles [121].

As GIT media is composed of several constituents, its composition is susceptible to large variability between different individuals leading to inter-subject variability. Day-to-day fluctuations may also lead to intra-subject variability [118]. Intra- and inter-subject variability in drug absorption are also affected by physicochemical conditions within GIT, which may vary with the position along the tract and the ingested food [122, 123]. The dynamic nature of GIT environment, mediated by meal intake and biliary and pancreatic secretions, further enhances the variability.

Therefore, simple aqueous solubility cannot reflect GIT solubility and to determine drug solubility in vitro. Therefore, the most obvious solvent for the in vitro study of drug solubility and dissolution are human gastric (HGF) and intestinal fluids (HIF) [124].

However, human fluids are not suitable for in vitro solubility studies being expensive, difficult to sample, variable in composition, unstable in air, difficult to obtain quantities for large scale studies and dependent upon the volunteer's fasted or fed state and collection technique employed [118, 122]. On the contrary, simulated GIT media are more easily prepared, and several modifications can be done to change its composition [125-127]. Systems were designed to reflect the in vivo pH as well as incorporate biological compounds in GIT such as bile salts and phospholipids to simulate fasted and fed intestinal fluid [128]. These initial simulated media were modified in an attempt to optimize the composition and the constituents especially to mimic the fed state [110, 127]. Among the most common components in simulated GIT media are bile salts, lecithin, cholesterol, fatty acids and monoglycerides [129-133].

Sodium oleate (SO) and glycerylmonooleate (GMO) are hydrolytic products from the digestion of meals. They influence the solubility and permeability of lipophilic drugs [120]. Cholesterol (CHL) is (3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-[(2R)-6-methylheptan-2-yl]-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H cyclopenta[a] phenanthren-3-ol [134]. It is an important lipid, being a main part of cell membrane and keeps membrane permeability and fluidity, it is the major sterol synthesized by animals [135]. CHL is a crucial biomarker for several diseases such as cardiovascular diseases and a precursor of different biological compounds such as hormones, vitamin D, and bile acid [136]. CHL is also found in foods from animal origin such as milk, meat, cheese, egg and seafood [137].

1.3.1. Analysis of intestinal media constituents

No analytical method was reported for the analysis of these three compounds in GIT fluids. Therefore, it was important to develop an analytical method for their quantitation.

1.4. Chromatographic Techniques

The International Union of Pure and Applied Chemistry (IUPAC) defines chromatography as '*a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (the stationary phase), while the other (the mobile phase) moves in a definite direction*'. A mobile phase is described as '*a fluid which percolates through or along the stationary bed in a definite direction*'. It may be a liquid, a gas or a supercritical fluid, while the stationary phase may be a solid, a gel or a liquid. If stationary phase is a liquid, it may be distributed on a solid, which may or may not contribute to the separation process [138]. A chromatographic system consists of four main parts: a device for sample introduction (injector), a mobile phase, a stationary phase, and a detector. The chromatographic separation occurs if the mixture components interact to different degrees with the mobile and/or stationary phases and therefore take different times to move from the injector to the detector [138].

1.4.1. Liquid chromatography (LC)

In liquid chromatography (LC), the mobile phase is a liquid. A variety of liquid chromatographic separation techniques exist for qualitative and quantitative sample analysis, most notably high-performance liquid chromatography (HPLC) and ultra-high-performance liquid chromatography (UHPLC) (**Figure 1.6**) [139]. HPLC uses high pressure (400 bar) to push the mobile phase through a compact stationary phase. Stationary phase particles (2–5 µm) are packed in a column which is typically 2.0–4.6

mm in diameter and 20–250 mm in length [140]. UHPLC achieves fast analysis times using minimal mobile phase solvents compared to HPLC. The crucial characteristic of UHPLC lies in the sub-2-micron stationary phase particles in contrast to the conventional particles utilized in HPLC systems [140]. Although the smaller particle size in UHPLC contributes to its superior efficiency, they demand higher operating pressures (1500 bar) [139].

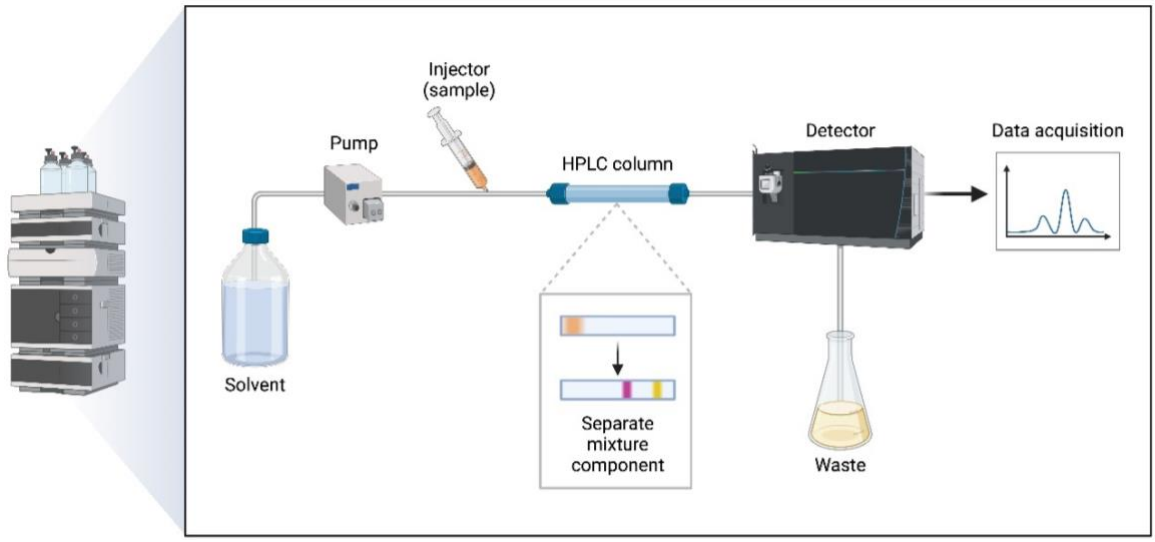


Figure 1.6. A typical HPLC system

In LC, it is the interaction of analyte with both the mobile and stationary phases that determines its retention in the column. Hence, it is the varying degrees of interaction of different analytes with the mobile and stationary phases which determines whether they will be separated by a particular LC system. This interaction depends on the relative polarities of the species involved. The majority of LC separations utilize reversed phase chromatography, in which the mobile phase is more polar than the stationary phase. In these systems, the more polar analytes elute more rapidly than the less polar ones [141]. It is not always possible to achieve an adequate separation

by using a mobile phase containing a single solvent and often mixtures of solvents are used. A separation involving a mobile phase of constant composition is termed isocratic elution, while that in which the composition of the mobile phase is changed is termed gradient elution [141].

Currently, several types of LC columns are available, such as normal phase and reversed phase columns. The most widely used LC columns contain a chemically modified silica stationary phase, with the chemical modification determining the polarity of the column. Normal phase chromatography employs polar stationary phases with a mobile phase that is typically a nonpolar solvent (hexane), causing non-polar analytes to elute earlier. On the contrary, reversed phase chromatography uses a non-polar stationary phase, paired with a polar mobile phase (methanol or water). A very popular stationary phase is one in which a C18 alkyl group is bonded to the silica surface forming the common octadecyl silane (ODS) stationary phase used in reversed phase chromatography. LC has diverse applications, including the separation of drugs, metabolites, and bioactive molecules [140].

1.4.2. Gas chromatography (GC)

Gas chromatography (GC) is that form of chromatography in which a gas is the mobile phase. A gas chromatograph (**Figure 1.7**) functions as follows, an inert carrier gas (e.g. helium) flows continuously from a large gas cylinder through the injection port, the column, and the detector. The sample is injected into the heated injection port, where it is vaporized and carried into the column. The sample partitions between the mobile and stationary phases, and it is separated into individual components based on relative solubility in the stationary phase and relative boiling points. After the column, the carrier gas and sample pass through a detector [142].

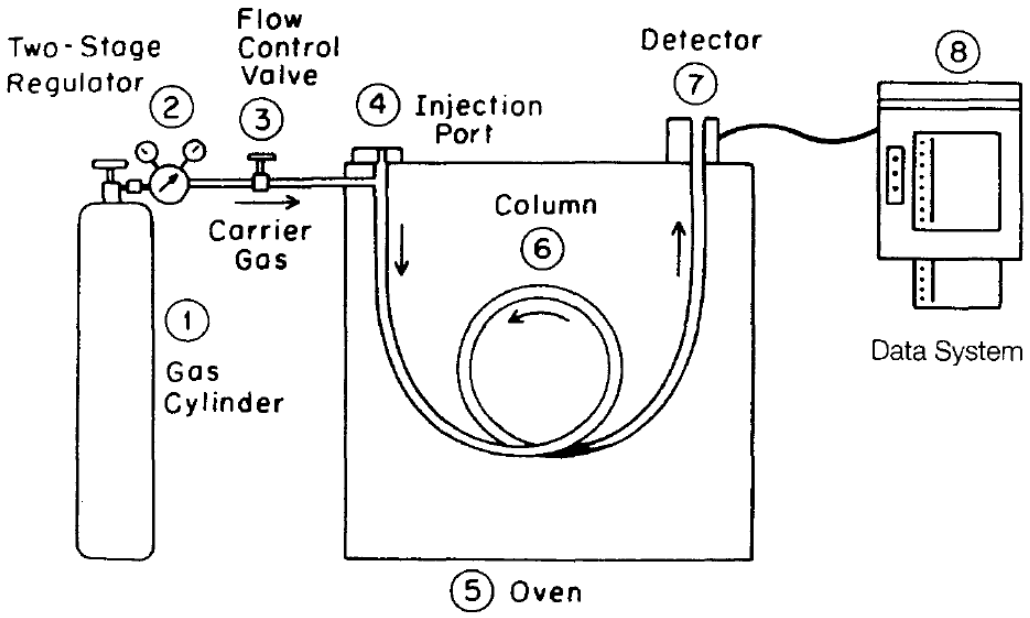


Figure 1.7. A typical gas chromatograph

The main purpose of the carrier gas is to carry the sample through the column. It is inert and does not interact with the sample on contrast to LC [142]. A variety of injectors are used in GC, with the most common, a split-splitless injector that can be used in two modes [143]. For solutions containing extremely concentrated levels of analytes, the injector is operated in the split mode where only a small fraction of the solution injected actually enters the separation column and the majority of the sample is vented to the atmosphere. For solutions containing lower levels of analytes, the injector is operated in a dual splitless-split mode. Upon injection of a sample, the injector is operated in a splitless mode where all of the injected volume is being pushed onto the column. Then, the split mode is switched on approximately 30-60 seconds after injection [143].

Separation columns are the heart of the GC and are housed in a temperature programmable oven. Considerable advances were made in GC columns, where packed columns were replaced by capillary columns that have dramatically more theoretical plates [143]. Capillary columns are typically 15 to 100 m long, coated on

the inside with a thin (0.2 µm) film of high boiling liquid (stationary phase) [142]. The column temperature must be carefully controlled so that a good separation occurs in a reasonable time. The control of temperature is one of the easiest and most effective ways to influence the separation in GC [142].

1.4.3. Hyphenated techniques (GC-MS and LC-MS)

The combination of chromatography and mass spectrometry (MS) is a technique that has drawn much interest over the years. The combination of gas chromatography with mass spectrometry (GC-MS) was first described in 1958 and was available commercially in 1967 [141]. Since then, it has become increasingly employed and is probably the most widely used hyphenated technique.

Mass spectrometer (MS) systems consist of ionization source, mass analyzer, and detector, and they require a low operating pressure, typically 10^{-5} to 10^{-6} Torr throughout the system (**Figure 1.8**) [142, 143]. The MS works by ionizing each analyte, accelerating and focusing the ionized compound and its fragments into the mass analyzer, where they are separated based on mass to charge (m/z) ratios, and finally fragments are detected, and mass spectrum is obtained. The mass spectrum is a plot between abundance of the ions (y-axis) and m/z (x-axis). There are a variety of ionization systems and mass analyzers that achieve these results [143].

Various ionization techniques have been invented to ionize molecules with diverse characteristics, including field desorption, electron ionization or electron impact (EI), chemical ionization (CI), electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), matrix assisted laser desorption/ionization (MALDI), proton transfer reaction ionization (PTR), and fast ion bombardment (FAB) [143].

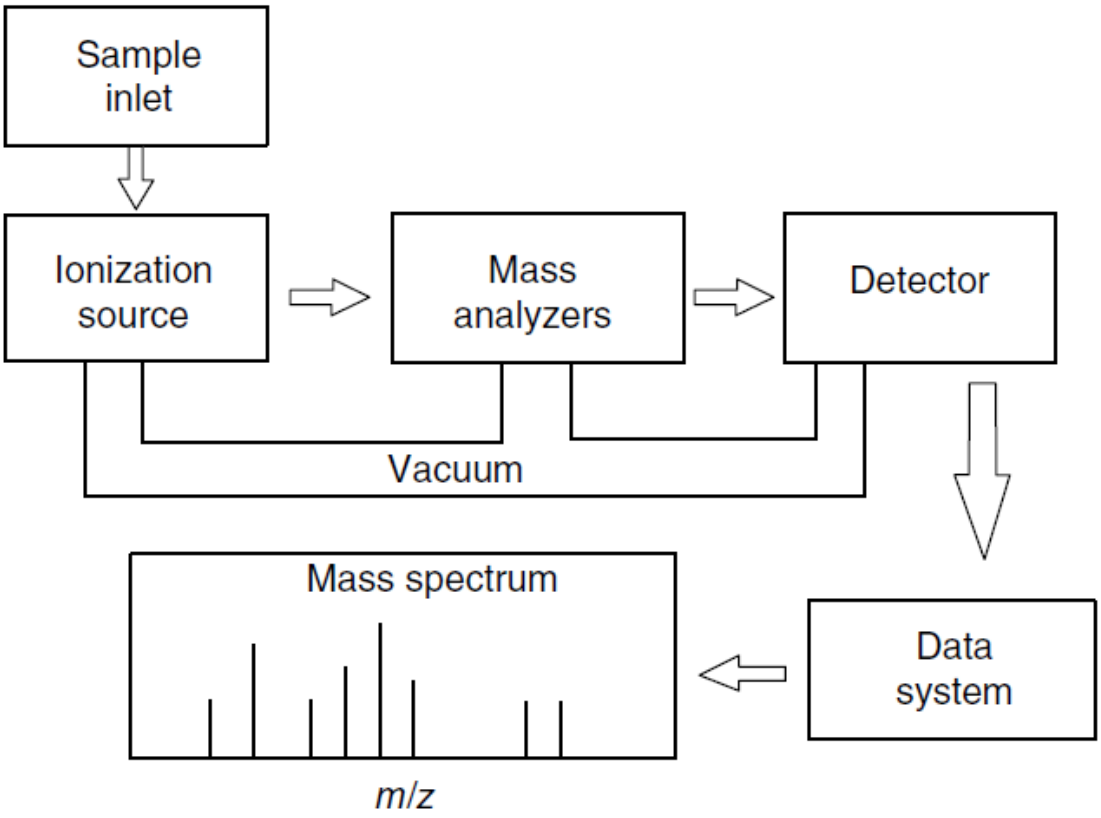


Figure 1.8. Overview of a mass spectrometer

The emergence and wide use of GC-MS (**Figure 1.9**) as a routine technique has been due to the fact that simple interfaces have been available for efficient transfer of compounds separated by GC to the mass spectrometer. Compounds liable to analysis by GC need to be both volatile, and thermally stable at the temperatures used in GC. These are the same requirements needed by MS using either electron impact (EI) or chemical ionization (CI). Therefore, virtually all compounds that can be separated through a GC column can be ionized and the full analytical capabilities of MS utilized [141]. The most common ionization technique in GC-MS is EI. This ionization technique works by forcing the stream of pure analytes exiting the GC through a beam of high energy electrons which are created by heating a metal filament [143]. EI is referred to as a hard ionization technique, since it causes fragmentation within a

sample molecule [143]. Another ionization technique in GC-MS is CI, which is referred to as a soft ionization technique as less fragmentation occurs. One of the main purposes of using CI is to observe the molecular ion [143].

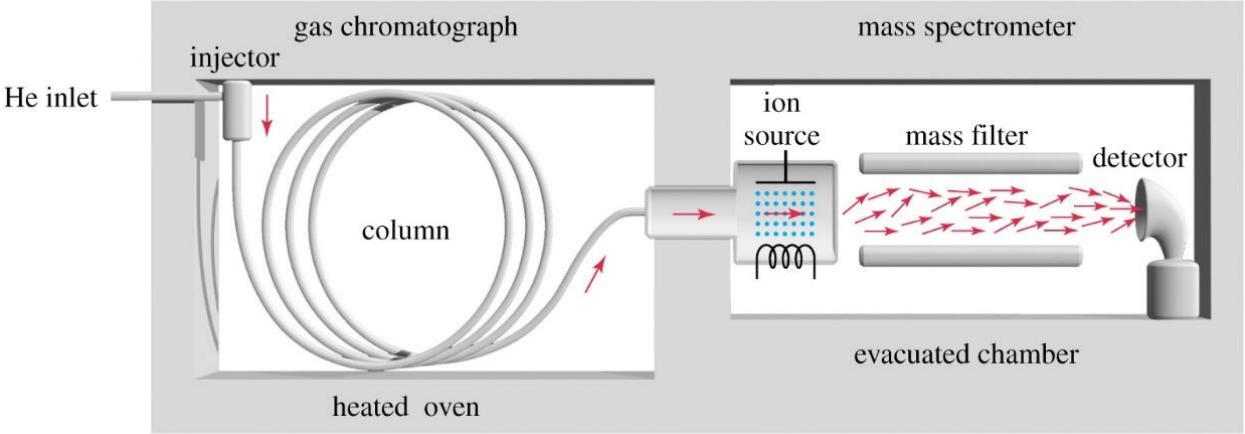


Figure 1.9. Illustration of a GC-MS

In contrast to GC, LC has incompatibilities with MS that require a special interface. The prime purpose of this interface is the removal of the chromatographic mobile phase and the transfer and ionization of non-volatile analyte molecules into the gas phase [141, 143]. The invention of ESI solved all of the major problems associated with sample introduction from LC to MS and became the most common form of LC-MS interfaces. In ESI (**Figure 1.10**), the eluate from LC is sprayed into a chamber at atmospheric pressure in the presence of a strong electrostatic field and a drying gas. The heated drying gas causes the solvent in the droplets to evaporate, and eventually, the ions are ejected into the gas phase. These ions are attracted to and passed through a capillary orifice into the mass analyzer [143]. **Figure 1.11** represents the components and steps of work on an LC-MS system.

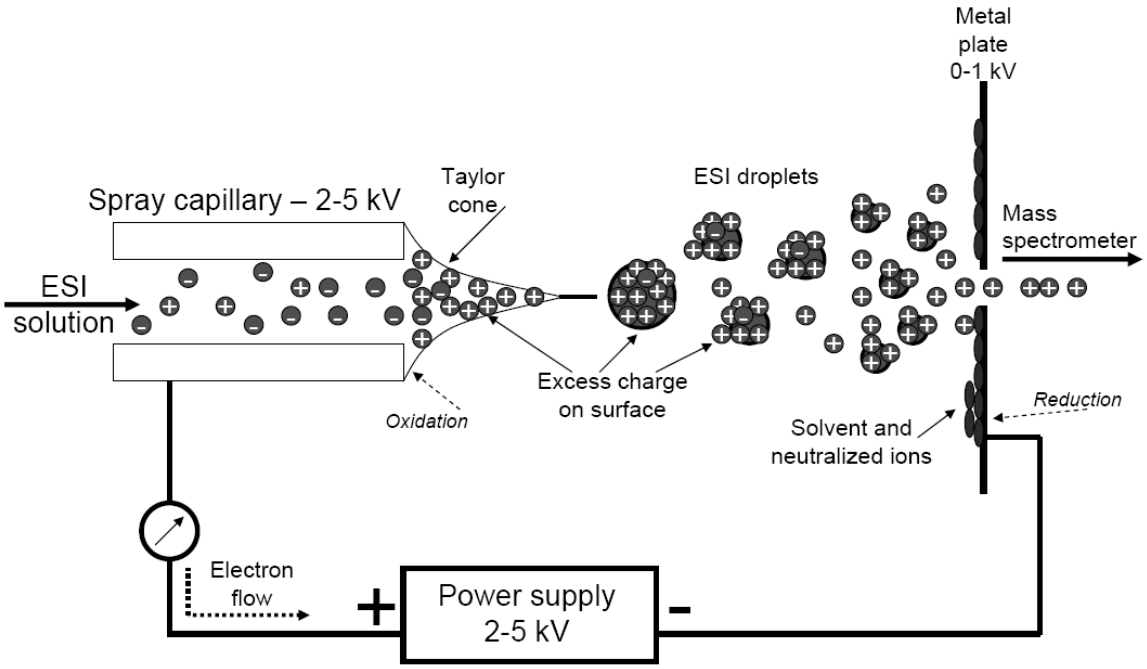


Figure 1.10. Overview of an Electrospray Ionization (LC-MS) Interface

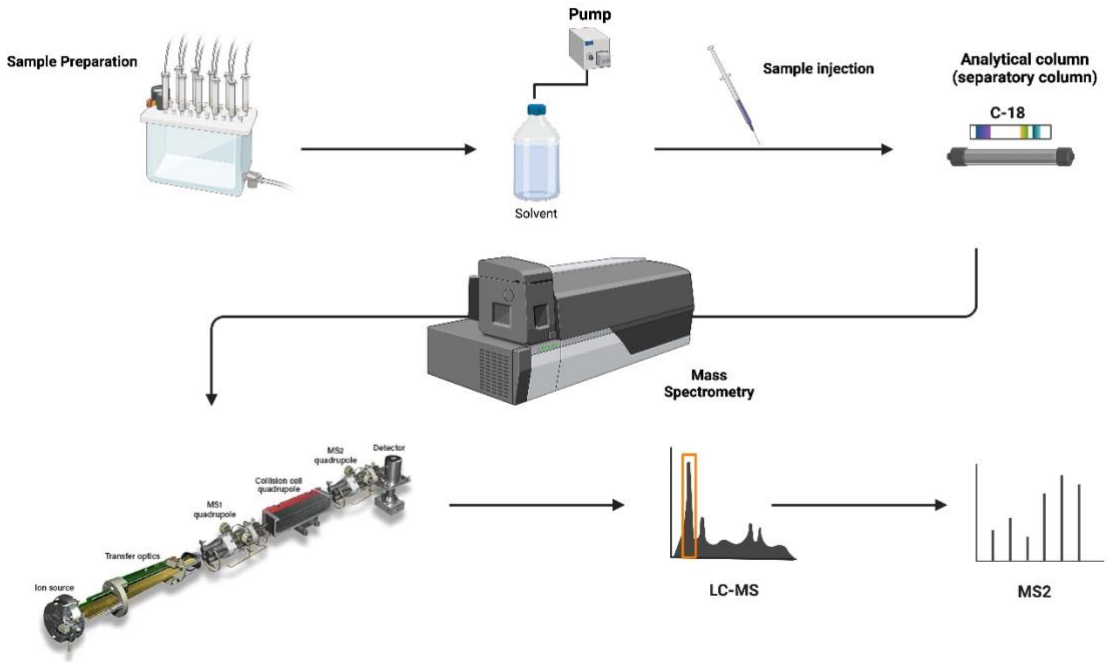


Figure 1.11. Schematic representation of LC-MS

Mass analyzer is responsible for sorting the ions and fragments according to their m/z. It comes in various forms, including quadrupole (Q), time-of-flight (TOF), ion mobility, magnetic sector, double focusing, ion trap, Fourier transform ion cyclotron, and orbitrap mass analyzers. Each type poses distinct characteristics in terms of resolution and mass accuracy that serve as key parameters for selecting the optimum mass analyzer [143]. TOF analyzer is commonly used in high resolution mass spectrometry (HRMS), while Q is the most common analyzer used in LC-MS and LC-MS/MS. TOF has high resolution that allows it to differentiate isobars, while Q has unit resolution only. Quadrupole mass filters have become the most common type of MS used in either GC-MS or LC-MS due to their relatively small size, light weight, low cost, and rapid scan times [143]. It consists of 4 parallel metal rods with different charges & 2 electric fields are applied (**Figure 1.12**). The applied fields are direct current (DC) and alternating current (AC) that affect the trajectory of ions. For given DC and AC voltages, only ions of a certain m/z pass through the quadrupole filter reaching the detector, while all other ions are thrown out of their original path [143].

The most commonly used detector in MS is electron multiplier, which can be a series of discrete or continuous dynodes [143]. Another form of MS detector is the Faraday cup that counts each ion entering the detector zone. It is less expensive but provides no amplification of the signal and hence has poor detection limits. One of the latest detectors is the electrooptical ion detector, which has the advantage of high-speed mass determinations. However, it has not been readily incorporated into instruments [143].

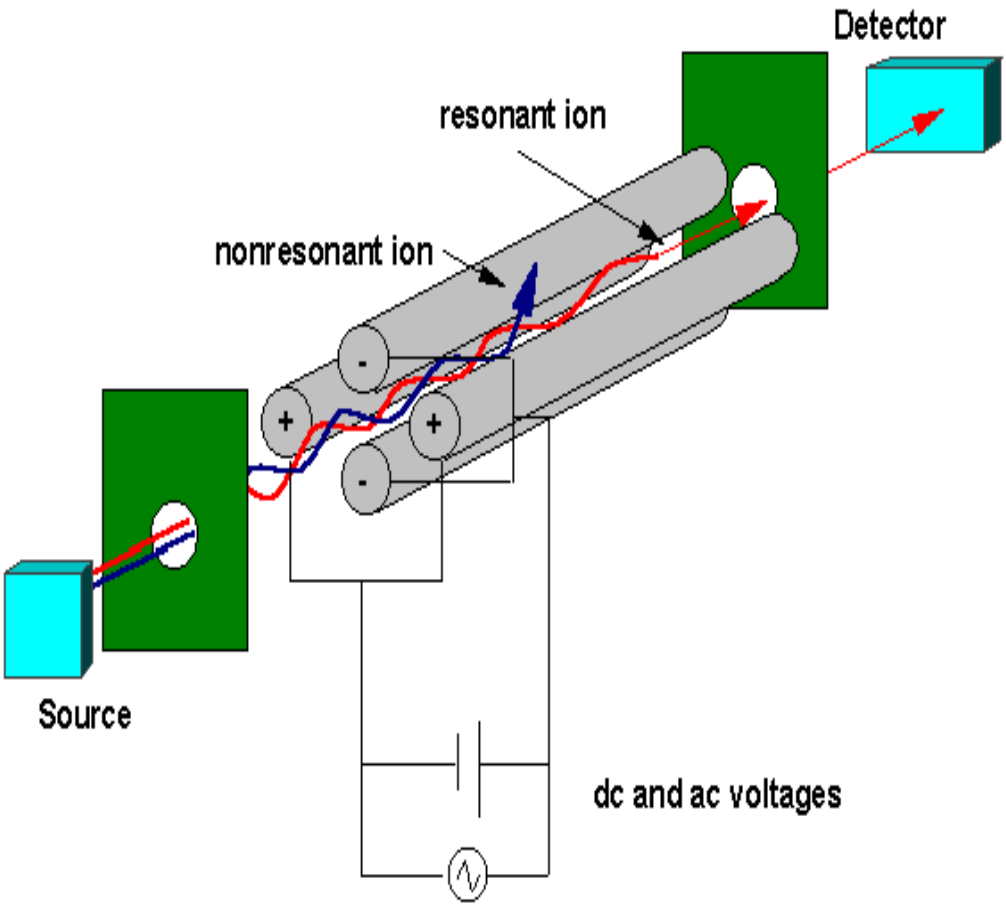


Figure 1.12. Illustration of a Quadrupole MS.

Integrating two mass analyzers into a single system is known as Tandem MS and usually referred to as MS-MS. This configuration offers enhanced capabilities for increased specificity and for structural analysis [144]. In this technique, parent ions passing through the first analyzer (MS1) are fragmented via a process known as collision-induced dissociation (CID) with an inert gas in a collision cell. These fragments, along with the parent ions, are then separated and measured by a second mass analyzer (MS2) [144]. This additional fragmentation step provides more detailed information about the molecule's structure. The MS-MS technique can be found in a range of combinations, such as triple quadrupole mass spectrometer (QqQ) that contains two quadrupole mass filters (**Figure 1.13**) and QToF which add a TOF

analyzer to a quadrupole mass filter [141]. Tandem MS systems offer various scanning modes. The precursor or product ion scan modes in which either the first stage or the second stage of MS are used to isolate an ion of interest, respectively. In MS-MS, quantitative determinations are often carried out by a mode known as selected reaction monitoring (SRM) or multiple reaction monitoring (MRM). In this mode, the fragmentation of a selected precursor ion to a selected product ion is monitored. This is achieved by setting each of the stages of mass spectrometry to transmit a single ion [141].

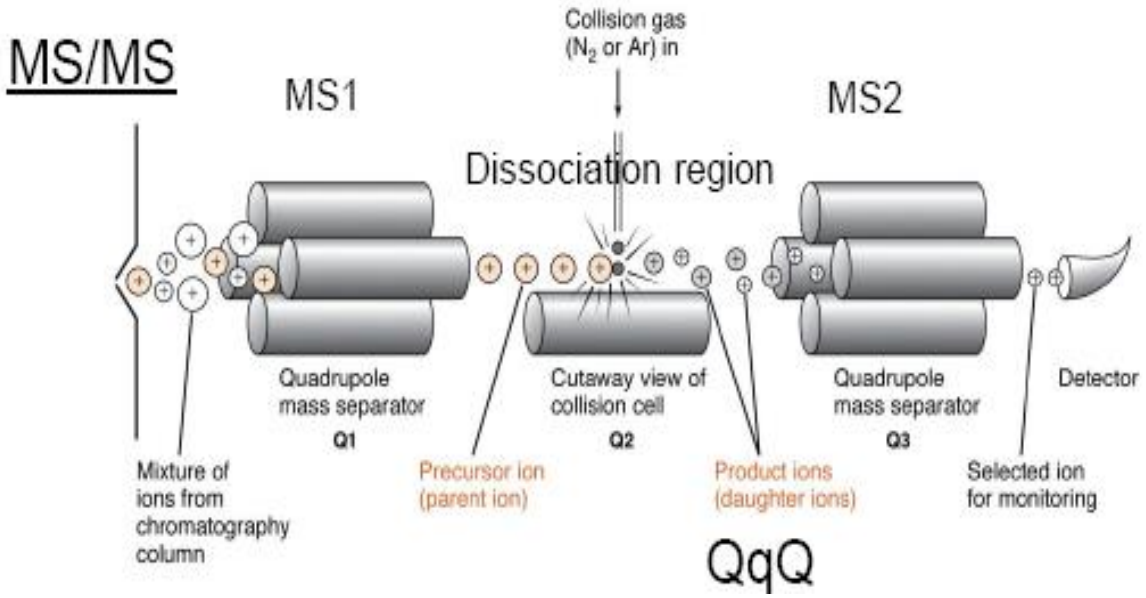


Figure 1.13. Illustration of a triple quadrupole mass spectrometer (QqQ)

1.5. Sample Preparation Methods

In order to ensure adequate selectivity, sensitivity, and reproducibility of the analytical methods in biological samples, sample preparation (known as sample pretreatment, sample extraction or sample cleanup) is an integral step of the analytical method. Sample preparation in hyphenated techniques is considered a pre-analytical separation process that involves isolation of analyte(s) of interest from the biological

matrix, minimization or elimination of matrix components, and enrichment of analyte(s) to achieve assay linearity range [145]. The optimal sample preparation method can minimize the matrix effect while maintaining a reasonable and consistent extraction recovery (R%). With many factors affecting matrix removal and analyte recovery, developing a sample preparation procedure can be difficult, tedious, and time consuming. Therefore, sample preparation is the most significant part in the development of a robust bioanalytical method. The most common sample preparation techniques are protein precipitation (PPT), liquid-liquid extraction (LLE), and solid phase extraction (SPE) [145].

1.5.1. Protein Precipitation (PPT)

Biological matrix, such as blood, plasma, or serum contains about 8% (w/w) proteins. Direct injection of these samples onto the analytical system could precipitate these proteins as a result of the organic solvents and/or buffers used in the mobile phase [145]. In PPT, a small volume of the sample is mixed with a certain volume of protein precipitant. As a result of conformation changes of the proteins induced by the precipitant, the analyte(s) of interest are released into the solution. Upon centrifugation, the precipitated proteins are separated from the supernatant containing the analyte(s). The optimization of PPT method includes selection of precipitant and its amount along with centrifugation. Protein precipitants used for PPT include water-miscible organic solvents (acetonitrile, acetone, ethanol, and methanol), acids (trichloroacetic acid and perchloric acid), metal ions, or salts. PPT is a quick and convenient sample preparation technique operating with low-cost equipment (centrifuge tubes and a centrifuge). A significant advantage of PPT is its high recovery as compared to LLE and SPE [145].

1.5.2. Liquid-Liquid Extraction (LLE)

LLE involves the extraction of the analyte(s) of interest from one aqueous liquid phase (biological samples) to another immiscible liquid phase (organic solvent). Biological samples are mixed with additives to adjust pH (buffer, acids, or bases) to ensure efficient extraction of the target molecules. This is followed by the addition of an organic solvent (extraction solvent). Then, the two-immiscible phase mixture is shaken or vortex-mixed in tubes for a certain period to mix the sample with the organic solvent. During the mixing process, the target molecules are usually transferred from the aqueous phase to the organic phase. This is followed by centrifugation for phase separation. After centrifugation, the phase containing the target molecules can be collected for analysis [146].

LLE is ideal for non-polar to moderately polar analyte(s) having higher affinity to water-immiscible organic solvents. However, when developing an assay for a drug with its metabolite, adjusting the LLE conditions may be difficult to achieve acceptable R% for both compounds as the metabolite is usually more polar than the drug. Therefore, other sample extractions such as PPT or SPE may be considered in this case [145].

1.5.3. Solid-phase Extraction (SPE)

SPE is an effective sample preparation technique that can be used for the extraction and enrichment of analyte(s) in various biological samples. It is based on the affinity between analyte(s) dissolved in a liquid and sorbent materials. The steps for performing SPE is illustrated in **Figure 1.14**.The liquid sample is loaded onto a preconditioned cartridge packed with appropriate sorbent materials. The analyte of interest is retained by interacting with the sorbent materials (stationary phase); while the interfering matrix components either directly pass through the cartridge during the loading step or are washed away during the wash steps with a proper solvent. Finally,

the analyte is eluted from the cartridge with a suitable elution solvent. SPE shows advantages over PPT and LLE including little matrix effect compared to PPT and better recovery compared to LLE [145].

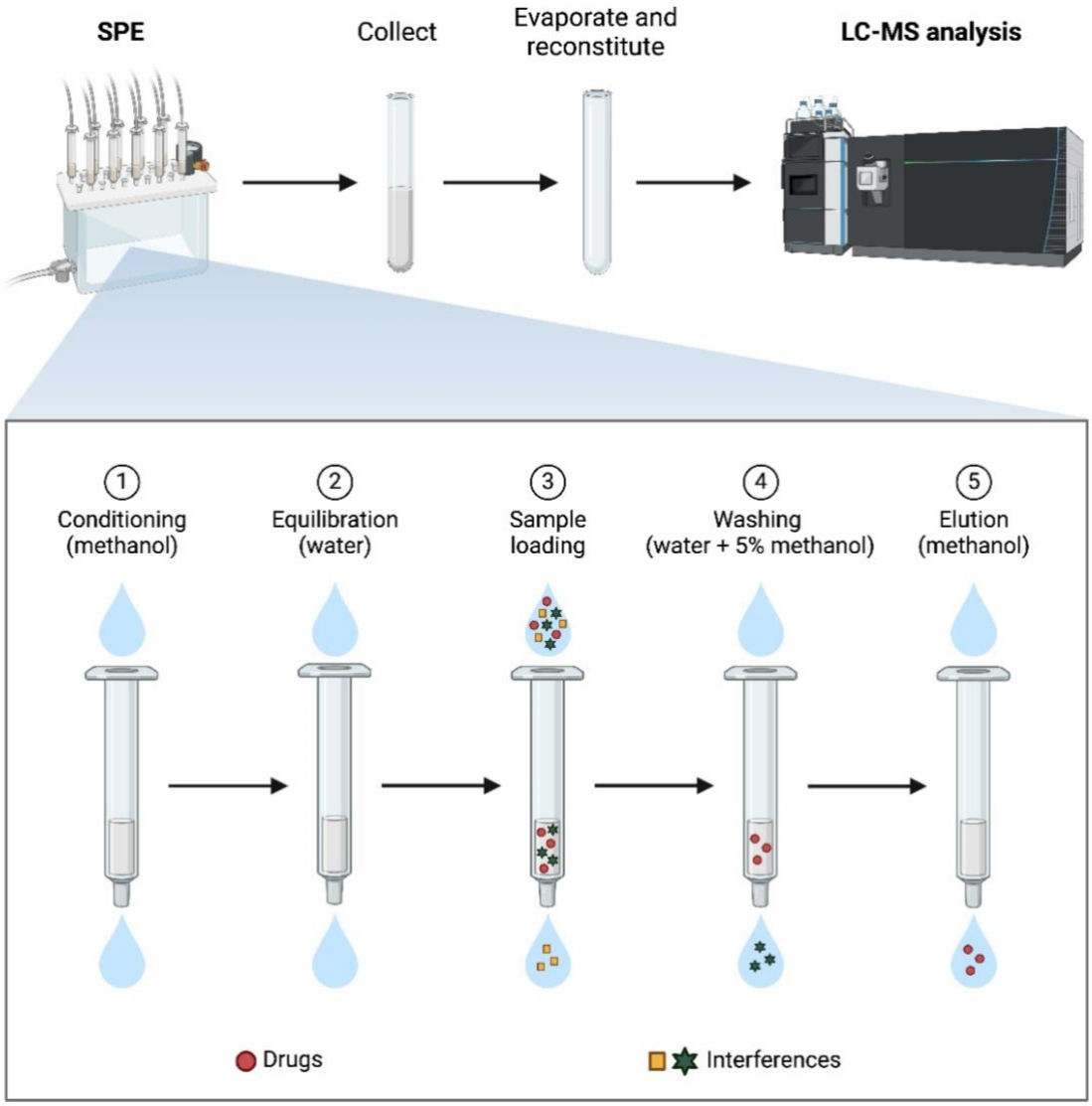


Figure 1.14. Steps of Solid-phase Extraction

SPE sorbent materials are commonly irregular-shaped rigid particles (8 to 70 µm), which allows for reasonable flow rates of the samples or solutions through the cartridge. Most SPE materials are fully porous in nature, which results in a higher total surface area and, consequently, a higher capacity for active adsorption. SPE sorbents can be broadly divided into silica-based sorbents and polymer-based sorbents [145].

According to the retention mechanisms, SPE can be classified into reversed-phase, ion-exchange, and mixed-mode SPE. Reversed-phase SPE employs non-polar stationary phases, which retain most molecules with any hydrophobic character. The common functional groups of silica-based non-polar sorbents include C18, C8, C6, C4, C2, C1, phenyl, cyclohexyl, and cyanopropyl. Being the least selective retention mechanism when compared to other SPE types, it is very useful for extracting analytes that are very diverse in their physicochemical properties [145]. Ion-exchange SPE utilizes ionic functional groups (strong or weak organic acids or bases bonded to a supporting base) of the sorbents and can be further classified as strong/weak cation/anion ion exchange SPE [145]. Mixed-mode SPE is an extraction approach involving sorbents that exhibit two or more primary interactions for retaining the analyte(s) of interest. Mixed-mode sorbents are typically produced by either bonding the sorbents concurrently with two different functional groups (e.g. C8 and sulfate) or by blending discrete sorbent chemistries in appropriate ratios to create the combination of retention properties. The most commonly used mixed-mode sorbents have a hydrophobic functional group in combination with an ion-exchange functional group [145].

The collected analyte(s) extract produced from any of the discussed extraction methods is either directly injected into the analysis instrument or subjected to evaporation process followed by reconstitution in the mobile phase. In most applications, especially LLE, evaporation/reconstitution process is recommended. This is because organic solvents dramatically weaken the analyte retention on the column. Furthermore, analyte concentrations in the final extract are generally lower (diluted) than those in the original samples, which impairs the sensitivity of the method [145].

1.6. Objectives of Thesis

The aim of work in this thesis was to identify common psychoactive compounds used in Kuwaiti market and to study the effect of gut media concentrations on drug bioavailability.

For the first aim, the following objectives were fulfilled:

- Ten SCs were investigated, and an LC-MS/MS method was developed to identify and quantify these SCs. The used SCs were 5F-AB-PINACA, 5F-ADBICA, AB-PINACA, 5F-AMB, 5F-MDMB-PICA, 4F-MDMB-BUTINACA, FUB-PB-22, MDMB-4en-PINACA, 5F-APICA and APICA. The developed method was used to analyse samples seized in the Kuwaiti streets recently, to give a clear picture on SCs sold in Kuwait.
- Three SCs were screened in urine samples, an LC-MS/MS method was developed and validated for their quantification. The three SCs were ADB-PINACA 5-Pentanoic Acid (ADB-PINACA-COOH), 5F-AB-PINACA, and 5F-AMB. The method was successfully utilized to analyze these SCs in urine samples collected in Kuwait.
- Six drugs of abuse were also investigated by developing an LC-MS/MS method for screening and quantifying them. The drugs were PGB, MOR, AMP, MAMP, COD, and DZP. The developed method was used to screen 150 urine samples collected from individuals suspected of drug abuse in Kuwait.
- We worked in cooperation with the toxicology department in the general department of criminal evidence in Kuwait to develop and validate the methods.
- The developed methods represented novel ones to determine the mixtures under investigation, as no reported method was identified for these mixtures.

For the second aim, the following objectives were fulfilled:

- Common constituents of synthetic gut media were investigated to develop a sensitive and specific GC-MS method. The investigated constituents were SO, GMO, and CHL.
- The method characterized the constituents of synthetic gut media and quantified them and can be used to study the effect of their concentrations on drug bioavailability.
- The developed method is considered a novel one, with no reported method for determination of this specific mixture of synthetic gut media constituents.

Chapter 2 Development and validation of a LC-MS/MS method for the screening of ten synthetic cannabinoids in Kuwaiti market during 2021-2022

2.1. Introduction

Synthetic cannabinoids (SCs) are a heterogeneous group of compounds synthesized to affect ECS or as therapeutic agents. In 2000s, SC were known as “legal highs” under several brand names such as Spice and K2, due to their ability to escape detection by standard cannabinoid screening tests. That necessitates the development of fast, specific, and sensitive analytical methods for the screening of such compounds. LC-MS technique offers a very specific and sensitive way to detect SCs in different matrices, such as powder, herbal and biological samples. In forensic laboratories in Kuwait, 10 SCs are commonly detected in the last 5 years. These SCs are usually received as powder, smoking equipment, blood, and urine samples. The SCs are 5F-AB-PINACA, 5F-ADBICA, AB-PINACA, 5F-AMB, 5F-MDMB-PICA, 4F-MDMB-BUTINACA, FUB-PB-22, MDMB-4en-PINACA, 5F-APICA and APICA. The structures of the 10 compounds are illustrated in **Figure 2.1**, and their chemical names and molecular weights are summarized in **Table 2.1**.

No single method is reported for the analysis of these compounds simultaneously. The aim of work in this section was to develop a LC-MS/MS method for screening of 10 different SCs common in street samples in Kuwaiti market. The method can be used to draw a picture of the SCs use and spread in Kuwait.

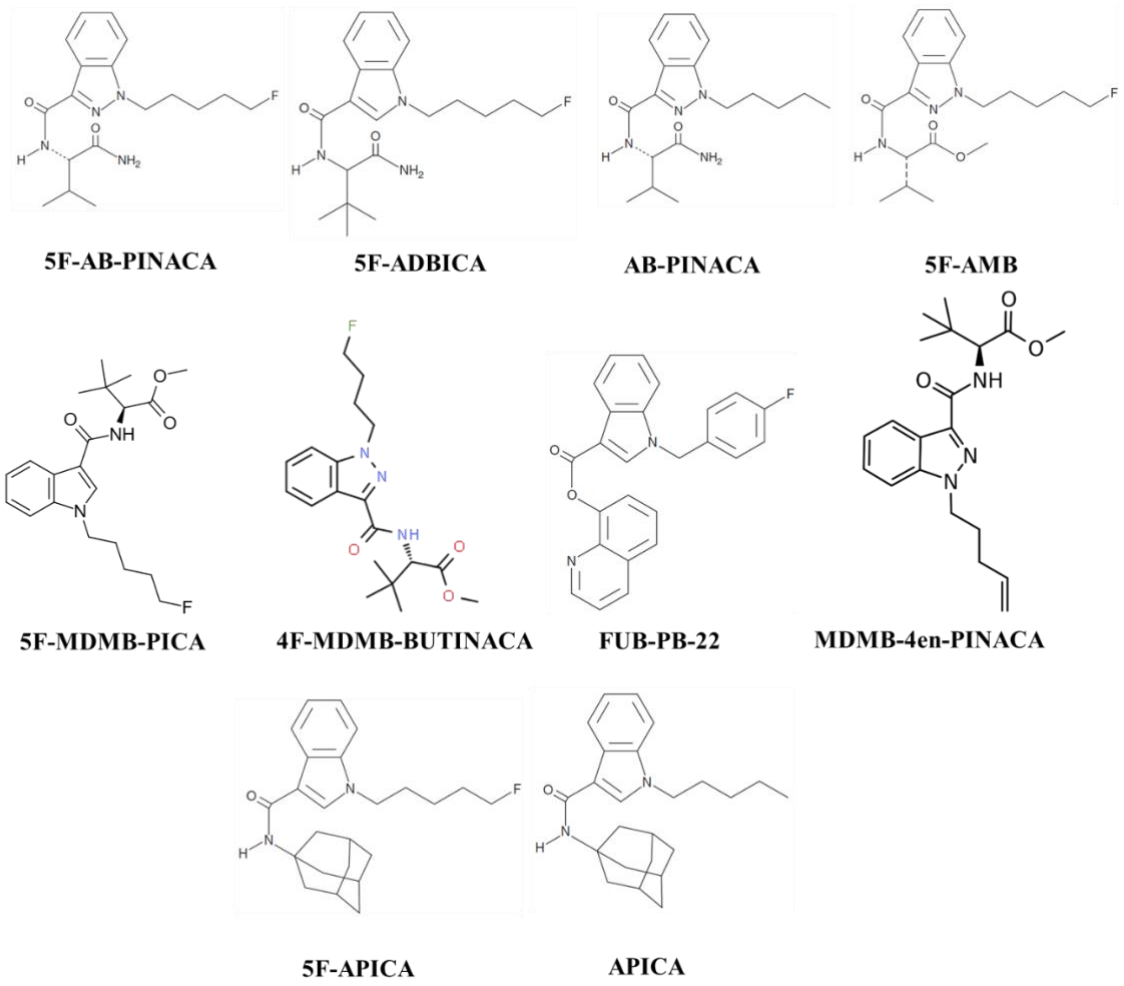


Figure 2.1. Chemical structures of the ten synthetic cannabinoids under investigation.

Table 2.1. chemical names and molecular weights of the ten synthetic cannabinoids under investigation.

Synthetic cannabinoid	Synonyms	Chemical name	Molecular formula	Molecular weight
5F-AB-PINACA		N-[(1S)-1-(aminocarbonyl)-2-methylpropyl]-1-(5-fluoropentyl)-1H-indazole-3-carboxamide	$C_{18}H_{25}FN_4O_2$	348.4
5F-ADBICA	5F-ADB-PICA	N-[1-(aminocarbonyl)-2,2-dimethylpropyl]-1-(5-fluoropentyl)-1H-indole-3-carboxamide	$C_{20}H_{28}FN_3O_2$	361.5
AB-PINACA		(S)-N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-pentyl-1H-indazole-3-carboxamide	$C_{18}H_{26}N_4O_2$	330.4
5F-AMB	5F-AMB-PINACA 5F-MMB-PINACA 5F-AMP	N-[[1-(5-fluoropentyl)-1H-indazol-3-yl]carbonyl]-L-valine, methyl ester	$C_{19}H_{26}FN_3O_3$	363.4
5F-MDMB-PICA	5F-MDMB-2201 MDMB-2201	N-[[1-(5-fluoropentyl)-1H-indol-3-yl]carbonyl]-3-methyl-L-valine, methyl ester	$C_{21}H_{29}FN_2O_3$	376.5
4F-MDMB-BUTINACA	4F-MDMB-BINACA	N-[[1-(4-fluorobutyl)-1H-indazol-3-yl]carbonyl]-3-methyl-L-valine, methyl ester	$C_{19}H_{26}FN_3O_3$	363.4
FUB-PB-22		1-[(4-fluorophenyl)methyl]-1H-indole-3-carboxylic acid, 8-quinolinyl ester	$C_{25}H_{17}FN_2O_2$	396.4
MDMB-4en-PINACA	MDMB-PENINACA	3-methyl-N-[[1-(4-penten-1-yl)-1H-indazol-3-yl]carbonyl]-L-valine, methyl ester	$C_{20}H_{27}N_3O_3$	357.5
5F-APICA	STS-135 N-adamantyl-1-fluoropentylindole-3-Carboxamide	1-(5-fluoropentyl)-N-tricyclo[3.3.1.13,7]dec-1-yl-1H-indole-3-carboxamide	$C_{24}H_{31}FN_2O$	382.5
APICA	JWH 018 adamantyl carboxamide 2NE1 SDB-001	1-pentyl-N-tricyclo[3.3.1.$1^{3,7}$]dec-1-yl-1H-indole-3-carboxamide	$C_{24}H_{32}N_2O$	364.5

2.2. Experimental

2.2.1. Materials and reagents

The SCs, 5F-AB-PINACA, 5F-ADBICA, AB-PINACA, 5F-AMB, 5F-MDMB-PICA, 4F-MDMB-BUTINACA, FUB-PB-22, MDMB-4en-PINACA, 5F-APICA, and APICA, were purchased from Cayman Chemicals (USA). Acetonitrile and methanol (LC-MS grade) and ammonium formate were purchased from Sigma Aldrich (USA). Deionized water was obtained from Purite Select Ondeo (Purite Limited, UK).

2.2.2. Instruments

Chromatographic analysis was conducted using a Nexera-i LC-2040C ultrahigh-performance liquid chromatography (UHPL) (Shimadzu Corporation, Japan) connected to a TQ-8050 triple quadrupole electrospray ionization (TQ-ESI) mass spectrometer (Shimadzu Corporation, Japan) operated in the ESI positive mode and multiple reaction monitoring (MRM) scan mode. The analysis was performed using Shimadzu Lab Solution software.

2.2.3. Liquid chromatographic and mass spectrometric conditions

Chromatographic separations were carried out using a Kinetex C18 column (50 × 3 mm, 2.6 um; Phenomenex Inc., USA). Gradient elution was used, wherein 5 mM ammonium formate was the mobile phase A, and acetonitrile was the mobile phase B. The gradient started with 20% B for 1 min, followed by a linear increase up to 90% within 10 min, held for 1 min, then a return to 20% B at 11.1 min, where it was maintained for 0.9 min for equilibration. The total run time was 12.0 min. The flow rate was 0.4 mL/min at a column temperature of 40 °C with an injection volume of 5 µL. Each component of the

mobile phase was degassed in an ultrasonic bath for 10 min before use. The interface was in positive ESI mode using nitrogen gas: nebulizing gas flow, 3 L/min; heating gas flow, 10 L/min; and drying gas flow, 10 L/min. The interface temperature was set to 300 °C, the desolvation line temperature to 300 °C, and the heat block temperature to 500 °C. The molecular ion transitions are summarized in **Table 2.2.**

2.2.4. Standard solutions

Stock solutions of 5F-AB-PINACA, 5F-ADBICA, AB-PINACA, 5F-AMB, 5F-MDMB-PICA, 4F-MDMB-BUTINACA, FUB-PB-22, MDMB-4en-PINACA, 5F-APICA, and APICA were prepared at a concentration of 1 ppm in LC-MS grade methanol.

2.2.5. Construction of calibration curves

Aliquots were taken from the stock solutions and diluted with LC-MS-grade methanol to prepare solutions in the range of 0.2–100 ppb for the ten SCs. The area under the curve was plotted against the corresponding concentrations, and regression equations were computed.

Table 2.2. LC-MS/MS parameters selected for the quantification of the ten synthetic cannabinoids.

Compound	Rt (min)	MRM (m/z)	Collision Energy (eV)
5F-AB-PINACA	5.80	349.5 > 145.4	−25
		349.5 > 304.1	−16
		349.5 > 233.2 *	−24
5F-ADIBICA	6.12	362.5 > 232.1 *	−23
		362.5 > 345.1	−11
		362.5 > 144.1	−40
AB-PINACA	6.76	331.5 > 215.1 *	−23
		331.5 > 286.2	−15
		331.5 > 145.1	−37
5F-AMB	7.86	364.1 > 233.3 *	−21
		364.1 > 213.3	−15
		364.1 > 69.3	−39
5F-MDMB-PICA	7.87	377.3 > 232.2 *	−21
		377.3 > 144.1	−40
		377.3 > 116.1	−55
4F-MDMB-BUTINACA	7.99	364.3 > 219.3 *	−26
		364.3 > 55.2	−47
FUB-PB-22	8.39	397.5 > 251.8	−15
		397.5 > 109.0 *	−35
MDMB-4en-PINACA	8.96	358.3 > 213.2 *	−24
		358.3 > 298.2	−16
		358.3 > 145.1	−42
5F-APICA	9.18	383.6 > 135.2 *	−26
		383.6 > 93.1	−47
		383.6 > 232.1	−24
APICA	10.11	365.5 > 135.1 *	−28
		365.5 > 214.1	−23
		365.5 > 107.1	−40

* The ion pair used for quantification

2.2.6. Validation

The proposed method was validated for specificity, accuracy, precision, linearity range, and robustness according to ICH guidelines [147]. The specificity of the method was assessed by analyzing various laboratory-prepared mixtures of the ten SCs. The accuracy was confirmed by applying the proposed method to determine three concentrations of each SC (20, 40, and 60 ppb; n = 3). The repeatability and intermediate precision were assessed using three concentrations (10, 50, and 100 ppb) of each standard SC solution (n = 3). The robustness of the proposed method was evaluated based on the reliability of the analysis concerning small variations in the experimental conditions. These parameters included oven temperature (45 ± 5 °C), and ammonium formate concentration (5 ± 1 mM). Three concentrations (10, 50, and 100 ppb) of standard SCs (n = 3) were analyzed, and only one parameter was changed for each experiment.

2.2.7. Application to street samples

The Toxicology Department of the General Department of Criminal Evidence in Kuwait kindly supplied 74 samples seized from Kuwaiti streets between September 2021 and March 2022. The method reported by UNODC was employed [148] . 2 mL of methanol as the extraction solvent was added to 20 mg of herbal sample. The mixture was sonicated in a water bath at 37 °C for 10 min and then centrifuged (3000 rpm, 5 min). The supernatant was filtered through a membrane filter (0.45 µm) and 5 µL of each solution was injected to LC/MS-MS. The SCs were identified, and their concentrations were calculated using the corresponding regression equations.

2.2.8. Ethics approval and consent to participate

Ethical approval for the collection and analysis of the samples has been granted by the Ministry of Justice and Ministry of Interior Ethical Committee. Permission to use samples and data was obtained from the General Department of Criminal Evidence, Ministry of Interior.

2.3. Results and dicussion

2.3.1. Method development and optimization

The detection of SCs is a challenging process as new compounds continuously evolve to bypass the restrictions on illicit drugs. LC-MS/MS is highly recommended because of its high specificity resulting from coupling LC separation with MS/MS detection. In MRM mode, only ions derived from the target analyte are detected; thus, interference from other compounds and endogenous matrix components is minimal [149].

Several columns were tried such as Phenomenex C8, ACE C8, Shim-pack XR-ODS, and Kinetex C18 columns. The optimum performance was obtained using Kinetex C18 column owing to good shape and symmetry of the obtained peaks, and reasonable retention time. Both isocratic and gradient elution methods were tested, but isocratic elution failed to separate the 10 SCs in a reasonable time, so gradient elution was chosen. Several solvents were tested as mobile phase combinations. Both methanol and acetonitrile are compatible with ESI, they were used in different gradient systems with ammonium formate and formic acid. Several concentrations of formic acid ranging from 0.01% to 0.1 were tested, also 5 and 10 mM ammonium formate were both tried. A gradient of acetonitrile and 5 mM ammonium formate was

found to yield superior results compared to methanol and formic acid regarding peak shape and run time. Optimal elution was achieved using a gradient with 5 mM ammonium formate as mobile phase A and acetonitrile as mobile phase B.

Under the optimal conditions, all SCs were separated except for 5-AMB, 5F-MDMB-PICA, and 4F-MDMB-BUTINACA. Overlapping of the peaks of 5-AMB and 5F-MDMB-PICA was not a problem because their mass transitions were different (364.1 is the parent for 5F-AMB, whereas the parent for 5F-MDMB-PICA is 377.3), and both compounds could be quantified without interference despite their coelution. However, 5-AMB and 4F-MDMB-BUTINACA share close parent peaks (364.1 for 5F-AMB and 364.3 for 4F-MDMB-BUTINACA) and similar daughter peaks (304.2 and 145.1, respectively). The gradient conditions and different mass transitions were optimized to allow their separation. The mass transitions were 364.1 > 233.3, 364.1 > 213.3, and 364.1 > 69.3 for 5F-AMB, whereas for 4F-MDMB-BUTINACA, the transitions were 364.3 > 219.3 and 364.3 > 55.2. **Figure 2.2-2.11** show the mass spectra of the ten SCs.

The protonated precursor ions $[M+H]^+$ of 5F-AB-PINACA, 5F-ADBICA, AB-PINACA, 5F-AMB, 5F-MDMB-PICA, 4F-MDMB-BUTINACA, FUB-PB-22, MDMB-4en-PINACA, 5F-APICA, and APICA were detected in the full-scan mass spectra at m/z values of 349.5, 362.5, 331.5, 364.1, 377.3, 364.3, 397.5, 358.3, 383.6, and 365.5, respectively. The collision energy was optimized, and MS/MS transitions were selected to determine the ten SCs, as summarized in **Table 2.2**.

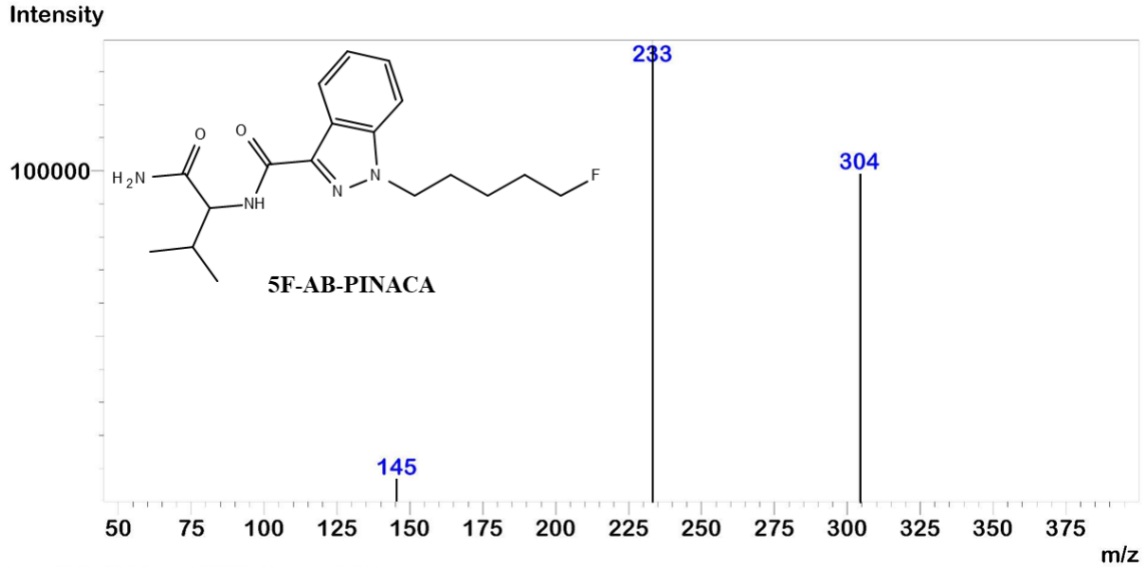


Figure 2.2. Mass Spectrum of 5F-AB-PINACA

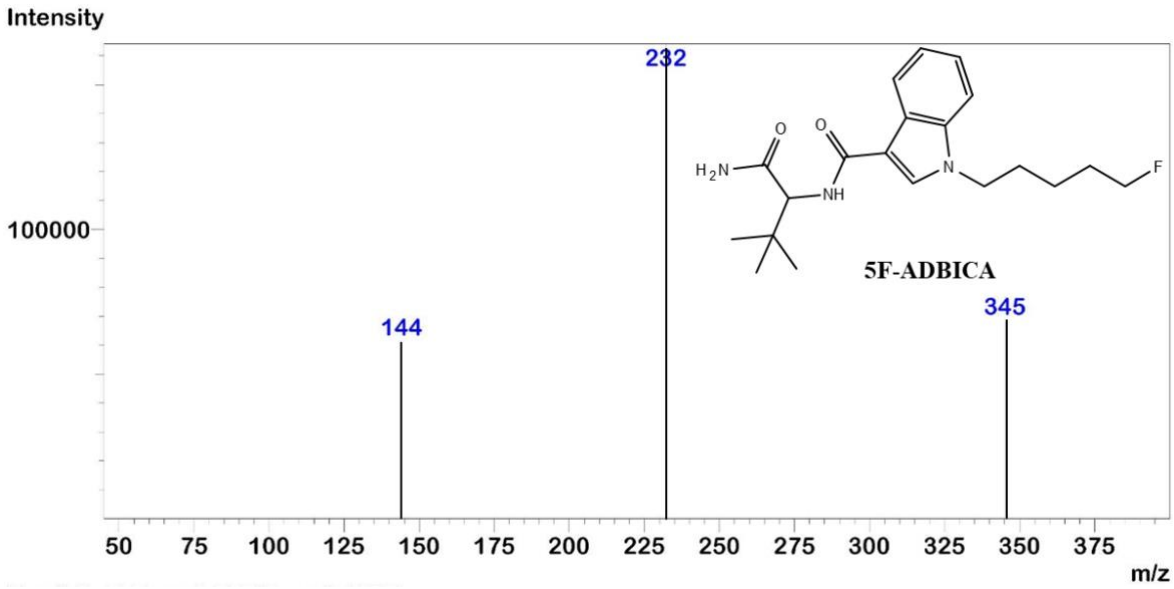


Figure 2.3. Mass Spectrum of 5F-ADBICA

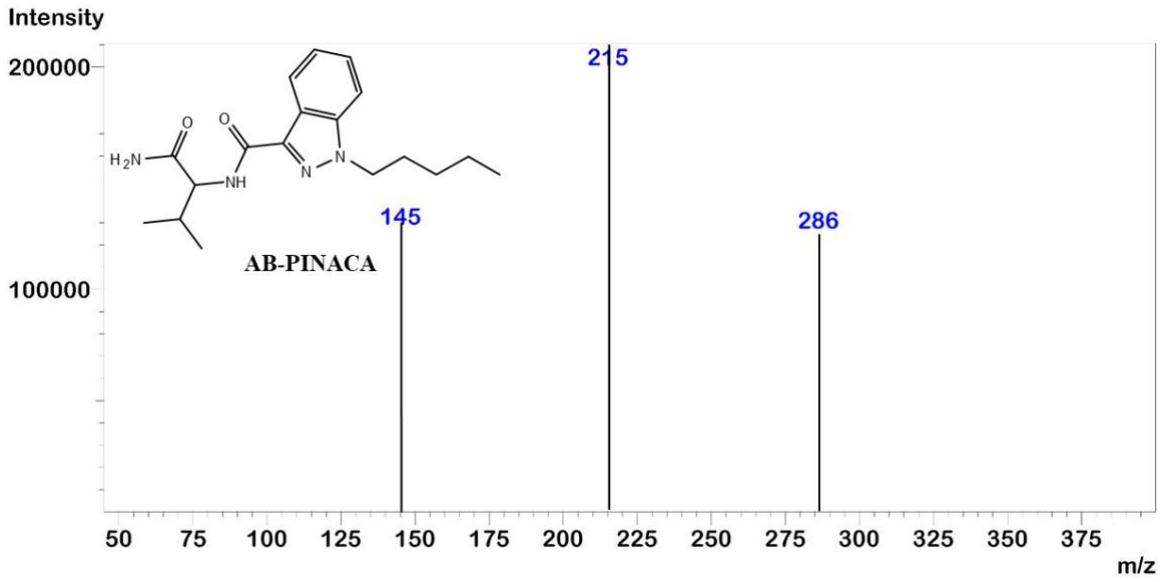


Figure 2.4. Mass Spectrum of AB-PINACA

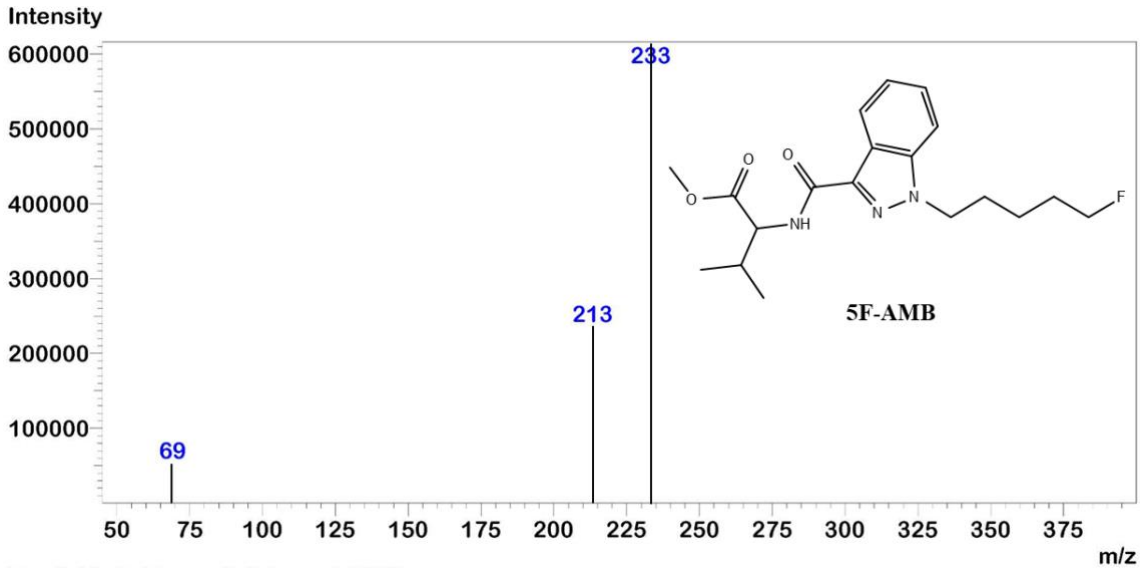


Figure 2.5. Mass Spectrum of 5F-AMB

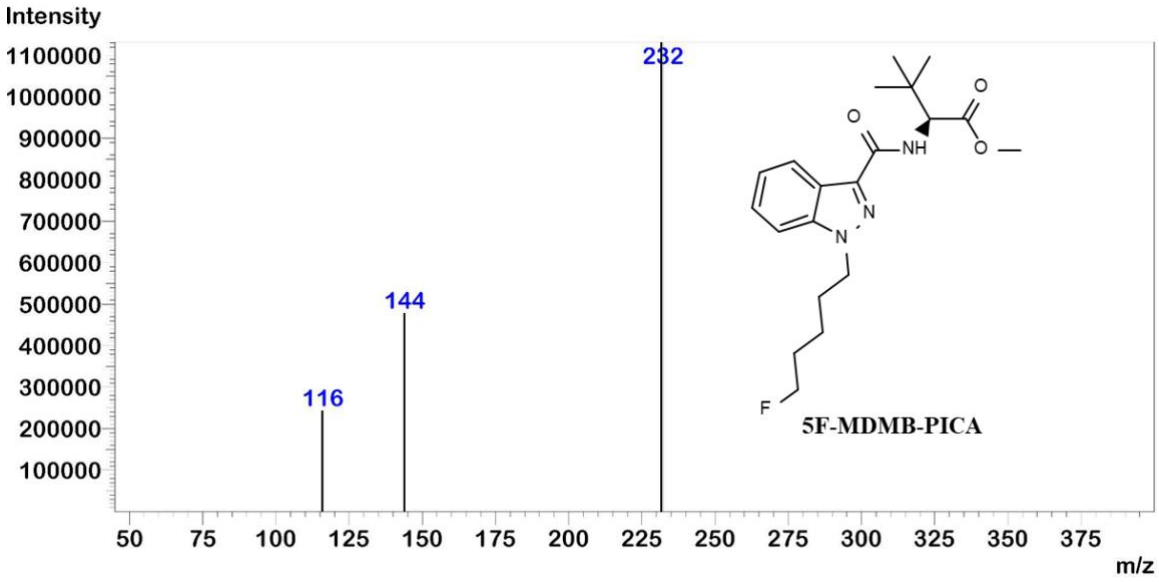


Figure 2.6. Mass Spectrum of 5F-MDMB-PICA

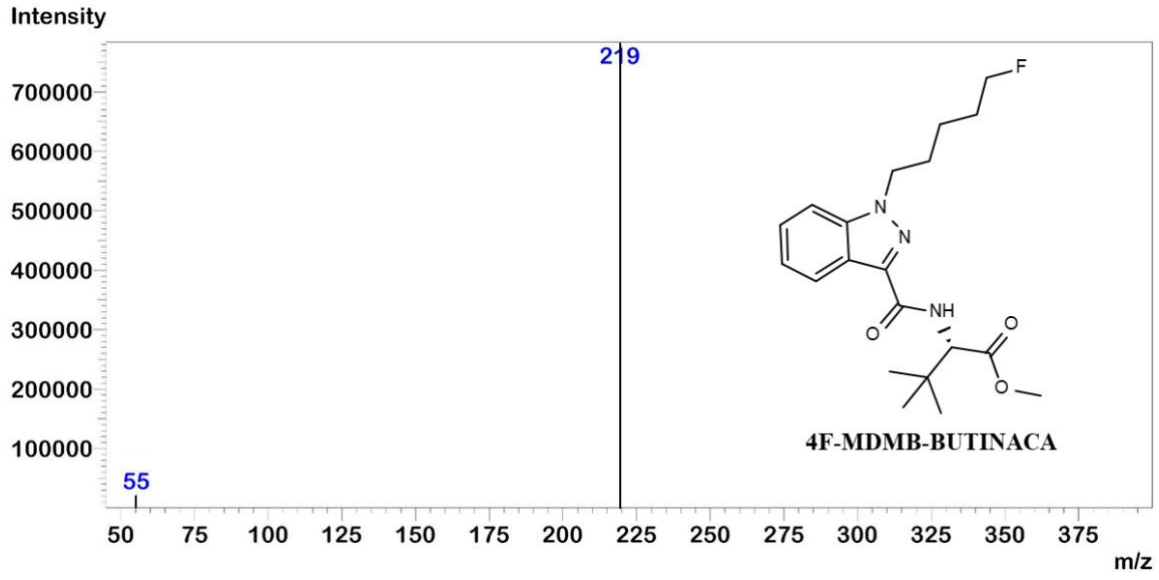


Figure 2.7. Mass Spectrum of 4F-MDMB-BUTINACA

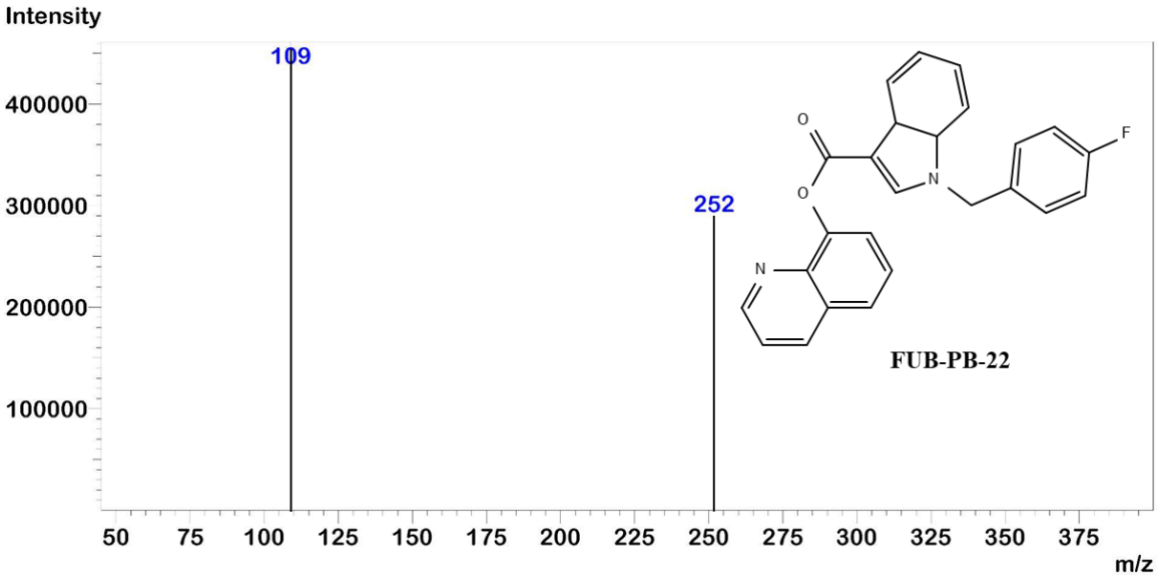


Figure 2.8. Mass Spectrum of FUB-PB-22

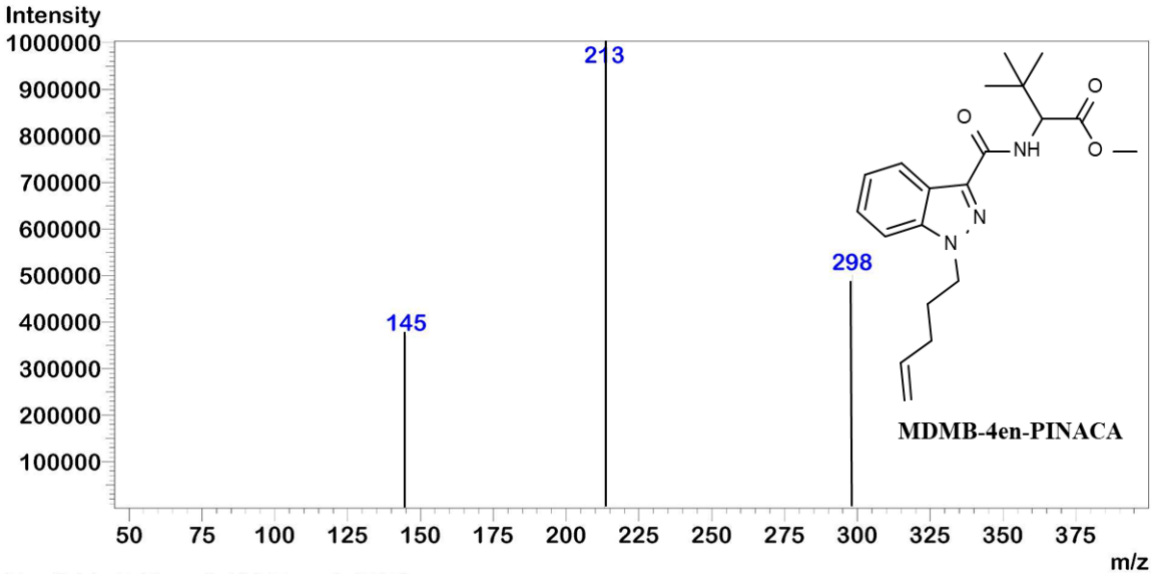


Figure 2.9. Mass Spectrum of MDMB-4en-PINACA

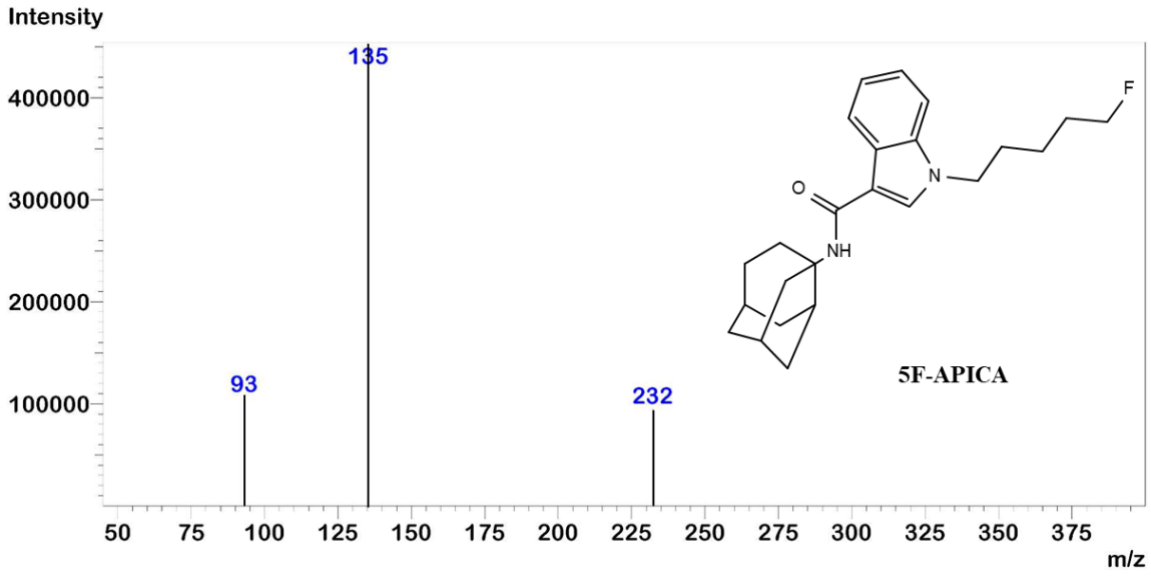


Figure 2.10. Mass Spectrum of 5F-APICA

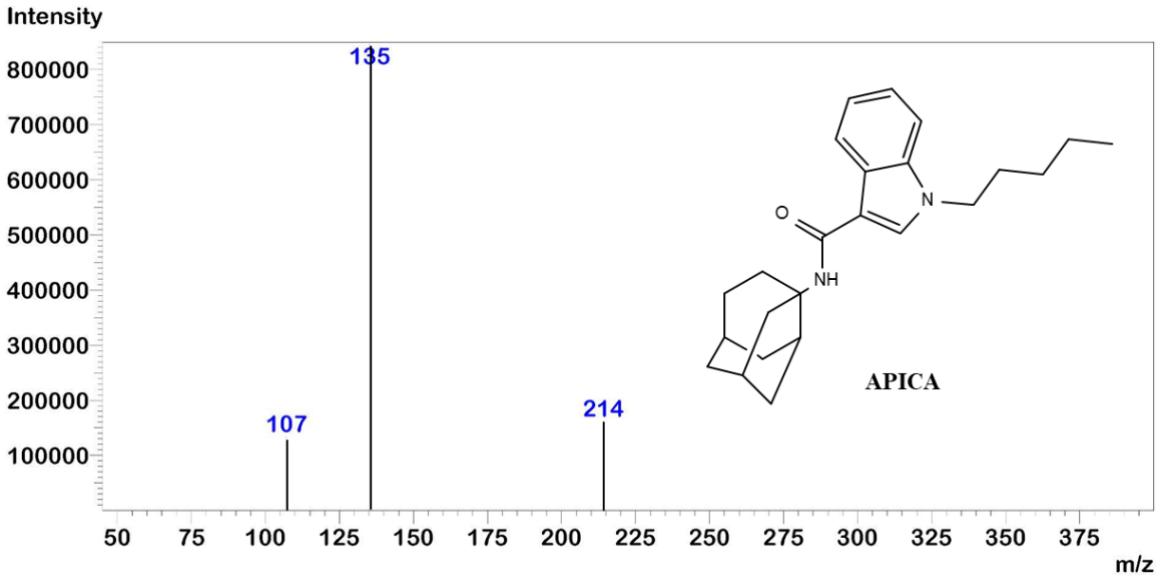


Figure 2.11. Mass Spectrum of APICA

The optimized method could quantify the ten compounds despite some overlapping of the peaks (**Figure 2.12**). This was attributed to specific MS transitions, proving that the method was specific for the ten SCs. The method was linear in the range of 0.2–100 ppb for the ten SCs. **Figure 2.13-22** show the calibration curves for the ten SCs and their regression equations. From the curves, it appears that the method can be used successfully to determine these SCs in different matrices.

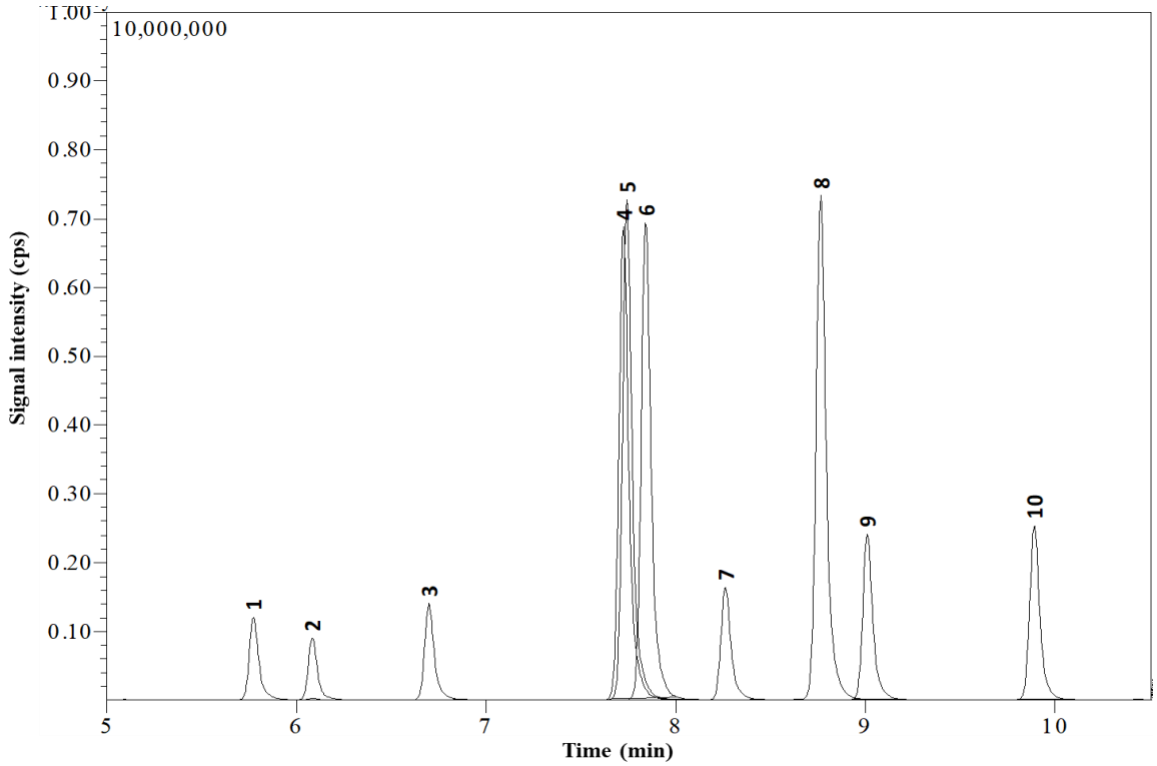


Figure 2.12. Mass chromatogram of the ten SCs under investigation at 10 ppb, 5F-AB-PINACA (1), 5F-ADIBICA (2), AB-PINACA (3), 5F-AMB (4), 5F-MDMB-PICA (5), 4F-MDMB-BUTINACA (6), FUB-PB-22 (7), MDMB-4en-PINACA (8), 5F-APICA (9) and APICA (10).

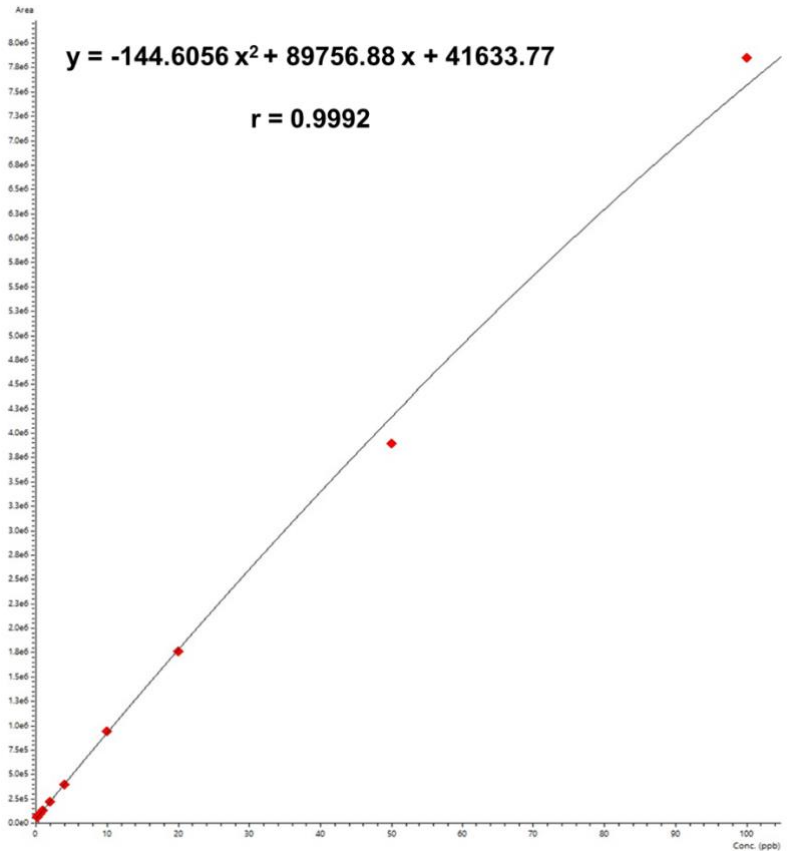


Figure 2.13. Linearity of AUC to the corresponding concentrations of 5F-AB-PINACA

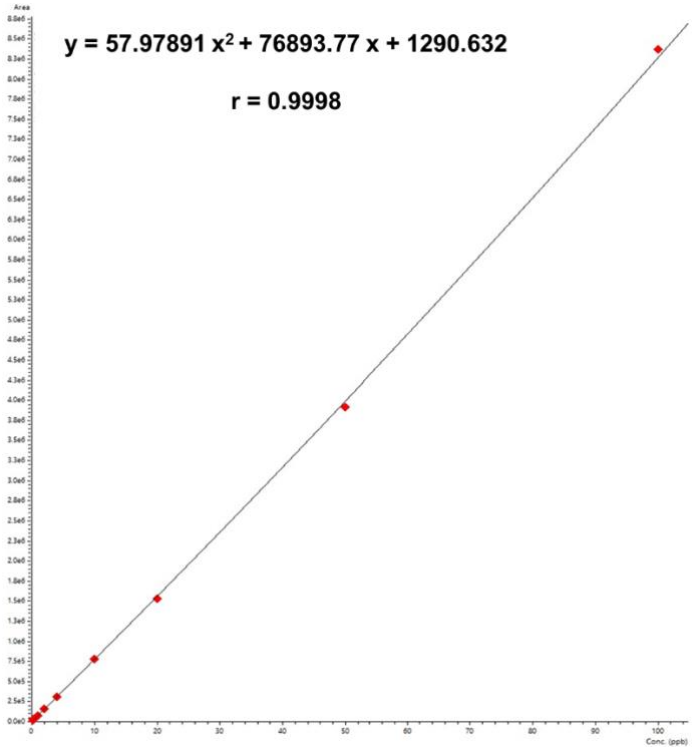


Figure 2.14. Linearity of AUC to the corresponding concentrations of 5F-ADBICA

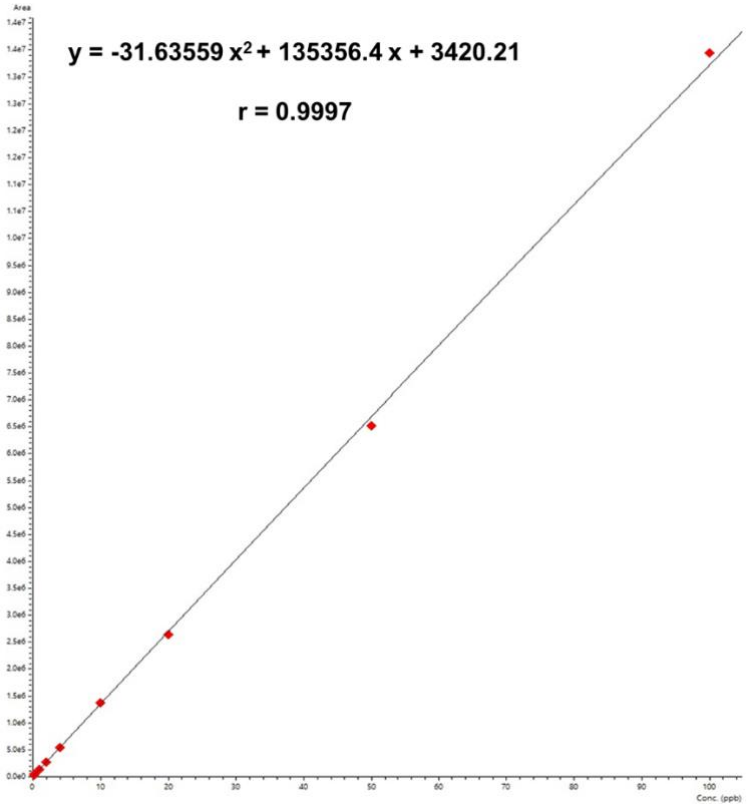


Figure 2.15. Linearity of AUC to the corresponding concentrations of AB-PINACA

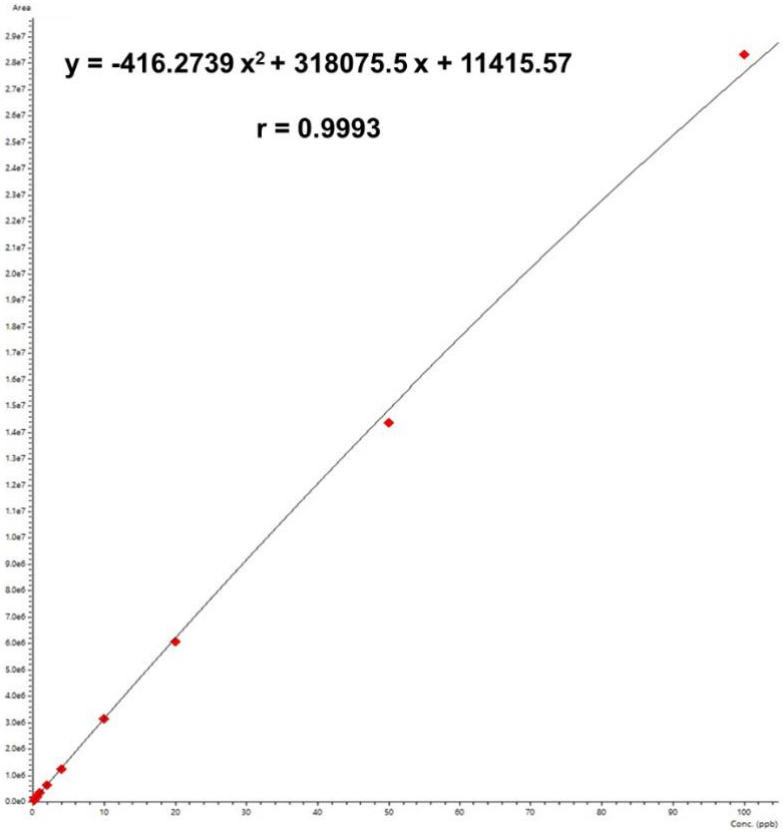


Figure 2.16. Linearity of AUC to the corresponding concentrations of 5F-AMB

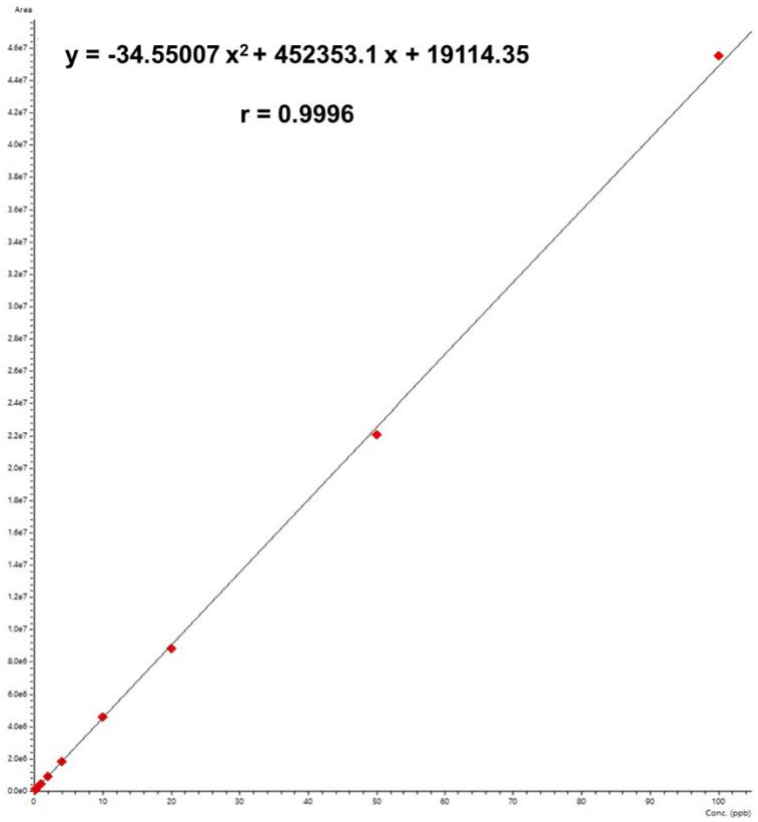


Figure 2.17. Linearity of AUC to the corresponding concentrations of 5F-MDMB-PICA

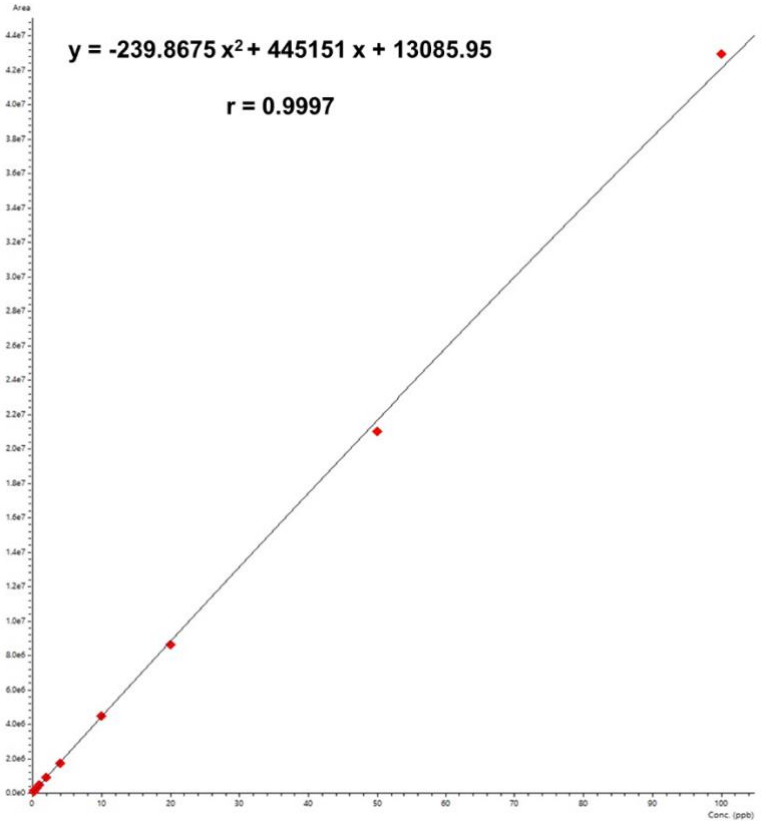


Figure 2.18. Linearity of AUC to the corresponding concentrations of 4F-MDMB-BUTINACA

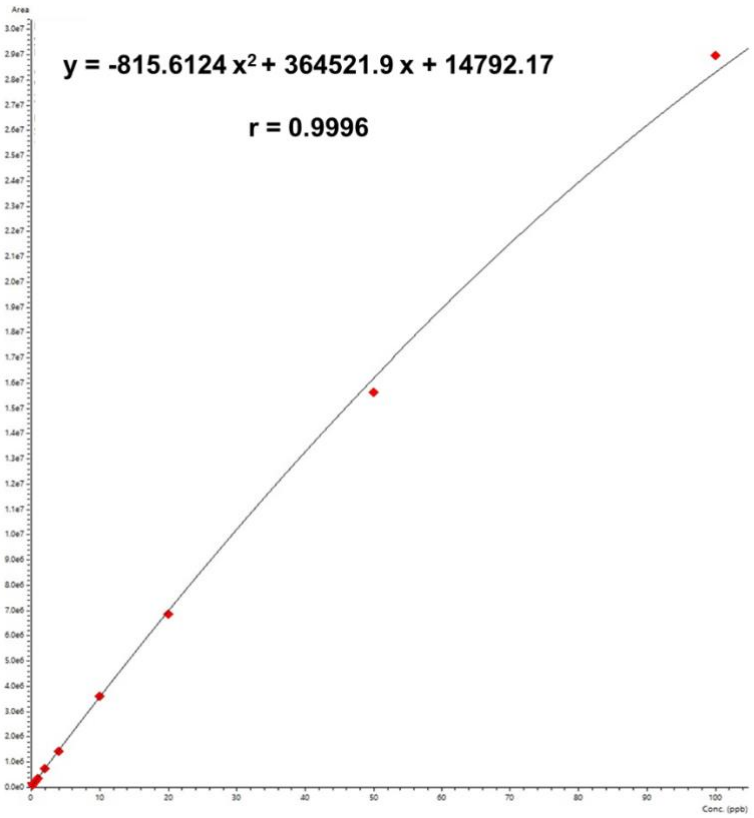


Figure 2.19. Linearity of AUC to the corresponding concentrations of FUB-PB-22

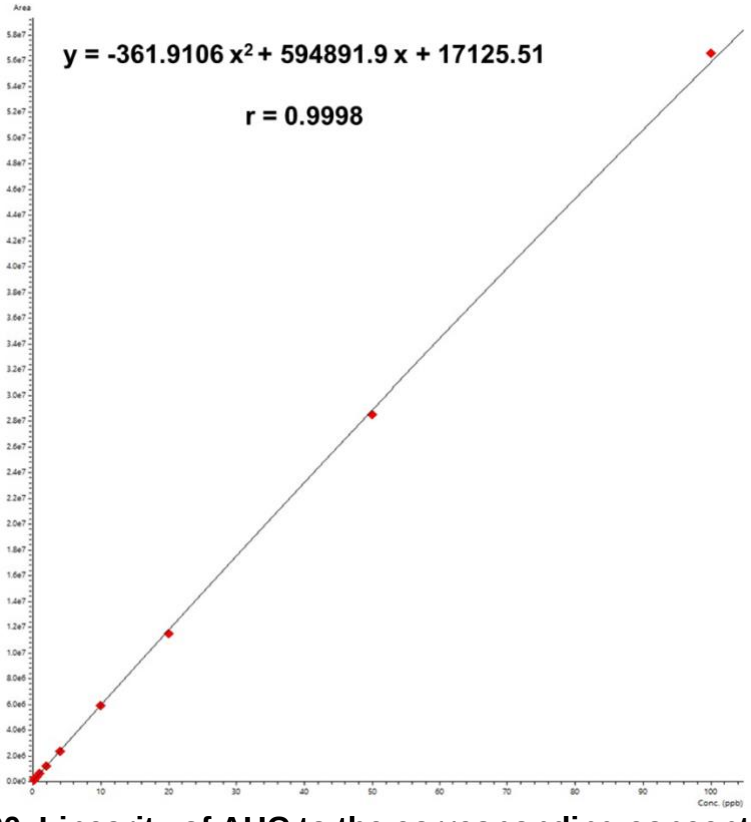


Figure 2.20. Linearity of AUC to the corresponding concentrations of MDMB-4en-PINACA

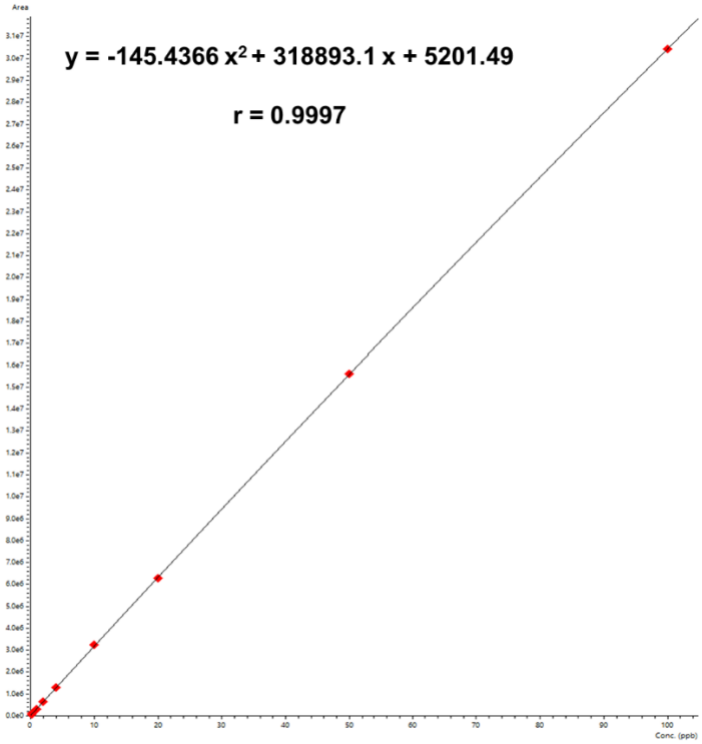


Figure 2.21. Linearity of AUC to the corresponding concentrations of 5F-APICA

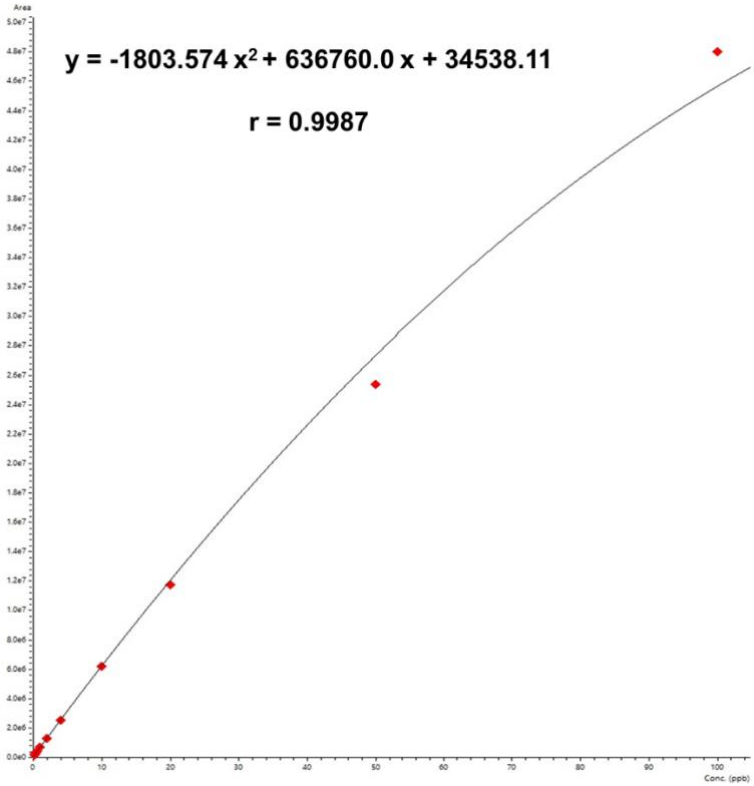


Figure 2.22. Linearity of AUC to the corresponding concentrations of APICA

2.3.2. Method Validation

The proposed LC-MS/MS method was validated for the ten SCs according to ICH guidelines [147]. The method was developed and validated in the Toxicology Department of the General Department of Criminal Evidence in Kuwait, and it was cross validated in our laboratory at the University of Strathclyde. In this study, we present the data obtained from the validations in our laboratory. For specificity, satisfactory recoveries were obtained to quantify SCs at various ratios in the laboratory-prepared mixtures, as shown in **Table 2.3**. For accuracy, good mean percentage recoveries were obtained to quantify the SCs at concentrations of 20, 40, and 60 ppb, as shown in **Table 2.4**. For precision, a satisfactory relative standard deviation (RSD%) was obtained from the analysis of the ten SCs at three concentration levels (10, 50, and 100 ppb), three times on the same day and three different days, as shown in **Table 2.5** indicating reasonable repeatability and intermediate precision. For robustness, minor variations in the experimental conditions, as defined in the materials and methods section, showed reasonable RSD of the responses of the SCs using the proposed method (**Table 2.6**), indicating that the method was sufficiently robust.

Table 2.3. Determination of SCs in laboratory prepared mixtures by the proposed method

	5F-AB-PINACA	5F-ADBICA	AB-PINACA	5F-AMB	5F-MDMB-PICA	4F-MDMB-BUTINACA	FUB-PB-22	MDMB-4en-PINACA	5F-APICA	APICA
Conc (ppb)	1	1	1	1	1	1	1	1	1	1
Recovery % [a]	87.7	98.9	101.2	99.6	100.0	98.6	98.4	99.4	100.5	101.6
Conc (ppb)	50	50	50	50	50	50	50	50	50	50
Recovery % [a]	96.8	100.3	99.3	98.7	99.7	100.1	96.9	99.4	102.0	93.0
Conc (ppb)	100	100	100	100	100	100	100	100	100	100
Recovery % [a]	103.3	101.4	101.3	103.4	101.6	102.9	101.5	101.4	100.5	107.2
Conc (ppb)	10	10	10	10	10	50	50	50	50	50
Recovery % [a]	79.9	93.9	89.6	89.8	93.5	87.7	100.5	104.0	100.1	90.6
Conc (ppb)	50	50	50	50	50	10	10	10	10	10
Recovery % [a]	97.0	109.9	102.2	106.8	103.4	90.0	93.3	96.0	101.0	87.7
Mean ± SD	92.9±9.2	100.9±5.8	98.7±5.2	99.7±6.4	99.6±3.7	95.9±6.6	98.1±3.2	100.0±2.9	100.8±0.7	96.0±8.1

[a] Recovery% = found/claimed conc.*100, n =3.

Table 2.4. Accuracy results of the proposed LC-MS/MS method for analysis of the ten SCs

Compound	Recovery%[a]			Accuracy
	20 ppb	40 ppb	60 ppb	Mean ± SD
5F-AB-PINACA	99.3	105.8	96.9	100.7±4.6
5F-ADBICA	101.4	104.3	100.9	102.2±1.8
AB-PINACA	100.8	103.3	99.2	101.1±2.1
5F-AMB	100.3	103.2	99.3	100.9±2.0
5F-MDMB-PICA	101.3	103.1	100.6	101.7±1.3
4F-MDMB-BUTINACA	100.9	104.3	100.0	101.7±2.3
FUB-PB-22	100.2	105.5	96.2	100.6±4.7
MDMB-4en-PINACA	101.2	103.4	100.1	101.6±1.7
5F-APICA	102.6	105.1	102.0	103.2±1.6
APICA	98.5	102.4	94.3	98.4±4.1

[a] Recovery% = found/claimed conc.*100, n =3.

Table 2.5. Precision results of the proposed LC-MS/MS method for analysis of the ten SCs

Compound	Intra-day Precision [a]			Inter-day Precision [b]		
	RSD%					
	10 ppb	50 ppb	100 ppb	10 ppb	50 ppb	100 ppb
5F-AB-PINACA	1.4	0.4	0.6	2.3	1.8	1.6
5F-ADBICA	0.4	1.3	1.1	2.3	1.4	1.4
AB-PINACA	0.1	1.3	0.7	1.4	1.5	1.3
5F-AMB	1.2	0.8	0.8	2.3	1.5	1.5
5F-MDMB-PICA	0.4	1.2	1.4	2.0	1.2	1.2
4F-MDMB-BUTINACA	1.1	0.9	0.9	1.2	1.9	1.3
FUB-PB-22	1.2	0.2	0.5	2.7	1.5	1.2
MDMB-4en-PINACA	1.0	0.4	1.0	2.3	2.0	1.4
5F-APICA	1.5	1.4	1.4	1.2	1.8	1.1
APICA	1.4	0.9	0.6	1.7	2.0	1.7

[a] Intra-day precision (n = 3), RSD of three concentrations repeated three times within the day.

[b] Inter-day precision (n = 3), RSD of three concentrations repeated three times in three different days.

Table 2.6. Robustness results of the proposed LC-MS/MS method for analysis of the ten SCs

Compound	Oven temperature 45±5 °C			Ammonium formate concentration 5±1 mM		
	RSD%[a]					
	10 ppb	50 ppb	100 ppb	10 ppb	50 ppb	100 ppb
5F-AB-PINACA	1.4	2.4	2.0	4.1	2.5	0.9
5F-ADBICA	1.4	1.4	0.8	2.3	2.2	1.9
AB-PINACA	2.1	1.2	1.3	2.5	2.5	2.6
5F-AMB	2.3	0.7	1.1	2.5	2.1	2.4
5F-MDMB-PICA	1.4	0.7	0.7	2.7	1.8	2.0
4F-MDMB-BUTINACA	1.3	1.3	1.0	2.8	1.5	1.9
FUB-PB-22	1.1	0.9	1.4	2.0	1.5	0.8
MDMB-4en-PINACA	1.1	1.1	1.3	1.8	2.0	1.3
5F-APICA	1.4	1.6	1.3	2.2	3.0	2.3
APICA	1.8	2.0	2.5	2.5	0.8	0.9

[a] Average of three determinations

2.3.3. Analysis of Kuwaiti street samples by the proposed method

The main aim of this study was to scan the Kuwaiti market, identify the common SCs used by Kuwaiti youth, and quantify their amounts in products sold on the streets. For this purpose, 74 samples of SC products were kindly provided by the Toxicology Department of the General Department of Criminal Evidence, Kuwait. The samples were collected from September 2021 to March 2022 (**Figure 2.23**). The validated LC-MS/MS method was applied to screen 74 samples to identify the SCs used in the sold products and to quantify their concentrations. Each sample was analyzed thrice, and the average concentrations are listed in **Table 2.7**. Approximately 25–28% of the samples contained MDMB-4EN-PINACA or FUB-PB-22. Most samples (approximately 46%) contained 4F-MDMB-BUTINACA. This suggests that the most common SC in the Kuwaiti market was 4F-MDMB-BUTINACA, followed by MDMB-4EN-PINACA or FUB-PB-22. The quantitative data of the samples indicate that the concentrations of these three SCs in the powdered samples ranged from 528.7 to 1416.2 ng/g (**Table 2.7**).

Figure 2.23. A picture of some of the seized samples from Kuwaiti market

Table 2.7. Analysis of Kuwaiti street samples using the proposed LC-MS/MS method

No.	Detected SC	Conc (ng/g) [a]
1	MDMB-4EN-PINACA	1416.2
2	4F-MDMB-BUTINACA	769.2
3	4F-MDMB-BUTINACA	594.5
4	MDMB-4EN-PINACA	889.2
5	FUB-PB-22	793.8
6	MDMB-4EN-PINACA	922.9
7	FUB-PB-22	809.8
8	MDMB-4EN-PINACA	528.7
9	FUB-PB-22	764.6
10	4F-MDMB-BUTINACA	715.2
11	FUB-PB-22	761
12	4F-MDMB-BUTINACA	875.9
13	MDMB-4EN-PINACA	890
14	4F-MDMB-BUTINACA	811.2
15	4F-MDMB-BUTINACA	1023.2
16	MDMB-4EN-PINACA	879.3
17	FUB-PB-22	774.9
18	4F-MDMB-BUTINACA	987.2
19	MDMB-4EN-PINACA	532.1
20	4F-MDMB-BUTINACA	1248.4
21	4F-MDMB-BUTINACA	786.6
22	4F-MDMB-BUTINACA	1000.4
23	MDMB-4EN-PINACA	901.5
24	MDMB-4EN-PINACA	927.7
25	4F-MDMB-BUTINACA	766.3
26	4F-MDMB-BUTINACA	834.5
27	FUB-PB-22	783.5
28	FUB-PB-22	831.9

Table 2.7. Analysis of Kuwaiti street samples using the proposed LC-MS/MS method, continued.

No.	Detected SC	Conc (ng/g) [a]
29	MDMB-4EN-PINACA	535.1
30	MDMB-4EN-PINACA	917.2
31	4F-MDMB-BUTINACA	845.6
32	MDMB-4EN-PINACA	889.2
33	MDMB-4EN-PINACA	930.4
34	FUB-PB-22	751.3
35	FUB-PB-22	1246.9
36	FUB-PB-22	772.3
37	4F-MDMB-BUTINACA	818.6
38	FUB-PB-22	763.9
39	FUB-PB-22	756.4
40	MDMB-4EN-PINACA	911.1
41	MDMB-4EN-PINACA	903.1
42	FUB-PB-22	771.3
43	MDMB-4EN-PINACA	536.3
44	FUB-PB-22	795.3
45	MDMB-4EN-PINACA	904.6
46	MDMB-4EN-PINACA	902.4
47	4F-MDMB-BUTINACA	951.9
48	4F-MDMB-BUTINACA	1101.1
49	4F-MDMB-BUTINACA	1269
50	4F-MDMB-BUTINACA	1007
51	FUB-PB-22	755.2
52	MDMB-4EN-PINACA	921.9
53	FUB-PB-22	778.6
54	4F-MDMB-BUTINACA	873.4
55	4F-MDMB-BUTINACA	750.5

Table 2.7. Analysis of Kuwaiti street samples using the proposed LC-MS/MS method, continued.

No.	Detected SC	Conc (ng/g) [a]
56	FUB-PB-22	1273.6
57	MDMB-4EN-PINACA	900.2
58	4F-MDMB-BUTINACA	1076.8
59	4F-MDMB-BUTINACA	1214.9
60	4F-MDMB-BUTINACA	826.2
61	FUB-PB-22	749.8
62	4F-MDMB-BUTINACA	628.8
63	4F-MDMB-BUTINACA	877.4
64	4F-MDMB-BUTINACA	940.7
65	4F-MDMB-BUTINACA	692
66	4F-MDMB-BUTINACA	806
67	4F-MDMB-BUTINACA	953.6
68	FUB-PB-22	768.5
69	4F-MDMB-BUTINACA	935.7
70	4F-MDMB-BUTINACA	941.9
71	4F-MDMB-BUTINACA	661.5
72	MDMB-4EN-PINACA	903.3
73	4F-MDMB-BUTINACA	1260.9
74	4F-MDMB-BUTINACA	808.9

[a] Concentration is expressed as nanogram SC per grams of street sample (n=3).

The concentrations of the three SCs in the seized samples varied significantly. As shown in **Figure 2.24**, the concentrations of MDMB-4EN-PINACA in the samples were ranging from 879.3 to 930.4 ng/g, while the concentrations of FUB-PB-22 in the samples was little lower than MDMB-4EN-PINACA ranging from 749.8 to 831.9 ng/g. The limited spread in the concentrations of these two SCs indicated that their samples contain the same concentration in Kuwaiti market. On the contrary, samples of 4F-MDMB-BUTINACA showed larger distribution of concentration from 594.5 to 1269 ng/g. This indicates the large variations in 4F-MDMB-BUTINACA concentrations in samples in Kuwaiti market which may indicate more danger from products containing this SC than other ones.

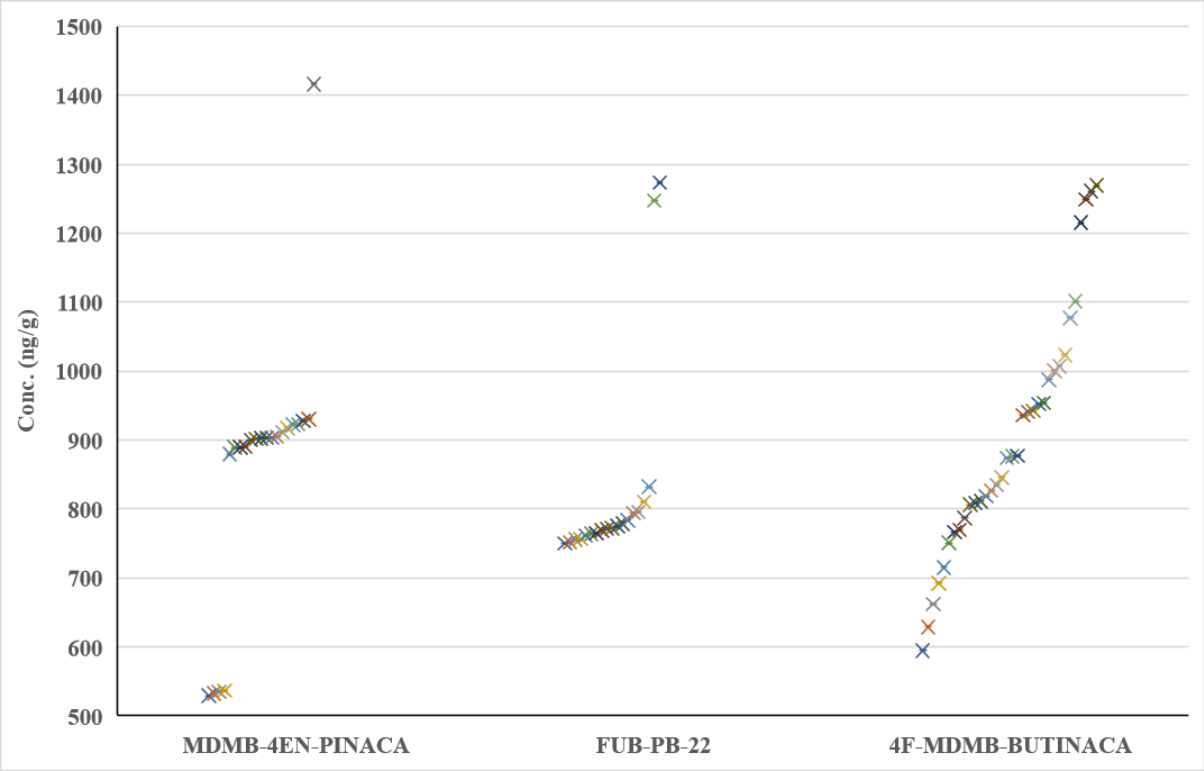


Figure 2.24. Concentrations of MDMB-4en-PINACA, FUB-PB-22, and 4F-MDMB-BUTINACA in the 74 samples collected in Kuwaiti streets.

In a previous screening study conducted in 2018 [74], 5F-AKB-48, 5Cl-AKB-48, 5F-ADB, FUB-AMB, and UR-144 were the most common SC detected in urine samples obtained from Kuwait. Another study conducted in 2018 and 2019 [150] revealed that single SCs and combinations of SCs with other illicit drugs were detected in the samples. The most commonly identified compounds were 5F-ADB, 5F-MDMB-PICA, FUB-AMB, and 5Cl-AKB-48. These studies were conducted on a larger number of samples, and they included postmortem urine samples to identify SCs commonly associated with death. Although we employed a smaller number of samples, our study had the advantage of SC quantitation. This is important because the level of SC in products may trigger side effects and, ultimately, death. Our future plans involve analyzing a larger number of samples, including urine and blood plasma, and incorporating other illicit drugs that may be combined with SCs to identify the most common illicit drug combinations in Kuwait. Postmortem samples will also be collected to investigate SCs associated with death.

2.4. Conclusion

A sensitive and specific LC-MS/MS method was developed to separate the ten SCs—5F-AB-PINACA, 5F-ADBICA, AB-PINACA, 5F-AMB, 5F-MDMB-PICA, 4F-MDMB-BUTINACA, FUB-PB-22, MDMB-4en-PINACA, 5F-APICA, and APICA. Further, this method was used to quantify the SCs, and the linearity range was 0.2–100 ppb. The method was cross validated by a laboratory at the University of Strathclyde and the Toxicology Department of the General Department of Criminal Evidence, Kuwait. Street samples seized from Kuwaiti streets from 2021 to 2022 were provided by the General Department of Criminal Evidence and screened for SCs. The analysis of the 74 street samples showed an abundance of 4F-MDMB-BUTINACA (46% of the seized samples) and the detection of MDMB-4EN-PINACA or FUB-PB-22 in less than 30%

of the samples. Quantitation of these SCs showed concentrations ranging from 528.7 to 1416.2 ng/g.

Chapter 3 Development and validation of LC-MS/MS method for analysis of three synthetic cannabinoids in urine samples from Kuwait

3.1. Introduction

Nowadays, SCs represent the most common group of NPS that is consumed by youth. SCs are more potent, toxic, and unpredictable than THC, which make them a substantial health concern. They can lead to acute CNS and CVS toxicity, which make SC a major public health concern. Also, possible adverse effects of SC exposure are tachycardia, anxiety, psychosis, and the their addiction potential [37]. The speed at which new SCs are emerging poses a significant challenge for forensic science. While authorities routinely review the legislation and add new substances to the schedules, manufacturers usually make only slight changes to SC structures to discriminate them from the listed ones. This challenge necessitates the development of new analytical methods for identifying of these new substances [47].

The aim of this work is to screen SCs abuse in Kuwait. For this purpose, urine samples collected from Kuwait were analyzed via a validated LC-MS/MS method to detect three of the common SCs abused in Middle East. The compounds under investigation are ADB-PINACA 5-Pentanoic Acid (ADB-PINACA-COOH), 5F-AB-PINACA, and 5F-AMB. The structures of the three SCs are illustrated in **Figure 3.1**, and their chemical names and molecular weights are summarized in **Table 3.1**.

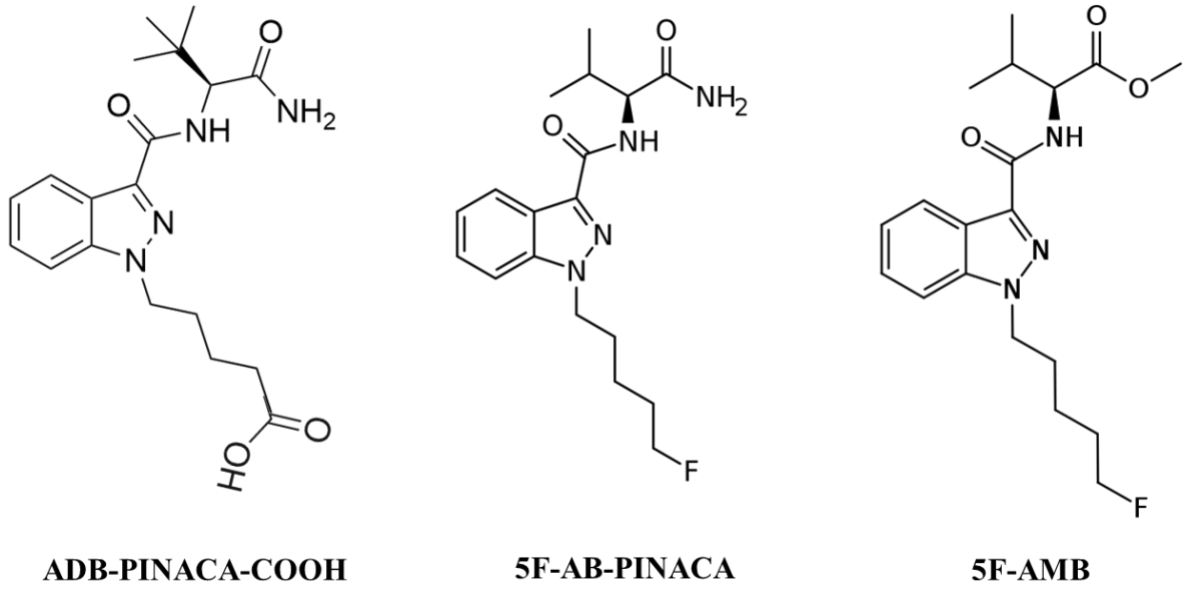


Figure 3.1. Chemical structures of the three SCs under investigation.

Table 3.1. Chemical names and molecular weights of the SCs under investigation.

Compound	Chemical name	Molecular formula	Molecular weight
ADB-PINACA-COOH	(S)-N-[1-(aminocarbonyl)-2,2-dimethylpropyl]-1-(5-pentanoic acid)-1H-indazole-3-carboxamide	$C_{19}H_{26}N_4O_4$	374.4
5F-AB-PINACA	N-[(1S)-1-(aminocarbonyl)-2-methylpropyl]-1-(5-fluoropentyl)-1H-indazole-3-carboxamide	$C_{18}H_{25}FN_4O_2$	348.4
5F-AMB	N-[[1-(5-fluoropentyl)-1H-indazol-3-yl]carbonyl]-L-valine, methyl ester	$C_{19}H_{26}FN_3O_3$	363.4

3.2. Experimental

3.2.1. Materials and reagents

The analytical Standards of 5F-AB-PINACA, and 5F-AMB were purchased from Cayman Chemicals (USA). The analytical standard of ADB-PINACA-COOH was purchased from Merck Life Science (UK). Acetonitrile, methanol (LC-MS grade) and Ammonium formate were obtained from Merck (UK). Sigmatrix Urine Diluent was obtained from Merck (UK). Deionized water was prepared from Purite Select Ondeo (Purite Limited, UK).

3.2.2. Instruments

Chromatographic analysis was conducted using Nexera-i LC-2040C UHPLC (Shimadzu Corporation, Japan) connected to a TQ-8050 triple quadrupole electrospray ionization (TQ-ESI) mass spectrometer (Shimadzu Corporation, Japan) operated in ESI positive mode and multiple reaction monitoring (MRM) scan mode. The analysis was performed using Shimadzu Lab Solution software.

3.2.3. Liquid chromatographic and mass spectrometric conditions

Chromatographic separation was carried out using Kinetex C18, (50 x 3 mm, 2.6 µm; Phenomenex Inc., USA). Gradient elution was used where mobile phase A consisted of 5 mM ammonium formate (v/v), and mobile phase B consisted of acetonitrile. The gradient started with 20% B for 1 min, followed by a linear increase up to 90% within 6 min, held for 2.5 min, followed by a return to 20% B at 9.6 min, where it was held for 2.4 min for equilibration. The total run time was 12.0 min. The flow rate was 0.40 mL/min at a column temperature of 45 °C with an injection volume of 2 µL. Each component of the

mobile phase was degassed in an ultrasonic bath for 10 min prior to use. The interface was on a positive ESI mode using nitrogen gas, nebulizing gas flow was 3 L/min; heating gas flow was 10 L/min; and drying gas flow was 10 L/min. The interface temperature was set to 300 °C, the desolvation line temperature was set to 300 °C, and the heat block temperature to 500 °C. The transitions of molecular ions are summarized in **Table 3.2**.

3.2.4. Standard solutions

Stock solutions of ADB-PINACA-COOH, 5F-AB-PINACA, and 5F-AMB were prepared at concentration 1 ppm in methanol. All solutions were stored at −20 °C in amber vials and were allowed to warm to room temperature prior to use.

3.2.5. Construction of calibration curves

Aliquots were taken from the stock solutions and diluted with methanol to prepare solutions in the range 1–100 ppb for the three compounds. Areas under the curve (AUCs) were plotted against the corresponding concentrations and regression equations were computed.

Table 3.2. LC-MS/MS parameters selected for the quantification of the SCs under investigation.

Compound	Rt (min)	+/-	MRM (m/z)	Collision Energy (eV)
ADB-PINACA-COOH	1.8	+	375.1 > 330.2* 375.1 > 217.1 375.1 > 358.2	−17 −32 −11
5F-AB-PINACA	5.0	+	349.5 > 145.4 349.5 > 304.1 349.5 > 233.2*	−25 −16 −24
5F-AMB	6.4	+	364.1 > 233.3* 364.1 > 213.3 364.1 > 69.3	−22 −29 −35

* The ion pair used for quantification.

3.2.6. Validation

Validation of the proposed method was performed with respect to specificity, accuracy, precision, and robustness according to the ICH guidelines [147]. Specificity of the method was assessed by the analysis of different laboratory prepared mixtures of the three compounds. The accuracy was confirmed by applying the proposed method to determination of three concentrations of each compound (10, 40 and 60 ppb) in three triplicates. Repeatability and intermediate precision were assessed using three concentrations (10, 20 and 40 ppb) of standard solutions (n=3). The robustness of the proposed method was evaluated by the reliability of the analysis with respect to small variations in the experimental conditions. These parameters included column temperature (45±5 °C), ammonium formate concentration (5±1 mM) and flow rate (0.40±0.02 mL/min). One concentration (40 ppb) of standard compounds (n=3) was analyzed and only one parameter was changed in the experiments at a time.

3.2.7. Application to urine samples

For this purpose, 49 urine samples were kindly received from the toxicology department in the general department of criminal evidence in Kuwait. These samples were collected from suspicious persons between December 2023 and January 2024. The samples were prepared and analysed in Kuwait.

Bond Elut C18 cartridges (3 mL, 500 mg bed size) were purchased from Agilent Technologies (USA). The cartridge was preconditioned with 1 mL of 100% methanol and 1 mL of water. Then, the sample (2 mL) was loaded into the cartridge and washed with 1 mL water followed by 1 mL of 5% methanol,

then dried for approximately 2 min. Retained drugs were eluted with 2 mL of 100% methanol and evaporated almost to dryness using nitrogen at about 45 °C. The extraction process was done through a vacuum master. After drying, the extract was reconstituted with 1 mL of methanol and transfer to an autosampler vial. The vial was vortexed for 2 min before transferring to the instrument where a volume of 2 uL was injected into the LC-MS/MS.

3.2.8. Ethics approval and consent to participate

Ethical approval for the collection and analysis of the samples has been granted by the Ministry of Justice and Ministry of Interior Ethical Committee. Permission to use samples and data was obtained from the General Department of Criminal Evidence, Ministry of Interior.

3.3. Results and dicussion

3.3.1. Method development and optimization

The detection of SCs is a challenging process as new compounds continuously evolve to bypass the restrictions on illicit drugs. LC-MS/MS is highly recommended because of its high specificity resulting from coupling LC separation with MS/MS detection. In MRM mode, only ions derived from the target analyte are detected; thus, interference from other compounds and endogenous matrix components is minimal [149].

Several columns were tried such as Phenomenex C8, ACE C8, Shim-pack XR-ODS, and Kinetex C18 columns. The optimum performance was obtained using Kinetex C18 column owing to good shape and symmetry of the peaks, and reasonable retention time.

Both isocratic and gradient elutions were tested, but isocratic elution failed to separate the three SCs in a reasonable time, so gradient elution was chosen. Several solvents were tested as mobile phase combinations. Both methanol and acetonitrile are compatible with ESI, they were used in different gradient systems with ammonium formate and formic acid. Several concentrations of formic acid ranging from 0.01% to 0.1 were tested, also 5 and 10 mM ammonium formate were both tried. A gradient of acetonitrile and 5 mM ammonium formate was found to yield superior results compared to methanol and formic acid regarding peak shape and run time.

Optimum performance was obtained using Kinetex C18 (50 x 3 mm, 2.6 µm) with mobile phase A consisting of 5 mM ammonium formate, and acetonitrile as mobile phase B. The gradient started with 20% B for 1 min, followed by a linear increase up to 90% within 6 min, held for 2.5 min, followed by a return to 20% B at 9.6 min, where it was held for 2.4 min for equilibration. Different flow rates were tested and the optimum one regarding run time and resolution was 0.40 mL/min. various column temperatures and injection volumes were investigated and best separation was achieved at 45 °C column temperature with an injection volume of 2 µL. Under the best of these conditions, the three SCs were separated within a 7 min run time as shown in **Figure** 3.2.

The protonated precursor ions $[M+H]^+$ of ADB-PINACA-COOH, 5F-AB-PINACA, and 5F-AMB were detected in the full scan mass spectra at m/z 358.2, 304.1, and 233.3, respectively. Three different collision energies and their corresponding MS/MS transitions were tested for each compound, and the optimum one regarding sensitivity were selected. The optimum MS/MS

transitions and collision energy selected for the determination of the three compounds are summarized in **Table 3.2**.

The optimized method could quantify the three compounds over the range 1-100 ppb. **Figure 3.3-3.5** show the calibration curves for the compounds and their regression equations. From the curves, it appears that the method can be used successfully to determine these compounds in different matrices.

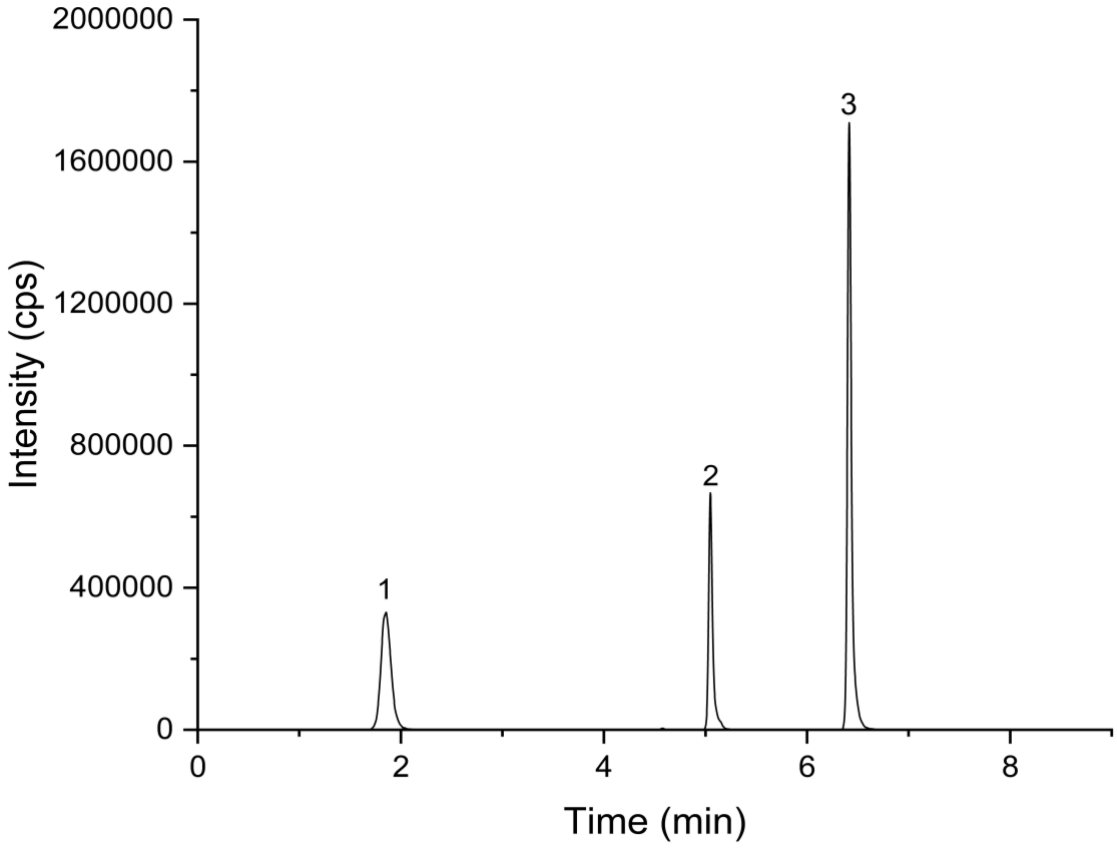


Figure 3.2. Mass chromatogram for 1) ADB-PINACA-COOH (60 ppb), 2) 5F-AB-PINACA (60 ppb), and 3) 5F-AMB (20 ppb).

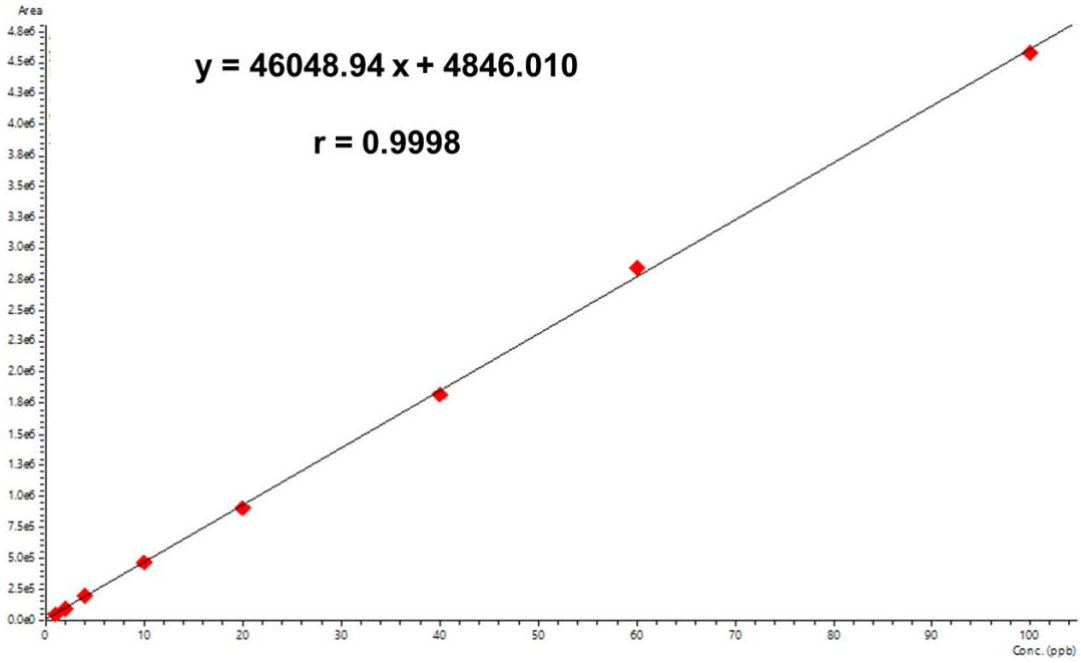


Figure 3.3. Linearity of AUC to the corresponding concentrations of ADB-PINACA-COOH

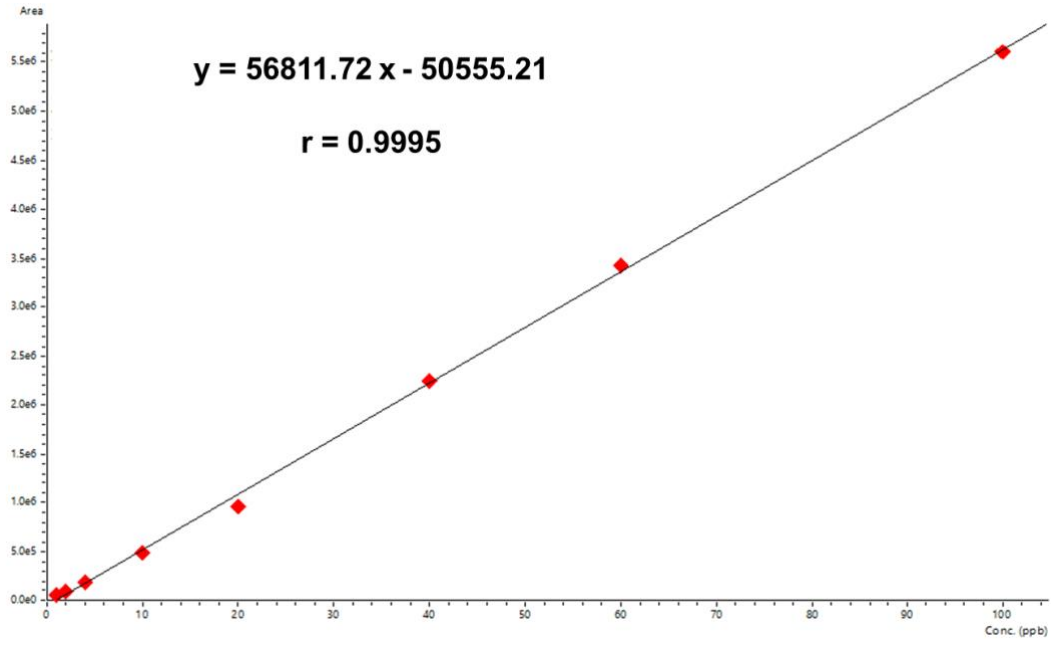


Figure 3.4. Linearity of AUC to the corresponding concentrations of 5F-AB-PINACA

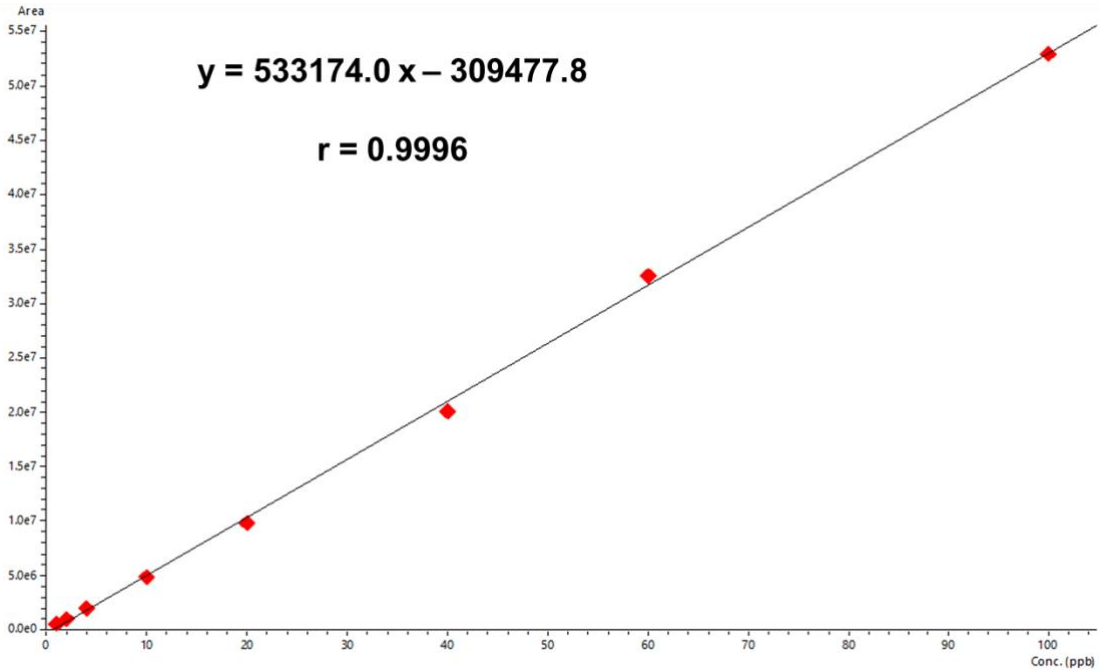


Figure 3.5. Linearity of AUC to the corresponding concentrations of 5F-AMB

3.3.2. Method Validation

The proposed LC-MS/MS method was validated for the three SCs according to ICH guidelines [147]. The method was developed and validated in the toxicology department in the general department of criminal evidence in Kuwait and was cross validated in our lab in the University of Strathclyde. In this thesis, we present the data obtained from validation in our lab. For specificity, satisfactory recoveries were obtained for quantitation of the drugs in their different ratios in laboratory prepared mixtures as shown in **Table 3.3**. For accuracy, reasonable mean percentage recoveries were obtained for quantitation of the drugs in the three concentrations 10, 40 and 60 ppb as shown in **Table 3.4**. For repeatability and intermediate precision, satisfactory RSD% was obtained from analysis of the three drugs in three concentration levels (10, 20 and 40 ppb) three times in the same day and on three different days as shown in **Table 3.5**, indicating reasonable precision. For robustness, small changes were imparted in column temperature, ammonium formate concentration, and flow rate. The method showed reasonable RSD% of the responses, indicating that the method was robust enough regarding these factors (**Table 3.6**).

Table 3.3. Determination of the three SCs in laboratory prepared mixtures by the proposed method

	ADB-PINACA-COOH	5F-AB-PINACA	5F-AMB
Concentration (ppb)	10	10	10
Recovery %[a]	100.9	96.3	97.8
Concentration (ppb)	60	60	60
Recovery %[a]	103.1	101.5	102.8
Concentration (ppb)	100	100	100
Recovery %[a]	98.6	101.5	99.6
Concentration (ppb)	40	60	40
Recovery %[a]	102.8	101.8	98.0
Concentration (ppb)	60	40	60
Recovery %[a]	103.3	103.0	99.1
Mean ± SD	100.9±2.6	101.3±2.6	98.7±2.6

[a] Average of three determinations.

Table 3.4. Accuracy results of the proposed LC-MS/MS method for analysis of the three SCs

Compound	Recovery% [a]			Accuracy
	10 ppb	40 ppb	60 ppb	Mean ± SD
ADB-PINACA-COOH	99.5	101.3	103.1	101.3±1.8
5F-AB-PINACA	98.3	88.1	101.5	96.0±7.0
5F-AMB	96.8	95.4	102.8	98.3±3.9

[a] Average of three determinations

Table 3.5. Precision results of the proposed LC-MS/MS method for analysis of the three SCs

Compound	Intra-day Precision [a]			Inter-day Precision [b]		
	RSD%					
	10 ppb	20 ppb	40 ppb	10 ppb	20 ppb	40 ppb
ADB-PINACA-COOH	1.9	1.2	1.5	2.0	1.6	1.2
5F-AB-PINACA	2.1	2.1	2.0	2.3	2.0	1.4
5F-AMB	2.2	1.8	0.5	2.1	1.5	1.9

[a] Intra-day precision (n = 3), RSD of three concentrations repeated three times within the day.
[b] Inter-day precision (n = 3), RSD of three concentrations repeated three times in three different days.

Table 3.6. Robustness results of the proposed LC-MS/MS method for analysis of the three SCs

Compound	Oven temperature 45±5 °C	Ammonium formate concentration 5±1 mM	Flow rate 0.4±0.02 mL/min
	RSD%[a]		
ADB-PINACA-COOH	1.9	1.2	0.8
5F-AB-PINACA	0.9	1.1	1.0
5F-AMB	1.1	1.1	1.1

[a] Average of three determinations

3.3.3. Application to urine samples from Kuwait

The aim of our work was to analyze the market of SCs abuse in Kuwait in the year 2023-2024. For that purpose, urine samples were received from the toxicology department in the general department of criminal evidence in Kuwait. The samples were seized and collected from suspicious persons between December 2023 and January 2024.

Several extraction procedures were tested by spiking the three SCs in Sigmatrix urine diluent and applying the extraction procedure. Liquid-liquid extraction with various solvents was tried, the recovery of the compounds from blank urine was not satisfactory. Therefore, solid phase extraction (SPE) was introduced with several cartridges tested. C8 cartridge did not recover the drugs adequately, so C18 was used. This may be attributed to the high lipophilicity of SCs, which will be more retained and extracted from the matrix on a less polar stationary phase as C18. The washing and elution solvents were also optimized where 5% and 100% methanol were optimum for washing and elution, respectively. The SPE recovery study showed very acceptable R% as shown in **Table *3.7***.

Table 3.7. Recovery study of the SCs under investigation from blank urine.

Compound	R% ± SD [a]
ADB-PINACA-COOH	99.3±1.7
5F-AB-PINACA	99.1±1.6
5F-AMB	98.5±1.3

[a] Average of six determinations.

The developed SPE and LC-MS/MS method was applied to screen 49 urine samples for the presence of SCs. When samples showed high concentrations of the studied drugs that exceeded our linear range, they were suitably diluted before injection. The results are shown in **Table 3.8**. The prevalence of the three SCs in the samples was investigated as shown in **Figure 3.6** and **Figure 3.7**.

From **Figure 3.6**, 5F-AMB was the most detected SCs in the samples with 42 of the analyzed 49 samples containing the SC representing 85.7% of the samples (**Table 3.9**). ADB-PINACA-COOH and 5F-AB-PINACA were detected in only 14.3 and 2.0 % of the samples, respectively (**Table 3.9**). 5F-AMB also showed higher concentrations in the samples up to 425.0 ng/mL as shown in **Figure 3.7**. The dominance of 5F-AMB with its higher concentrations is reflected in its inclusion in Schedule 2 drugs act in Kuwait in 2021. 5F-AMB is reported for its high toxicity and can be a cause of death [151, 152].

While ADB-PINACA-COOH is a known metabolite for the SC ADB-PINACA, it's also considered one of the metabolites of the SC 5F-ADB-PINACA [153]. Therefore, detection of ADB-PINACA-COOH in urine may indicate ingestion of either ADB-PINACA or 5F-ADB-PINACA.

Table 3.8. Analysis of urine samples using the proposed LC-MS/MS method.

No.	ADB-PINACA-COOH	5F-AB-PINACA	5F-AMB
	Concentration (ng/mL)[a]		
1	--	--	107.9
2	--	--	90.6
3	--	--	54.8
4	81.4	--	--
5	172.4	--	--
6	150.5	--	--
7	--	--	147.1
8	43.2	--	48
9	--	--	45.1
10	--	--	24.6
11	--	--	57.5
12	19.1	--	--
13	--	--	223.1
14	--	--	155.2
15	--	--	345.8
16	--	--	71.6
17	--	--	155.2
18	--	--	292.3
19	--	--	240.6
20	--	--	141.3
21	--	--	1.4
22	--	--	390.7
23	--	--	125.6
24	--	--	116.5
25	--	--	63.8
26	--	--	153.7

Table 3.8. Analysis of urine samples using the proposed LC-MS/MS method, continued.

No.	ADB-PINACA-COOH	5F-AB-PINACA	5F-AMB
	Concentration (ng/mL)[a]		
27	--	--	19.9
28	--	--	77
29	--	--	403.8
30	--	--	377.5
31	--	--	193.6
32	--	--	425
33	--	--	285.7
34	--	--	246.2
35	--	--	140.7
36	--	12.6	--
37	89	--	--
38	67	--	--
39	--	--	393.7
40	--	--	129.7
41	--	--	112.2
42	--	--	63.1
43	--	--	152.2
44	--	--	14.8
45	--	--	75.7
46	--	--	393
47	--	--	327.7
48	--	--	183.2
49	--	--	421.1

[a] Average of three determinations

Table 3.9. Prevalence and concentrations measured for SCs under investigation in 49 urine samples

	ADB-PINACA-COOH	5F-AB-PINACA	5F-AMB
Positive samples (%)	7 (14.3%)	1 (2.0%)	42 (85.7%)
Range (ng/mL)	19.1-172.4	12.6	1.4-425.0
Mean ± SD (ng/mL)	88.9 ± 55.2	NA	178.3 ± 130.2
Median (ng/mL)	81.4	12.6	144.2

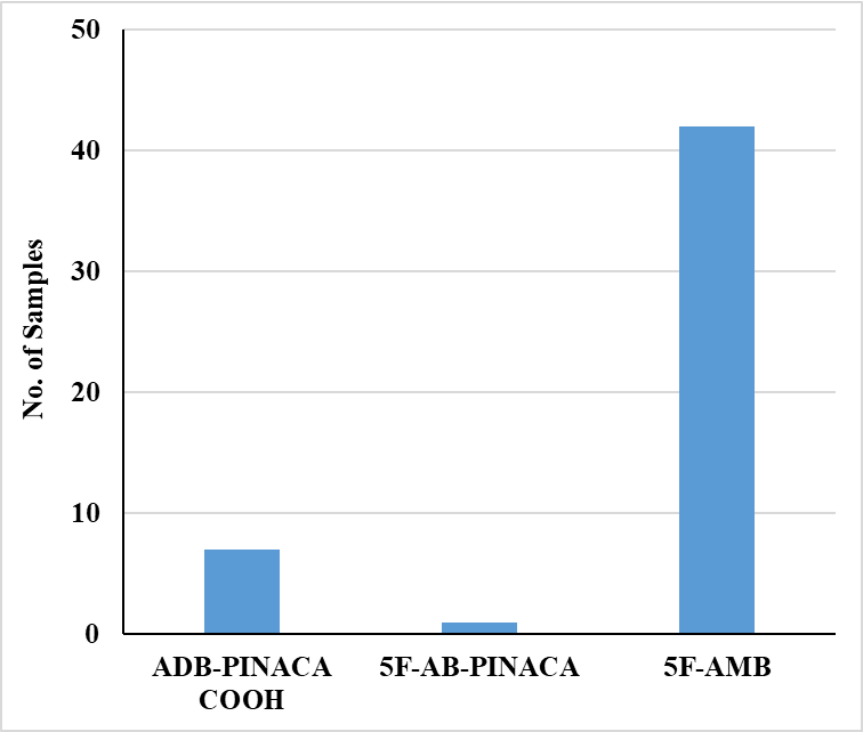


Figure 3.6. Results of analysis of the SCs under investigation in Kuwaiti urine samples showing the prevalence of each SC.

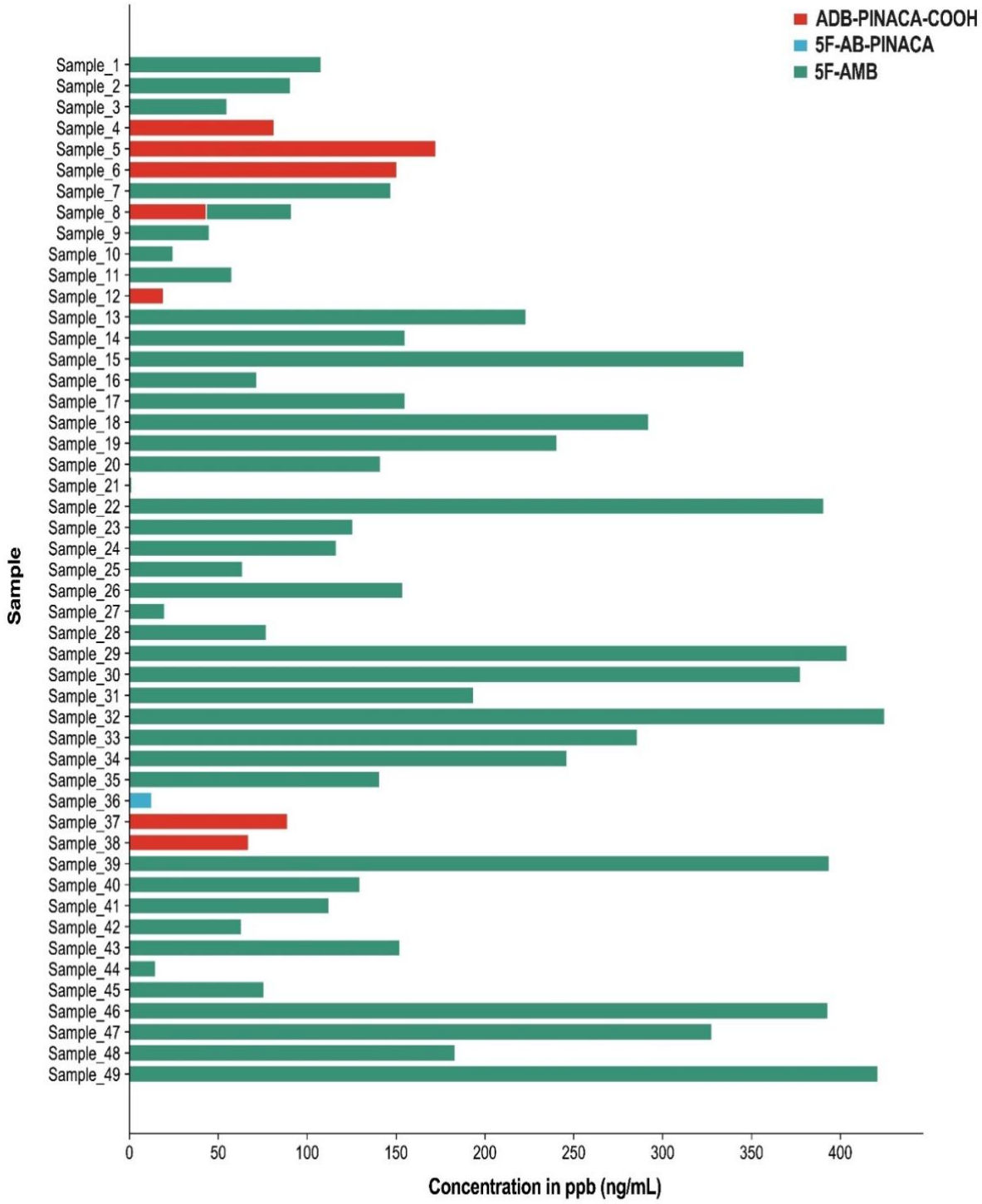


Figure 3.7. Results of analysis of the SCs under investigation in Kuwaiti urine samples showing concentrations of the SCs in urine samples.

3.4. Conclusion

A sensitive and specific LC-MS/MS method was developed for the detection of three SCs common in Kuwaiti market namely ADB-PINACA-COOH, 5F-AB-PINACA, and 5F-AMB. The method was cross validated between the lab in University of Strathclyde and the toxicology department in the general department of criminal evidence in Kuwait according to ICH guidelines and was found to be accurate, precise, sensitive, and robust. A SPE procedure was developed for extraction of the three SCs from urine, and satisfactory recoveries were obtained. The method was used for screening and quantification of the SCs in 49 urine samples collected from suspicious people in Kuwait in 2023-2024 to draw a picture of the market of SCs. By analyzing the results, it was found that 5F-AMB was the most common SC detected in the collected samples, and also showed the highest concentrations in urine.

Chapter 4 Development and validation of LC-MS/MS method for analysis of six drugs of abuse in urine: Screening of abused drugs in Kuwait during 2022

4.1. Introduction

Individuals with substance use disorders often engage in polydrug abuse, where they combine multiple substances to enhance or alter their effects. In cases of polydrug abuse, it is common to encounter mixtures of pregabalin (PGB), morphine (MOR), amphetamine (AMP), methamphetamine (MAMP), codeine (COD), and diazepam (DZP), which can lead to increased risks of addiction, overdose, and other negative outcomes. When different substances are combined, there can be synergistic effects, meaning that the combined effect is greater than the sum of the individual effects. For instance, if PGB is mixed with opioids like MOR or COD, it can intensify sedation, respiratory depression, and CNS depression, which in turn raises the risk of overdose and death [154]. The structures of the six compounds are illustrated in **Figure 4.1**, and their chemical names and molecular weights are summarized in **Table 4.1**.

The aim of work in this chapter was to identify the polydrug abuse patterns in Kuwait. Six compounds known for their abuse and addiction tendency were investigated, and a LC-MS/MS method was developed to identify and quantify these drugs. The used drugs are PGB, MOR, AMP, MAMP, COD, and DZP. This work was done in cooperation with the toxicology department in the general department of criminal evidence in Kuwait to develop and validate the method. The developed method was used to analyse urine samples collected from suspicious persons in the Kuwaiti streets recently, to give a clear picture on polydrug abuse in Kuwait.

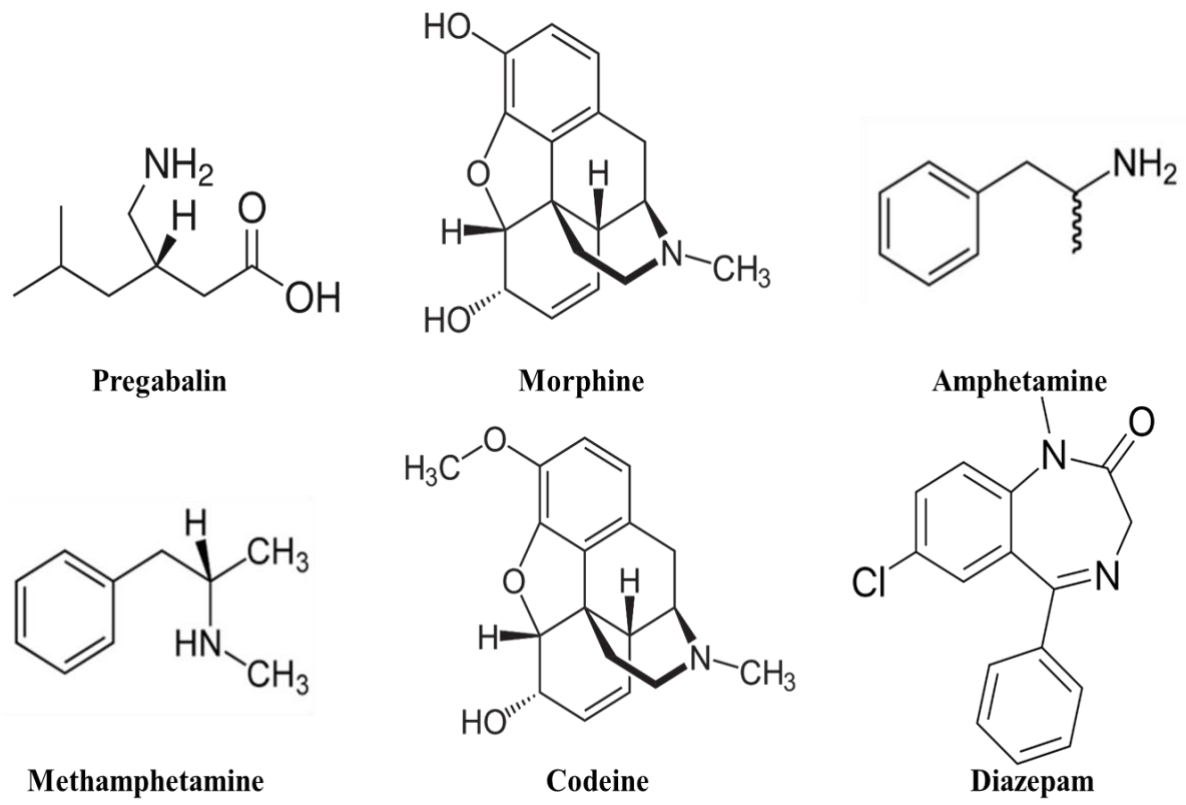


Figure 4.1. Chemical structures of the six drugs of abuse under investigation (PGB, MOR, AMP, MAMP, COD, and DZP).

Table 4.1. Chemical names and molecular weights of the drugs of abuse under investigation.

Compound	Chemical name	Molecular formula	Molecular weight
Pregabalin	(*S*)-3-(Aminomethyl)-5-methylhexanoic acid	$C_8H_{17}NO_2$	159.23
Morphine	7,8-Didehydro-4,5α-epoxy-17-methylmorphinan-3,6α-diol	$C_{17}H_{19}NO_3$	285.34
Amphetamine	(±)-α-Methylphenethylamine	$C_9H_{13}N$	135.21
Methamphetamine HCl	(+)-(*S*)-*N*,α-Dimethylphenethylamine hydrochloride	$C_{10}H_{15}N$	185.69
Codeine	7,8-Didehydro-4,5α-epoxy-3-methoxy-17-methylmorphinan-6α-ol	$C_{18}H_{21}NO_3$	299.36
Diazepam	7-Chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one	$C_{16}H_{13}ClN_2O$	284.74

4.2. Experimental

4.2.1. Materials and reagents

The drug Standards were all purchased from LGC standards (UK), with PGB and AMP as 1.0 mg/mL solution in methanol, while MOR, MAMP, COD, and DZP as 0.1 mg/mL solution in methanol. Acetonitrile and methanol (LC-MS grade) and ammonium formate were obtained from Merck (UK). Sigmatrix Urine Diluent was obtained from Merck (UK). Deionized water was prepared from Purite Select Ondeo (Purite Limited, UK). Bond Elut C18 cartridges (3 mL, 500 mg bed size) were purchased from Agilent Technologies (USA).

4.2.2. Instruments

Chromatographic analysis was conducted using Nexera-i LC-2040C UHPLC (Shimadzu Corporation, Japan) connected to a TQ-8050 triple quadrupole electrospray ionization (TQ-ESI) mass spectrometer (Shimadzu Corporation, Japan) operated in ESI positive mode and MRM scan mode. The analysis was performed using Shimadzu Lab Solution software.

4.2.3. Liquid chromatographic and mass spectrometric conditions

Chromatographic separation was carried out using Kinetex C18, (50 x 3 mm, 2.6 µm; Phenomenex Inc., USA). Gradient elution was used where mobile phase A consisted of 5 mM ammonium formate (v/v), and mobile phase B consisted of acetonitrile.

The gradient started with 10% B for 4.5 mins, followed by a linear increase up to 90% within 1.5 min, held for 2.8 min, followed by a return to 10% B at 9 min, where it was held for 5 min for equilibration. The total run time was 14.0 min.

Different flow rates were tested starting from 3 mL/min to 0.6 mL/min and the best separation and peak shape were obtained on 0.47 mL/min. The separation was performed at 50 °C column temperature with an injection volume of 5 µL. Each component of the mobile phase was degassed in an ultrasonic bath for 10 min prior to use. The interface was on a positive ESI mode using nitrogen gas, nebulizing gas flow was 2.5 L/min, heating gas flow was 5 L/min and drying gas flow was 10 L/min. Interface temperature was set at 300 ºC, DL temperature was 280 ºC and heat block temperature was 500 ºC. The transitions of molecular ions are summarized in **Table 4.2**.

4.2.4. Standard solutions

Stock solutions of PGB, MOR, COD, and DZP were prepared at a concentration of 1 ppm, whereas AMP was prepared at a concentration of 1 mg/mL and MAMP at a concentration of 0.1 mg/mL. All solutions were prepared using LC-MS grade methanol and stored at a temperature of -20 °C in amber vials. Prior to usage, the solutions were thawed to a temperature of 25 °C.

4.2.5. Construction of calibration curves

Aliquots were taken from the stock solutions and diluted with methanol to prepare solutions in the range 0.5–100 ppb for the six compounds. AUCs were plotted against the corresponding concentrations and regression equations were computed.

Table 4.2. LC-MS/MS parameters selected for the quantification of the compounds under investigation.

Compound	Rt (min)	+/-	MRM (m/z)	Collision Energy (eV)
Pregabalin	1.1	+	160.3 > 54.9*	−23
			160.3 > 142.1	−14
			160.3 > 82.9	−16
Morphine	2.3	+	286.2 > 200.9	−23
			286.2 > 165.0*	−38
			286.2 > 152.0	−50
Amphetamine	3.6	+	136.2 > 91.0*	−21
			136.2 > 65.1	−37
			136.2 > 119.1	−13
Methamphetamine	4.7	+	150.3 > 90.9*	−21
			150.3 > 64.9	−40
			150.3 > 119.1	−13
Codeine	6.3	+	300.2 > 165.1*	−40
			300.2 > 215.0	−25
			300.2 > 199.0	−28
Diazepam	7.1	+	285.2 > 193.0*	−31
			285.2 > 154.0	−25
			285.2 > 221.9	−27

* The ion pair used for quantification.

4.2.6. Validation

The proposed method was validated following the guidelines set by the ICH [147]. The specificity of the method was evaluated by analyzing different laboratory-prepared mixtures containing the six compounds. To confirm accuracy, the proposed method was applied to determine three concentrations (10, 20, and 40 ppb) of each compound in triplicate. Repeatability and intermediate precision were assessed by analyzing three concentrations (10, 20, and 40 ppb) of standard solutions (n=3). The robustness of the method was evaluated by analyzing three concentrations (10, 20, and 40 ppb) of standard compounds (n=3) while introducing small variations in experimental conditions. The parameters tested included column temperature (50±5 °C), ammonium formate concentration (5±1 mM), and flow rate (0.47±0.1 mL/min). In each experiment, only one parameter was altered while the others remained constant.

4.2.7. Application to urine samples

For this study, a total of 150 urine samples were obtained from the toxicology department within the general department of criminal evidence in Kuwait. These samples were collected from individuals deemed suspicious between the months of March and May 2022. The subsequent sample preparation and analysis were conducted within Kuwait.

To carry out the extraction process, SPE was applied using Bond Elut C18 cartridges (3 mL, 500 mg bed size). Prior to use, each cartridge was pre-conditioned with 1 mL of 100% methanol followed by 1 mL of water. Subsequently, a sample size of 2 mL was loaded into the cartridge and

subjected to a washing step involving 1 mL of water followed by 1 mL of 5% methanol. The cartridge was then allowed to dry for approximately 2 minutes. The retained drugs were subsequently eluted using 2 mL of 100% methanol and subsequently evaporated almost to dryness using nitrogen at a temperature of approximately 45 °C. The extraction process was performed utilizing a vacuum master system. Once dried, the resultant extract was reconstituted with 1 mL of methanol and transferred to an autosampler vial. The vial was vortexed for 2 minutes before being transferred to the instrument, where a volume of 5 uL was subsequently injected into the LC-MS/MS system.

4.2.8. Ethics approval and consent to participate

Ethical approval for the collection and analysis of the samples has been granted by the Ministry of Justice and Ministry of Interior Ethical Committee. Permission to use samples and data was obtained from the General Department of Criminal Evidence, Ministry of Interior.

4.3. Results and dicussion

4.3.1. Method development and optimization

The detection of the six compounds PGB, MOR, AMP, MAMP, COD, and DZP in urine samples presents a considerable challenge due to their significant variation in physicochemical properties. For this reason, it is highly recommended to employ LC-MS/MS, which offers a high level of specificity resulting from the coupling of LC with MS/MS detection. The MRM mode enables the detection of only ions derived from the target analyte, thereby minimizing interference from other compounds and endogenous matrix components [149].

To achieve optimum performance, Kinetex C18 (50 x 3 mm, 2.6 µm) was utilized. Various mobile phases were employed under both isocratic and gradient conditions. The most favorable elution, characterized by high resolution, desirable peak shapes, and reasonable run time, was attained through a gradient with mobile phase A composed of 5 mM ammonium formate, while acetonitrile was used as mobile phase B. The gradient commenced with 10% B for 4.5 minutes, followed by a linear increase up to 90% within 1.5 minutes. This composition was maintained for 2.8 minutes before returning to 10% B at the 9-minute mark, where it was held for 5 minutes to ensure adequate equilibration. Different flow rates were tested, and the optimal flow rate of 0.47 mL/min was determined based on considerations of run time and resolution. Furthermore, various column temperatures and injection volumes were investigated, with the most effective separation achieved at a column temperature of 50°C and an injection volume of 5 µL. Under these optimal conditions, all six compounds were successfully separated, with a total run time of 14.0 min, as depicted in **Figure 4.2.**

The protonated precursor ions $[M+H]^+$ of PGB, MOR, AMP, MAMP, COD, and DZP were detected in the full scan mass spectra at m/z 160.25, 286.20, 136.20, 150.25, 300.20, and 285.20, respectively. Three different collision energies and their corresponding MS/MS transitions were tested for each compound, and the optimum one regarding sensitivity were selected. The optimum MS/MS transitions and collision energy selected for the determination of the six compounds are summarized in **Table 4.2**.

The optimized method could quantify the six compounds over the range 0.5-100 ppb. **Figure 4.3-4.8.** show the calibration curves for the compounds and their regression equations. Form the curves, it appears that the method can be used successfully to determine these compounds in different matrices.

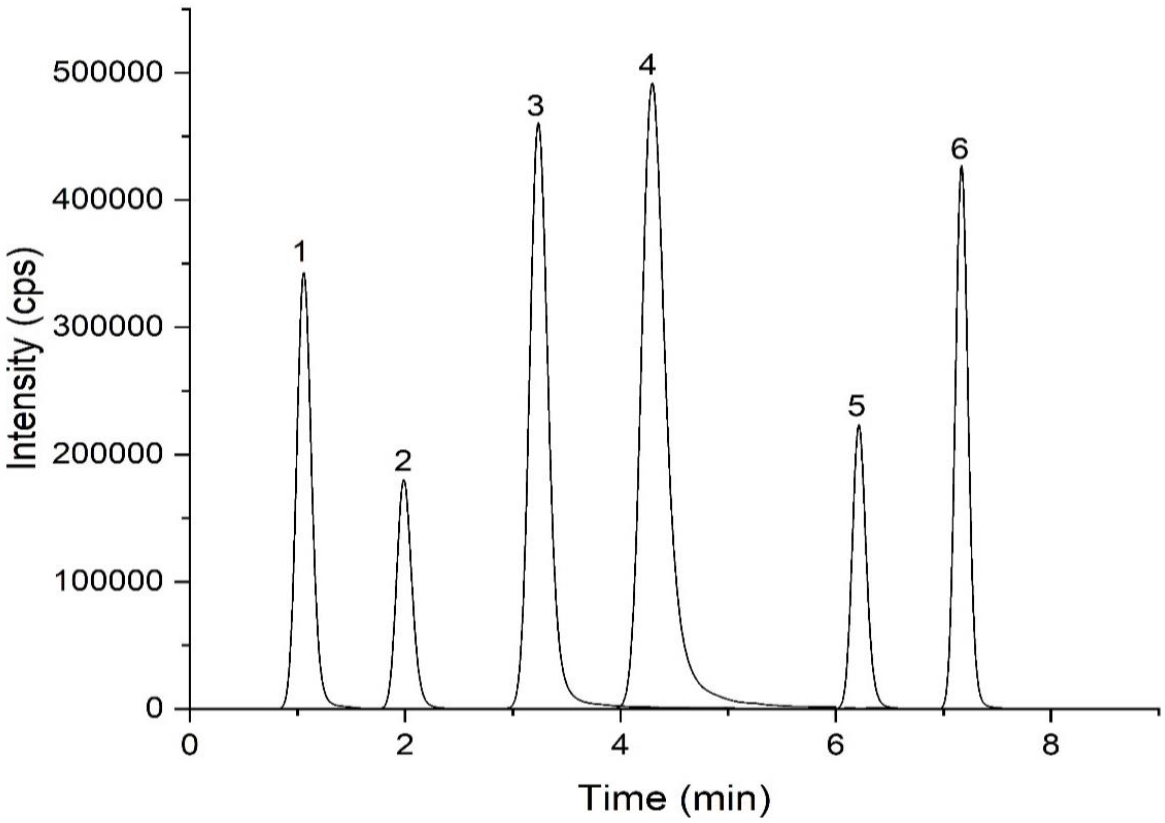


Figure 4.2. Mass chromatogram for PGB (1), MOR (2), AMP (3), MAMP (4), COD (5), and DZP (6) at 60 ppb.

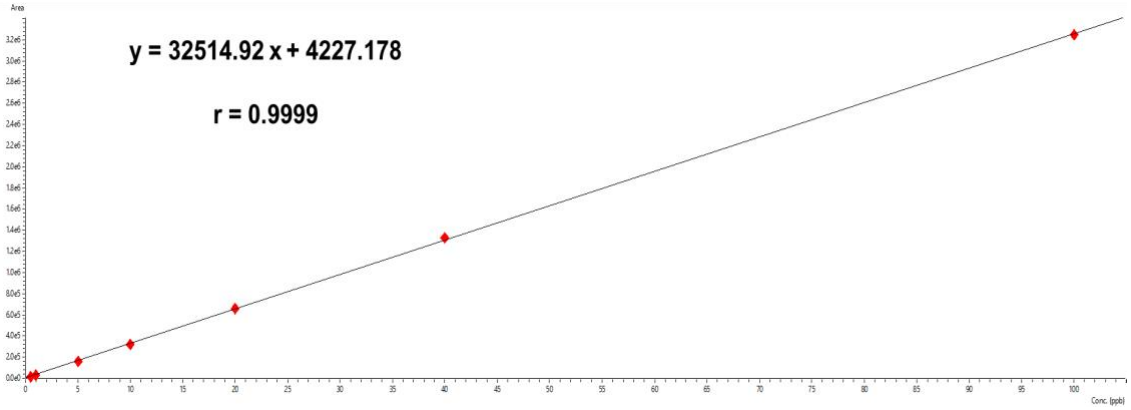


Figure 4.3. Linearity of AUC to the corresponding concentrations of Pregabalin

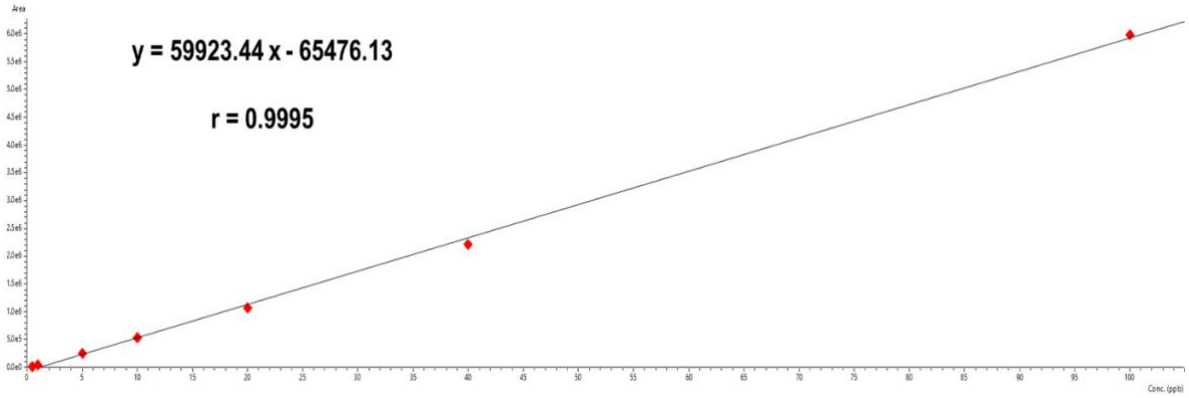


Figure 4.4. Linearity of AUC to the corresponding concentrations of Morphine

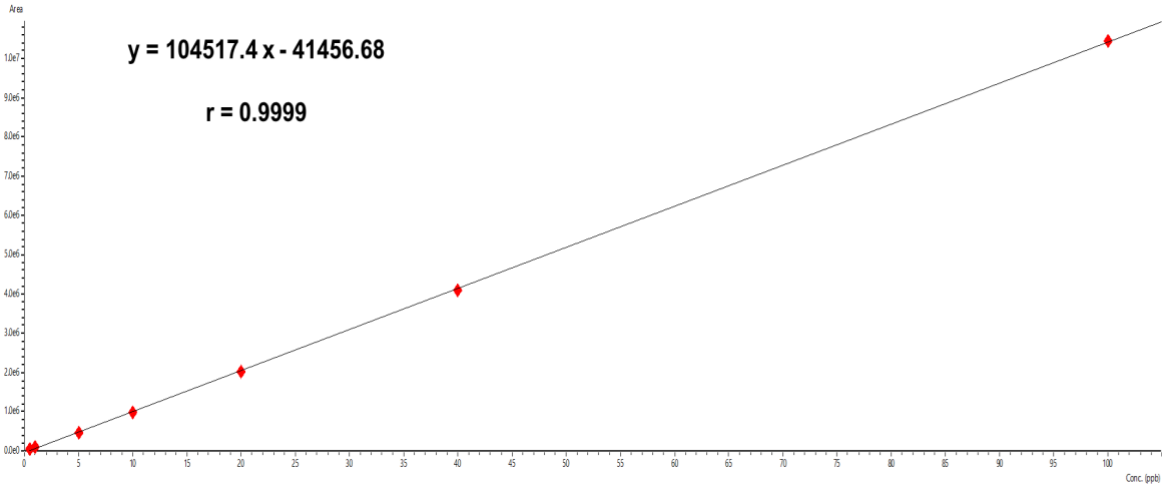


Figure 4.5. Linearity of AUC to the corresponding concentrations of Amphetamine

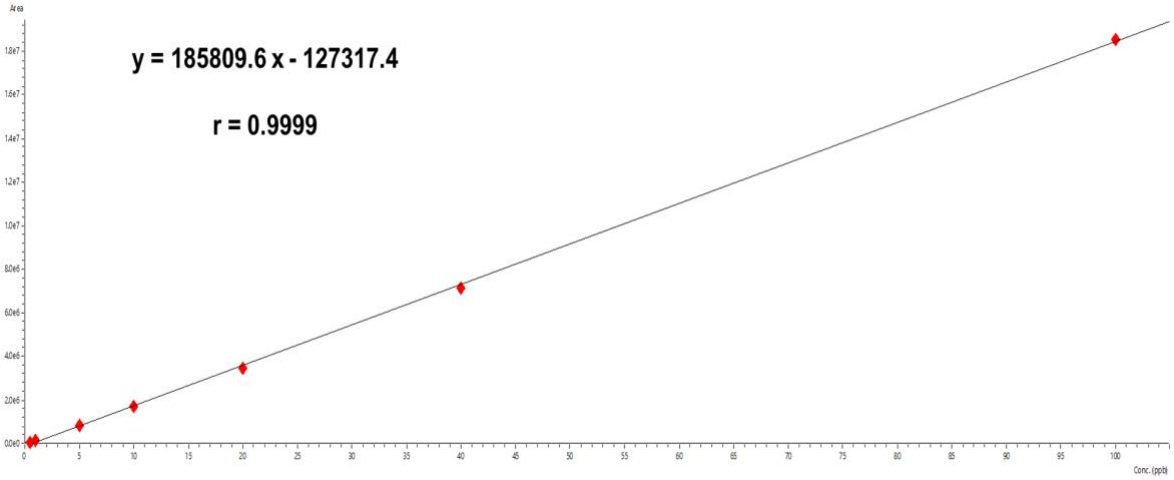


Figure 4.6. Linearity of AUC to the corresponding concentrations of Methamphetamine

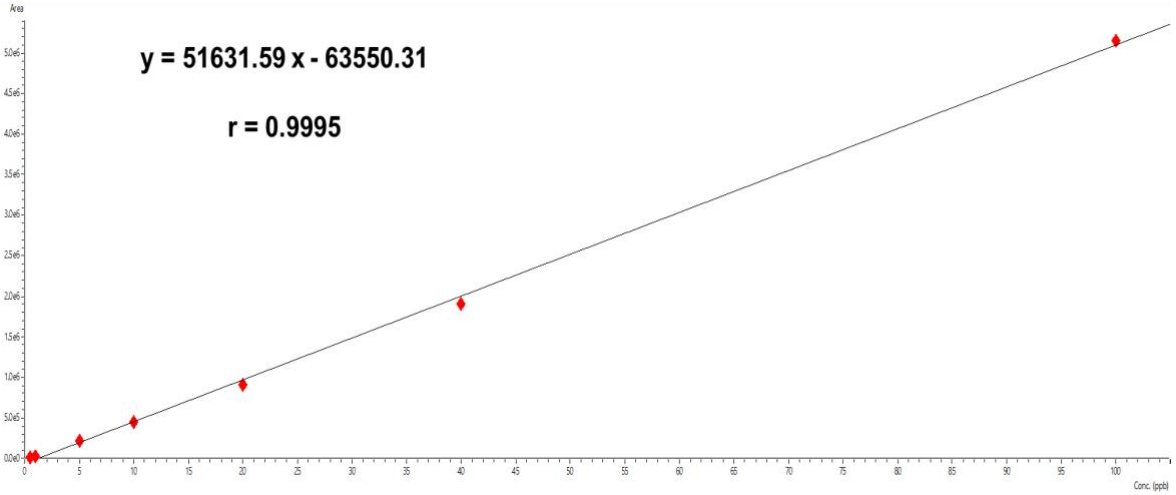


Figure 4.7. Linearity of AUC to the corresponding concentrations of Codeine

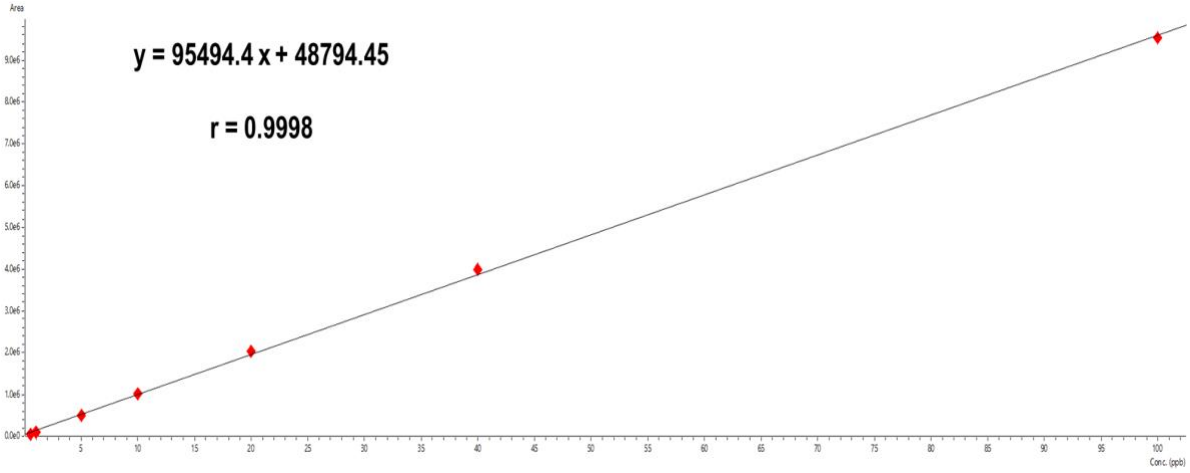


Figure 4.8. Linearity of AUC to the corresponding concentrations of Diazepam

4.3.2. Method Validation

The proposed LC-MS/MS method was validated for the six compounds in accordance with the guidelines set forth by ICH *[147]*. The method was developed and validated by the toxicology department in the general department of criminal evidence in Kuwait, and it was further cross-validated in our laboratory at the University of Strathclyde. In terms of specificity, satisfactory recoveries were achieved for the quantitation of the drugs in various ratios within laboratory-prepared mixtures, as presented in **Table 4.3**. Accuracy was assessed by obtaining reasonable mean percentage recoveries for the quantitation of the drugs at three concentrations (10, 20, and 40 ppb), as shown in **Table 4.4**.Repeatability and intermediate precision were evaluated by analyzing the six drugs at three concentration levels (10, 20, and 40 ppb) on three separate occasions within the same day and on three different days, as indicated in **Table 4.5**.The obtained RSD% values indicated a satisfactory level of precision. To assess robustness, minor alterations were made to the column temperature, ammonium formate concentration, and flow rate. The method demonstrated reasonable RSD% values for the responses, suggesting that it exhibited sufficient robustness with respect to these factors (**Table 4.6**).

Table 4.3. Determination of the six compounds in laboratory prepared mixtures by the proposed method

	PGB	MOR	AMP	MAMP	COD	DZP
Concentration (ppb)	10	10	10	10	10	10
Recovery %[a]	100.5	98.3	101.5	97.3	106.4	98.3
Concentration (ppb)	20	20	20	20	20	20
Recovery %[a]	104.3	100.6	96.5	101.1	98.9	100.3
Concentration (ppb)	40	40	40	40	40	40
Recovery %[a]	101.2	102.0	100.5	99.1	99.1	102.4
Concentration (ppb)	100	100	100	100	100	100
Recovery %[a]	91.0	90.4	98.2	98.3	93.6	94.0
Concentration (ppb)	40	20	40	20	40	20
Recovery %[a]	98.1	100.7	93.7	91.6	95.8	98.0
Concentration (ppb)	20	40	20	40	20	40
Recovery %[a]	97.3	97.9	93.4	90.7	88.2	94.2
Mean ± SD	98.7±4.5	98.3±4.2	97.3±3.4	96.4±4.2	97.0±6.1	97.9±3.3

[a] Average of three determinations.

Table 4.4. Accuracy results of the proposed LC-MS/MS method for analysis of the six compounds

Compound	Recovery% [a]			Accuracy
	10 ppb	20 ppb	40 ppb	Mean ± SD
PGB	96.9	101.1	107.3	101.8±5.2
MOR	102.1	101.0	105.7	102.9±2.5
AMP	103.5	97.2	102.0	100.9±3.3
MAMP	97.6	99.9	102.3	99.9±2.4
COD	105.6	103.8	104.6	104.7±0.9
DZP	99.8	101.8	104.2	101.9±2.2

[a] Average of three determinations

Table 4.5. Precision results of the proposed LC-MS/MS method for analysis of the ten SCs

Compound	Intra-day Precision [a]			Inter-day Precision [b]		
	RSD%					
	10 ppb	20 ppb	40 ppb	10 ppb	20 ppb	40 ppb
PGB	0.9	0.9	0.3	2.0	1.8	2.1
MOR	0.6	0.5	0.4	2.3	1.7	1.5
AMP	0.9	0.9	0.1	2.5	1.3	1.5
MAMP	0.7	0.3	0.3	1.1	1.4	1.1
COD	0.8	0.3	0.4	1.6	1.5	1.6
DZP	0.9	0.7	0.6	1.3	1.4	1.2

[a] Intra-day precision (n = 3), RSD of three concentrations repeated three times within the day.
[b] Inter-day precision (n = 3), RSD of three concentrations repeated three times in three different days.

Table 4.6. Robustness results of the proposed LC-MS/MS method for analysis of the ten SCs

Compound	Oven temperature 50±5 °C			Ammonium formate concentration 5±1 mM			Flow rate 0.47±0.1 mL/min		
	RSD%[a]								
	10 ppb	20 ppb	40 ppb	10 ppb	20 ppb	40 ppb	10 ppb	20 ppb	40 ppb
PGB	1.8	1.7	1.9	1.8	1.9	0.6	1.8	1.7	1.5
MOR	1.8	1.9	0.9	1.8	1.3	1.6	0.8	0.9	1.0
AMP	1.4	1.2	1.8	0.6	1.1	2.0	0.9	1.4	1.2
MAMP	1.6	0.8	1.9	1.7	2	2.4	2.0	1.8	1.9
COD	1.2	1.4	1.6	1.3	1.1	2.0	2.1	0.9	0.9
DZP	1.6	1.1	1.9	0.8	1.4	1.0	0.8	1.0	0.8

[a] Average of three determinations

4.3.3. Application to urine samples from Kuwait

The aim of our work was to analyze the market of drugs of abuse in Kuwait in the year 2022. For that purpose, urine samples were received from the toxicology department in the general department of criminal evidence in Kuwait. A total of 150 urine samples were collected from subjects under investigation for suspected drug abuse violations during 2022.

Different methods of extraction were tested by spiking the six drugs in blank urine and applying the extraction procedure. Liquid-liquid extraction with various solvents was tried, the recovery of the compounds from blank urine was not satisfactory. Therefore, SPE was introduced with several cartridges tested. C8 cartridge did not recover the drugs adequately, so C18 cartridge was used. This may be due to the high lipophilicity of the studied drugs, which allowed them more retention on C18 than C8 and hence better separation from the matrix components. The washing and elution solvents were also optimized where 5% and 100% methanol were optimum for washing and elution, respectively. The SPE recovery study showed very acceptable R% as shown in **Table 4.7**.

The developed SPE and LC-MS/MS method was applied to screen 150 urine samples for the presence of the six drugs. When samples showed high concentrations of the studied drugs that exceeded our linear range, they were suitably diluted before injection. The results are shown in **Table 4.8**. From the analysis of the samples, only one sample (No. 34) gave no indication of any of the drugs under study, while all the other 149 samples gave a positive

indication of one or more of the drugs. The individual and in combination prevalence of each drug in the samples was also analyzed (**Table 4.9**).

Table 4.7. Recovery study of the compounds under investigation from blank urine.

Compound	R%±SD [a]
PGB	98.8±2.0
MOR	98.7±2.1
AMP	100.7±0.9
MAMP	99.9±1.2
COD	98.4±2.2
DZP	97.8±0.6

[a] Average of three determinations

Table 4.8. Analysis of urine samples using the proposed LC-MS/MS method.

Sample	PGB	MOR	AMP	MAMP	COD	DZP
	Concentration (ng/mL)[a]					
1	217.4	--	1257	1693.2	--	--
2	--	--	309.8	864.8	--	--
3	--	20913	--	--	3105	--
4	6408.2	--	7063.8	--	--	--
5	9453.2	--	6981.4	17546.6	--	--
6	--	1600	--	--	--	--
7	160162	--	11552.4	11804	--	--
8	--	--	1475.4	13564.2	--	--
9	--	--	1631.6	14978	--	--
10	19364.6	--	--	--	--	--
11	--	--	1116	7807	--	--
12	391062	--	8270	--	--	--
13	27129	--	1912.2	9307.6	--	--
14	396050	--	13386	114654	--	--
15	--	--	13254	87216	--	--
16	29860	8064	13720	66370	--	--
17	76847.6	1601.4	980.6	3459	--	--
18	--	--	4753.6	35790	--	--
19	--	--	898.8	22558.4	--	--
20	250444	--	--	--	--	--
21	1545.2	--	--	--	--	--
22	--	--	1657.2	3707.4	--	--
23	16050.2	--	--	--	--	--
24	828.6	--	--	--	--	--
25	--	--	7342	54008	--	--
26	243010	--	7060	--	--	--

Table 4.8. Analysis of urine samples using the proposed LC-MS/MS method, continued.

Sample	PGB	MOR	AMP	MAMP	COD	DZP
	Concentration (ng/mL)[a]					
27	16030.4	--	12597.8	25752.4	--	--
28	59266.8	--	--	--	--	--
29	--	--	18243	22801.4	--	--
30	353.2	--	--	--	--	--
31	--	--	13432	60114	--	--
32	782.6	--	3413.8	29222	--	--
33	--	--	851.1	3254.8	--	--
34	--	--	--	--	--	--
35	304438	--	--	--	--	--
36	52350.4	--	1589.5	4960.2	--	--
37	809.7	--	1407.1	--	--	--
38	1241.1	3736.2	467.2	997.2	381.3	--
39	437	6382.2	949.6	6127.4	100.9	--
40	28518.6	--	--	22186.4	--	--
41	16010.4	680.6	220.8	1175.5	112	--
42	299354	--	--	--	--	--
43	653.9	--	--	--	--	--
44	--	--	--	2713.7	--	--
45	327.9	--	2799.6	46112	--	--
46	164.5	--	--	--	--	--
47	290.8	--	3408	34416.8	--	--
48	--	--	2244.1	14725.8	--	--
49	15626	--	8306	64808	--	--
50	343.7	--	11282.6	55747.6	--	--
51	724	--	--	613.3	--	--
52	34057.2	--	--	--	--	--
53	355.1	12614	75842	--	--	--

Table 4.8. Analysis of urine samples using the proposed LC-MS/MS method, continued.

Sample	PGB	MOR	AMP	MAMP	COD	DZP
	Concentration (ng/mL)[a]					
54	--	11314.6	41836.6	--	--	--
55	--	--	554.7	--	--	--
56	3607.8	--	3628.2	--	--	--
57	--	--	2852.1	--	--	--
58	417.8	--	12544	59070	--	--
59	--	--	3960.9	10368.2	--	--
60	513.4	784.2	6224.2	34417.4	--	--
61	294.2	--	--	--	--	--
62	3484.9	--	7902	74120	--	--
63	3678.4	--	17306	75290	--	--
64	54022	--	--	440.5	--	--
65	--	166.3	--	--	--	--
66	277	--	--	--	--	--
67	--	--	--	1044.3	--	--
68	340.7	--	--	--	--	--
69	142470	--	--	--	--	--
70	86080	--	2585.6	--	--	--
71	--	--	11034.4	--	--	--
72	222788	--	3670	--	--	--
73	242630	--	2905.5	30804	--	--
74	63549.6	--	2657.4	39663.4	--	--
75	1354.9	--	--	--	--	--
76	9266	--	14170	61110	--	--
77	--	886.6	3685.5	13045.2	--	--
78	854.6	--	--	--	--	--
79	46.6	--	11688	114444	--	--
80	19846.4	--	--	--	--	--

Table 4.8. Analysis of urine samples using the proposed LC-MS/MS method, continued.

Sample	PGB	MOR	AMP	MAMP	COD	DZP
	Concentration (ng/mL)[a]					
81	915.4	--	10142	52374	--	--
82	1437.6	--	2054.3	468.9	--	--
83	--	--	1047.7	2740.4	--	--
84	1093.6	--	6949	22452.2	--	--
85	2138.6	--	--	--	--	--
86	130.7	--	8519.2	27236.2	--	--
87	67250	--	--	--	--	--
88	--	--	2308.2	9479.8	--	--
89	3610.9	--	6323	42666	--	--
90	659.4	--	--	--	--	--
91	--	--	1832.9	5167.6	--	--
92	--	--	89.1	577.6	--	--
93	--	--	2766.5	15952.4	--	--
94	285.3	--	2727.1	12042.6	--	--
95	--	--	791.6	5757.4	--	--
96	8666	--	2591.5	24366	--	--
97	--	--	287.7	--	--	--
98	--	--	1106.9	3558.8	--	--
99	47726	--	10228	72254	--	--
100	112.1	--	8500.4	--	--	--
101	84935	--	--	--	--	--
102	--	--	1932	6509	--	--
103	--	--	--	4089	--	--
104	69743	--	--	--	--	--
105	268910	--	--	--	--	--
106	--	--	4529	23215	--	--
107	86.4	--	--	--	--	--

Table 4.8. Analysis of urine samples using the proposed LC-MS/MS method, continued.

Sample	PGB	MOR	AMP	MAMP	COD	DZP
	Concentration (ng/mL)[a]					
108	348010	--	1445	3706	--	--
109	69289	--	--	--	--	--
110	96676	--	--	--	--	--
111	--	--	3558	11202	--	--
112	--	--	3435	18609	--	--
113	40.7	--	--	--	--	--
114	--	--	4519	16872	--	--
115	6034	--	--	3817	--	--
116	--	--	1738	7736	--	--
117	1356.6	--	--	--	--	--
118	--	--	--	3766	--	--
119	--	--	581.8	967	--	--
120	--	--	2666	--	--	--
121	--	--	6785	8787	--	--
122	2.7	--	101.9	1096.7	--	--
123	38181	--	9114	78804	--	--
124	--	--	4050	--	--	--
125	--	--	6494	43663	--	--
126	9706	--	6173	8742	--	--
127	--	--	3379	--	--	--
128	64639	--	3566	33558	--	--
129	11746	--	6761	9620	--	--
130	5.4	--	397.6	657.8	--	--
131	33599	--	--	--	--	--
132	124549	--	11021	5771	--	--
133	3223	--	5485	4641	--	--
134	--	--	2094	7878	--	--

Table 4.8. Analysis of urine samples using the proposed LC-MS/MS method, continued.

Sample	PGB	MOR	AMP	MAMP	COD	DZP
	Concentration (ng/mL)[a]					
135	--	--	--	1408.8	--	--
136	299.2	--	4616	3660	--	--
137	--	--	74331	105569	--	--
138	--	--	6510	--	--	--
139	--	--	5867	69549	--	--
140	51001	--	2724	14670	--	--
141	--	--	4155	5203	--	--
142	--	--	--	3902	--	--
143	209369	--	1476	4040	--	--
144	199.8	--	--	--	--	--
145	736240	--	--	4049	--	--
146	--	--	2417	5227	--	--
147	137853	--	3319	24155	--	--
148	49073	--	30417	5285	--	--
149	1519	--	1971	5415	--	--
150	573.2	--	546.3	1148.8	--	--

[a] Average of three determinations.

Table 4.9. Prevalence and concentrations measured for drugs under investigation in 150 urine samples

	PGB	MOR	AMP	MAMP	COD	DZP
Positive samples (%)	95 (63.3%)	12 (8%)	103 (68.7%)	96 (64%)	4 (2.7%)	0 (0%)
Range (ng/mL)	2.7-736240	166.3-20913	89.1-75842	440.5-114654	100.9-3105	NA
Mean ± SD (ng/mL)	61757.6 ± 117471.6	5728.6 ± 6448.6	6867.2 ± 11398.9	23218.3 ± 27508.8	924.8 ± 1459.2	NA
Median (ng/mL)	8666	2668.8	3558	10785.1	246.7	NA

AMP, MAMP, and PGB are the most detected drugs in the samples either individually (**Figure 4.9**) or in combination (**Figure 4.10**) with 67.3, 64.7, and 62.7% of the analyzed 150 samples, respectively. Accordingly, they showed the highest total prevalence of the six drugs as shown in **Figure 4.11**. The most widely detected individual drug is PGB (20.7%) followed by MAMP (4.7%), AMP (4%), and MOR (2.7%). This agrees with the literature that PGB is a major cause of concern in Kuwait due to its abuse potential [155]. This was shown in its inclusion in Schedule 4 drugs act in Kuwait in 2021.

The mixture of AMP and MAMP was detected in 85 samples, which represents 75.2% of the total samples containing AMP and/or MAMP (113). AMP alone was detected in 16 samples (14.2%), and MAMP alone was detected in 12 samples (10.6%). The interpretation of samples containing AMP and/or MAMP is closely related to their metabolic pathway. Since MAMP is converted to AMP by cytochrome P450, it is often challenging to determine whether the detected AMP came from the ingestion of the drug itself or from the ingestion of MAMP metabolized into AMP [156].

Both drugs were detected in the majority of their samples (75.2%), which may indicate that they are commonly used together in addictive preparations. Another possibility is that the preparations containing only MAMP were ingested, and urine was collected shortly after ingestion. This may have allowed metabolism of some MAMP into AMP, resulting in detection of both AMP and MAMP in the urine. The situation is more complex with the samples containing only AMP. This could indicate ingestion of AMP preparations, but it could also mean that the samples were collected long after the ingestion of

either single MAMP or AMP/MAMP preparations, allowing all MAMP to be metabolized and excreted in urine as AMP. The detection time for AMP in urine ranges from 1 day to 4.8 days, while the detection time for MAMP ranges from 42.7-87.2 hr [157].

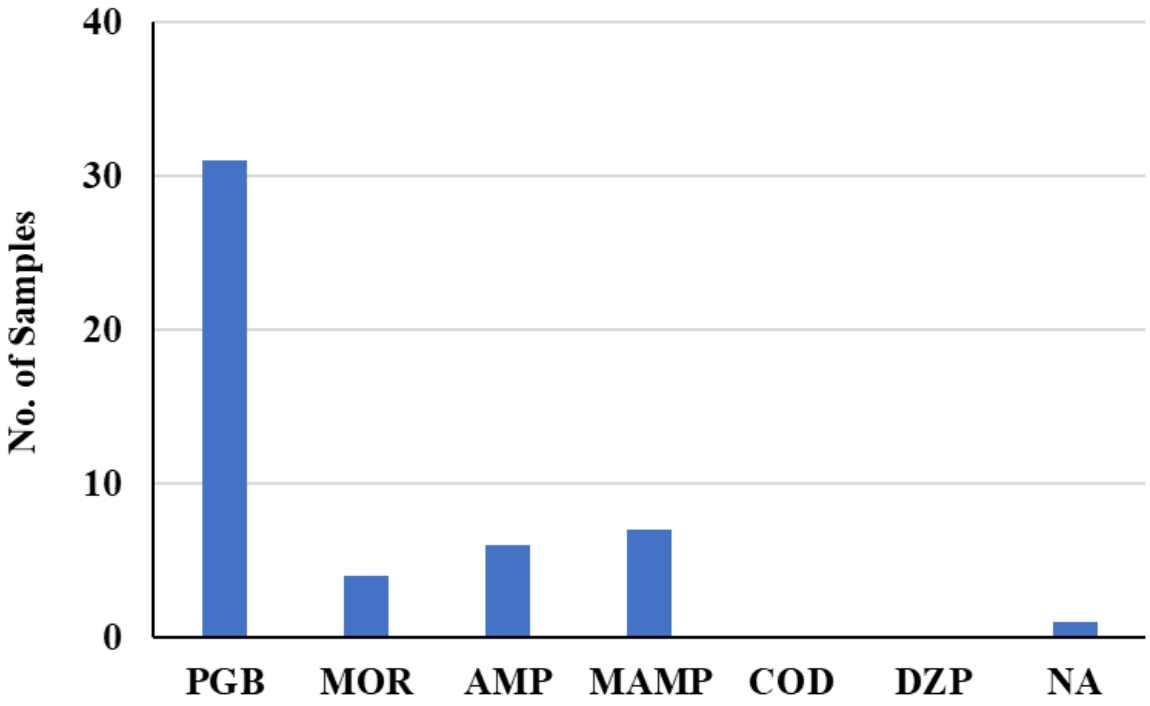


Figure 4.9. Results of the analysis of the drugs under investigation in urine samples from Kuwait revealing the prevalence of each drug individually.

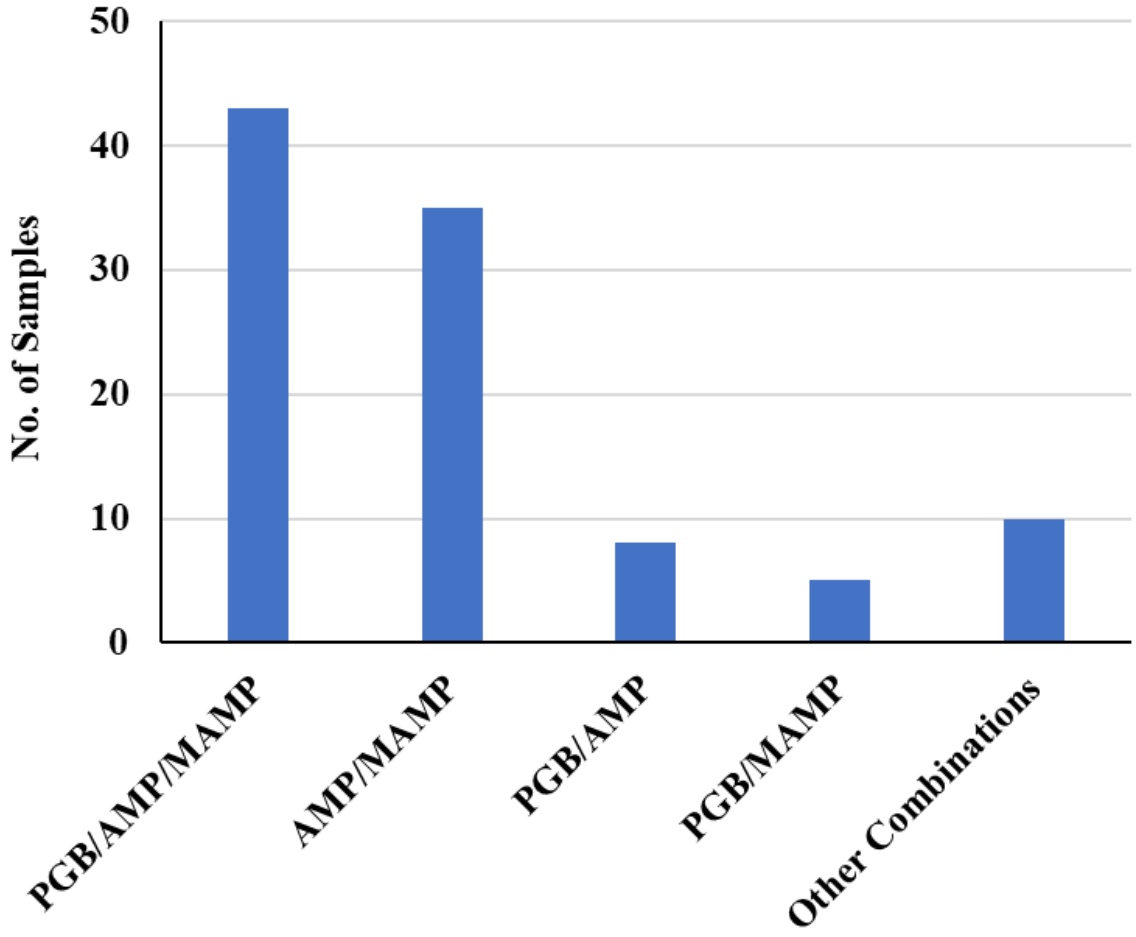


Figure 4.10. Results of the analysis of the drugs under investigation in urine samples from Kuwait revealing the prevalence of drug combinations.

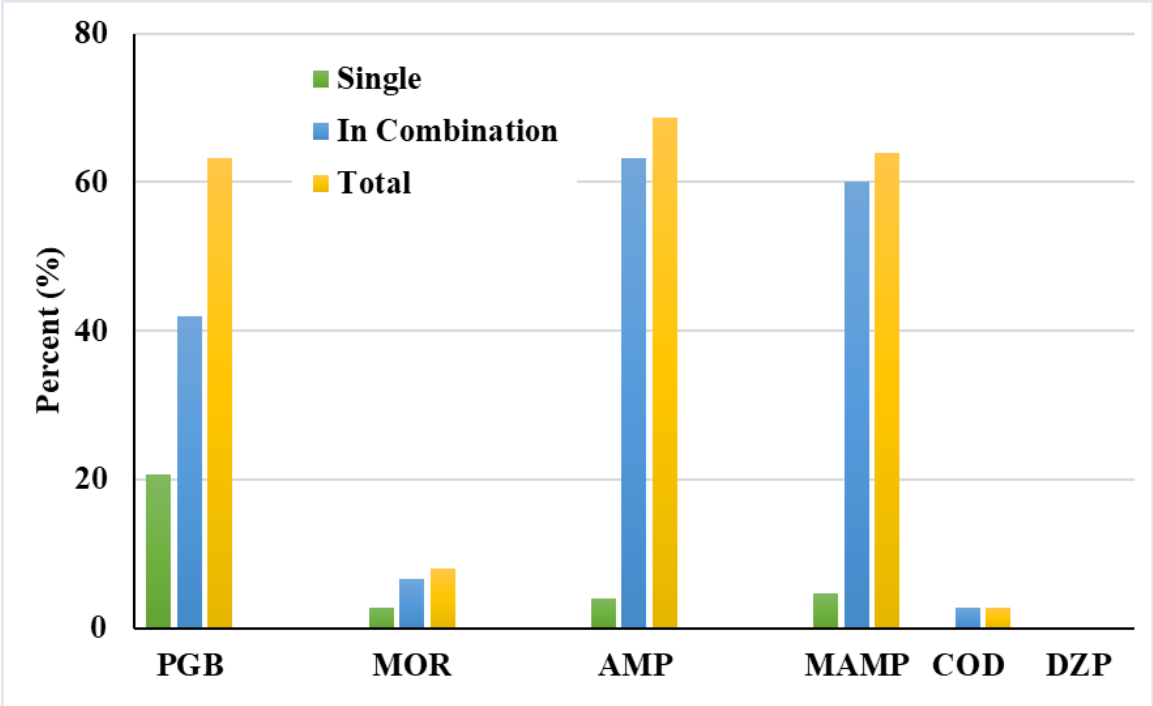


Figure 4.11. Results of the analysis of the drugs under investigation in urine samples from Kuwait revealing the percentage prevalence of each drug individually, in combinations and its total prevalence.

MOR was only detected in fewer samples, 14 (9.3%) of the total 150 samples. Of that number, in only 4 cases it was detected on its own. In the other 10 cases it was detected in combination with PGB, AMP/MAMP or COD. The interpretation of samples with MOR is complicated as it depends on the manufacturing process of MOR and HER, and it is also largely influenced by metabolism.

MOR shares the same metabolic pathway with several heroin related drugs [158, 159]. HER (diacetylmorphine) is rapidly metabolized to 6-acetylmorphine which is then converted to MOR. Ethylmorphine and COD also are converted to MOR by cytochrome P-450 [159]. Therefore, the detection of MOR can indicate the ingestion of MOR itself, COD, ethylmorphine, or HER which is more readily available on the drugs market than MOR.

Opium contains several alkaloids including MOR and COD, therefore pharmaceutical grade MOR purified from opium contains low level contamination of COD, where up to 0.5% is allowed [160, 161]. HER is a semi synthetic drug obtained by the acetylation of MOR, and this process typically utilizes opium as its main source of MOR. Therefore, street HER can contain significantly more COD as a contaminant than medical MOR due to less stringent purification processes. This results in the formation of acetylcodeine, which has been suggested as an indicator of HER abuse. Upon ingestion, acetylcodeine similarly undergoes rapid metabolism by cytochrome P-450 to produce COD.

Therefore, it is likely that the presence of MOR detected in the samples could be a result of HER ingestion. This is supported by the data shown in samples 3, 38, and 41, where the percentage of COD to MOR is significantly higher (14.8%, 10.2%, and 16.5%, respectively) compared to the expected percentage in pharmaceutical grade MOR (0.5%). In contrast, sample 39 may indicate ingestion of medical MOR, as the percentage of COD was 1.6%. This result could also suggest that the sample was taken a long time after HER ingestion, allowing COD to be metabolized into MOR. It is worth noting that HER and opium have been reported as some of the top drugs of abuse seized by Kuwait authorities [162].

4.4. Conclusion

A sensitive and specific LC-MS/MS method was developed for the separation of six drugs of abuse common in Kuwaiti market namely PGB, MOR, AMP, MAMP, COD, and DZP. The method was cross-validated between our laboratory at the University of Strathclyde and the toxicology department within the general department of criminal evidence in Kuwait, following the ICH guidelines. The validation results demonstrated that the method is accurate, precise, sensitive, and robust. A SPE procedure was developed for extraction of the six drugs from urine, and satisfactory recoveries were obtained. The method was used for screening and quantification of the six drugs in 150 urine samples collected from subjects under investigation for suspected drug abuse violations in Kuwait in 2022 to draw a picture of the market of drugs of abuse. Through the analysis of the screening results, it was determined that PGB, AMP, and MAMP were the most frequently detected

drugs in the collected samples, either individually or in combination. Notably, PGB emerged as the most prevalent individual drug. MOR was detected in 9.3% of the total samples, indicating that the ingestion of HER was the primary cause in the majority of these samples.

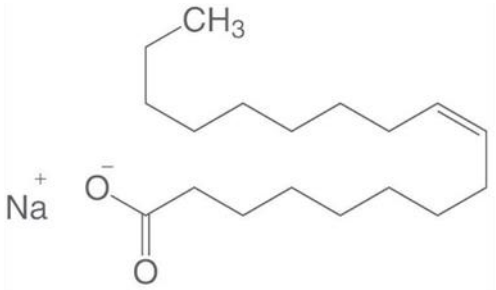
Chapter 5 Development and validation of GC-MS method for intestinal media characterization

5.1. Introduction

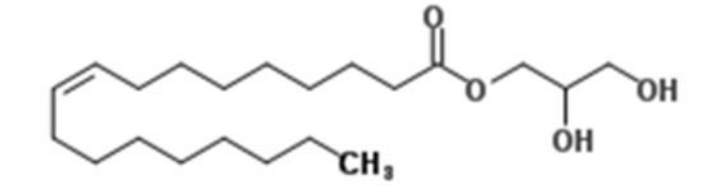
Simple aqueous solubility cannot reflect GIT solubility anymore and to determine drug solubility in vitro, human gastric (HGF) and intestinal fluids (HIF) should be used [124]. Due to the disadvantages of human fluids, simulated GIT media are taking more role in solubility studies [127]. Bile salts, lecithin, cholesterol, fatty acids and monoglycerides are very common components in simulated GIT media [130]. GIT media is susceptible to large variability between individuals and day-to-day fluctuations, in addition to meal intake and biliary and pancreatic secretions. These facts shows the importance of characterization of GIT fluid components for accurate determination of drug bioavailability [118].

The aim of work in this chapter was to develop accurate and specific GC-MS method for the determination of common GIT fluid components. A simulated GIT media was used for the method development, it contained sodium oleate (SO), glyceryl monooleate (GMO), and cholesterol (CHL). The structures of the three compounds are illustrated in **Figure 5.1**, and their chemical names and molecular weights are summarized in

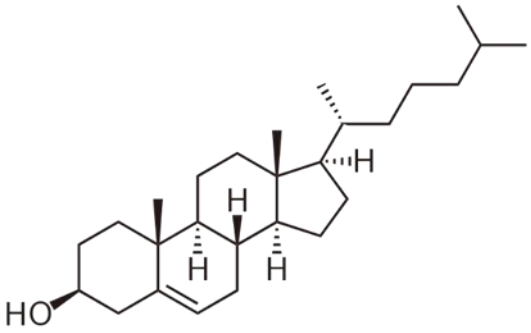
Table 5.1.



Sodium oleate



Glyceryl monooleate



Cholesterol

Figure 5.1. Structural formulae of sodium oleate, glyceryl monooleate, and cholesterol

Table 5.1. Chemical names and molecular weights of the compounds under investigation.

Compound	Chemical name	Molecular formula	Molecular weight
Sodium Oleate	Oleic acid sodium salt	$C_{18}H_{33}NaO_2$	304.4
Glycerol monooleate	2,3-dihydroxypropyl octadec-9-enoate	$C_{21}H_{40}O_4$	356.5
Cholesterol	(3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-[(2R)-6-methylheptan-2-yl]-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H cyclopenta[a] phenanthren-3-ol	$C_{27}H_{46}O$	386.7

5.2. Experimental

5.2.1. Materials and reagents

Chloroform was of LC-MS grade. Methyl tert-butyl ether (MTBE) was an ACS reagent. Trimethylsilyldiazomethane (TMSDM) was approximately 10% hexane solution. The previous chemicals were purchased from Merck (UK), in addition to benzene, pyridine, trimethylchlorosilane (TMCS), N-trimethylsilylimidazole (TMSI), CHL, and SO. GMO was purchased from Croda (UK). N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), N-methyl-N-(trimethylsilyl)trifluoroacetamie (MSTFA), ammonium iodide (NH_4I), and dithioerythritol (DTE) were supplied by Fluorochem (UK). Deionized water was prepared from Purite Select Ondeo (Purite Limited, UK).

5.2.2. Instruments and operating conditions

GC-MS analyses were carried out with Shimadzu Nexis GC-2030, equipped with an AOC-20i Plus auto-sampler, and coupled to a mass spectrometer (GCMS-TQ8040NX). LabSolutions GCMS (version 4.53SP1) was used for instrument control, and data acquisition and processing.

The separation was performed on SH-1-5MS, 30 m × 0.25 mm, 0.25 µm, (Shimadzu, UK). Helium was used as carrier gas at a constant pressure flow rate of 1.30 mL/min. The injector temperature was set at 280 °C. Split mode injection was applied with an injection volume of 1 uL the purge flow rate was 3.0 mL/min. The column oven temperature program was as follows: the initial temperature was set at 180 °C and maintained for 2 min, then it was increased to 320 °C at a rate of 24 °C/min and held for 5 min. The total run time was 12

min. The ion source temperature was set at 220 °C. GC operating conditions are summarized in
Table 5.2. The electron impact (EI) ionization mode was used, and all the ions were monitored in the selected ion monitoring (SIM) mode. Mass spectrometric conditions are given in
Table 5.2 and **Table 5.3**.

5.2.3. Derivatization procedure and preparation of standard solutions

An aliquot of 160 µL of the derivatization reagent MSTFA: NH_4I: DTE (500: 4: 2, v/w/w) was added to the 2 mg of each standard, and incubated for 60 min at 60 °C. The mixture was cooled and diluted with MTBE to prepare the stock solutions (1 mg/mL).

5.2.4. Construction of calibration curves

Aliquots were taken from the stock solutions and diluted with MTBE to prepare solutions in the range 2 - 100 µg/mL for the three compounds. AUCs were plotted against the corresponding concentrations and regression equations were computed.

5.2.5. Validation

Validation of the proposed method was performed with respect to specificity, accuracy, and precision according to the ICH guidelines [147]. Specificity of the method was assessed by the analysis of different laboratory prepared mixtures of the three compounds. The accuracy was confirmed by applying the proposed method to determination of three concentrations of each compound (20, 40 and 100 ppm) in triplicates. Repeatability and intermediate precision were assessed using three concentrations (10, 20 and 40 ppm) of standard solutions (n=3).

Table 5.2. GC and MS parameters used for quantification of the compounds under investigation.

Parameter	Conditions
GC	
Injection mode	Split
Injector temp	280 °C
Column oven temp.	180 °C (2min) → (24 °C/min) → 320 °C (5min)
Control mode	Linear velocity (43.7 cm/s)
Purge flow rate	3.0 mL/min
Injection pressure	100 kPa
MS	
Measurement mode	Scan or SIM (Scan range: *m/z* 45-600)
Ion source temp.	220 °C
Interface temp.	300 °C
Event time	0.3 s
Voltage	0.1 kV

Table 5.3. GC-MS/MS parameters selected for the quantification of the derivatized compounds.

Derivative	Rt (min)	[M]	Daughter ions		SIM (m/z)
			Ion-1	Ion-2	
O-TMS	6.01	354.3	339.2	264.2	339.2
GMO-TMS	8.01	500.3	485.3	397.3	397.3
CHL-TMS	9.83	458.3	368.3	329.3	368.3

5.3. Results and dicussion

The detection of SO, GMO, and CHL is a challenging process as they all show similar lipophilic properties. GC is a common technique used for determination of fatty compounds such as fatty acids and sterols. The main disadvantage of GC technique is the derivatization step essential for non-volatile compounds.

5.3.1. Derivatization method optimization

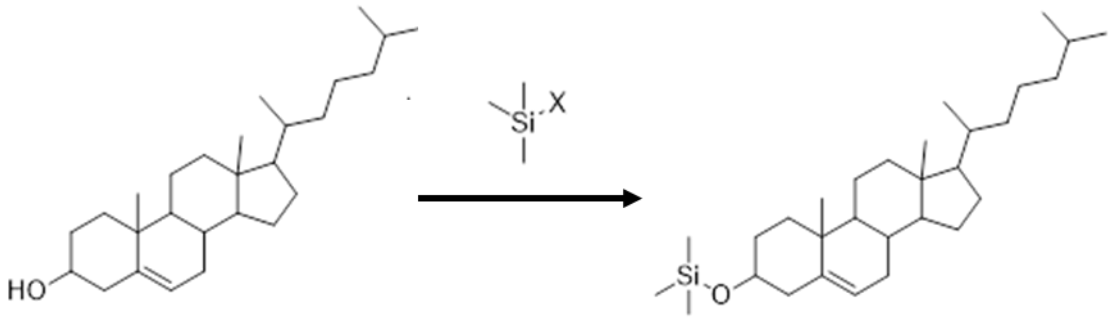
Among the common derivatization techniques in GC are alkylation and silylation. Both methods depend on the reaction between the free hydroxyl group in the target molecules with the derivatizing agents. The two methods were tested while the derivatization step was carefully optimized before the GC-MS analysis. Different derivatization reagents were tested to find the optimal one. The derivatization reagents were used to produce derivatized compounds and their performance was compared using GC-MS. The reagents were compared regarding the appearance of the peaks on GC-MS, which meant a successful derivatization step with this reagent. Another point of comparison was the AUCs of the peaks, which can be used to assess the reaction yields and sensitivity of the detector to the produced derivatives.

Firstly, a mixed methylation and silylation derivatization protocol developed by Shimadzu was tested [147]. This protocol involved successive derivatization with TMSDM and TMSI to act on both carboxylic and hydroxylic hydrogens of the three compounds. The derivatization process was not successful, as none of the three compounds was derivatized and no peaks were observed on GC-MS. Then, either single methylation or silylation processes were tested. A

methylation reagent (TMSDM) was tried and failed to produce satisfactory results, as no GC-MS were observed.

Silylation process usually involves introduction of a trimethylsilyl group (TMS) to a free hydroxyl group via various reagents such as BSTFA, TMCS, MSTFA or TMSI which are used either individually or as combinations (**Scheme 1**). Several reports on using these reagents were reported in literature including Kunz et al. [163], Castellaneta et al. [164], Kumar et al. [165], and Lian et al. [166] among others.

Some silylation reagents suggested by Kumar et al. [165] were tested. The first reagent was BSTFA: TMCS (100: 1, v/v), but this reagent failed to derivatize any of the three compounds and no peaks were observed on GC-MS. Other reagent mixtures such as MSTFA: TMCS (100: 1, v/v) and MSTFA: TMSI: TMCS (100: 2: 5, v/v/v) were also used. Both SO and CHL were successfully derivatized by these two reagents, and it was observed that MSTFA: TMSI: TMCS resulted in a better reaction yield giving higher peaks than MSTFA: TMCS. Finally, MSTFA: NH_4I: DTE (500: 4: 2, v/w/w) was used. The three compounds were successfully derivatized by this reagent, with even higher reaction yield than the previous reagents (**Figure 5.2**). Therefore, MSTFA: NH_4I: DTE was the derivatization reagent of choice for the three compounds.



Scheme 1. General reaction of Cholesterol with silylating reagents (Me_3-Si-X), where X is the leaving group to form TMS-Cholesterol.

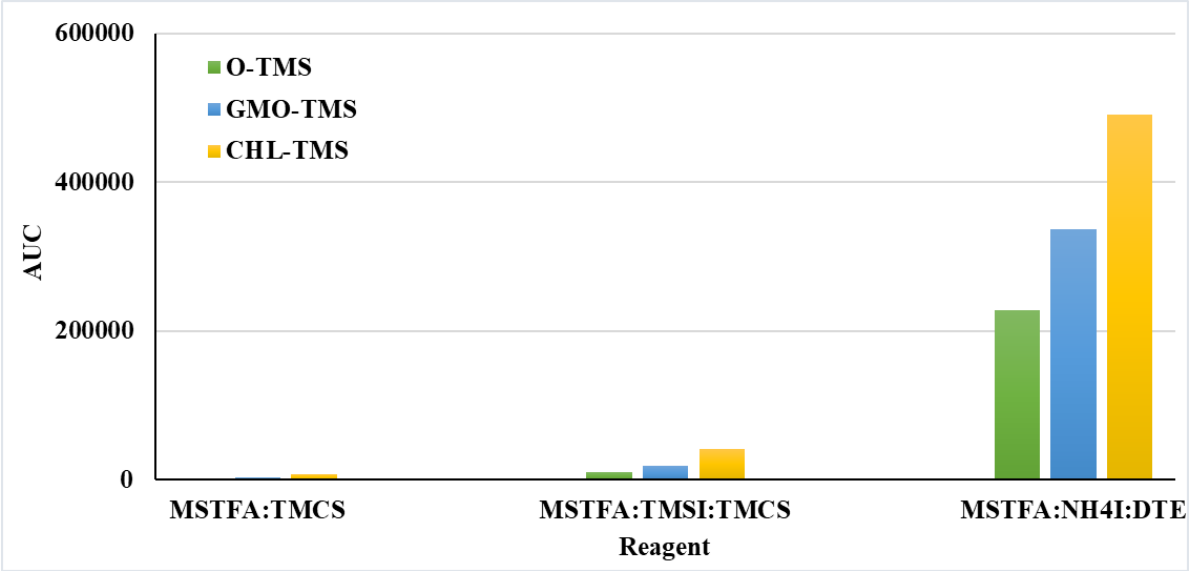


Figure 5.2. Results of optimization of the derivatization reaction showing AUCs obtained with each reagent mixture for the three compounds.

The type of solvent represented a significant factor affecting the success of derivatization reactions. The majority of the derivatization protocols mentioned above used alcohols in their work, therefore, methanol, ethanol, and isopropanol were all tested with different derivatizing reagents. No GC-MS peaks were observed upon using alcohols as solvents. Therefore, different solvent mixtures were introduced such as chloroform: methanol (2:1) and dichloromethane: isopropanol (4:1), but they also failed in the derivatization reactions. The solvent that showed successful derivatization with several derivatizing reagents was MTBE. The failure of alcohols or mixtures containing alcohols may be attributed to the interference of their free hydroxyls in the derivatization reaction (**Scheme 1**). MTBE is also a suitable solvent for extraction of the three compounds from aqueous samples, so it would help simplify the extraction process of more complex biological samples such as gut media or blood.

The volume of derivatization reagent added to the compounds was also optimized. For every 2 mg of analyte, different reagent volumes were tried (40, 80, 160, and 200 µL), the volume 160 µL was the best regarding peak area in GC-MS (**Figure** 5.3). The derivatization incubation time was also considered, as 30, 40, and 60 min were allowed for the reaction between the analytes and the reagent mixture. The best reaction time with the highest abundance was 60 min (**Figure** 5.4).

Therefore, the best result based on the presence of peaks and highest intensity was observed with the reagent mixture MSTFA: NH_4I: DTE, from which 160 µL were added to each 2 mg analyte and incubated at 60 °C for 60

min, and then diluting the product with MTBE. The target analytes SO, GMO, and CHL were successfully derivatized using the mentioned conditions into new silyl derivatives, Oleate-TMS (O-TMS), GMO-TMS, and CHL-TMS.

5.3.2. GC-MS method development and optimization

The silyl derivatives were separated using GC and detected with MS detector. GC conditions were optimized. The separation was achieved on SH-1-5MS, 30 m × 0.25 mm, 0.25 µm, with helium as the carrier gas. The injector parameters were optimized, where the best temperature regarding resolution and peak shape was 280 °C, and the injection mode was a split mode with a split ratio of 10. The injection volume was 1 µL with a purge flow rate of 3.0 mL/min and injection pressure of 100 kPa. The column temperature was also optimized, where isothermal program did not achieve satisfactory results; so, temperature programming was applied. The best temperature program was as follows: initial temperature was set at 180 °C (for 2 min), then it was increased to 320 °C (at a rate of 24 °C/min) and then it was held for 5 min. The total run time was 12 min. GC best operating conditions are summarized in

Table 5.2.

The mass spectrometry parameters were also optimized. The best ion source temperature was 220 °C. The EI ionization mode was used, and all the ions were monitored in the SIM mode. Mass spectrometric conditions are given in **Table 5.3**. The quantification of the derivatized compounds was optimized by comparing three characteristic ions for each derivative (the parent ion and two daughter ions), as shown in **Table 5.3**. Among them, the ion that had the

highest intensity or stability was selected for SIM as the quantitative ion for this derivative (**Table 5.3**).

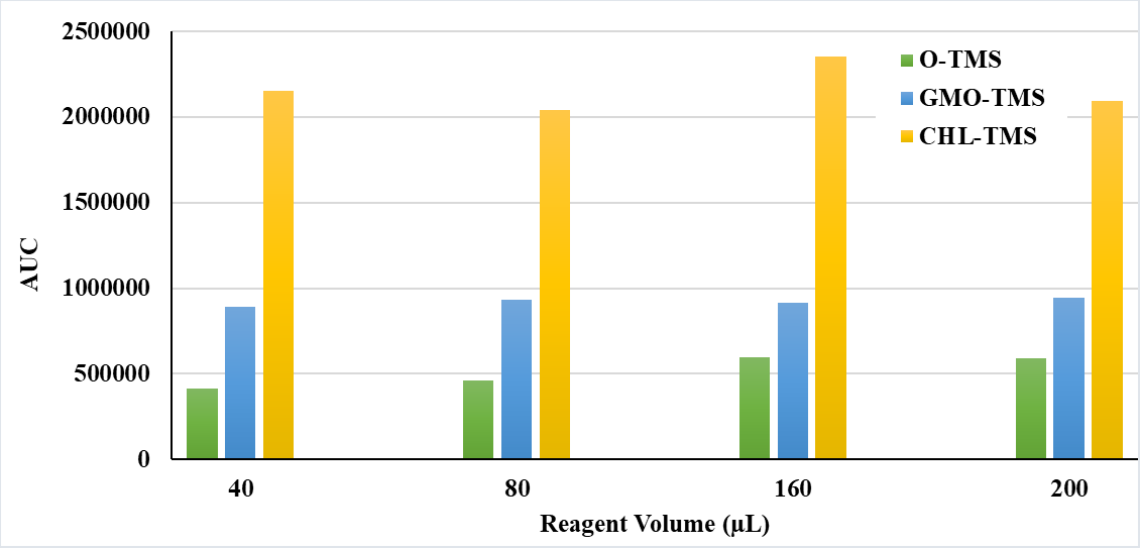


Figure 5.3. Results of optimization of the derivatization reaction showing AUCs obtained with different reagent volumes for the three compounds.

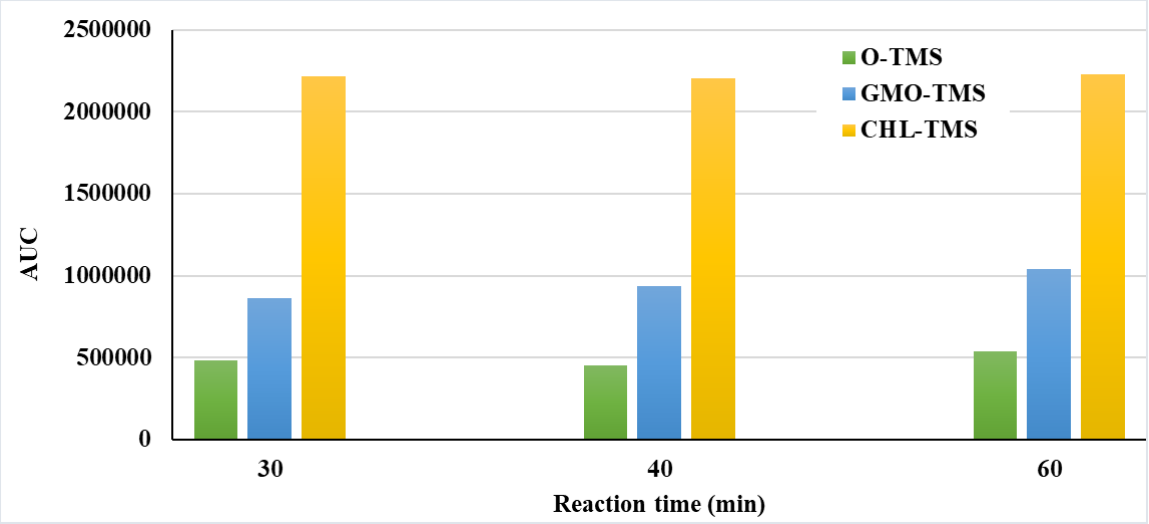


Figure 5.4. Results of optimization of the derivatization reaction showing AUCs obtained with different reaction time for the three compounds.

With the optimized derivatization, GC, and MS conditions, the three compounds were separated successfully with retention times of 6.01, 8.01, and 9.83 min for O-TMS, GMO-TMS, and CHL-TMS, respectively (**Figure 5.5**). The method was linear over the concentration range 2-100 µg/mL as shown in **Figure 5.6-5.8**.

5.3.3. Method Validation

The proposed GC-MS method was validated for the three compounds according to ICH guidelines [147]. For specificity, satisfactory recoveries were obtained for quantitation of the drugs in their different ratios in laboratory prepared mixtures as shown in

Table 5.4. For accuracy, reasonable mean percentage recoveries were obtained for quantitation of the drugs in three concentrations levels (10, 40 and 60 ppm) as shown in

Table 5.5. For repeatability and intermediate precision, satisfactory RSD% was obtained from analysis of the three drugs in the three concentration levels 10, 20 and 40 ppm, three times in the same day and on three different days as shown in **Table 5.6**, indicating reasonable precision.

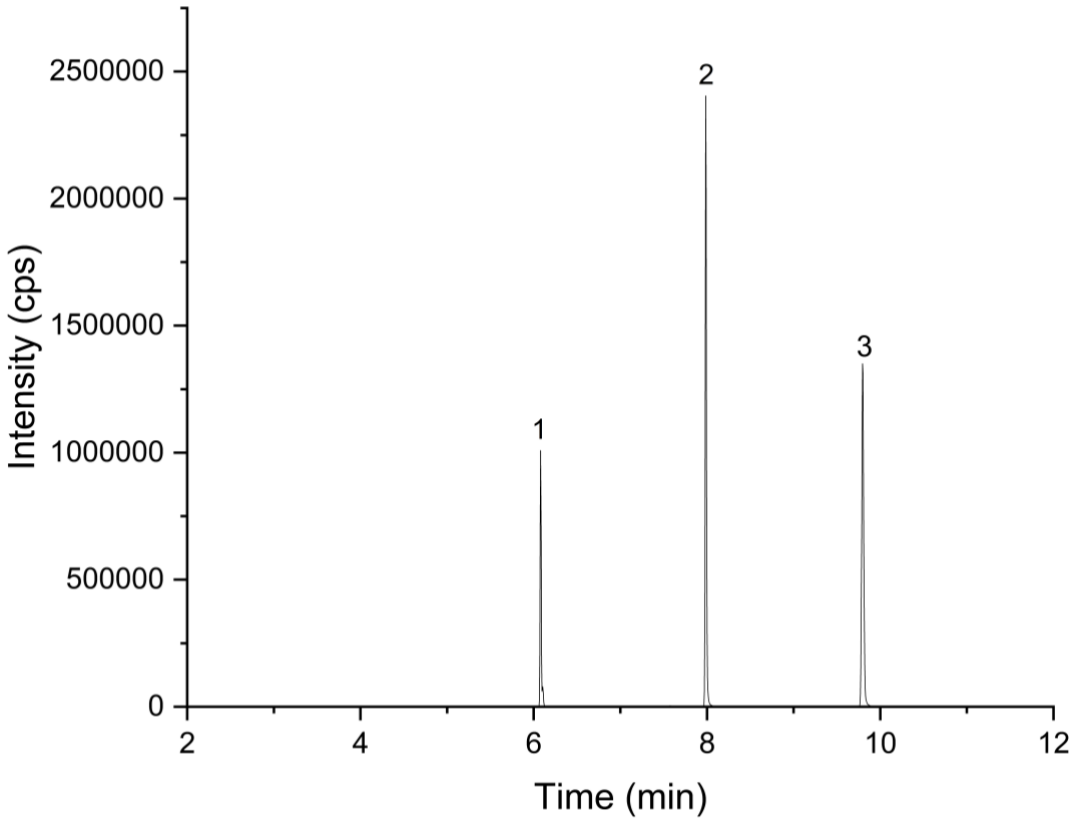


Figure 5.5. GC-MS chromatogram for 1) O-TMS, 2) GMO-TMS, and 3) CHL-TMS at 40 ppm.

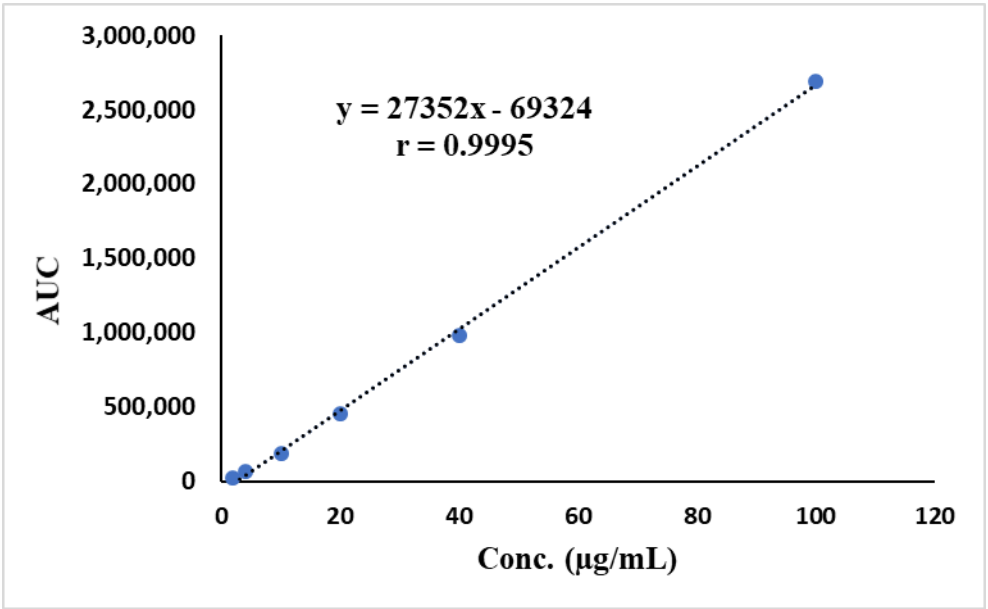


Figure 5.6. Linearity of AUC to the corresponding concentrations of SO

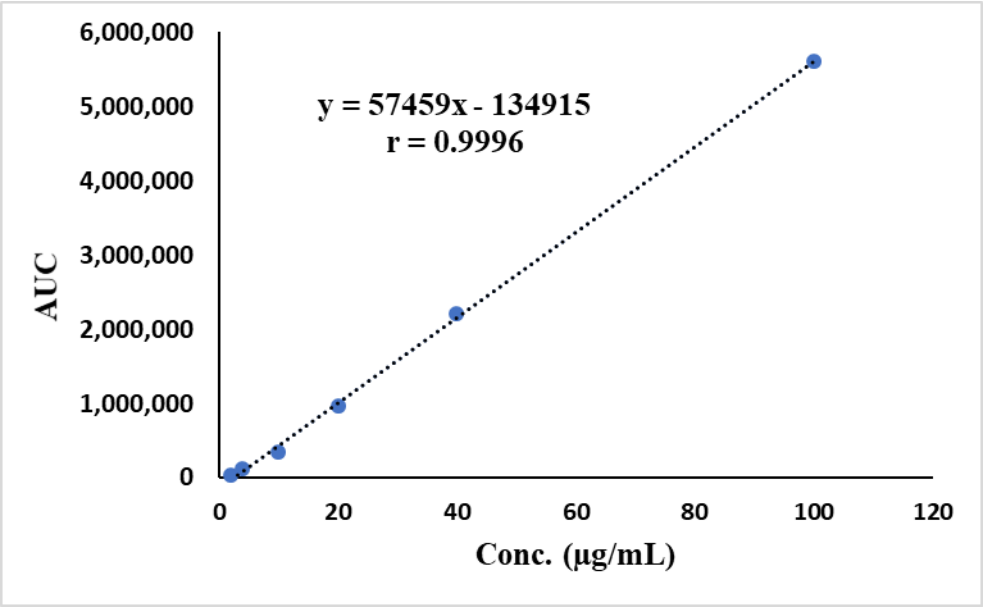


Figure 5.7. Linearity of AUC to the corresponding concentrations of GMO

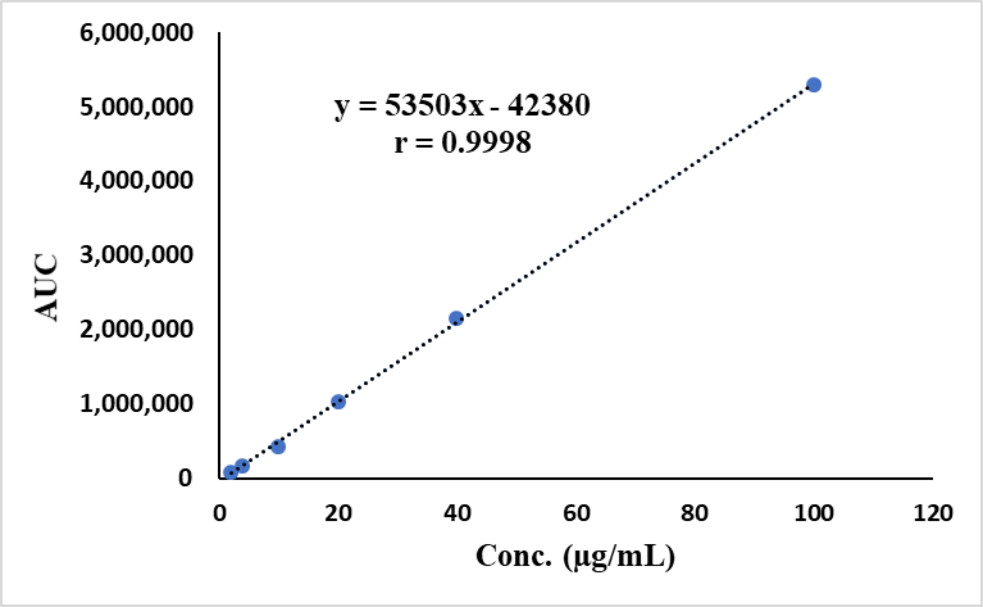


Figure 5.8. Linearity of AUC to the corresponding concentrations of CHL

Table 5.4. Determination of the three compounds in laboratory prepared mixtures by the proposed method

	SO	GMO	CHL
Concentration (ppm)	2	2	2
Recovery %[a]	95.3	101.6	101.3
Concentration (ppm)	20	20	20
Recovery %[a]	101.6	103.4	101.7
Concentration (ppm)	100	100	100
Recovery %[a]	99.8	99.8	99.8
Concentration (ppm)	90	40	90
Recovery %[a]	101	104	102
Mean ± SD	99.4±2.9	102.2±1.9	101.2±1.0

[a] Average of three determinations

Table 5.5. Accuracy results of the proposed GC-MS/MS method for analysis of the three compounds

Compound	Recovery% [a]			Accuracy
	20 ppm	40 ppm	100 ppm	Mean ± SD
SO	101.6	103.4	101.7	100.9±0.8
GMO	100.9	101.1	101.1	100.7±2.9
CHL	100.1	97.6	97.3	100.0±2.4

[a] Average of three determinations

Table 5.6. Precision results of the proposed GC-MS/MS method for analysis of the three compounds

Compound	Intra-day Precision [a]			Inter-day Precision [b]		
	RSD%					
	10 ppm	20 ppm	40 ppm	10 ppm	20 ppm	40 ppm
SO	1.4	0.1	1.3	1.3	1.5	1.7
GMO	1.4	1.0	1.5	1.1	1.7	1.3
CHL	0.9	0.7	1.5	1.6	1.4	1.3

[a] Intra-day precision (n = 3), RSD of three concentrations repeated three times within the day.
[b] Inter-day precision (n = 3), RSD of three concentrations repeated three times in three different days.

5.4. Conclusion

A specific and simple GC-MS method was developed for the detection of three compounds common in simulated GIT media, namely sodium oleate (SO), glyceryl monooleate (GMO), and cholesterol (CHL). The method was validated according to ICH guidelines and was found to be accurate, specific, precise, and sensitive. The method can be used for screening and quantification of the GIT media used in bioavailability studies for pharmaceuticals. This can help understand and consider the variability between individuals and day-to-day fluctuations in these studies.

Chapter 6 General Conclusion and Future Work

6.1. General Conclusion

Three sensitive and specific LC-MS/MS methods have been developed for the screening and quantitation of illicit drugs in Kuwait. The three methods were cross-validated between our laboratory at the University of Strathclyde and the toxicology department within the general department of criminal evidence in Kuwait, following the ICH guidelines. The methods were used successfully for determination of the target drugs of abuse either in street samples or in urine samples collected from subjects under investigation for suspected drug abuse violations in Kuwait.

The first method has successfully separated ten SCs—5F-AB-PINACA, 5F-ADBICA, AB-PINACA, 5F-AMB, 5F-MDMB-PICA, 4F-MDMB-BUTINACA, FUB-PB-22, MDMB-4en-PINACA, 5F-APICA, and APICA. The developed method can quantify the SCs within a linearity range of 0.2–100 ppb. The method was applied for the analysis of 74 street samples collected in Kuwait between September 2021 and March 2022. The samples showed an abundance of 4F-MDMB-BUTINACA (46% of the seized samples) and the detection of MDMB-4EN-PINACA or FUB-PB-22 in less than 30% of the samples. Quantitation of these SCs showed concentrations ranging from 528.7 to 1416.2 ng/g. The concentrations of the three SCs in the seized samples varied significantly, where MDMB-4EN-PINACA and FUB-PB-22 showed limited spread in their concentrations. On the contrary, samples containing 4F-MDMB-BUTINACA showed larger distribution of its concentration indicating more danger from products containing this SC than the other two.

Another sensitive and specific LC-MS/MS method was developed for the detection of three SCs, namely ADB-PINACA-COOH, 5F-AB-PINACA, and 5F-AMB. A SPE procedure was developed for extraction of the three SCs from urine, and satisfactory recoveries were obtained. The method was used for screening and quantification of the target SCs in 49 urine samples collected in Kuwait between December 2023 and January 2024. By evaluating the analysis results, it was found that 5F-AMB was the most common SC detected showing the highest concentrations in urine.

Six drugs of abuse common in Kuwaiti market, namely PGB, MOR, AMP, MAMP, COD, and DZP, were separated by a sensitive and specific LC-MS/MS method. The six drugs were successfully extracted from urine by a SPE procedure showing satisfactory recoveries. The method was used for screening and quantification of the six drugs in 150 urine samples collected in Kuwait between the months of March and May 2022. Through the analysis of the screening results, it was determined that PGB, AMP, and MAMP were the most frequently detected drugs in the collected samples, either individually or in combination. Notably, PGB emerged as the most prevalent individual drug. MOR was detected in 9.3% of the total samples, indicating that the ingestion of HER was the primary cause in the majority of these samples.

SO, GMO, and CHL, common surfactants in simulated GIT media, were successfully separated by a specific and simple GC-MS method. Being non-volatile, a derivatization step was essential for GC separation of the three compounds. An optimized derivatization procedure was developed for the silylation of the three compounds using a reagent mixture consisting of

MSTFA: NH_4I: DTE (500: 4: 2, v/w/w). The procedure involved the addition of 160 µL of the reagent to each 2 mg analyte and incubation at 60 °C for 60 min, and then diluting the product with MTBE. The silyl derivatives, O-TMS, GMO-TMS, and CHL-TMS showed good linearity with the developed GC-MS method over the concentration range 2-100 µg/mL. The method was validated according to ICH guidelines and was found to be accurate, specific, precise, and sensitive.

6.2. Future Work

The developed LC-MS/MS methods for screening of illicit drugs will be extended to analysis of the target drugs in biological matrices such as blood, saliva, and hair. The methods will be used for analysis of samples collected in Kuwait to draw an idea of the evolution of illicit drugs abuse in Kuwait. Complete bioanalytical procedures will be developed including internal standards to compensate analytes loss in extraction procedures. Full bioanalytical validation will be applied following the new ICH guideline M10 [167].

Future work will also include possible metabolites in addition to parent compounds to present a general method that can be used to detect drugs and/or their metabolites. Furthermore, we will screen drugs of abuse emerging everyday to develop new methods for their detection. For the novel drugs, metabolic studies will be conducted for detection of possible metabolites for these drugs.

In future work, the GC-MS method developed for gut media constituents will be further optimized and more media constituents will be included in the method such as lecithin and sodium taurocholate.

The optimized and validated method will be used for the analysis of GIT fluids' components in different synthetic media purchased from different sources and in real samples from volunteers. The method will be used to measure the intra- and inter-subject variability in gut media, to assist in the in vitro determination of drug GIT solubility and the subsequent effect on drug efficacy.

Appendices

Articles

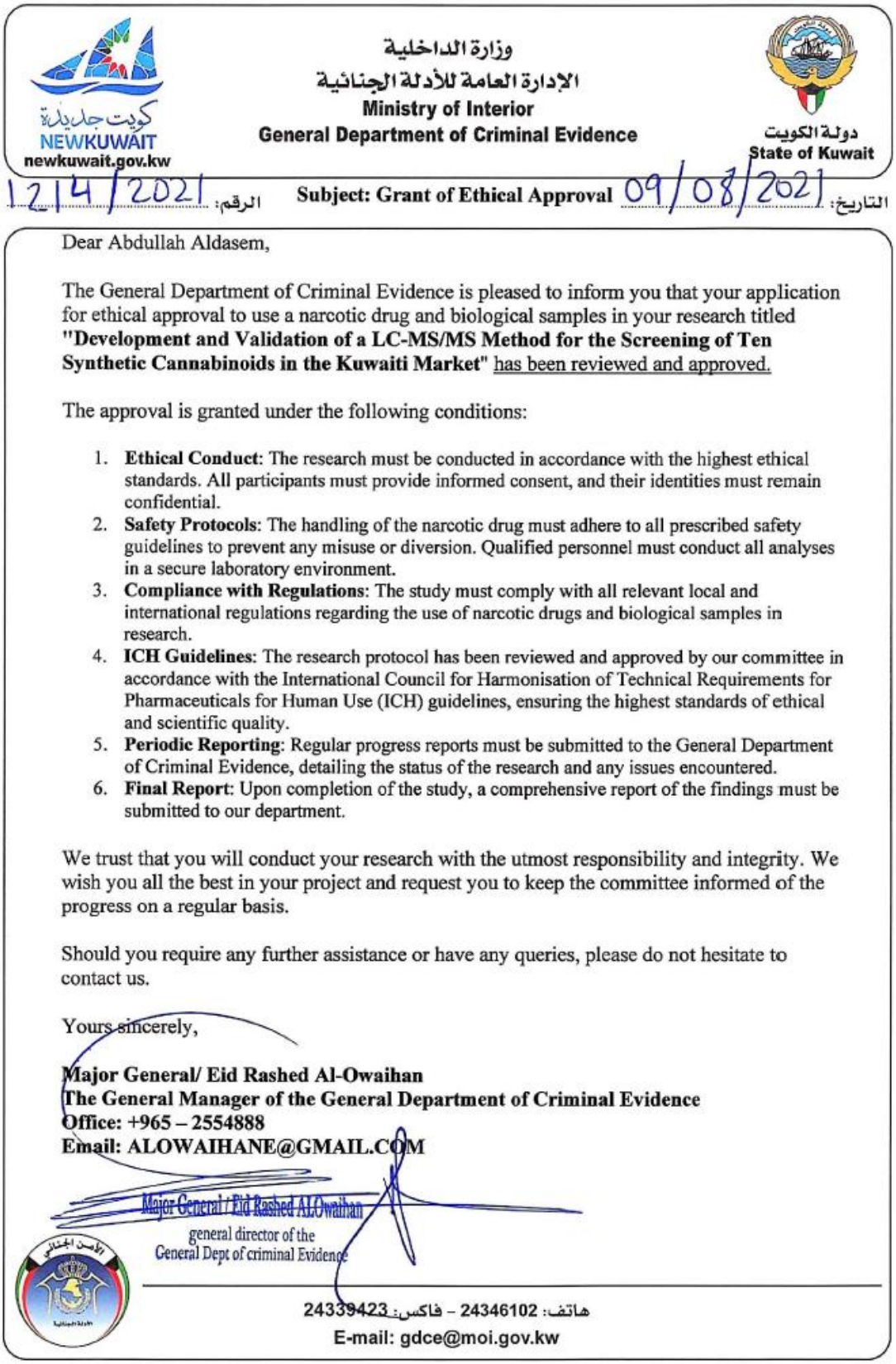
1. **Abdullah F. Aldasem**, Lina Akil, Abdullah Al-Matrouk and Ibrahim Khadra, *Development and validation of a LC-MS/MS method for the screening of ten synthetic cannabinoids in Kuwaiti market during 2021-2022*, Under Review in BMC Chemistry.
2. **Abdullah F. Aldasem**, Mohammad H. Al-Hasan, Abdullah Al Sultan and Ibrahim Khadra, *Development and validation of LC-MS/MS method for analysis of six drugs of abuse in urine: Screening of abused drugs in Kuwait during 2022*, Under Review in Journal of Pharmaceutical and Biomedical Analysis.

Conferences

1. 19th World Congress of Basic & Clinical Pharmacology**,** Glasgow, Scotland**,** July 2023. **(**attendance**)**
2. Strathclyde Institute of Pharmacy & Biomedical Sciences (SIPBS) Research Day, February 2023**. (Poster presentation)**

   Abdullah F. Aldasem, Lina Akil, Nicholas Rattray, Ibrahim Khadra. *Development and Validation of LC-MS/MS Method for Analysis of Ten Synthetic Cannabinoids in Kuwaiti Illicit Drugs Market*.

Ethical Approval for Chapter 2



وزارة الداخلية
الإدارة العامة للأدلة الجنائية
Ministry of Interior
General Department of Criminal Evidence

كويت جديدة
NEWKUWAIT
newkuwait.gov.kw

دولة الكويت
State of Kuwait

1214/2021 الرقم: **Subject: Grant of Ethical Approval** 09/08/2021 التاريخ:

Dear Abdullah Aldasem,

The General Department of Criminal Evidence is pleased to inform you that your application for ethical approval to use a narcotic drug and biological samples in your research titled **"Development and Validation of a LC-MS/MS Method for the Screening of Ten Synthetic Cannabinoids in the Kuwaiti Market"** has been reviewed and approved.

The approval is granted under the following conditions:

1. **Ethical Conduct**: The research must be conducted in accordance with the highest ethical standards. All participants must provide informed consent, and their identities must remain confidential.
2. **Safety Protocols**: The handling of the narcotic drug must adhere to all prescribed safety guidelines to prevent any misuse or diversion. Qualified personnel must conduct all analyses in a secure laboratory environment.
3. **Compliance with Regulations**: The study must comply with all relevant local and international regulations regarding the use of narcotic drugs and biological samples in research.
4. **ICH Guidelines**: The research protocol has been reviewed and approved by our committee in accordance with the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines, ensuring the highest standards of ethical and scientific quality.
5. **Periodic Reporting**: Regular progress reports must be submitted to the General Department of Criminal Evidence, detailing the status of the research and any issues encountered.
6. **Final Report**: Upon completion of the study, a comprehensive report of the findings must be submitted to our department.

We trust that you will conduct your research with the utmost responsibility and integrity. We wish you all the best in your project and request you to keep the committee informed of the progress on a regular basis.

Should you require any further assistance or have any queries, please do not hesitate to contact us.

Yours sincerely,

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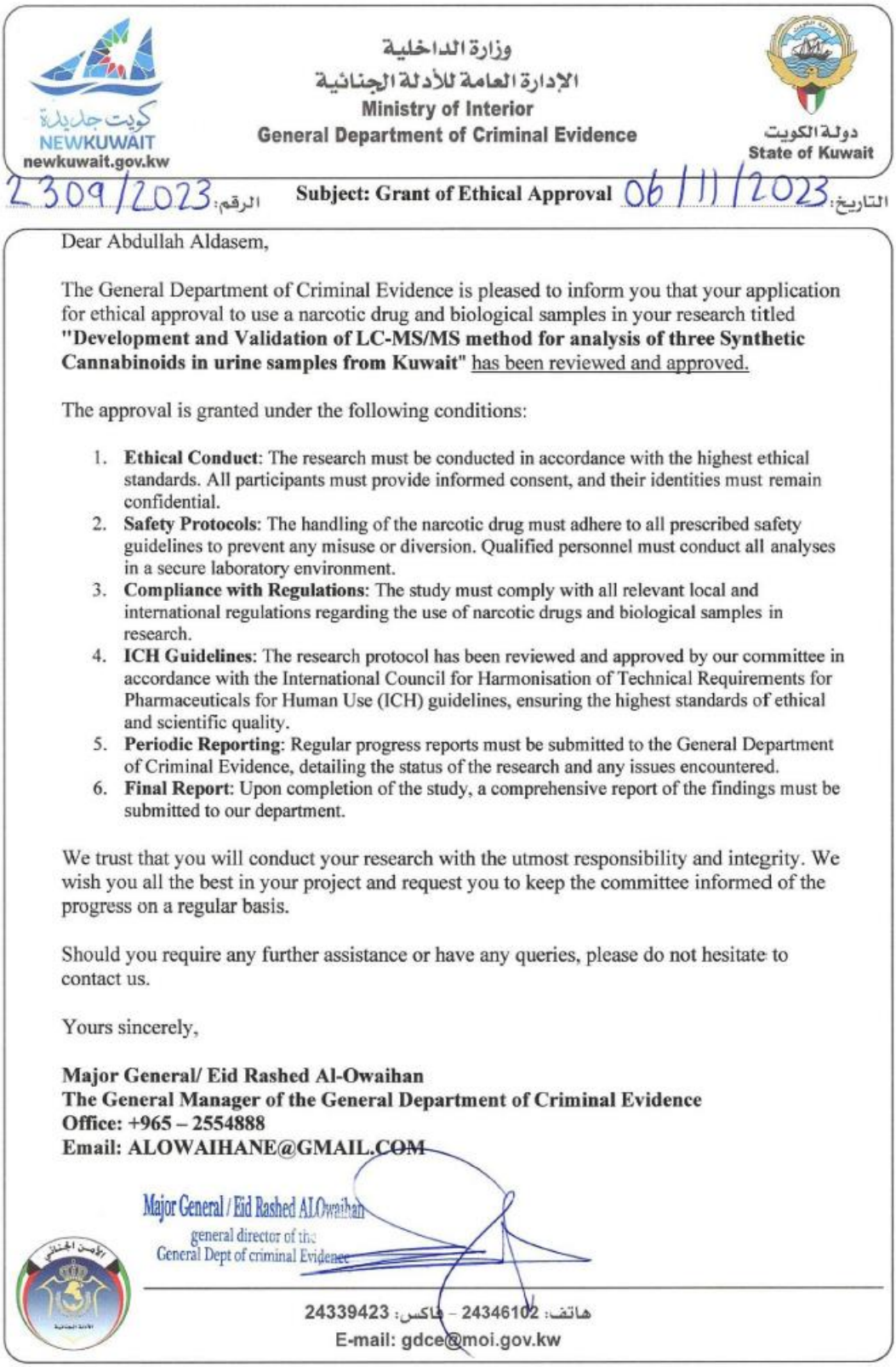
Ethical Approval for Chapter 3



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الإدارة العامة للأدلة الجنائية
Ministry of Interior
General Department of Criminal Evidence

دولة الكويت
State of Kuwait

2309/2023 الرقم: **Subject: Grant of Ethical Approval** 06/11/2023 التاريخ:

Dear Abdullah Aldasem,

The General Department of Criminal Evidence is pleased to inform you that your application for ethical approval to use a narcotic drug and biological samples in your research titled **"Development and Validation of LC-MS/MS method for analysis of three Synthetic Cannabinoids in urine samples from Kuwait"** has been reviewed and approved.

The approval is granted under the following conditions:

1. **Ethical Conduct**: The research must be conducted in accordance with the highest ethical standards. All participants must provide informed consent, and their identities must remain confidential.
2. **Safety Protocols**: The handling of the narcotic drug must adhere to all prescribed safety guidelines to prevent any misuse or diversion. Qualified personnel must conduct all analyses in a secure laboratory environment.
3. **Compliance with Regulations**: The study must comply with all relevant local and international regulations regarding the use of narcotic drugs and biological samples in research.
4. **ICH Guidelines**: The research protocol has been reviewed and approved by our committee in accordance with the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines, ensuring the highest standards of ethical and scientific quality.
5. **Periodic Reporting**: Regular progress reports must be submitted to the General Department of Criminal Evidence, detailing the status of the research and any issues encountered.
6. **Final Report**: Upon completion of the study, a comprehensive report of the findings must be submitted to our department.

We trust that you will conduct your research with the utmost responsibility and integrity. We wish you all the best in your project and request you to keep the committee informed of the progress on a regular basis.

Should you require any further assistance or have any queries, please do not hesitate to contact us.

Yours sincerely,

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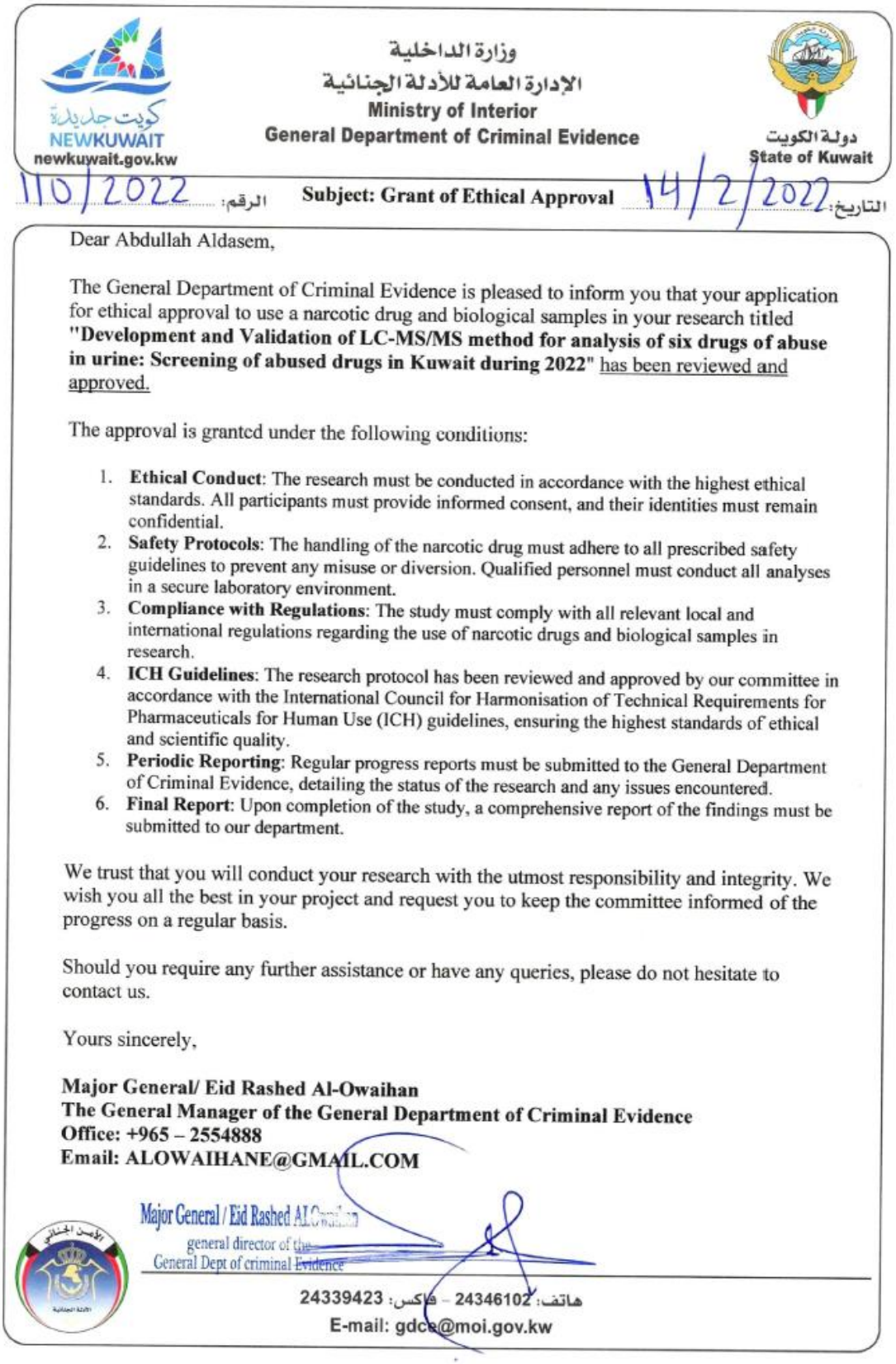
Ethical Approval for Chapter 4



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State of Kuwait

الرقم: 110/2022 Subject: Grant of Ethical Approval التاريخ: 14/2/2022

Dear Abdullah Aldasem,

The General Department of Criminal Evidence is pleased to inform you that your application for ethical approval to use a narcotic drug and biological samples in your research titled **"Development and Validation of LC-MS/MS method for analysis of six drugs of abuse in urine: Screening of abused drugs in Kuwait during 2022"** has been reviewed and approved.

The approval is granted under the following conditions:

1. **Ethical Conduct**: The research must be conducted in accordance with the highest ethical standards. All participants must provide informed consent, and their identities must remain confidential.
2. **Safety Protocols**: The handling of the narcotic drug must adhere to all prescribed safety guidelines to prevent any misuse or diversion. Qualified personnel must conduct all analyses in a secure laboratory environment.
3. **Compliance with Regulations**: The study must comply with all relevant local and international regulations regarding the use of narcotic drugs and biological samples in research.
4. **ICH Guidelines**: The research protocol has been reviewed and approved by our committee in accordance with the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines, ensuring the highest standards of ethical and scientific quality.
5. **Periodic Reporting**: Regular progress reports must be submitted to the General Department of Criminal Evidence, detailing the status of the research and any issues encountered.
6. **Final Report**: Upon completion of the study, a comprehensive report of the findings must be submitted to our department.

We trust that you will conduct your research with the utmost responsibility and integrity. We wish you all the best in your project and request you to keep the committee informed of the progress on a regular basis.

Should you require any further assistance or have any queries, please do not hesitate to contact us.

Yours sincerely,

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