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Comparison of sample preparation techniques for the detection and quantification of twenty-three drugs in oral fluid

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SCHOOL OF MEDICINE

Thesis

**COMPARISON OF SAMPLE PREPARATION TECHNIQUES FOR THE
DETECTION AND QUANTIFICATION OF TWENTY-THREE DRUGS IN
ORAL FLUID**

by

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**COMPARISON OF SAMPLE PREPARATION TECHNIQUES FOR THE
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ABSTRACT

Forensic toxicology is a branch of science that involves the analysis of drugs and other substances in biological fluids and tissues such as blood, urine, and oral fluid to aid medical or legal investigation of death, poisoning, and drug use. Due to the various components of different matrices, efficient and effective sample preparation techniques are necessary for reliable and accurate analysis. Following sample clean-up, a sensitive, specific, and robust method is ideal for consistent detection, identification, and quantitation of analytes. With the rise of drug abuse, there is a growing need to develop a single method that can target multiple classes of drugs quickly and effectively.

This study validated two different sample preparation techniques for the detection and quantitation of six drug classes comprised of twenty-three drugs and metabolites in oral fluid. The drug classes were as follows: amphetamines, local anesthetics, opioids, hallucinogens, antidepressants, and novel psychoactive substances (NPS). Amphetamines used were amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxy-N-ethylamphetamine (MDEA), and 3,4-methylenedioxymethamphetamine (MDMA). Local anesthetics contained benzocaine (BZE), cocaine, and lidocaine. Opioids included codeine, methadone, morphine, 6-monoacetylmorphine (6-MAM), fentanyl, and oxycodone. Hallucinogens

included lysergic acid diethylamide (LSD) and phencyclidine (PCP). Antidepressants were amitriptyline, citalopram, fluoxetine, and trazodone. Lastly, NPS included ethylone, α -pyrrolidinopentiophenone (α -PVP), and 2,5-dimethoxy-4-iodophenethylamine N-(2-methoxybenzyl) (25I-NBOMe). Supported liquid extraction (SLE) and solid phase extraction (SPE) were assessed followed by confirmatory analysis by liquid chromatography (LC)-tandem mass spectrometry (MS/MS).

Both methods were validated according to guidelines in the Standard Practices for Method Validation in Forensic Toxicology set by the American Academy of Forensic Science (AAFS) Standards Board (ASB). Parameters assessed include calibration model, bias, precision, limit of detection (LOD), limit of quantitation (LOQ), dilution integrity, ion suppression/enhancement, interference studies, and stability. Matrix recovery was added as another parameter. All calibration models were 0.99 or greater and all compounds were stable for at least 72 hours. Bias, precision, LOD, LOQ, dilution integrity, and interferences were similar between both methods. SLE yielded slightly better LOD and LOQ values. SLE had greater values of matrix recovery as well as lower levels of ionization suppression/enhancement.

Overall, SLE was determined to be the better method of sample preparation for this panel of drugs in oral fluid. Not only did it yield higher values for several of the parameters assessed but it also was more efficient (1 hour versus 2 hours) while using less solvent.

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LIST OF ABBREVIATIONS

μg	Microgram
μL	Microliter
25I-NBOMe	N-(2-methoxybenzyl)-2,5-dimethoxy-4-iodophenethylamine
6-MAM	6-monoacetylmorphine
AAFS	American Academy of Forensic Science
ACN	Acetonitrile
API	Atmospheric Pressure Ionization
BZE	Benzoyllecgonine
°	Celsius
CE	Collision Energy
CNS	Central Nervous System
CV	Coefficient of Variation
CXP	Cell Exit Potential
DC	Direct Current
DCM	Methylene Chloride, Dichloromethane
DI	Deionized
DP	Declustering Potential
ESI	Electrospray Ionization
H ₂ NaO ₄ P*H ₂ O	Sodium Phosphate Monobasic Monohydrate
HPLC	High Performance Liquid Chromatography
IPA	2-Propanol, Isopropanol

ISTD	Internal Standard
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantitation
LSD	Lysergic Acid Diethylamide
m	Mass
MDA	3,4-Methylenedioxyamphetamine
MDEA	3,4-Methylenedioxy-N-ethylamphetamine
MDMA	3,4-Methylenedioxymethamphetamine
MeOH	Methanol
mg	Milligram
Min	Minute
mL	Milliliter
mM	Millimolar
mm	Millimeter
MS	Mass Spectroscopy
MS/MS	Tandem Mass Spectroscopy
Na ₂ HPO ₄	Anhydrous Sodium Phosphate Dibasic
NAIU	Monoamine Oxidase Inhibitor
NFLIS	National Forensic Laboratory Information System
ng	Nanogram
NH ₄ OH	Ammonium Hydroxide

NPS	Novel Psychoactive Substances
PCP	Phencyclidine
psi	Pound-Force Per Square Inch
Q	Quadrupole
Qual	Qualitative
Quant	Quantitation
R ²	Correlation Coefficient
RF	Radio Frequency
SAMHSA	Substance Abuse and Mental Health Services Administration
SLE	Supported Liquid Extraction
SMx	Special Matrix
SPE	Solid Phase Extraction
SSRI	Selective Serotonin Reuptake Inhibitor
TCA	Tricyclic Antidepressant
U.S.A	United States of America
UCT	United Chemical Technologies
UFLC	Ultra-Fast Liquid Chromatogram
UNODC	United Nations Office on Drugs and Crime
v	Volume
V	Voltage
z	Charge
α-PVP	Alpha-Pyrrolidinopentiophenone

1. INTRODUCTION

1.1. Background

Drugs are used for a myriad of reasons: to relieve pain, to treat or cure illnesses, to promote well-being, or to produce other effects. Upon ingestion, a drug is capable of inducing a physiological change to the body.¹ Desired effects upon consumption can lead to abuse, increasing prevalence in forensic casework. Forensic toxicology is the analysis of bodily fluids such as blood, urine, vitreous humor and tissues for toxic substances which can include drugs and poisons.² For confirmatory testing, the process begins with sample preparation. There are several techniques that range from simple to rigorous clean-up of the matrix. Volatile substances can be separated from an aqueous matrix by heating it in a sealed container.² Protein precipitation uses inorganic acids or solvents such as tungstic acid, acetone, chloroform, or methanol to physically remove proteins from the matrix.^{2,3} Supported liquid extraction and solid phase extraction are more robust methods of sample preparation that can remove not only proteins but also other compounds including drugs.² Certain compounds may extract better with one technique over another. Certain techniques can target a diverse group of compounds while others are more specific. Choosing the ideal sample preparation technique for the situation can lead to successful analysis.

For this research, two different sample preparation techniques were assessed: supported liquid extraction and solid phase extraction. SPE is a commonly used and well-established technique while SLE is recent but powerful technique for sample extraction.

Both techniques can target multiple classes of drugs while removing the matrix components.

Typical biological fluids analyzed for forensic toxicology are blood and urine. This project explored oral fluid as an alternative matrix. Oral fluid (saliva) is produced by mainly three glands in the mouth: the parotid, submaxillary, and sublingual.⁴ It is a much cleaner matrix when compared to blood with a low protein content of approximately 0.3%.⁴ The collection of oral fluid is not as invasive as collection of blood or urine. Unlike urine, it also cannot be substituted by common liquids or chemicals. Oral fluid analysis has become a growing area of interest for testing drugs of abuse. Screening and confirmation testing has been done using oral fluid in five Australian states.⁵ According to the Substance Abuse and Mental Health Services Administration (SAMHSA), federal agencies can collect oral fluid as an alternative specimen when someone is unable to provide a sufficient urine specimen.⁶

Collection of oral fluid can be done with several different methods. A person can simply expectorate or spit into a collection tube but this may result in a viscous fluid with food contaminants.⁴ Commercial collection devices that use a diluent such as an extraction buffer can help create an easier matrix to work with.⁴ Certain commercial devices may have better recovery for specific drugs or classes of drugs than others. Oral fluid production can be stimulated by agents such as gum or citric acid which can also affect the pH or concentration of the drug in the mouth.⁴ Additionally, certain drugs can also increase or

decrease oral fluid production such as codeine, amphetamines, and antidepressants.⁴ Ultimately, it can depend on the individual as well.

The concentration of drugs in oral fluid is reported to be similar to that of blood and plasma.^{4,7} The detection of drugs in oral fluid can be affected by different factors such as pKa, physical size, degree of protein binding, and lipophilicity of the drug.⁸ The most common method of drug transport from blood to oral fluid is passive diffusion.⁹ Typically, the parent drug is found rather than the metabolite because it is more lipid soluble.⁸ Its lipophilicity allows the parent drug to diffuse through the capillary and acinar cell membranes into the oral fluid.⁸ Drugs that are weak bases are found in higher concentrations in oral fluid than in plasma due to ion trapping.¹⁰ Concentrations of specific drugs in oral fluid will vary between each class and type since they have their own unique structures that affect size, polarity, and lipophilicity. In a study performed by Verstraete, amphetamine was detectable for 20 to 50 hours while cocaine was only found for 5 to 12 hours in oral fluid.¹¹

This project included twenty-three different drugs for extraction by SLE and SPE. The classes of drugs included amphetamines, local anesthetics, opioids, hallucinogens, antidepressants, and novel psychoactive substances. It is important to be able to test for multiple drugs in one method. Abusers may be taking multiple illicit drugs at once. Some may be ingesting prescription drugs as well as illicit drugs. Victims of sexual assault can

have multiple drugs in their system. In forensic toxicology casework, testing for multiple drugs at once can expedite analysis and prevent or aid in backlogs.

1.2. Drugs

1.2.1 Amphetamines

Amphetamines are a group of synthetic drugs derived from phenethylamines, which are structurally similar to adrenaline.¹² These drugs illicit varying degrees of sympathomimetic activity when ingested by mimicking the actions of endogenous neurotransmitters that stimulate the sympathetic nervous system or by affecting the release of endogenous neurotransmitters or inhibiting their reuptake.² These central nervous stimulants (CNS) were first designed for medicinal use to treat narcolepsy and depression but their ability to alleviate fatigue, elevate mood, increase confidence, and produce euphoria resulted in their abuse.² This class of drugs include amphetamine, methamphetamine, and their methylenedioxy analogs: 3,4-methylenedioxyamphetamine, 3,4-methylenedioxymethamphetamine, and 3,4-methylenedioxy-N-ethylamphetamine.²

Currently, amphetamines are used clinically to treat exogenous obesity through appetite suppression, attention deficit disorder, and narcolepsy.² The United Nations Office on Drugs and Crime (UNODC) World Drug Report 2019 show the steady yearly rise of amphetamine use since 2002 with methamphetamine as the drug of choice.¹³ According to the 2001-2017 National Forensic Laboratory Information System (NFLIS) Special Report of Methamphetamine, cocaine and cannabis use have declined through the years while

methamphetamine use has increased.¹⁴ Similarly, MDA and MDMA have become more popular as drugs of abuse due to their hallucinogenic and psychoactive properties.²

1.2.2. Local Anesthetics

Local anesthetics interrupt neural conductivity by inhibiting the influx of sodium ions through channels or ionophores within neuronal membranes, resulting in loss of senses and motor function.¹⁵ All local anesthetics contain a lipophilic aromatic ring, intermediate ester or amide linkage, and tertiary amine, which contribute to their distinct clinical properties.¹⁵ Lidocaine and cocaine are examples of local anesthetics while benzoylecgonine is a major metabolite of cocaine.² Cocaine's limited clinical use is as topical administration as a local anesthetic in ear, nose, and throat surgery and in ophthalmologic procedures.² Lidocaine is the most widely used local anesthetic through topical use and intravenous injection.^{15,16}

Cocaine is a natural alkaloid isolated from the *Erythroxylon coca* plant, mainly found in the South American Andes.² It is abused by users to experience effects such as intense euphoria, heightened sexual excitement, psychic energy, and self-confidence.² Although medicinally used as a local anesthetic, illicit cocaine is used as a stimulant and can be found in two forms: hydrochloride salt and freebase (crack).² The purity of salt form is generally higher than 50% and can be cut with compounds to increase bulk such as mannitol, lactose, and sucrose or to stimulate the effects of cocaine such as caffeine,

ephedrine, lidocaine, and procaine.² According to the NFLIS-Drug 2018 Annual Report, cocaine is one of the top five most abused drugs.¹⁷

1.2.3. Opioids

Opioid is a general term for alkaloids derived from the opium poppy, *Papaver somniferum*, their synthetic analogues, and compounds synthesized in the body that bind to the opioid receptors found in the brain.¹⁸ Opioid use results in an analgesic effect by blocking the transmission of painful stimuli as well as a state of euphoria.² The primary use for opioids is pain management for postoperative analgesia and chronic pain associated with cancer or other terminal illnesses.² Other uses include cough suppressant, sedation, and antidiarrheal; methadone can be used for detoxification and to lessen opioid withdrawal symptoms.² Risks of opioid use include respiratory depression, hypothermia, seizure, hypotension, and coma.²

Natural opioids include morphine and codeine, which can be found in the latex or milky substance from the poppy seed pod.¹ Heroin, hydrocodone, and oxycodone, which are derived from morphine, codeine, and thebaine, respectively, are examples of semi-synthetic opioids. 6-acetylmorphine is an active metabolite of heroin and can be detected in oral fluid.^{10,19} Synthetic opioids are designed to mimic the effects of naturally occurring opioids and include fentanyl and methadone.²

1.2.4. Hallucinogens

Hallucinogens are drugs that can alter one's perception of reality; they can also be referred to as psychedelic, psychotomimetic or psychotogen drugs.² Although there are several classes of drugs that can cause illusion, hallucinations, or delusions, psychedelics

differ in that they can produce states of altered perception, thought, and feeling². Hallucinogens, on the other hand, induce a heightened awareness of sensory input and diminished control over what is experienced.²

Phencyclidine is a dissociative anesthetic but it can also act as a stimulant, depressant, hallucinogen, and anesthetic depending on the dose, route of administration, personality of the individual, and genetic predisposition.² At low doses, PCP can cause perspiration, agitated behavior, anxiety, and dissociation; at moderate doses, rage, insomnia, fever, amnesia and at high doses, hypertension, catatonia, coma, and death.² Although there is a decline in PCP use over the years, it is still one of the top twenty-five identified drugs in 2018 according to the NFLIS-Drug 2018 Annual Report.¹⁷

Lysergic acid diethylamide is a semi-synthetic drug created from lysergic acid and dimethylamine.^{1,2} Lysergic acid is an alkaloid found in the grain parasitized by the fungus *Claviceps purpurea* and the similar compound lysergic acid amide can be found in morning glory seeds and the Hawaiian baby wood rose; both can be used to synthesize LSD.² LSD can illicit both physiological such as mydriasis, tachycardia, and hyperglycemia and psychological effects such as visual illusions and altered hearing.² It is extremely potent with low microgram dosages leading to hallucinogenic “trips” that can last up to fifteen hours.²⁰ Due to its potency, LSD is almost always diluted before ingestion; the colorless, odorless, and tasteless liquid can be impregnated onto blotter paper and microdots at dosages between 0.05 – 0.1 milligram (mg).² In the 1950s, LSD was used clinically to treat

alcoholism, opioid addiction and sexual disorders but present day, it has no accepted medical use.²

1.2.5. Antidepressants

Antidepressants are one of the most commonly prescribed drugs in the United States. In the United States alone, about nineteen million Americans suffer from depression.² The use of antidepressants amongst adults eighteen years of age or older increased fivefold during 1988 – 1994 and 2005 – 2008.²¹ Antidepressants are primarily used to treat depression but they can also be used to treat eating disorders, panic disorders, posttraumatic stress disorders, anxiety, and chronic pain.^{2,21}

Antidepressants can be classified into first-generation, second-generation, selective serotonin reuptake inhibitors (SSRIs) or third-generation.² First-generation antidepressants include tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs).^{2,22} TCAs all have a three-ring structure; amitriptyline is an example of a TCA.² They act by preventing the reuptake of either norepinephrine or serotonin while MAOIs inhibit their metabolism.²

Second-generation antidepressants such as trazodone do not have a similar basic structure but can work similarly to TCAs and also exhibit a sedating effect.² In the United States, SSRIs such as citalopram and fluoxetine are the most prescribed class of antidepressants.² They not only inhibit the reuptake of serotonin but also act on with a number of serotonin receptors.² Unlike TCAs, they are not adrenergic, antihistaminic or anticholinergic and are better suited for most individuals.² Third-generation

antidepressants include a number of chemically and pharmacologically different compounds.²

1.2.6. Novel Psychoactive Substances

To achieve “legal highs,” novel psychoactive substances are created in an effort to evade the law. NPS are generally analogues or chemical derivatives of already controlled substances made to produce similar effects but they can also have completely new structures.²² They can include a variety of different classes of drugs such as synthetic cannabinoids, cathinones, phenethylamines, and opioids.^{23,24} Ethylone, α -pyrrolidinopentiphenone, and 2,5-dimethoxy-4-iodophenethylamine N-(2-methoxybenzyl) examples of NPS.

Ethylone and α -PVP are both synthetic cathinones, derived from the naturally occurring cathinone found in the khat plant (*Catha edulis*).²³ Synthetic cathinones are β -ketoamphetamines with structures similar to dopamine and MDMA; they can produce properties similar to amphetamine and interact with serotonin.^{25,26} They were originally synthesized to treat depression and anorexia but due to their severe side effects and abuse potential, only bupropion is used medicinally in the United States and Europe.^{26,27} The use of synthetic cathinones can lead to stimulant and hallucinogenic effects and are often used in replace of MDMA and cocaine.²⁵ They also labeled as “bath salts,” “plant food,” “fertilizer,” or “not for human consumption.”^{25,26} As more regulations are put into place, the rise of NPS follows. In the NFLIS Special Report on Synthetic Cannabinoids and

Synthetic Cathinones, ethylone and α -PVP can both be found in the top twenty-five most frequently reported synthetic cathinones.²⁸

25I-NBOMe is part of an expanding class of NBOMes, which have the addition of a 2-methoxybenzyl functional group to the nitrogen of the phenethylamine backbone.²⁹ They are mainly sued for their hallucinogenic properties and have a high affinity for the serotonin 5-HT_{2A} receptor.^{30,31} The effects are similar to that of LSD and can be impregnated onto blotter paper.³² The drug can be taken sublingually, by insufflation, or through the buccal cavity.³² The use of 25I-NBOMe can lead to not only hallucinations but also tachycardia, hypertension, agitations, and seizures.²⁹ A clinical effect of 25I-NBOMe that can lead to death is excited delirium, which is described as delirium with agitation, violence, hyperactivity, and hyperthermia.³³ These effects can ultimately result in sudden cardiopulmonary arrest.³³

1.3. Sample Preparation

1.3.1. Supported Liquid Extraction

Supported liquid extraction is a technique that isolate compounds based on their affinity between two immiscible solvents.³⁴ It has a packed column made up of diatomaceous earth, which is a natural silica based sediment from under the sea.³⁴ Due to its polar nature and large irregular surface area, it is an ideal material for analytes to adsorb onto.³⁴ First, the sample should be prepared so that it is un-ionized. Then, it should be put onto the bed of the column and allowed to absorb completely. Next, a nonpolar organic solvent is applied to remove compounds of interest. Finally, pressure or vacuum can be applied to the column to push through all the eluent and collected for analysis.

1.3.2. Solid Phase Extraction

Solid phase extraction utilizes solid particles to separate components of a sample or mixture.³⁵ It was first developed during the 1960s and early 1970s but is still very popular to this day.² Columns generally have a purified silica base with different chemical groups such as hydrocarbon chains, phenyl groups, or polar groups covalently bonded to it.² Solvents are used to wash and elute the columns of the analyte of interest. First, the column must be conditioned with a number of solvents to wet the packing material. After, the sample can be loaded onto the column. Then solvents wash the column to rid of any unwanted compounds or materials. Finally, the analyte of interest can be eluted and prepared for analysis. SPE selectively allows certain molecules to bind to the packing material while eliminating carbohydrates, proteins, polar lipids, and other unwanted compounds from the final sample.²

1.4. Instrumentation Theory

1.4.1. Liquid Chromatography

To accurately and reliably detect and identify an analyte of interest, samples should be relatively pure. Qualitative and quantitative analysis of a mixture can lead to the wrong conclusions. Liquid chromatography allows for the separation of components in a mixture based on its affinity to the mobile and stationary phase. The sample is dissolved and carried through a solid stationary phase by the liquid mobile phase.³⁶ Compounds will be adsorbed onto the stationary phase. The LC column is tightly packed with solid material of varying size and composition. Compounds are separated based on their interactions with both the mobile and stationary phase. LC can be paired up with different types of detectors such as

ultraviolet-visible spectroscopy or mass spectrometry.³⁶ The components of an LC are the mobile phase reservoir/pumps, pump, injector, column, and detector.³⁶

In an LC, separation does not always happen isocratically, which is the use of an unchanging mobile-phase.² The mobile phase can be manipulated during the course of a run to improve chromatographic separation. The mobile phase is organic for normal phase separation while it is aqueous for reverse phase. Reverse phase is much more common for LC. Mixtures of solvents as well as gradients during the run can be utilized to aid in better separation. Commonly used solvents include water, methanol, acetonitrile, and tetrahydrofuran.²

The first packing material was made up of silica or alumina and were very polar; this was called normal phase.² Later, the packing material was further developed so that the silica base held chains of C₁₈; now, they can not only hold various chain lengths of hydrocarbons but also different chemical groups.²

Both mobile phase and stationary phase affect chromatographic separation. Molecules with a higher affinity towards the stationary phase travel more slowly while those with lower affinity will travel quickly, reaching the detector sooner. Additionally, the stationary phase particle size and column length can also impact separation.³⁶ Smaller particles lead to an improvement in separation efficiency but results in higher pressure. A longer column gives an analyte more time to elute which could lead to better separation but can lead to broadening of peaks.

1.4.2. Mass Spectrometry

Mass spectrometry is a technique that relies on the ionization and subsequent fragmentation of an analyte in the gas phase.³⁶ It can help identify a compound's structure through mass data analysis. First, the analyte must be a gaseous ion. Based on its mass (m) to charge (z) ratio, it can be isolated through the manipulation of magnetic and electrostatic fields.² The mass analyzer and detector can translate the ions into a signal that is read by the data system.² The basic components of a mass spectrometer include the sample inlet, ion source, mass analyzer, detector, and vacuum system.² The ion source, mass analyzer, and detector are kept under vacuum when in use.²

Before a sample is introduced to the mass spectrometer after the LC, the liquid sample must be converted to the gas phase and ionized through atmospheric pressure ionization (API).² Electrospray ionization (ESI) is the most common method of API.² This technique has three main components: the nebulizer, desolvation assembly, and mesh electrode or repeller.² A strong electric field is applied to the liquid sample, which results in the accumulation of charge and the breaking of the sample into highly charged droplets.³⁷ Heated nitrogen gas aid in converting the liquid sample into gas.^{2,36} As the solvent evaporates, droplets become smaller in size; repulsion forces between like charges break the surface tension of droplets and result in even smaller droplets.³⁶ This process repeats until ions are formed. Ions are drawn to capillary due to a charge difference and are lead into the mass analyzer.² ESI is a soft ionization technique, which results in more parent ion and less fragmentation.³⁶

The quadrupole is the most common mass analyzer used in MS. It consists of four parallel rods of hyperbolic section placed about a central axis.^{36,37} Two diagonal rods are connected to radio frequency (RF) while the other two are connected to direct current (DC).³⁶ Specific RF and DC allow ions with a certain mass-to-charge ratio (m/z) to pass through the analyzer without touching the rods and to the detector.³⁶

Tandem mass spectrometry utilizes three quadrupoles in a series as the mass analyzer.² The first (Q1) and third (Q3) quadrupoles act as mass spectrometers, utilizing both RF and DC; the second quadrupole (Q2), however, only uses RF and acts as a collision cell.³⁷ Q1 allows only the parent ion to pass through.^{2,37} An inert gas causes collisions between gas molecules in Q2 to create fragments of the parent ion, known as product ions.² Product ions formed in Q2 move onto Q3, which can scan for all the ions or only allow specific ions to the detector.² Tandem mass spectrometry can identify compounds with greater accuracy and reliability since it can isolate a single parent ion and its fragments.

1.5. Research Objective

The purpose of this research is to compare two different sample preparation techniques for the detection and quantitation of twenty-three different drugs in oral fluid. The analytes of interest included amphetamines, local anesthetics, opioids, hallucinogens, antidepressants, and novel psychoactive drugs. The goal was to develop a method that could efficiently target all the drugs while minimizing as much time, effort, and solvent use as possible. Method validation of each technique was done for both evaluation and comparison purposes according to the American Academy of Forensic Science Standards Board final draft of Standard Practices for Method Validation in Forensic Toxicology.³⁸

Evaluated parameters include calibration model, bias and precision, matrix recovery, limit of detection, limit of quantitation, carryover, dilution integrity, ionization suppression/enhancement, and stability.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Standards and Reagents

Drug and deuterated standards were purchased from Cerilliant (Round Rock, Texas, U.S.A), Cayman Chemical Company (Ann Arbor, Michigan, U.S.A.), and Toronto Research Chemicals (North York, Ontario, Canada). Table 1 contains the list of drugs, lot numbers, and source of purchase. Optima grade methanol (MeOH), optima grade acetonitrile (ACN), optima grade isopropanol (2-propanol, IPA), optima grade methylene chloride/dichloromethane (DCM) and American Chemical Society (ACS) certified ammonium hydroxide (NH₄OH) were purchased from Fischer Scientific (Fair Lawn, New Jersey, U.S.A). ACS reagent grade glacial acetic acid, anhydrous sodium phosphate dibasic (Na₂HPO₄), and sodium phosphate monobasic monohydrate (H₂NaO₄P*H₂O) were purchased from Acros Organics (New Jersey, U.S.A). All deionized (DI) water used during this research was Milli-Q water from an Ultrapure (type 1) water system from Millipore Sigma (Burlington, Massachusetts, U.S.A). 1% ammonium hydroxide aqueous, 1% hydrochloric acid in methanol, 100 mM (millimolar) phosphate buffer (pH 6.0), 100 mM glacial acetic acid, 78/20/2 (v/v/v) DCM/IPA/NH₄OH, and 95/5 (v/v) DCM/IPA reagents were made in the laboratory. 0.1% formic acid in water and 0.1% formic acid in ACN were prepared to use as mobile phases A and B.

Table 1: Lot Numbers for Drug Standards

Compound	Lot #
Amphetamine	FE05241804
Methamphetamine	FE08101708

Compound	Lot #	
MDA	FE03161701	0523071
MDEA	FE04021801	
MDMA	0518489	
Benzoylecgonine	FE01061604	
Cocaine	FE07191702	
Lidocaine	FN08101706	
Codeine	0523684	
Methadone	0505453	
Morphine	FE6231704	
6-MAM	0535035	
Fentanyl	FE06151802	
Oxycodone	0506358	
LSD	FE12141601	
PCP	FE06201602	
Amitriptyline	FN06131706	
Citalopram	FN08141804	
Fluoxetine	FN06281603	
Trazodone	FN07251703	
Ethylone	FE06301504	
Alpha-PVP	0517087	
25I-NBOMe	0529372	
Amphetamine-d6	FE08301801	
Methamphetamine-d5	FE03411801	
MDA-d5	FE05031601	
MDEA-d5	FE08251701	
MDMA-d5	0504233	
Benzoylecgonine-d8	FE06071702	
Cocaine-d6	0491697	
Lidocaine-d6	27-GHZ-14-1	
Codeine-d6	FE06221701	
Methadone-d9	FE01251601	
Morphine-d6	FE10241701	
6-MAM-d6	FE10061501	FE08101706
Fentanyl-d5	FE07281604	
Oxycodone-d6	FE09201701	
LSD-d3	FE06301503	

Compound	Lot #	
PCP-d5	FE12271704	
Amitriptyline-d3	FN04151601	
Citalopram-d6	FN01301701	
Fluoxetine-d6	FN12281701	
Trazodone-d6	FN08051801	
Ethylone-d5	FE12131601	
Alpha-PVP-d8	0503495	
25I-NBOMe-d3	FE05021601	

2.1.2. Extraction Columns and Buffer

ISOELUTE SLE+ (1 mL) columns were purchased from Biotage, LLC (Charlotte, North Carolina, U.S.A). Clean Screen® DAU (CSDAU133) SPE columns were purchased from United Chemical Technologies (UCT) (Bristol, Pennsylvania, U.S.A.). Extraction buffer (MS-EXTBUF) was purchased from Immunoanalysis Corporation (Pomona, California, U.S.A).

2.1.3. Specimens

Special Matrix (SMx) Oral Fluid was purchased from UTAK (Valencia, California, U.S.A). Drug-free oral fluid was donated and collected following approved Institutional Review Board requirements at Boston University School of Medicine Biomedical Forensic Science Program (Boston, Massachusetts, U.S.A.).

2.1.4. Equipment

The pH of the phosphate buffer (pH 6.0) was verified using an Oakton pH 700 meter from Fisher Scientific. Sample preparation was aided by UCT Positive Pressure manifold. Samples were dried down using an Organomation (Berlin, Massachusetts) Multivap Nitrogen Evaporator.

2.1.5. Instrumentation and Software

A Shimadzu (Kyoto, Japan) Ultra-Fast Liquid Chromatogram (UFLC) system and a SCIEX (Framingham, Massachusetts, U.S.A.) 4000 QTRAP tandem mass spectrometer with ESI was used for all analysis during the research. The column used was a Phenomenex (Torrance, California, U.S.A) Kinetex F5 2.6 μ 100 Å 50 x 3.0 mm. SCIEX Analyst® (version 1.6.2) software was used for all data collection and SCIEX MultiQuant™ 3.0 (version 3.0.5373.0) software was used for all quantitation.

2.2. Methods

2.2.1. Sample Preparation

2.2.1.1. Biotage SLE+

The following method was adapted from Courtney McGowan's thesis project.³⁹ Samples were prepared in 12 x 75 millimeter (mm) test tubes. Oral fluid was vortexed with buffer in a 1:3 ratio for at least 15 seconds. 250 microliters (μ L) of the oral fluid mixture was spiked with 20 μ L of the appropriate working stock solution and 25 μ L of 300 ng/mL (nanogram/milliliter) internal standard stock solution. 250 μ L of 1% ammonium hydroxide aqueous solution (made fresh daily) was added to each sample and then vortexed for at least 30 seconds.

After, samples were loaded onto the Biotage SLE+ columns using plastic pipettes. Pressure was applied using a bulb so that the sample can be absorbed by the bed of the column. The sample was absorbed by the column for 5 minutes (min). Before elution, 100 μ L of 1% hydrochloric acid in methanol (made fresh daily) was pipetted into each collection test tube to stabilize analytes when drying. 3 mL of elution solvent 95:5 (v:v)

DCM/IPA was added to columns and allowed to flow by gravity for five minutes. Another 3 mL of elution solvent was added and allowed to flow by gravity for five minutes. Then positive pressure was applied to the columns 10 – 20 seconds to collect the remaining elution solvent. The collected eluent was dried under nitrogen at ≤ 40 °C (celcius). Dry samples were reconstituted with 125 μ L of 0.1% formic acid in 95:5 (v:v) water:ACN and vortexed for at least 30 seconds. The total sample preparation technique took approximately 1 hour.

Table 2: Solvent Use for SLE

SLE Solvent Use	
Preparation	250 μ L of 1% Ammonium Hydroxide
	100 μ L of 1% Methanolic HCl
Elution	6 mL of DCM/IPA
Reconstitution	125 μ L of Mobile Phase
Total	6.475 mL

2.2.1.2 UCT Clean Screen® DAU SPE

The following method was adapted from Courtney McGowan’s thesis project.³⁹ Samples were prepared in 12 x 75 mm test tubes. Oral fluid was vortexed with buffer in a 1:3 ratio for at least 15 seconds. 200 μ L of the oral fluid mixture was spiked with 20 μ L of the appropriate working stock solution and 25 μ L of 300 ng/mL internal standard stock solution. 200 μ L of 100 mM phosphate buffer (pH 6.0) was added to each sample and vortexed for at least 30 seconds.

SPE columns were conditioned with 2 mL of methanol, 2 mL of deionized water, and 2 mL of 100 mM phosphate buffer (pH 6.0). Each solution was allowed to flow by gravity before the addition of the next solvent. The sample was loaded onto the columns

and allowed to flow by gravity. Columns were washed with 2 mL DI water, 1 mL of 100 mM acetic acid, and 1 mL of methanol. The column was dried via five-minute full flow, >25 pound-force per square inch (psi) on the vacuum manifold. Then, 3 mL of elution solvent 78:20:2 (v:v:v) DCM:IPA:NH₄OH was added to the column on regulated flow on the vacuum manifold or allowed to flow by gravity. The collected eluent was dried under nitrogen at ≤ 40 °C. Dry samples were reconstituted with 125 μL of 0.1% formic acid in 95:5 (v:v) water:ACN and vortexed for at least 30 seconds. The total sample preparation technique took approximately 2.5 hour.

Table 3: Solvent Use for SPE

SPE Solvennt Use	
Preparation	200 μL of 100 mM Phosphate Buffer
Conditioning	2 mL of Methanol
	2 mL of DI Water
	2 mL of 100 mM Phosphate Buffer
Wash	2 mL of DI Water
	1 mL of 100 mM Acetic Acid
	1 mL of Methanol
Elution	3 mL of DCM/IPA/NH ₄ OH
Reconstitution	125 μL of Mobile Phase
Total	13.325 mL

2.2.2. Standard Preparation

Drug standards at a concentration of 1 mg/mL were purchased and used to create three initial working stocks at 100,000 ng/mL in methanol, which can be seen in Table 4. These three stock solutions were then combined and diluted to make three additional working stocks at 10,000 ng/mL, 1,000 ng/mL, and 100 ng/mL. These three were used to create solutions for spiking such as calibrators and quality controls.

Table 4: Stock Solution Analytes

Stock	Analytes
Stock 1	Amphetamine, Methamphetamine, MDA, MDEA, MDMA, Amitriptyline, Citalopram, Fluoxetine, Trazodone
Stock 2	Codeine, Methadone, Morphine, Oxycodone, 6-MAM, PCP, Benzoyllecognine, Cocaine, Lidocaine
Stock 3	LSD, Ethylone, Alpha-PVP, 25I-NBOMe & Fentanyl

Seven calibrations points were prepared for both SLE and SPE method validation at concentrations of 2, 5, 10, 25, 50, 150, and 250 ng/mL. Low, medium, and high quality controls were made at 6, 125, and 200 ng/mL. Additionally, working stock solutions used for matrix recovery, limit of detection, limit of quantitation, and dilution integrity were made from the three solutions mentioned earlier.

Deuterated drug standards were used to make the internal standard stock (ISTD) solution. All but lidocaine-d6 was purchased at 100 µg/mL. Lidocaine-d6 was purchased as a 1 mg standard therefore, 1 mL of methanol was added to create a 1 mg/mL standard. All deuterated compounds were combined to create an ISTD working stock solution at 300 ng/mL.

2.2.3. Method Validation

The method for SLE and SPE analysis was validated according to guidelines set by ASB³⁸. The following parameters were assessed: calibration model, bias and precision, limit of detection, limit of quantitation, carryover, dilution integrity, interference studies,

ionization suppression/enhancement, and stability; matrix recovery was included as an additional parameter to assess. Accepted values fell within $\pm 20\%$ of expected values.

2.2.3.1. Calibration Model

For any quantitation method, a calibration model must be determined for analytes of interest. The working range demonstrates that there exists a relationship between the signal response and analyte concentration in the sample.³⁸ The appropriate mathematical model that best represents the correlation is the calibration model. At least, six calibration points should be included in the model; they should also be evenly distributed over the curve. Calibrators should be based on the expected or commonly encountered concentrations of day-to-day samples. A minimum of five replicates for each point must be analyzed.³⁸ A simple linear regression or weighted least squares model can be used to best illustrate the relationship.³⁸

2.2.3.2. Bias and Precision

Bias is a measurement of how close the calculated concentration is to the known “true” value while precision is how close repeated measurements of the same concentration are to each other.³⁸ These values were determined from the same set of data. Each of the five runs had its own prepared calibration curve and triplicates of low, middle, and high (6, 125, and 200 ng/mL) quality controls.

Acceptable values must be calculated to be within $\pm 20\%$ of the expected concentration. Bias was calculated for each concentration from every run and an average bias was determined. Precision was calculated within and between each run for all concentrations. The following equations used:

$$\text{Coefficient of variation (\%CV)} = \left(\frac{\text{standard deviation}}{\text{average}} \right) \times 100$$

$$\text{Bias(\%)} = \left(\frac{\text{grand mean of calculated concentration} - \text{nominal concentration}}{\text{nominal concentration}} \right) \times 100$$

$$\text{Within - run \%CV} = \left(\frac{\text{standard deviation of single run of samples}}{\text{average calculated concentration of single run of samples}} \right) \times 100$$

$$\text{Between - run \%CV} = \left(\frac{\text{standard deviation of grand average for each concentration}}{\text{grand average for each concentration}} \right) \times 100$$

2.2.3.3. Matrix Recovery

Matrix recovery evaluates the efficiency of the extraction method for the analyte of interest. Pre-extraction and post-extraction spiked samples at low (20 ng/mL) and high (175 ng/mL) concentrations were assessed. The equation used to calculate matrix recovery can be seen below:

$$\text{Recovery (\%)} = \left(\frac{\text{Average Area of Pre Extraction Spikes}}{\text{Average Area of Post Extraction Spikes}} \right) \times 100$$

2.2.3.4. Ionization Suppression/Enhancement

Analytes of interest can undergo ionization suppression/enhancement from co-eluting compounds.³⁸ Neat standards at low (20 ng/mL) and high (175 ng/mL) concentrations were injected six times alongside duplicates of ten different lots of matrix spiked post-extraction. The equation used to calculate ionization suppression/enhancement can be seen below:

$$\text{Ionization Suppression/Enhancement (\%)} = \left(\frac{\text{Average Area of Neat Standard}}{\text{Average Area of Post Extraction Spikes}} - 1 \right) \times 100$$

Negative values showed ion suppression while positive values indicated ion enhancement.

2.2.3.5. Limit of Detection and Limit of Quantitation

The limit of detection is the lowest concentration that can be reliably distinguished from the blank matrix and identified by the method.³⁸ For three different lots of matrix, a calibration curve, series of duplicate low concentration samples, and duplicate double blanks were analyzed; quality control samples were also included. The LOD samples were at concentrations: 0.05, 0.1, 0.5, 1, and 2 ng/mL. LOD was determined by the concentration that reliably yielded a signal greater than “the average signal of the blank samples plus 3.3 times the standard deviation.”³⁸ The lowest concentration that can be reliably measured while having acceptable identification, bias, and precision is the limit of quantitation.³⁸

2.2.3.6. Carryover

Carryover is the detection of unwanted analyte in a sample after the analysis of a positive sample.³⁸ It should be assessed for both qualitative and quantitative methods to ensure accurate and reliable results. To evaluate the presence of carryover, a blank matrix sample was analyzed after the highest calibration point. Carryover was present if a signal greater than 10% of the lowest calibrator was detected in the blank matrix sample.

2.2.3.7. Dilution Integrity

Since it is possible to test samples that are outside of the established calibration curve, dilutions are necessary for accurate analysis. Two common dilutions (1:10 and 1:50) in triplicate with bias and precision calculations were evaluated to assess dilution integrity.

2.2.3.8. Interference Studies

Samples that contain compounds that are not target analytes can lead to interference in detection, identification, and quantification. Interfering compounds can include matrix

components, other drugs and metabolites, and internal standard.³⁸ Matrix interference was assessed. Three mixes, listed in Table 5, containing drugs commonly found in forensic toxicology casework were analyzed. Interference from product ions of drugs used and internal standards were also assessed.

First, the matrix was tested to see if it interfered with any of the compounds. Ten different lots were tested and did not cause any false positives for either method. Drug-free oral fluid spiked only with internal standard was analyzed next. To check if drug ions interfered with their internal standard ions, the highest calibration point was run without any internal standard. Lastly, three mixes that contain commonly encountered drugs in forensic casework was spiked into drug-free oral fluid.

Table 5: Mixes Used for Interference Assessment

Mix 1	Mix 2	Mix 3
Alpha-hydroxyalprazolam	Amobarbital	THC
Clonazepam	Butalbital	11-Hydroxy- Δ^9 -THC
7-aminoclonazepam	Phenobarbital	11-nor-9-Carboxy- Δ^9 -THC
Diazepam		AB-FUBINACA
Etizolam		AB-FUBINACA metabolite 3
Hydrocodone		AB-FUBINACA metabolite 2a
Norcocaine		AB-PINACA
		AB-PINACA pentatonic acid metabolite

2.2.3.9. Stability

Under certain circumstances, processed samples may not always be processed within the ideal time frame. It is important to evaluate how long a processed sample can be retained before it cannot be detected, identified, or accurately and reliably quantified.³⁸

Triplicate samples of low, medium, and high quality controls were assessed at the 0, 24, 48, and 72 hour time points. Samples were stored in the chilled autosampler of the LC while in between time points. At each time point, the analyte signal ratio to the internal standard was compared to the ratio at the zero hour.

2.2.4. LC-QTRAP Instrument Parameters

UFLC (Shimadzu, Kyoto, Japan) paired with a 4000 Q-Trap Electrospray Ionization Tandem Mass Spectrometry (ESI/MS/MS, SCIEX, Waltham, Massachusetts, U.S.A.) in positive ionization mode was used for all sample analysis. The injection volume was 5 μ L with a flow rate of 0.6 mL/min. The column was a Kinetex F5 2.6 μ 100 \AA 50 x 3.0 mm column (Phenomenex, Torrance, California, USA). A binary gradient of 0.1% Millipore water and acetonitrile was used for analysis. The gradient used during the LC method can be seen in Table 6.

Table 6: LC Binary Gradient

Time (min)	%A	%B
0.01	90	10
0.50	90	10
3.00	80	20
5.00	60	40
6.50	30	70
7.50	0	100
9.00	0	100
9.01	95	5
10.00	Stop	

Mass spectrometry parameters were determined through optimization of each analyte.³⁹ The following instrument settings were used: curtain gas 25 psi, collision gas

high, ion spray voltage 2500 voltage (V), temperature 550°C, and ion source gas 1 at 245.0 psi. An entrance potential voltage of 10 V was used for all analytes. Each analyte had a product ion for quantitation (quant) and a second ion for qualitative (qual) assessment while deuterated compounds had one product ion. Declustering potential (DP), collision energy (CE), and cell exit potential (CXP) values for all compounds are listed in Table 7.

Table 7: MS/MS Method Parameters

Compound ID	Q1 Mass (Da)	Q3 Mass (Da)	DP (V)	CE (V)	CXP (V)
Amphetamine qual	136.1	119.3	40	12.61	20.07
Amphetamine quant	136.1	91	40	22.16	15.7
Amphetamine-d6	142.2	93.1	40	21.31	15.41
Methamphetamine quant	150.1	91.1	51	25	16
Methamphetamine qual	150.1	119.3	51	15	10
Methamphetamine-d5	155.1	92.2	51	27	6
MDA qual	180.1	105.2	41	35	8
MDA quant	180.1	135.2	41	29	10
MDA-d5	185.1	168.1	26	15	10
MDEA quant	208.2	163.1	58	18.27	27.1
MDEA qual	208.2	135.3	58	29.71	22.9
MDEA-d5	213.2	163	57	19.15	27.17
MDMA quant	194.1	163.2	40	18	12
MDMA qual	194.1	133.1	40	28	10
MDMA-d5	199.1	165.2	61	19	14
Benzoylcegonine quant	290.1	168.1	70	27.93	29.12
Benzoylcegonine qual	290.1	77.3	70	79.72	13.61
Benzoylcegonine-d8	298.2	171.4	75	28.81	29.37
Cocaine quant	304.1	182	96	29	14
Cocaine qual	304.1	76.9	96	87	12
Cocaine-d3	307.1	185	76	29	16
Lidocaine quant	235.2	86.1	60	25.26	14.65
Lidocaine qual	235.2	58.1	60	52.43	8.66
Lidocaine-d6	241.2	86.2	60	25.26	14.65
Codeine qual	300.3	115.3	95	101.6	17.56
Codeine quant	300.3	152.1	95	86.6	25.05

Compound ID	Q1 Mass (Da)	Q3 Mass (Da)	DP (V)	CE (V)	CXP (V)
Codeine-d6	306.2	152.2	100	89.56	25.15
Methadone qual	310.3	265.3	60	21.02	47.82
Methadone quant	310.3	105.2	60	38.3	17.23
Methadone-d9	319.3	105.1	66	39	17.27
Morphine quant	286.3	152.1	91	99	12
Morphine qual	286.3	115.2	91	101	18
Morphine-d6	292.3	152	85	80.93	25.49
Fentanyl qual	337.2	188.3	86	33	14
Fentanyl quant	337.2	105.1	86	55	16
Fentanyl-d5	342.2	105.2	90	55.74	17.21
Oxycodone quant	316.2	298.5	80	26.81	23.57
Oxycodone qual	316.2	241.3	80	41.15	38.73
Oxycodone-d6	322.1	304.1	80	26.61	17.89
LSD quant	324.3	223.3	80	32.13	37
LSD qual	324.3	207.3	80	56.52	34.92
LSD-d3	327.3	226.3	85	33.36	37.46
PCP quant	244.2	86.2	46	17.29	14.39
PCP qual	244.2	91.1	46	41.39	15.69
PCP-d5	249.2	86.2	45	18.06	14.43
Amitriptyline quant	278.1	91	74	37.13	14.36
Amitriptyline qual	278.1	117.1	74	30.71	20.53
Amitriptyline-d3	281.2	91.3	75	36.14	14.76
Citalopram quant	325.2	109.1	80	37.19	17.12
Citalopram qual	325.2	262.4	80	27.35	43.87
Citalopram-d6	331.3	109.2	90	36.06	18.48
Fluoxetine quant	310.3	44.2	52	43.15	6.22
Fluoxetine qual	310.3	148.4	52	12.3	25.14
Fluoxetine-d6	316.3	44.2	60	43.28	5.84
Trazodone quant	372.3	176.2	85	34.56	31.16
Trazodone qual	372.3	148.2	85	48.71	24.7
Trazodone-d6	378.2	182.3	86	35.58	30.33
6-MAM quant	328	165.3	100	54.25	29.53
6-MAM qual	328	152.1	100	93.16	25.84
6-MAM-d6	334.1	211.1	100	37.02	35.28
Ethylone qual	222.2	204.2	64	26.3	29.46
Ethylone quant	222.2	174.2	64	18.7	35.16

Compound ID	Q1 Mass (Da)	Q3 Mass (Da)	DP (V)	CE (V)	CXP (V)
Ethylone-d5	227.2	179.1	64	28.25	30.46
Alpha-PVP qual	232.3	126.2	70	37.1	20.48
Alpha-PVP quant	232.3	91.2	70	31.78	15.79
Alpha-PVP-d8	240.1	77.1	88	71.33	12.22
25I-NBOMe quant	428.1	121.2	74	32.15	20.4
25I-NBOMe qual	428.1	91.1	74	73	14.17
25I-NBOMe-d3	431.1	124.2	125	53	10

3. RESULTS AND DISCUSSION

3.1. Calibration Model

An appropriate calibration model is necessary for the validation of any method and future analysis. A calibration model represents the correlation between the instrument signal response and analyte concentration based on the working range.³⁸

Working ranges are typically determined by looking at expected or commonly encountered drug concentrations. The same working range was used for all analytes and for both supported liquid extraction and solid phase extraction methods. Seven calibration points were used at 2, 5, 10, 25, 50, 150, and 250 ng/mL. All compounds except for codeine, morphine, and 6-acetylmorphine were determined to have a quadratic fit with $\frac{1}{x}$ weighting. Codeine, morphine, and 6-MAM have a linear fit with $\frac{1}{x}$ weighting.

The average correlation coefficient was calculated for each analyte and method. These values are listed in Table 8. R^2 values for every analyte and method were all above 0.99 or greater. Solid phase extraction generated more calibration curves with a R^2 value of 0.999 or greater than supported liquid extraction. Amphetamine is used as the example calibration curve, which are shown for both methods in Figure 1 and 2.

Figure 1: SLE Calibration Curve for Amphetamine

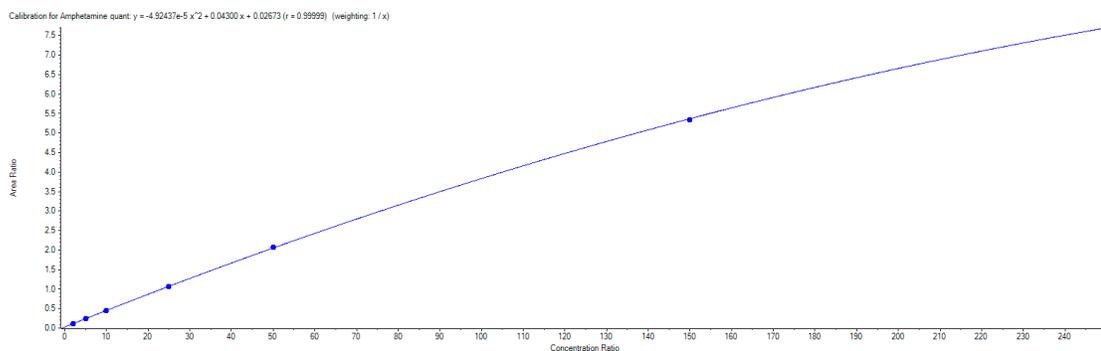


Figure 2: SPE Calibration Curve for Amphetamine (SPE)

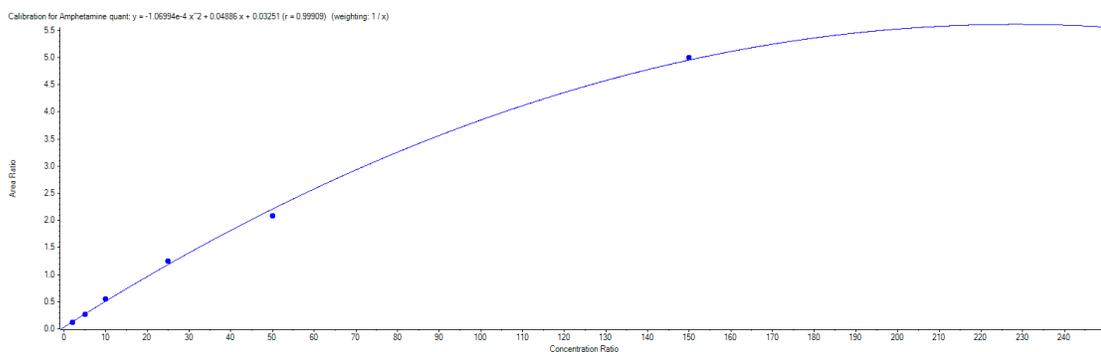


Table 8: Average Calibration Model Correlation Coefficient (R²) Values

Compound	SLE Average R ²	SPE Average R ²
Amphetamine	0.99999	0.99924
Methamphetamine	0.99929	0.99927
MDA	0.99992	0.99970
MDEA	0.99787	0.99937
MDMA	0.99977	0.99885
Benzoylecgonine	0.99878	0.99963
Cocaine	0.99885	0.99947
Lidocaine	0.99954	0.99931
Codeine	0.99922	0.99621

Compound	SLE Average R ²	SPE Average R ²
Methadone	0.99678	0.99821
Morphine	0.99794	0.99967
Fentanyl	0.99889	0.99941
Oxycodone	0.99982	0.99900
6-MAM	0.99881	0.99912
LSD	0.99892	0.99795
PCP	0.99879	0.99902
Amitriptyline	0.99962	0.99904
Citalopram	0.99828	0.99921
Fluoxetine	0.99919	0.99853
Trazodone	0.99986	0.99980
Ethylone	0.99899	0.99948
Alpha-PVP	0.99971	0.99964
25I-NBOMe	0.99894	0.99905

3.2. Bias and Precision

According to ASB guidelines, bias is a measurement of how close the calculated concentration is to the known “true” value while precision is how close repeated measurements of the same concentration are to each other.³⁸ Both were determined from the same set of five separate calibration curves with triplicates of low, middle, and high concentration (6, 125, and 200 ng/mL) quality controls.

Values for both parameters must be within 20% of expected concentrations. Table 9 contains bias calculated for low, middle, and high quality controls. All values were within 20% for SLE and SPE analysis. Average within, between, and grand run precision values are in Table 10. All, except citalopram, were found to be <20%. The SPE method for citalopram yielded a 20.87% for the average between run. Average bias values were similar

between the two methods. Average within and between precision values for both methods were comparable but SLE generally had lower values for grand run precision.

Table 9: Average Bias Values

Compound	SLE Bias			SPE Bias		
	L	M	H	L	M	H
Amphetamine	0.83	2.97	1.50	0.25	1.15	0.83
Methamphetamine	-0.37	4.19	2.67	1.83	0.64	0.77
MDA	-1.14	-2.61	-5.25	0.27	-0.15	4.11
MDEA	0.32	5.74	6.25	1.57	3.92	0.74
MDMA	1.54	4.74	4.46	0.28	1.94	2.05
Benzoylcegonine	-1.36	1.64	2.31	-1.45	3.38	5.25
Cocaine	-1.04	6.93	7.70	-1.08	6.71	1.25
Lidocaine	-4.25	-1.65	2.49	2.81	8.15	8.22
Codeine	-0.22	2.84	2.98	6.12	1.10	2.94
Methadone	-0.52	4.30	6.14	5.00	2.68	7.20
Morphine	2.06	4.52	-0.39	-1.80	0.31	4.80
Fentanyl	1.64	6.18	3.18	2.73	1.85	4.17
Oxycodone	-2.04	5.88	2.41	2.88	0.02	5.35
6-MAM	4.46	0.76	4.77	3.44	-2.43	2.78
LSD	4.62	2.73	4.40	0.22	1.55	6.85
PCP	1.30	1.13	3.18	1.13	3.26	3.97
Amitriptyline	2.41	0.27	7.48	2.27	1.73	-2.05
Citalopram	0.78	6.12	8.73	1.79	3.05	-11.09
Fluoxetine	1.67	5.24	1.33	7.00	5.58	0.74
Trazodone	-0.17	4.44	1.77	5.10	2.60	4.53
Ethylone	3.78	-0.10	-0.89	2.72	1.33	6.00
Alpha-PVP	3.87	4.53	3.04	0.04	3.41	-0.66
25I-NBOMe	-4.59	4.68	4.25	-3.96	2.16	4.41

Table 10: Average Precision Values

Compound	Avg Within Precision %CV		Avg Between Precision %CV		Grand Run Precision %CV	
	SLE	SPE	SLE	SPE	SLE	SPE
Amphetamine	6.05	7.13	7.44	11.26	7.52	11.01
Methamphetamine	6.80	9.12	9.04	13.83	9.25	14.31
MDA	7.36	11.37	10.22	14.73	10.52	15.33
MDEA	5.81	5.06	8.53	7.80	8.82	7.79
MDMA	6.39	7.27	8.38	8.99	8.25	8.84
Benzoyllecgonine	7.94	5.60	10.13	10.18	10.31	10.42
Cocaine	5.69	6.60	6.99	7.94	7.82	7.85
Lidocaine	6.21	11.36	7.42	14.42	7.85	16.74
Codeine	7.85	6.79	12.51	8.62	12.28	8.77
Methadone	4.79	7.59	11.18	11.54	9.09	11.42
Morphine	8.34	8.86	12.77	12.50	13.27	12.66
Fentanyl	5.49	10.12	10.81	13.14	10.44	13.59
Oxycodone	8.56	9.40	9.46	15.07	9.56	15.43
6-MAM	9.02	6.87	11.96	7.59	11.26	8.20
LSD	6.53	8.08	9.74	10.37	9.60	10.66
PCP	5.20	5.75	7.50	9.31	7.51	9.61
Amitriptyline	5.79	11.60	7.45	12.26	7.98	12.58
Citalopram	6.63	11.62	7.87	20.87	7.86	27.79
Fluoxetine	6.77	11.83	7.91	14.41	8.05	14.37
Trazodone	5.31	9.24	8.95	12.44	8.87	12.38
Ethylone	6.65	10.01	8.63	13.73	8.63	14.81
Alpha-PVP	5.30	6.81	8.66	9.59	8.51	9.74
25I-NBOMe	6.25	7.47	9.30	9.53	9.99	10.00

3.3. Matrix Recovery

Matrix recovery details the efficiency of the extraction method for each analyte.

Pre-extraction and post-extraction spiked samples at low (20 ng/mL) and high (175 ng/mL)

concentrations were assessed. The % recoveries for low and high concentrations are listed in Table 11.

Most recovery values for SLE and SPE were between 80-120%; some values were outside this accepted range. SLE had better recovery values than SPE. For SPE, the recovery values for antidepressants were not as high as those of SLE. LSD and PCP could not be recovered well by either method.

Table 11: Matrix Recovery Values

Compound	SLE %Recovery		SPE %Recovery	
	Low	High	Low	High
Amphetamine	110.86	107.29	98.14	105.30
Methamphetamine	113.86	94.60	77.37	105.91
MDA	93.45	109.23	112.01	118.99
MDEA	120.20	103.71	92.57	101.49
MDMA	114.96	95.86	93.81	107.81
Benzoylcegonine	131.84	108.81	74.11	80.45
Cocaine	104.35	122.06	85.49	87.46
Lidocaine	108.60	99.26	101.66	91.45
Codeine	106.06	104.06	121.53	119.51
Methadone	121.46	98.05	108.77	98.98
Morphine	125.47	100.15	108.19	114.15
Fentanyl	122.18	103.00	58.08	70.11
Oxycodone	109.81	95.89	104.33	119.42
6-MAM	100.93	97.95	76.81	124.22
LSD	66.58	75.65	46.21	55.81
PCP	57.02	67.45	63.27	67.22
Amitriptyline	101.41	84.49	50.16	33.62
Citalopram	111.44	85.94	46.53	52.35
Fluoxetine	100.97	81.52	45.75	36.44
Trazodone	113.91	107.05	51.53	55.29
Ethylone	138.27	109.74	99.92	121.46
Alpha-PVP	143.21	98.71	83.16	81.97
25I-NBOMe	94.21	105.70	86.01	33.93

3.4. Ionization Suppression/Enhancement

Ionization suppression and enhancement occur from co-eluting compounds from the matrix or extraction method. Neat standards at low (20 ng/mL) and high (175 ng/mL) concentrations were injected six times alongside duplicates of ten different lots of matrix spiked post-extraction, a calibration curve, and quality controls. Data within a method can be affected since it relies on analyte signal. Values can be seen in Table 12 Ionization suppression/enhancement values were better for SLE than for SPE. For both methods, compounds generally experienced suppression rather than enhancement. For SPE, the antidepressants had high values of suppression compared to that of SLE.

Table 12: Ionization Suppression/Enhancement Values

Compound	SLE Suppression/Enhancement %		SPE Suppression/Enhancement %	
	Low	High	Low	High
Amphetamine	-11.27	-11.35	0.44	-5.50
Methamphetamine	-9.01	-10.02	10.89	-5.98
MDA	9.72	0.21	-9.38	-7.93
MDEA	-5.26	-15.66	-4.86	-1.4
MDMA	-3.40	-9.96	-7.05	-5.07
Benzoylcegonine	-4.70	-0.76	-0.61	-2.36
Cocaine	-14.99	-26.03	-30.16	-20.09
Lidocaine	-8.03	-5.40	-17.66	-0.92
Codeine	-9.70	-7.61	-10.37	7.31
Methadone	-3.90	-0.07	-54.90	-28.25
Morphine	4.09	-9.95	-36.74	-21.60
Fentanyl	5.07	-12.86	-60.25	-48.03
Oxycodone	2.31	-0.84	2.86	8.06
6-MAM	-1.94	-3.97	1.25	-32.56
LSD	7.89	-5.93	-56.13	-41.58
PCP	-18.39	-13.99	-41.33	-18.04
Amitriptyline	-6.48	6.15	-72.74	-46.63

Compound	SLE Suppression/Enhancement %		SPE Suppression/Enhancement %	
	Low	High	Low	High
Citalopram	-22.04	-16.72	-59.85	-35.77
Fluoxetine	-1.35	-4.12	-73.69	-55.47
Trazodone	6.22	-14.28	-64.88	-45.91
Ethylone	-15.77	-5.06	-2.92	5.73
Alpha-PVP	-11.71	-6.61	-19.59	-11.45
25I-NBOMe	-8.83	-13.32	-75.27	-60.50

3.5. Limit of Detection and Limit of Quantitation

The limit of detection is the lowest concentration that can be consistently distinguished and identified from the blank matrix by the method.³⁸ For three different lots of matrix, a calibration curve, series of duplicate low concentration samples, and duplicate double blanks were analyzed; quality control samples were also included. The LOD samples were at concentrations: 0.05, 0.1, 0.5, 1, and 2 ng/mL. Peak shape was also assessed to ensure that it was distinguishable from the base line. Table 13 contains LOD values. Both SLE and SPE methods yielded similar LOD values. Some compounds like lidocaine and codeine may extract better with SLE while others like methadone and amitriptyline are better with SPE.

Table 13: Limit of Detection Values

Compound	SLE	SPE
Amphetamine	0.1	0.1
Methamphetamine	0.5	0.5
MDA	0.5	0.5
MDMA	0.5	0.5
MDEA	1	1

Compound	SLE	SPE
Benzoylecgonine	1	1
Cocaine	0.5	0.5
Lidocaine	1	2
Codeine	0.5	2
Methadone	1	0.5
Morphine	0.5	1
Fentanyl	0.5	1
Oxycodone	0.5	1
6-MAM	1	0.5
LSD	0.5	2
PCP	0.5	1
Amitriptyline	0.1	0.5
Citalopram	0.5	0.5
Fluoxetine	0.5	0.5
Trazodone	0.1	0.5
Ethylone	0.5	1
Alpha-PVP	1	1
25I-NBOMe	2	2

The lowest concentration that can be reliably measured while having acceptable identification, bias, and precision is the limit of quantitation.³⁸ A calibration curve and series of duplicate low concentration samples in three different lots of matrix were used to assess LOQ. LOQ values were determined as the concentration which had average calculated concentration accuracy between 80-120%. Table 14 has the values of LOQ for each analyte. LOQ values for generally lower for SLE than the SPE method.

Table 14: Limit of Quantitation Values

Compound	SLE	SPE
Amphetamine	0.1	0.5
Methamphetamine	1	1
MDA	0.5	1
MDEA	1	2
MDMA	0.5	1
Benzoyllecgonine	2	2
Cocaine	1	0.5
Lidocaine	2	2
Codeine	1	2
Methadone	2	1
Morphine	2	1
Fentanyl	0.5	1
Oxycodone	1	1
6-MAM	2	1
LSD	0.5	2
PCP	0.5	2
Amitriptyline	0.5	1
Citalopram	0.5	0.5
Fluoxetine	1	0.5
Trazodone	0.5	0.5
Ethylone	1	2
Alpha-PVP	1	1
25I-NBOMe	2	2

3.6. Carryover

Carryover is the presence of unwanted analyte in samples preceding positive analysis. It was seen with several drugs for both SLE and SPE methods. Methadone and 6-

MAM carryover was present in SPE but not SLE. For SLE, 25I-NBOMe had carryover. For both methods, fentanyl carryover was present.

Following a high concentration sample with solvent blank is recommended and more can be run as necessary. Another solution is to add a longer 100% organic mobile phase period at the end of the method to aid in complete elution of compounds.

3.7. Dilution Integrity

Dilution of samples may be necessary when their concentrations are outside of the working range. Precision was assessed after dilution. Two common dilutions (1:10 and 1:50) in triplicate were analyzed five times alongside five separate calibration curves. Table 15 lists values for each analyte. Most of the values for the dilutions were within $\pm 20\%$. Morphine 1:50 dilution for SLE and 25I-NBOMe 1:10 dilution for SPE did not meet the accepted values (bolded in table below). Both SLE and SPE methods yielded similar precision values but SLE had slightly lower numbers.

Figure 3: SLE 1:10 Dilution of Amphetamine

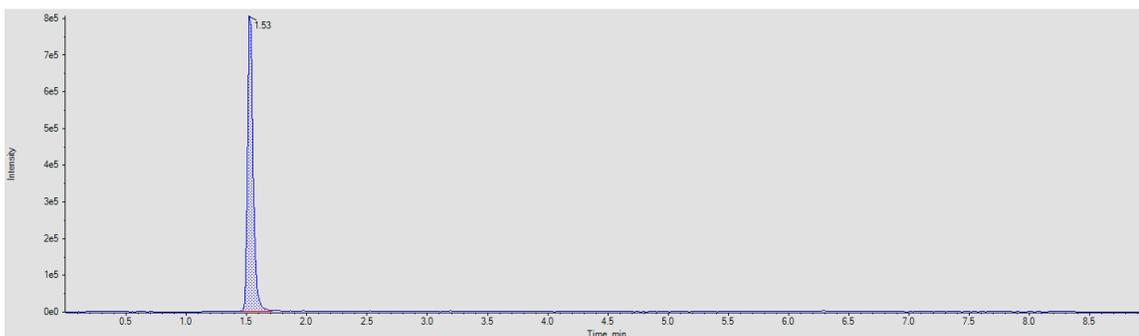


Figure 4: SLE 1:50 Dilution of Amphetamine

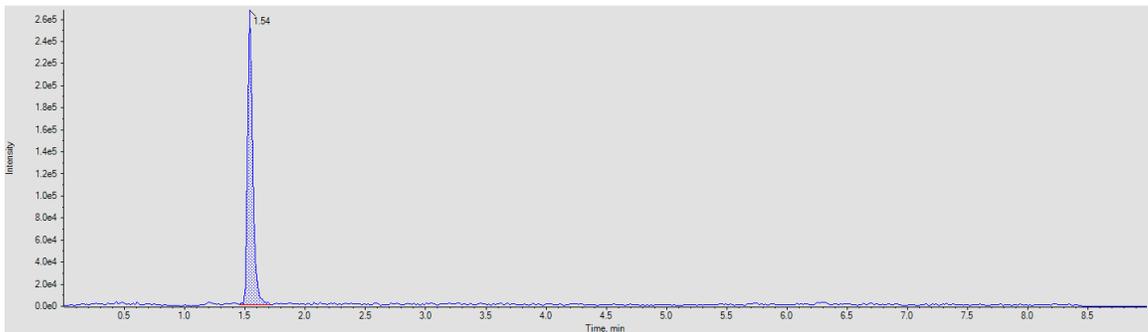


Figure 5: SPE 1:10 Dilution of Amphetamine

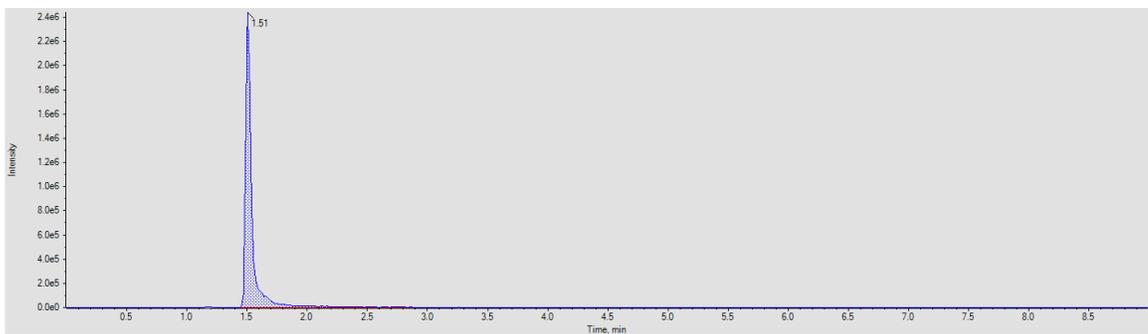


Figure 6: SPE 1:50 Dilution of Amphetamine

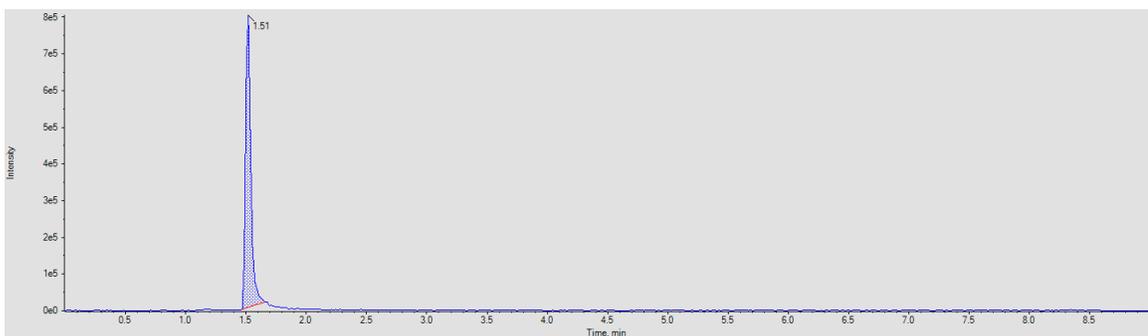


Table 15: Dilution Integrity Precision Values

Compound	%CV for SLE		%CV for SPE	
	1:10	1:50	1:10	1:50
Amphetamine	8.72	6.73	5.10	5.36
Methamphetamine	5.50	4.88	5.67	6.08
MDA	4.68	7.53	6.54	4.20
MDEA	7.15	9.47	3.97	5.14
MDMA	7.26	4.52	5.71	5.76
Benzoyllecgonine	6.91	7.38	5.89	5.55
Cocaine	5.77	5.56	7.82	5.45
Lidocaine	4.96	3.54	4.43	7.33
Codeine	7.81	9.77	4.16	6.40
Methadone	9.02	3.95	6.74	5.67
Morphine	6.91	31.48	4.01	4.06
Fentanyl	6.47	4.60	8.52	5.10
Oxycodone	9.60	9.12	6.73	4.66
6-MAM	7.64	14.08	7.49	4.78
LSD	5.15	4.60	8.73	4.89
PCP	5.07	7.30	8.85	7.53
Amitriptyline	4.29	6.06	5.46	7.25
Citalopram	5.28	3.77	5.11	5.24
Fluoxetine	7.46	6.61	5.79	10.26
Trazodone	4.99	3.15	7.43	13.18
Ethylone	6.87	4.29	7.05	6.28
Alpha-PVP	9.07	6.03	6.38	5.35
25I-NBOMe	4.37	3.55	20.68	4.58

3.8. Interference Studies

Interferences are the presence of compounds that are not specifically targeted by the method.³⁸ This can affect the integrity to detect, identify, and quantitate an analyte of

interest. Commonly encountered drugs, matrix, target analyte ions, and internal standard ions can all be sources of interference.

To check if the matrix could interfere with the analytes, ten different lots of double blanks were assessed. No interferences were seen with either method. Then drug-free matrix with only internal standard was analyzed and no interferences were found. The highest calibration standard was analyzed without adding any internal standard; ions from PCP were discovered to interfere with PCP-d5. Lastly, three mixes were spiked into drug-free oral fluid. Compounds in each of the three mixes can be seen in Table 3. Norcocaine, a metabolite of cocaine, interfered with cocaine; they had the same transitions and retention times. Additionally, hydrocodone was found to interfere with codeine; they also had the same transitions and eluted at similar times.

3.9. Stability

Stability is important to consider when samples cannot be run immediately. Average values of drug to internal standard ratio at 24, 48, and 72 hours were compared to value at 0 hours and accepted if they fell within $\pm 20\%$ of the expected range. For both sample preparation methods, all analytes were stable for at least 72 hours.

4. CONCLUSIONS

This study compared supported liquid extraction and solid phase extraction for the detection and quantitation of twenty-three different drugs. These two methods were assessed through method validation results. Guidelines set by ASB were used.³⁸ All drugs were validated for both SLE and SPE. Overall, SLE was the preferred method of extraction for all classes of drugs.

Matrix recovery and ionization suppression/enhancement varied greatly between the two methods. Recovery values were generally higher for SLE than SPE. For SLE, most values were between the acceptable values of 80 – 120%. SPE had more compounds that did not have acceptable recovery values. The antidepressants, in particular, did not extract very well for SPE; they also experienced high ion suppression following SPE. Both LSD and PCP did not extract to acceptable standards from either method. Although recovery was low, LSD and PCP had results for bias, precision, and dilution integrity that fell within acceptable values. Since ion suppression/enhancement can affect the results and interpretation of other parameters, it is important to develop a method that can provide acceptable values. Most SLE values for ionization suppression/enhancement were within $\pm 20\%$ but many drugs exceeded $\pm 20\%$ for SPE.

Limit of detection and quantitation values were similar between SLE and SPE. SLE did have slightly lower values for both LOD and LOQ. Sample preparation technique did not greatly affect parameters such as the calibration model, stability, interferences, and carryover. Bias, precision, and dilution integrity values were also similar between the methods.

Furthermore, it is important to consider both the time and cost of each sample preparation technique. SLE took approximate 1 hour while SPE took roughly 2.5 hours due to additional conditioning and wash steps. SLE cartridges were also more expensive than SPE cartridges; one SLE cartridge was approximately \$2.50 while a single SPE cartridge cost about \$1.74. SPE, however, has added costs due to heavy solvent use when conditioning, washing, and eluting. Based on the overall results, SLE is determined to be the preferred method of sample preparation due to its ease of use, speedier extraction, and better results.

5. FUTURE RESEARCH

A possible area for further research is to collect oral fluid donations using kits such as Quantisal®, Accu·Sal™, or Intercept®. In this project, oral fluid was collected by expectorating into collection tubes. Buffer was added when starting the sample preparation technique. In the field, oral fluid is collected using the kits and some even have buffer already added. Utilizing these kits most closely mimic these conditions. The effect of buffer can also be explored since it could lead to better or worse extraction of certain compounds. Another future direction is to study different groups of drugs that are not already included or even add them to the current method. Other drugs classes to consider include synthetic and natural cannabinoids, benzodiazepines, and barbiturates. Additionally, different brands or materials for the packing material in the SLE and SPE columns can be explored.

LIST OF JOURNAL ABBREVIATIONS

Anal Bioanal Chem	Analytical and Bioanalytical Chemistry
Arch Toxicol	Archives of Toxicology
Clin Biochem Rev	The Clinical Biochemist. Reviews
J Anal Toxicol	Journal of Analytical Toxicology
J Med Toxicol	Journal of Medical Toxicology
Mol Pharmacol	Molecular Pharmacology

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CURRICULUM VITAE

