An Optimized and Validated Method for Screening and Quantification of Commonly Encountered Stimulant Drugs and Selected Metabolites in Dried Blood Spot (DBS) samples by Ultra Performance Liquid Chromatography-Quadrupole Time of Flight-High Resolution Mass Spectroscopy (UPLC QTOF-HRMS) Analysis

by

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Abstract

Blood microsampling techniques, like Dried Blood Spots (DBS), have seen recent development as an alternative to wet blood samples and offer numerous advantages. Quantification of ng/mL-range concentrations using DBS microvolumes (10-20 µL) is made possible with cutting edge instrumentation. Worldwide establishment of *per se* limits for drug impaired driving requires timely methods of sampling blood for accurate drug concentration measurements and interpretation. The method validation for the analysis of stimulants and metabolites in DBS samples by Ultra Performance Liquid Chromatography-Quadrupole Time of Flight-High Resolution Mass Spectroscopy (UPLC-QTOF-HRMS) is presented. Limits of detection and quantitation to 10 ng/mL was achieved. Method validation criteria was satisfied for 10 of 14 analytes. DBS drug stability over 8 weeks varied by analyte. DBS samples may assist in overcoming challenges in blood sampling as they are less invasive, easily transported and stored, and accurate drug quantitation even at low concentrations in DBS samples is possible.

Keywords

Dried Blood Spots, DBS, microvolume sampling, QTOF, high resolution mass spectrometry, forensic toxicology.

Co-Authorship Statement

Chapter 1 of this thesis reviews the technological and chemical concepts relevant to this project. I am the sole author of this chapter. James Watterson contributed to experimental design and concepts, along with structural guidance through this thesis.

Chapter 2 is in part a restructured document of a poster presentation at the Canadian Society of Forensic Science 2018 Annual Meeting entitled, Optimization of sample preparation for analysis of selected stimulants in Dried Blood Spots (DBS) by high-resolution UPLC-QTOF-MS. I am the first author and James Watterson is the second author. Chapter 2 also contains additional, unpublished data describing the optimization of extraction solvent, solid phase extraction, liquid chromatography mass spectrometry optimization.

Chapter 3 is in part a manuscript to be submitted to a peer review scientific journal describing the validation of the analytical method developed in this work. I am the first author on this manuscript and James Watterson is the second author.

Chapter 4 discusses the conclusion of this thesis and suggests avenues for future research in this area. I am the sole author with conceptual guidance from James Watterson.

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This work would not be possible without the project supervisor Dr. James Watterson and his outstanding wealth of knowledge, vision, and inspiring commitment to research, progress and growth. Dr. Watterson is a credit to his profession and to science as a whole. Thank you, James. Thank you very much.

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Abbreviations

μ	Interstitial linear velocity/flow rate
μMCX	Microelution mixed-mode cation exchange
5-HT	Serotonin
A	Eddy diffusion term
AC	Alternating current
ACN	Acetonitrile
ADC	Analog to digital converter
AME	Anhydroecgonine methyl ester
AmmF	Ammonium formate
AMP	Amphetamine
AMP-d8	Amphetamine, deuterated
API	Atmospheric pressure ionization
B	Longitudinal diffusion term
BBB	Blood-brain barrier
BUP	Bupropion
BUP-d9	Bupropion, deuterated
BZE	Benzoylecgonine
BZE-d3	Benzoylecgonine, deuterated
CE	Cocaethylene
CE-d3	Cocaethylene, deuterated
C_M	Mobile phase mass transfer resistance coefficient
CN	Cocaine
CN-d3	Cocaine, deuterated
CNS	Central nervous system
C_S	Stationary phase mass transfer resistance coefficient
CYP	Cytochrome P450 enzyme
d	Distance
DA	Dopamine
DAT	Dopamine transporter

DC	Direct current
E_k	Kinetic energy
EME	Ecgonine methyl ester
EME-d3	Ecgonine methyl ester, deuterated
ESI	Electrospray ionization
FA	Formic acid
GC	Gas chromatography
GI	Gastrointestinal tract
HCT	Hematocrit
HETP	Height equivalent of theoretical plate
HIV	Human immunodeficiency virus
HLB	Hydrophilic-lipophilic balance
HPLC	High-performance liquid chromatography
IM	Intramuscular administration
IN	Insufflation administration
IPA	2-Propanol
IV	Intravenous administration
LC	Liquid chromatography
m	Mass of ion
MA	Methamphetamine
MA-d11	Methamphetamine, deuterated
MCX	Mixed-mode cation exchange
MDA	3,4-Methylenedioxyamphetamine
MDA-d5	3,4-Methylenedixoyamphetamine, deuterated
MDD	Major depression disorder
MDEA	3,4-Methylenedioxyethylamphetamine
MDEA-d5	3,4-Methylenedioxyethylamphetamine, deuterated
MDMA	3,4-Methylenedioxymethamphetamine
MDMA-d5	3,4-Methylenedioxymethamphetamine, deuterated
MeOH	Methanol

MP	Methylphenidate
MPA	Mobile phase A
MPB	Mobile phase B
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MS^E	High and low collision energy data independent acquisition
nAChR	Nicotinic acetylcholine receptor
NE	Norepinephrine
NET	Norepinephrine transporter
OH-BUP	Hydroxybupropion
OH-BUP-d6	Hydroxybupropion, deuterated
OTC	Over the counter
PBS	Phosphate buffer solution
PES	Post extraction spiked samples
PET	Positron emission tomography
PH	Phentermine
pKa	Acid dissociation constant
PO	Oral administration
Q1	Quadrupole 1/mass filter
Q2	Quadrupole 2/collision cell
Q3	Quadrupole 3
QTOF	Quadrupole time-of-flight
QTrap	Quadrupole linear ion trap
RBC	Red blood cells
RF	Radio frequency voltage
RoA	Route of administration
RPM	Rotations per minute
RT	Retention time
SERT	Serotonin transporter

SM	Smoking administration
SPE	Solid phase extraction
SSRIs	Selective serotonin reuptake inhibitors
SWGTOX	Scientific Working Group for Forensic Toxicology
TARR1	Trace amine-associated receptor-1
TCAs	Tricyclic antidepressants
THC	Δ9-tetrahyrdocannabinol
TLC	Thin-layer chromatography
TMBE	*tert*-Methyl butyl ester
TOF	Time-of-flight
U	Electric field strength
UPLC	Ultra-performance liquid chromatography
v	Velocity
VMAT	Vesicular monoamine transporters
WB	Whole blood
z	Electric charge of ion

Chapter 1

1 Introduction

1.1 Forensic Toxicology

A drug is a compound that elicits a change in biologic function through its chemical actions in the body.[1] Forensic toxicology is the study of the toxic effects of drugs and poisons on the human body in a medicolegal, or "forensic", context.[2] Like pharmacology, the study of the therapeutic effects of drugs, toxicology applies the disciplines of chemistry, biology, biochemistry and physics to separate, isolate, detect, and identify drugs in biological samples and to determine the pharmacokinetics and pharmacodynamics of drugs and poisons in the human body.

There are 3 main fields in forensic toxicology: human performance testing, postmortem toxicology, and forensic drug testing.[2] Human performance testing investigates performance enhancing drugs in athletics. Postmortem toxicology determines the presence and concentrations of drugs in the body to aid in death investigations. Forensic drug testing is used for a wide variety of investigations including criminal investigations, such as alcohol or drug impaired driving. Many biological matrices, including organ tissues, urine, hair, oral fluid, finger nails, and bone, have been used in forensic toxicological analyses.[3–18] However, drug positive results in these sample matrices only indicate past exposure or use of drugs and cannot give information about dose or impairment. Since blood carries drugs to the brain, concentrations of drugs in blood are a surrogate estimate of brain drug concentration and is why blood is the matrix of choice for forensic drug testing to assess allegations of impairment.

1.2 Blood Physiology and Drug Disposition in Blood

Blood is the fluid connective tissue within the vasculature that transports nutrients, wastes and respiratory gases throughout the body.[19] Blood is composed of erythrocytes, leukocytes, thrombocytes, and plasma proteins, suspended in serum fluid. Dissolved respiratory gases, electrolytes, nitrogenous metabolic waste products, hormones, fatty acids and other components, including drugs, are circulated to and from cells in blood's support of life function.

Whole blood can be separated into 3 fractions via centrifugation: plasma, erythrocytes, and a "buffy coat", made of leukocytes and thrombocytes. Plasma is approximately 90% water and makes up roughly 55% of the volume of whole blood. Plasma is a straw coloured fluid that contains plasma proteins, dissolved electrolytes, and other components that cannot be separated by centrifugation. Erythrocytes, also known as red blood cells (RBC), make up roughly 45% of whole blood volume. Erythrocytes are anucleate, concave disc-shaped "cells" made of a plasma membrane and cytoplasm containing high amounts of hemoglobin for gas transport. Discounting water, hemoglobin makes up roughly 97% of RBC. Erythrocyte content is expressed as the hematocrit (HCT) value, which is the fraction of volume in whole blood made up of RBCs. The value of hematocrit can vary greatly. The HCT range of 0.19 to 0.63 covers 99.5% of the population, though typical male and female HCTs range from 0.41 to 0.50, and 0.36 to 0.44, respectively.[20,21] The viscosity of blood is largely due to RBC content. Blood with a low HCT will be less viscous than a blood with a high HCT. Leucocytes, or white blood cells (WBCs), critical in immunoresponses, are the only complete cell component in blood tissue and make up less than 1% of whole blood volume. Thrombocytes, or platelets, are essential for the clotting response in plasma to ruptured or injured blood vessels and make up less than 1% of blood volume.

Once a drug enters the body, blood concentrations of drugs change as the body exerts the pharmacokinetic processes of absorption, distribution, metabolism and elimination. In order to be absorbed, a drug must be soluble in blood. A drug's absorption is highly dependent on the route of administration (RoA); a drug injected intravenously (IV) enters systemic circulation immediately, while a drug administered orally (PO) is subject to first pass metabolism in the liver and is delayed prior to entering blood circulation.

Once in systemic circulation, blood transports the drug throughout the body to the site of action and all other tissues, in proportion to the degree of a tissue's vascularization.[1,2,22,23] The chemical nature of a drug will dictate distribution. Lipophilic drugs uncharged at blood pH will readily partition from blood across cellular membranes.[1] The degree to which a drug is bound to plasma proteins can also influence distribution. A drug bound to plasma proteins is excluded from having clinical effect at the drug's site of action.[1,24] Warfarin is highly bound to plasma proteins and remains largely in blood; however, Δ9-tetrahyrdocannabinol (THC), also highly bound to plasma proteins, distributes readily into highly perfused tissues and adipose due to its lipophilicity.[2,22–25] The chemistry of a drug and that of the blood environment will largely determine drug distribution in the body; however, physical barriers like the blood brain barrier (BBB) excludes some drugs from reaching sites of action in the central nervous system.

Metabolism is the biochemical process of altering the structure of a drug to facilitate elimination.[1,2] The metabolism of a drug can occur simultaneously with its absorption and distribution, and the RoA will determine the extent of metabolism prior to a drug entering systemic circulation. Metabolism occurs principally in the liver, though metabolism can occur in the brain, blood, and other tissues.[1,26–29] Drugs taken orally will undergo first pass metabolism by hepatic and gastrointestinal enzymes prior to entering blood circulation while IV administration

bypasses first pass metabolism all together.[1,2] There are two general phases of metabolism: phase I is the enzymatic conversion of a drug to a more polar metabolite by introducing or unmasking a polar functional group; phase II metabolism is the enzymatic conjugation of a drug or phase I metabolite with a highly polar endogenous substrate such as acetic acid or glucuronic acid.[1,2] Increasing the polarity of a drug reduces its ability to distribute across plasma membranes and facilitates excretion from the body.[1,2] Drug activity is changed by metabolism. Generally, drug activity is reduced or eliminated; however, some metabolites can have equivalent or greater activity than the parent drug.[30–38] Metabolism can be exploited for drug therapy. A prodrug is an inactive compound that has pharmacological activity after metabolism.[39–41] Once transformed, metabolites circulate in the blood to reach drug targets, and if active, display activity. Given that metabolites can be active, pharmacogenetics and drug interactions are important considerations as toxic outcomes can arise from the inhibition or inducement of enzymatic activity, especially in individuals with atypical metabolism due to polymorphisms in genes relevant to drug disposition.[38,42–47] Age and hepatic disease states can affect metabolic rates, thereby influencing drug, prodrug, or metabolite concentrations in blood.[48–52]

Elimination of drugs occurs by biotransformation into inactive metabolites and excretion from the body. Excretion of drugs and metabolites from blood occurs primarily in liver and kidneys, the rates of which are influenced by liver and kidney health.[48–52] Small amounts of volatiles like ethanol can be eliminated in breath as volatiles diffuse from capillary beds to lung air across the alveolar membrane.[53] Drugs and metabolites eliminated by the liver form part of the bile, which enters the gastrointestinal tract (GI) for elimination in feces.[2,19] Drugs are also eliminated in by passive filtration or active secretion into urine in the kidneys. Molecules less than 65,000 Da are passively filtered from blood in the glomerulus of the kidneys.[1,19] Drugs bound to plasma

proteins larger than 65,000 Da are eliminated by active transportation in the proximal tubule by specific carrier proteins secreting drugs and metabolites into the urine.[1,2] Lipophilic or uncharged drugs may reabsorb into the blood from kidney tubules. Altering the pH of urine can increase elimination by trapping drugs and metabolites by exploiting their pKa values to create charged drug and metabolite ions that can't cross plasma membranes of renal tubule cells.[1,2]

Route of administration is an important factor in drug disposition in blood. The time to effect, time course, and bioavailability, the amount of drug in blood available to reach a site of action, is directly related to the RoA, therefore, the RoA used has important therapeutic, toxicologic and public health implications.[54–67] Intravenous administration allows the full dose to enter circulation immediately. Other RoAs require the drug to cross biological membranes which can limit drug adsorption into blood or subject a drug to metabolism.[1,2,60–63] Other common routes of administration are intramuscular injection (IM), smoking (SM), and nasal insufflation (IN). Time to effect and drug bioavailability for RoAs is presented in Table 1.

Table 1: Time to effect and drug bioavailability of common routes of administration.

Route of administration	Time to effect	Bioavailability (%)	References
Intravenous (IV)	< 1 to 3 minutes	100	1, 60, 61
Smoking (SM)	1 to 3 minutes	5 to < 100	1, 60, 61
Insufflation (IN)	6 to 20 minutes	68 to 100	60, 61
Intramuscular (IM)	10 to 30 minutes	75 to 100	1, 67
Oral (PO)	30 to > 90 minutes	5 to < 100	1, 2, 64-66
Transdermal	< 60 minutes to hours	80 to ≤ 100	1, 62, 63

1.3 Blood Sampling Techniques

Blood sampling is an invasive procedure that requires training and consideration for patient and sampler safety. Typically, blood is drawn by medical professionals or trained phlebotomists in a

controlled setting. With respect to allegations of drug impaired driving, the time between typical blood sampling and incident can be hours, which can render interpretation of drug concentrations in blood at the time of the incident impossible.

1.3.1 Liquid Blood Sampling

Liquid whole blood sampling is one of the most common invasive medical procedures performed.[68] Blood is drawn from veins in volumes from a few to tens of mL by trained phlebotomists or medical professionals with hypodermic needles, syringes, or vacuum extraction vessels, and then stored in a liquid state.[69] Preservatives and refrigeration are required to inhibit analyte and sample degradation of liquid blood during transport and storage.[14,70–74] Maintaining sample stability is an important consideration in forensic analyses as analyte loss or generation in blood samples complicate drug concentration interpretations.[2,70,73,74]

Whole blood sampling exposes the sampler to blood from other people, putting them at risk for bloodborne pathogens, such as hepatitis B and C viruses, human immunodeficiency virus (HIV), malaria and viral hemorrhagic fevers.[69,75–77] Serious blood sampling events in people being sampled, though rare, include tonic-clonic seizures and loss of consciousness.[69] Other unwanted outcomes of blood sampling include pain, anxiety, nerve damage, and hematoma.[69,78] Because blood sampling is necessary in clinical and forensic practices, these hazards must be considered and best practices followed when sampling whole blood.

1.3.2 Microvolume Dried Blood Spot Sampling

Dried blood spots (DBS) have been used as an economical and easily collected alternative to drawn venous blood samples for a variety of biochemical and biological analyses.[79–81] DBS samples capture microvolumes of capillary blood (10 to 30 μL) on an adsorbent material from a

small puncture in a finger or heel. Devices that collect a fixed microvolume sample of blood in an absorbent media are available. The first published use of DBS was in 1913, when Ivar Bang established microsampling as a reliable and practical method of measuring blood glucose levels.[82,83] DBS samples saw widespread use after Guthrie and Susi used the sampling technique in a rapid screening method for phenylketonuria in newborns in 1963.[79] Not only is DBS a simple and cost effective technique, DBS samples confer greater stability relative to liquid blood samples for a number of analytes prone to *ex vivo* production and degradation.[70,84–88] DBS samples have displayed equivalent or better analyte recoveries than wet whole blood samples, an important consideration for samples with potentially low analyte concentrations.[85,86,88–90] Published ratios of whole blood to DBS sample concentrations are close to unity. Mean ratios of whole blood/DBS blood concentrations for dexamphetamine (n = 29), MDMA (n = 36), and MDA (n = 32) were 0.95, 0.99 and 0.98, respectively.[91] Recent studies have investigated quantifying drugs in DBS, and a comparison of different methods for the analyses of drugs in DBS samples is presented in Table 2.

DBS samples also exhibit significant improvements in biohazard safety over liquid blood samples. Pathogens like HIV-1 have been shown to lose their virulence upon sample drying, while retaining sample stability permitting virus detection, genotyping, and viral load monitoring.[92–96] Further, DBS samples do not require the same travel and storage considerations as typical liquid blood, and DBS samples have been used successfully in resource limited locations.[92,96,97] Superior biohazard safety, transportation and storage considerations of DBS samples over liquid blood samples make them an ideal method for sampling blood in field locations without phlebotomists for a number of analytes, including drugs.

Table 2: Comparison of different microvolume DBS methods for selected drug analyses in humans.

Author and year	Drugs	Absorptive medium	Analytical method	Extraction method	Analytical range	Blood type, volume	Punch size
Thomas, *et al.*, 2012 [98]	CN, MDMA, MDA, MP, THC, others	Sartorius TFN cards	UPLC-Orbitrap MS/MS	45 min sonication in 0.5 mL 1:4 MeOH TMBE; 13000g centrifuge 5 min; 30 min sonication with 0.3 mL acetone; dried; recon in 35 µL 6:4 ACN-H_2O	0.5–20 ng/mL	Human WB, 10 µL	Whole spot
Ambach, *et al.*, 2013 [99]	AMP, MA, MDMA, MDEA, others	Bioanalysis 226-1004 cards	HPLC-QTrap MS/MS	15 min vortex in 0.5 mL MeOH, dried, recon 100 µL H_2O + 1% FA	2.5–1000 ng/mL	Human WB, 10 µL	Whole spot
Kyriakou *et al.*, 2016 [100]	AMP, MA, CN, MDMA, MDA, BZE, THC, others	Whatman® 903 Protein Saver Cards	UPLC-MS/MS	15 min sonication in 1 mL MeOH; 3500g centrifuge 5 min; dried; recon 80:20 MPA-MPB	5-500 ng/mL	Human HB, 30 µL	Whole spot
Moretti *et al.*, 2018 [88]	CN, BZE, CE, EME	Whatman® 903 Cards	HPLC-QTrap MS/MS	10 min sonication in 1 mL PBS; 4000g centrifuge 5 min; SPE; dried; recon 200 µL 0.1% FA	10-500 ng/mL	Postmortem heart blood, 85 µL	Whole spot
Ambach & Stove, 2019 [101]	CN, BZE, CE, EME, norcocaine, OH-BZE	Whatman® 903 Specimen Paper	HPLC-QTrap MS/MS	15 min shake at 1000 rpm in 0.2 mL 2 mM ammonium acetate, shake at 1000 rpm + 1 mL ACN; centrifuge 14000g for 20 min; dried; recon 50 µL 10 mM AmmF + 0.1% FA	1-1000 ng/mL	Human WB, 25 µL	6 mm
Ambach *et al.*, 2019 [102]	*Controlled cocaine administration:* CN, BZE, CE, EME, norcocaine, OH-BZE	Whatman® 903 Specimen Paper	HPLC-QTrap MS/MS	15 min shake at 1000 rpm in 0.2 mL 2 mM ammonium acetate, shake at 1000 rpm + 1 mL ACN; centrifuge 14000g for 20 min; dried; recon 50 µL 10 mM AmmF + 0.1% FA	1-1000 ng/mL	Human WB, 25 µL	6 mm
de Lima Feltraco Lizot *et al.*, 2019 [103]	CN, BZE, CE, EME, norcocaine	Whatman® 903 Paper	UPLC-MS/MS	45 min vortex at 1000 rpm in 0.5 mL 3:1 MeOH-ACN; dried, recon 0.1 mL 5 mM AmmF (pH 3)	10-1000 ng/mL	Whole blood, 50 µL	8 mm

1.4 Quantitative Analysis of Drugs in Blood

Drug concentrations in biological samples are quantitated following the extraction, isolation, and detection of the analyte. Drugs are separated from interfering compounds in biological samples by extraction methods such as liquid-liquid or solid phase extraction (SPE). Drugs are isolated from other compounds in a sample using chromatographic methods, such as liquid chromatography (LC). Identification and quantitation of drugs is typically performed by mass spectrometry (MS) instrumentation. A review of the methods and instrumentation used in this study is presented.

1.4.1 Drug Extraction I: Sonication and DBS Drug Extraction

Drugs are extracted from DBS samples by emersion and agitation in a solvent. The nature of the solvent used will depend on the chemistry of targeted analytes, the presence of matrix interferents, and downstream sample preparation conditions. Mechanical agitation (shaking and vortex stirring) increases the surface area interactions of the sample with solvent and provides mechanical energy, facilitating extraction of components off the DBS spot. Extracted compounds include drugs and blood components. Vortex stirring or mechanical shaking has successfully extracted a wide range of drugs from DBS samples in methanol, 1:5 2 mM ammonium formate-acetonitrile, and 3:1 methanol-acetonitrile extraction solvents.[99,101,103] Extraction is complete between 15 and 35 minutes at 1000 RPM using mechanical agitation methods.[99,101,103] The low volumes of DBS samples require correspondingly small volumes of extraction solvents. DBS drug extraction is possible in as little as 0.5 mL extraction solvent. Selected methods of DBS analysis using physical agitation extraction is presented in Table 2.

Sonication is the irradiation of a liquid system with ultrasonic acoustic frequencies, and was first described by Richards and Loomis in 1927.[104] Ultrasonic frequencies span 15 kHz to 10 MHz with respective acoustic wavelengths of 10 to 0.1 cm.[105] The physical effects of sonication is due to cavitation, the generation of short duration microbubbles (10^{-6} to 10^{-4} seconds) within the liquid and on the surfaces of immersed solids that release high energy and pressures when the bubbles collapse.[104–106] The high pressures and energy create microstreaming and localized high shear stress fields that alters chemical bonds, and also disrupts cell membranes and can cause cell death, mechanisms that have been exploited in drug extraction from plants and biological samples.[88,98,100,104–109] Selected sonication extraction methods used in DBS samples are summarized in Table 2.

Cavitation is the result of three distinct microbubble phases: nucleation, growth, and collapse. In a homogenous fluid, nucleation occurs at localized points of weakness like a gas-filled void in or on suspended matter, or the microbubbles from previous cavitation events.[105] Rapid microbubble growth occurs inertially when cavity expansion is faster than the recompression during the positive half of the pressure cycle.[105] Slow microbubble growth is the result of "rectified diffusion", where slow growth occurs over many pressure cycles as the microbubble growth is incrementally faster than shrinking, since microbubble surface area is slightly greater during expansion than compression.[105] A microbubble will grow until it reaches a resonant size determined by ultrasound frequency (e.g., roughly 170 μm at 20 kHz) at which it efficiently absorbs acoustic energy; a microbubble at this size and in phase with the ultrasound field will rapidly expand over a single expansion cycle and lose its ability to absorb acoustic energy, and become unstable and implode.[105] The implosion of a microbubble can generate localized, short-lived, extraordinary heat and pressure on the order of 5,000 °C and 500 atm.[105] Cavitation on

liquid-solid interfaces is different from that in homogenous liquids. Irregular interface environments cause self-reinforcing deformation as the microbubble collapses, creating a fast moving "microjet" stream of liquid (greater than 100 m/s) through the collapsing cavity and impact the surface of the solid.[105,110] Cavitation also causes shock waves that together with the fluid microjet impacts results in ultrasonic cleaning, dissolution of compounds, breakup and ejection of material off solid surfaces, and catalysis of surface chemistry reactions.[105,111,112] Surface-liquid sonication effects make it a useful technique in a wide variety of chemical and biochemical applications.

Sonication is a high energy process that disrupts protein-drug bonds and frees analytes from within mammalian cells.[106,113,114] Cavitation forces along liquid-solid interfaces will also breakup dried blood, facilitating extraction from DBS samples. Liberating and recovering as much analyte as possible is of importance in small volume, potentially low concentration DBS samples. Successful sonication extraction of DBS samples has been demonstrated down to 0.5 ng/mL in volumes as small as 10 μL, making sonication a sound method for extraction of drugs from DBS samples.[88,98,100]

1.4.2 Drug Extraction II: Solid Phase Extraction

Solid phase extraction (SPE) is a technique used to separate components in a fluid mixture by exploiting a components chemistry to retain it on a solid sorbent phase. Separation is achieved by altering the SPE conditions to promote analyte interaction with the sorbent phase while potentially interfering mixture components are removed.

SPE can be divided into three general mechanisms of extraction: reversed phase, ion exchange, and mixed-mode SPE. Reversed phase SPE consists of a nonpolar solid phase used with a polar

sample matrix.[115,116] Nonpolar sorbent beds made from silica or polymer backbones are surface modified with alkyl or aryl groups, and nonpolar analytes adsorb onto the sorbent material via van der Waals forces while polar compounds are retained in the polar sample matrix.[115] Ion exchange SPE is made of either anion or cation exchange functional groups bonded onto a silica or polymer backbone material.[115] Compounds are retained through ionic interactions with the sorbent material. Positively charged analytes are retained by anion exchange groups made of either quaternary or secondary amine groups, and negatively charged analytes are sequestered on the sorbent bed by their interactions with cation exchange groups made from sulfonic or carboxylic acid groups.[115,116] Generally, cation or anion exchange interaction with the sorbent bed requires preconditioned sorbent beds and solvent environments at pH conditions such that both analyte and sorbent groups are sufficiently charged to facilitate ionic interactions.[115] For cation exchange SPE, the pH should be 2 below the acid dissociation constant of targeted analytes. Mixed-mode SPE utilizes reverse-phase and either anion or cation exchange functional surface modifications on sorbent backbone material. Mixed-mode SPE allows extraction of neutral compounds via hydrophobic interactions with revere phase alkyl functional groups and simultaneous extraction of either basic or acidic analytes via ionic interactions with their respective cationic (acidic) or anionic (amine) exchange functional groups.[115] Like ion exchange SPE, mixed-mode requires sorbent and solvent environments at appropriate pH to allow both reverse and ionic extraction interactions.

Traditionally, SPE is performed in five steps: condition, equilibration, load, wash, and elution. Each step can be performed under gravity or negative pressure drawing solvents through the sorbent bed. A sorbent is conditioned with a strong organic solvent to wet and activate the surface and create a path between sorbent and sample.[115] A buffer solution or the sample solvent

is used to equilibrate the sorbent environment to an appropriate pH and allow sample analytes access to the sorbent surfaces.[115] The sample is loaded and targeted analytes and other compounds with similar chemistry adsorb onto sorbent surfaces as the sample passes through the sorbent bed.[115] Unwanted components interacting with the sorbent bed are washed off to isolate analytes remaining on the sorbent bed; this may take a series of steps of increasingly strong wash solvents to sufficiently remove unwanted sample components.[115] In the final step, analytes are eluted from the sorbent material with a solvent capable of disrupting the bonds between analyte and sorbent bed, by either solvent polarity, solvent pH, or both.[115] The elution solvent should be strong enough to remove the analytes of interest but not so strong as to remove all sample compounds that remain after washing.

Recent development of water-wettable SPE sorbent materials that don't require sorbent bed conditioning or equilibration, reduce the time and solvent volumes required for SPE. Two such wettable SPE sorbents are Oasis® PRiME HLB, or "Hydrophilic-Lipophilic Balanced" reversed phase (HLB), and Oasis® PRiME MCX, a novel "Mixed-mode Cation eXchange" (MCX) sorbent, manufactured by Waters Corporation (Milford, Massachusetts). HLB is composed of a proprietary blend of hydrophilic N-vinylpyrrolidone groups and lipophilic divinylbenzene functionalities, allowing for simultaneous extraction of neutral, basic and acidic analytes from sample extracts.[117] MCX is a mixed-mode cation exchange with proprietary ratios of reverse phase and sulfonic acid ion exchange functionalities that offer improved selectivity for basic analytes than HLB sorbent.[118] SPE apparatus are available as individual cartridges or well plate forms with different sorbent bed masses that allow tailored sample and elution volumes for large or small sample types.

1.4.3 Drug Isolation: Liquid Chromatography

Chromatography is a technique used to isolate compounds in a mixture by the basic principle of "like attracts like". A fluid mobile phase carries the sample through the instrument in a defined direction. Separation of compounds in a mixture occurs via chemical interactions with a stationary phase within a column. A compound similar to the chemistry of the stationary phase will preferentially interact with the stationary phase relative to a compound with a chemistry dissimilar to the stationary phase. The analyte's degree of interaction with the stationary phase depends on adsorption, partition, polarity, ion exchange, and analyte exclusion.[119] The degree of interaction will determine how long a compound will take to travel through the column; a compound dissimilar to the stationary phase will move through the column more quickly than a compound that is complementary to the chemistry of the stationary phase. Similarly, the composition of the mobile phase can be adjusted so that compounds with similar chemistry will preferentially interact with the mobile phase and travel through the column in less time. Compounds exit the column and are carried in the mobile phase to detection instrumentation for identification and quantitation. The period of time a compound takes to travel through the instrument and reach the detector is the retention time (RT). Retention time is characteristic for compounds at given liquid chromatography (LC) conditions and is used to identify analytes.

Liquid chromatography uses liquid mobile phase(s) to drive differential distribution of components in a mixture between the mobile and stationary phases and can be used to separate compounds that are soluble in a liquid sample.[2] Liquid chromatography was first described by Mikail Tsvet in 1903 in the separation of leaf extracts using a calcium carbonate-packed tube and petrol ether in a technique now known as column chromatography.[2,120] Separation was carried out using gravity, and later vacuum, to drive mobile phase flow through the column.[121] Tsvet

correctly identified the role of adsorption of analytes on solid phase matrices, both sample and stationary phase, and highlighted the importance of appropriate solvent strength for both the extraction and separation of compounds from a sample.[120,121] Isolation of plant pigments using Tsvet's methods was better than other isolation techniques used at that time.[122] Analyte adsorption on cellulose and the importance of appropriate solvent selection identified by Tsvet in his discovery of chromatography is an important consideration in the extraction of DBS samples today.[120,121]

Liquid chromatography methods were improved in the 1930's with the development of thin layer chromatography (TLC). The thin layer stationary phase is made from small silica particles affixed to glass plates, which allowed the simultaneous separation of several samples.[2] When compared to chromatographic columns of that time, TLC methods achieve more efficient separation of samples due to the small particle size of TLC stationary phase material.[2]

Significant advancements in LC were seen in the 1960's with the breakthrough development of high-pressure/high-performance liquid chromatography (HPLC).[2] HPLC was made possible after the development of HPLC columns and liquid mobile phase pumps and instrumentation capable of operating reliably at high pressures, and offered improved analyte resolution, peak shapes, reproducibility, and analysis time over earlier LC techniques.[119] Early HPLC columns were made from relatively coarse (44 μm) porous silica or alumina with strongly polar hydroxyl surface functional groups; separation was achieved with selective adsorption and polar interactions.[2] Methods to alter the surface functional groups of HPLC column packing was developed in the 1960's by reacting octadecyl-chlorosilanes with surface hydroxyl groups on silica packing.[2] The new C_{18} "reverse-phase" columns became widely used. Reverse-phase separation is achieved through weaker intermolecular hydrophobic interactions vs. ion-exchange and polar interactions

in early unmodified column packing.[2] Column particle size dropped from 10 μm in the 1970's, and again to 3.5 μm particles in the 1990's.[123] As column particle size decreases, column efficiency (height equivalent of theoretical plates) increases. Smaller and smaller column particle sizes improved peak capacity (the number of peaks resolved per unit time) and separation efficiency of HPLC. The efficiency of a chromatographic column with respect to particle size and flow rate can be described by the van Deemter equation below:[124]

$$HETP = A + \frac{B}{\mu} + (C_S + C_M)\, x\, \mu \qquad (1)$$

HETP: Height equivalent of theoretical plate (column efficiency, m)

A: eddy diffusion term (m)

B: longitudinal diffusion coefficient (m^2/s)

μ: interstitial linear velocity (flow rate; m/s)

C_S: stationary phase mass transfer resistance coefficient (s)

C_M: mobile phase mass transfer resistance coefficient (s)

Within a column, the eddy (A) and longitudinal dispersion (B) terms decrease in concert with the particle size of column packing material. From the van Deemter equation above, we see decreasing particle size results in improved separation efficiency (HETP) and improvements in resolution due to increased flow rate (μ).[119] Column efficiency is then inversely proportional to particle size. A van Deemter plot illustrating the relationship between column efficiency, flow rate, and column particle size is presented in Figure 1.[123] However, as particle size decreases, increasingly extreme pressures are required for adequate flow rates through the column. Pressures can reach several thousand psi in HPLC columns, and pressure increases exponentially with decreasing column particle size.[119]

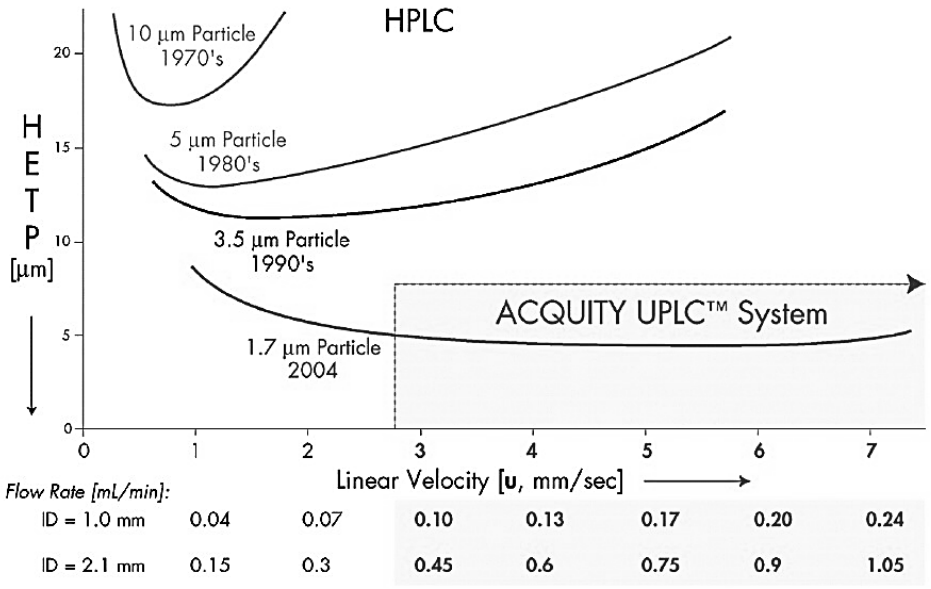

Figure 1: van Deemter plot comparison of column efficiency and column particle sizes. Adapted from Reference 123.

To take advantage of small particle sizes, high strength column materials had to be developed. In 2004, Waters synthesized a novel 1.7 µm column material with ethane-linked silanes with sufficient strength to withstand ultra-high pressures (15,000 psi) created in columns packed with the new material.[123] LC techniques using small particle columns and ultra-high pressures is called ultra-performance liquid chromatography (UPLC). The ultra-high pressures and small column packing in UPLC have made for improvements in solvent consumption and narrowed peak widths with a corresponding increase in detector sensitivity, improved peak resolution, and increased peak capacity.[119,123] UPLC techniques allow for increased flow rates without loss of chromatographic performance, giving high quality data in shorter run times than HPLC methods.[123] These advantages make UPLC more practical and cost effective vs. HPLC, and with the development of high quality <2 µm columns, UPLC has been adopted worldwide.

A variety of adsorbent surfaces are currently available in UPLC columns, allowing users to optimize chromatographic separations. Reverse-phase columns separate components via hydrophobic interactions with compounds dissolved in an aqueous mobile phase; normal-phase separates compounds by adsorptive and polar interactions with compounds carried in a nonpolar organic mobile phase.[2] An advantage of LC methods is the use of binary solvent systems. Binary solvent systems can have two mobile phases with different properties on board the instrument, for example, an aqueous mobile phase "A" (MPA), and an organic mobile phase "B" (MPB). This allows a user to further optimize separation, peak characteristics, and runtime by setting isocratic conditions with static mobile phase composition, ramp conditions with changing mobile phase composition, or a combination of isocratic and ramp conditions, to exploit analyte chemistries promoting the column retention or elution of an analyte.[123,125] This makes selecting appropriate column and mobile phases with the chemistries of the targeted analytes in mind a critical step for method development and optimization.

1.4.4 Introduction to Mass Spectroscopy

Mass spectrometry (MS) is a technique that is used to determine the masses of atoms, ionized molecules and molecular fragments.[126] Fragmentation of a "parent" or molecular ion gives structural information which, together with parent ion mass and retention time, can determine the chemical structure and elemental composition of analytes.[126] The fundamental properties of mass (m) and charge (z) are used to separate and detect parent and molecular ions in a mass spectrometer by manipulating ion paths by magnetic and electrostatic fields.[126] Mass spectrometry coupled with chromatography has become a common technique for the analysis of biological fluids in pharmacology and toxicology laboratories.[2,127]

A typical mass spectrometer will consist of an ion source, at least one mass analyzer, a detector, and a computer for instrument control and data processing.[126] The ion source produces gas phase ions required for MS analysis. A mass analyzer, or "quadrupole", can isolate ions based on their mass-to-charge ratio (*m*/*z*). For example, a molecule with a mass of 200 Da and molecular charge of +1 will have an *m*/*z* (200 Da / +1) of 200 Da; a molecule of 200 Da and +2 charge will have an *m*/*z* (200 Da / +2) of 100 Da. The detector will count the number of ions of selected *m*/*z* that exit the quadrupole. The number of ions that reach the detector is proportional to the concentration for the source of that ion in the analyzed sample.[2] Computer software will convert data from the quadrupole and detector to generate mass spectrum and chromatogram, when paired with chromatography instrumentation. The mass spectrum is presented as a bar graph of relative abundances of different *m*/*z* ions.

Mass spectrometry was developed in the late 19th and early 20th century by Wien and Thompson and used in the measurement of atomic masses and the discovery of elemental isotopes.[128] Charged ions that pass through an electromagnetic field are deflected relative to an ion's *m*/*z*.[128] Improvements in MS design were developed by Arthur Demptser and Francis Aston through 1917 and 1919, for which Aston won the Nobel Prize in 1922 for the discovery of isotopes and the whole number rule.[128] Subsequent mass spectrometers relied on the designs of Demptser and Francis, with considerable work done by Harold Washburn using magnetic sector MS for the characterization and refinement of petroleum in the 1940's.[128] Washburn compiled reference spectra from pure hydrocarbons and organic compounds, and serendipitously detected and identified trace levels of acetone in petroleum analyses. Washburn did not know sample vessels were cleaned with acetone, and today the ability to identify unknown compounds using MS is now widely regarded as one of the greatest strengths of the technique.[128]

In early MS, the mass analyzer was generally a magnetic sector apparatus. The development of the quadrupole mass analyzer in 1953 by Wolfgang Paul and Helmut Steinwedel revolutionized MS methods.[129] The breakthrough of Paul and Steinwedel allowed mass filtering of ions passing through the quadrupole. The quadrupole is made from 4 precisely-machined, parallel electrode rods mounted in a square configuration.[129] Opposing rods are paired and applied with radio frequency (RF) voltage, which is an alternating current (AC) electric potential that oscillates at radio frequencies.[129] Direct current (DC) voltages are also applied, and bias the potential between opposing rods in either the x or y axis.[129]

Ions that enter the MS are directed down the central axis of the quadrupole. RF and DC potentials applied to the electrode rods generate time-dependent electric fields that alter the trajectory of ions within the quadrupole.[129] Ion trajectories are complex, and can be described by second-order Mathieu differential equations. Mathieu equations for RF and DC potentials give either stable and periodic solutions or unbounded and unstable solutions.[129] Ions within stable solutions in both x and y directions travel through the quadrupole to reach the detector while ions with unstable solutions will collide with the electrodes and are removed from MS analysis.[129] The stability of an ion with a given m/z depends on the amplitude of RF and DC potentials and the frequency of the oscillating electric field.[129] By altering the RF and DC, ions of specific m/z will reach the detector while all other ions are filtered out. By narrowing the mass filter resolution, fewer and fewer trajectories will be stable; lighter ions are ejected by x-axis instability while heavier ions are lost through y-axis instability.[129] Conversely, by operating in RF-only (zero DC potential), almost all ions are transmitted through the quadrupole.[129] Mass filtration can be set for unit mass resolution or a range of masses.

Computing advancements in the 1960's and 1970's allowed for computerized instrument control, which allowed for very fast, high quality spectral data acquisition and precise RF and DC control.[129] In addition to computerized control, setting quadrupoles in series further augmented the capabilities of MS. Multi-stage MS, also called tandem MS, "triple quad" (MS/MS), allow for sensitive and accurate characterization and identification of analytes. In MS/MS, the first quadrupole (Q1) functions as a mass filter, allowing only specific *m*/*z* ions, or "parent ions", through to the instrument. The second quadrupole (Q2), also called a "collision cell", operates in RF-only; parent ions in Q2 are fragmented by kinetic interactions with a collision gas that produce fragment ions at diagnostic, reproducible masses and relative ratios for given collision energies and cell conditions.[2] Fragment ions generated in Q2 are transmitted to the third quadrupole (Q3), which acts as a second mass filter, segregating specific fragment ions from all others. Q3 selects for diagnostic ions produced by the parent ion for identification and quantitation. By selecting specific parent ions in Q1, fragmenting the parent in Q2, and selecting fragment ions unique to the parent ion in Q3, MS/MS methods offer highly selective and sensitive techniques for quantitation of targeted analytes in a wide variety of samples, including biological matrices for toxicological analysis.

The most common detector in mass spectrometry is the electron multiplier detector, first patented in 1930.[130] Electron multipliers are ideal for MS analyses due to their wide dynamic range, very fast response, and high sensitivity.[130] The detector functions by multiplying incident charges that strike a dynode surface of the detector. Ions are accelerated into the detector by significant electrical potential (-2 to 5 kV) applied at the entrance of the multiplier. Ions strike a dynode surface made of an emissive material which ejects multiple electrons for each ion striking the dynode. In a discrete electron multiplier, emitted electrons strike the surfaces of a series of

dynode plates of increasing potential. Each electron strike emits multiple electrons, resulting in a cascade of electrons several orders in magnitude more (~10^6-fold gain) reaching the detector than the number of ions that initially strike the first dynode.[130] The electron cascade terminates at an output generating an output current of several mA. The continuous electron multiplier was developed in 1962, and is constructed in a horn or cornucopia shape, lined with a continuous dynode made of proprietary electron emissive surfaces.[130] The electron cascade propagates to the narrow output end with negligible lag time, making continuous electron multipliers sensitive and fast enough for LC-MS applications.[130]

The electrons at the end of the multiplied electron cascade generate a current at the terminal electrode of the detector.[130] This current is proportional to the amount of ions striking the dynode; therefore, the current generated is assumed to be proportional to the concentration of the source of the ion in the sample. The current is a continuous, analog signal and is converted to a numerical digital output by an analog to digital converter circuit (ADC). The digital signal is a discrete value calculated in the ADC over a very short window of time. The speed at which digital outputs are calculated from the analog input is the sampling rate. The resolution of an ADC is defined by the range of discrete values produced over the range of voltages generated by the ADC. The ADC allows for very fast acquisition of digital data and simultaneous data processing by instrument computing, ideal for high throughput analytical laboratories.

1.4.5 Electrospray Ionization

Because ions must be in a gaseous state for MS analysis, ionization of analytes within a liquid solvent at atmospheric pressures was a significant challenge in the development of LC-MS systems. Today, atmospheric pressure ionization (API) sources are used to ionize and transfer analyte ions into MS instrumentation. Electrospray ionization (ESI) is the most regularly used

API method in forensic toxicology LC applications.[2] Many drugs are, or are derived from, plant alkaloids; alkaloids generally contain polar functionalities in their structure, and therefore are readily charged using ESI.[2] Electrospray is also capable of creating gas phase ions of high molecular weight analytes. ESI has been used for mass measurement and structural determination of proteins and virions up to MDa masses, like Hepatitis B, Norovirus, and Tobacco Mosaic Virus, even in native conformations.[131–136] Analytes and mobile phase exit the LC system into the ESI source enclosure through a narrow sample capillary housed within the electrospray probe for desolvation and ionization. Desolvation of mobile phase in ESI is achieved by nebulizing gas and desolvation gas. Nebulizing gas is pumped parallel with LC flow through a cylinder enclosing the sample capillary. As liquid droplets exit the capillary, nebulizing gas creates a turbulent aerosol "plume". At the electrospray probe tip, desolvation gas well above vaporization temperatures of the mobile phase is directed at the electrospray plume to further promote desolvation of mobile phase solvents.

Ionization of analytes can occur by two mechanisms: acid-base reactions of analytes in solution, and at the capillary tip where protons are generated by redox reactions at the liquid-metal interface (e.g., $2\ H_2O \rightarrow 4H^+ + 4\ e^- + 2\ O_2$).[137] A strong electric potential (up to several kV) is carried along the capillary, generating a strong electric potential between the capillary and MS orifice. At the capillary tip, surface tension and electrostatic forces pull the liquid into an ellipse that becomes a pointed cone at a threshold voltage called the Taylor Cone voltage.[138] Prior to reaching the Taylor Cone voltage, surface tension and Coulombic forces are balanced for specific radius curvatures at the apex of the fluid ellipse. However, the radius of apex curvature approaches zero as the applied voltage nears the Taylor Cone voltage.[138] At the Taylor Cone voltage, the radius of the apex becomes a point, forming a Taylor Cone from which droplets are

emitted.[138] The charge density at the point of the Taylor Cone is very high. Ejected droplets are close to their Rayleigh limit, the point at which a droplet's surface tension is in equilibrium with its Coulombic repulsion.[137] Droplets emitted from the Taylor cone undergo desolvation while in flight. Desolvation drives the droplet towards the Rayleigh limit, and Taylor Cones form highly charged smaller secondary droplets with radii approximately $1/10^{th}$ that of the ejecting droplet.[137,138] Charged droplets split into very small droplets only a few nm in diameter with continued desolvation and droplet fission. Nanodroplets are the source of gas phase ions analyzed by the MS.[137,138]

There are two proposed mechanisms of gas-phase ion release: ion evaporation and charge residue mechanisms. In ion evaporation, the surface of nanodroplets is sufficiently charged that individual ions are expelled in a solvated state by Coulombic repulsion.[138] In this model, electrical field strength of the droplet surface is so high that the energy needed to increase the droplet size during ion ejection is quickly compensated for due to the reduction of Coulombic repulsion from the loss of the ejected ion.[137,138] Ion evaporation rates are highly dependent on the chemical properties of the ion and the Gibbs Free Energy required for ion evaporation.[137,138] Ions that are highly "surface active" are more likely to undergo ion evaporation, and there is some evidence that low molecular weight molecules are more likely to undergo ion evaporation.[137] In the charged residue mechanism, nanodroplets that contain a single charged ion evaporate to dryness, leaving only the molecular ion.[137,138] It is not unreasonable to expect a single droplet to contain one molecule of analyte. For an analyte at 1 pmol/μL, a droplet with a diameter of 200 nm would contain on average less than one molecule.[138] Charged residue mechanism is widely accepted as the mechanism by which large, globular molecules like proteins in native conformation are released into the gas phase; however, it is likely the two mechanisms work in

concert.[137,138] The ESI process from plume to gaseous ion occurs within milliseconds.[139] Once in gaseous state, ions are drawn into the mass spectrometer along the electric potential between the source capillary and MS orifice for mass analysis.

It is important to note that the ESI source is essentially an electrochemical cell capable of operating in positive (POS) and negative (NEG) ion modes.[126,137] In POS, the electrospray plume carries a net positive charge with analytes in a cation state. To maintain electrical balance in POS, an electron must move from the sample solution to the electrode (the capillary) for each positive ion produced in the ESI source, and the reverse holds true for electronic balance in NEG.[126] Furthermore, components in the sample solution that generate ion current can disrupt the electrospray, leading to ionization suppression and potential loss of analyte signal.[126] It is therefore necessary to remove salts and other nonvolatile components in biological samples during sample preparation to minimize ionization suppression in biological sample extracts.[127]

1.4.6 Quadrupole Time of Flight Mass Spectroscopy

The quadrupole time of flight instrument (QTOF) is a high-resolution mass spectrometer. At its simplest, the QTOF consists of an ion source, quadrupole mass analyzer, ion drift tube, and detector with a sensitive and accurate timer. The first time of flight (TOF) MS was built in 1948 by A. E. Cameron and D. F. Eggers at the Tennessee Eastman Corporation.[140] The principle of TOF mass measurement is simple. Ions in a vacuum are accelerated down the axis of a drift tube by an electric field of known energy from a “pusher plate” pulse. The kinetic energy an ion receives is equivalent to the electric potential energy of a charged ion in an electric field, which is the product of the ion’s charge (z) and electric field potential (V). Ions travel at speeds inversely proportional to an ion’s m/z and will reach the detector at different times. The time from ion acceleration to detector impact is recorded, and ion velocity is calculated from the

measured time and the known drift tube length. Since we know the accelerating electric field potential, we can calculate an ion's *m*/*z* using simple Newtonian kinetics:

$$qE_k = zV \; ; \; and \; qE_k = \frac{1}{2}mv^2 \; ; \leftrightarrow \; m = {}^{2qE_k}/_{v^2} \; ; \tag{2.1}$$

$$v = {}^{d}/_{TOF} \; ; \quad TOF = {}^{d}/_{v} \; ; \quad and \; v = \sqrt{\left({}^{2qE_k}/_{m}\right)} \; ; \quad v = \sqrt{{}^{2qzV}/_{m}} \; ; \tag{2.2}$$

$$TOF = \left({}^{d}/_{\sqrt{(2qzV/m)}}\right) \; ; \; TOF^2 = {}^{md^2}/_{2qzV} \; ; \; TOF^2 = \frac{m}{z}\left(\frac{d^2}{2qV}\right) \tag{2.3}$$

$$therefore, \quad \boldsymbol{m/z = (2qV \, x \, TOF^2)/d^2} \tag{2.4}$$

E_k: Kinetic energy (J, or C·V, or kg·m^2/s^2)

z: ion charge number

q: elemental charge (1.602 × 10^{-19} C)

V: electric field potential (V, or kg·m^2/A·s^3, where 1 A = 1 C/s)

m: mass (kg, or Da)

v: velocity (m/s)

d: distance (drift tube length, m)

TOF: time of flight (ion travel time through the drift tube, (s)

Simplifying the units of formula 2.4 gives kg/z, or simply, mass to charge number.

Two major TOF advancements were developed in 1955; electric field-free ion gate pulse that allowed for measuring a full spectra between pulses, and two-grid pusher plate that compensated for kinetic energy differences in earlier single grid pusher plates.[140] Coupling TOF with MS first occurred with a gas chromatograph (GC) in 1956, with fast and accurate spectra measurements

from different organic compounds.[140] Early TOF instruments had resolutions (the mass of an ion/difference in mass at 50% peak height, or m/Δm) around 300.[140] The development of the "reflectron" in 1973 greatly increased mass resolution by functionally lengthening the flight tube and minimizing the spread of ions of given *m*/*z*'s over time.[140] Developments in computing and alternative ion sources such as ESI advanced TOF further by increasing the dynamic range and resolution of TOF MS, and allowing for spectral measurements of high mass molecules, even singly-charged proteins with masses greater than 100,000 Da.[140]

The Waters Xevo G2-XS (Waters Corporation, Milford, Massachusetts), is a quadrupole time-of-flight high-resolution mass spectrometer (QTOF-HRMS). The Xevo G2-XS can operate in positive or negative ion modes, and in MS^E or multiple reaction monitoring (MRM) mass spectra modes. MS^E mode can be thought of MS-"every" data independent acquisition, as all ions reaching the quadrupole pass through to the detector. Ions scanned in MS^E are fragmented by collisions with argon gas; collisions within alternating narrow time bins of "high energy" and "low energy" scans that permits simultaneous mass spectra data for parent ions from low energy scans, and fragment ion data from high energy scans. MS^E records all ion data without mass segregation allowing for retrospective MS analysis for unknowns or novel substances. The modern QTOF is an incredibly capable and robust instrument and is ideal for a wide range of applications like metabolomics or forensic toxicology analyses.

1.5 Pharmacology and Toxicology of Selected Stimulants

Central nervous system stimulants are a class of drugs that typically act on dopamine, norepinephrine, and/or serotonin neurotransmitter pathways in neuronal synapses to increase dopamine (DA), serotonin (5-HT), and norepinephrine (NE) concentrations in neuronal clefts, eliciting sympathomimetic effects.[141] Effects of CNS stimulants include increased alertness and

vigilance, improved accuracy and speed in certain task performances, reduced fatigue, improved concentration, increased blood pressure and heart rate, and elevated mood and euphoric effects.[141–146] Central nervous system stimulants have been used for thousands of years. Spanish conquistadors witnessed South American peoples chewing coca leaves to ward off hunger, and lift mood and energy.[147] Coca leaves were, and continue to be, chewed with an alkali, like lime from burnt shells, to help extract cocaine from the leaves.[147,148] In Ecuador and northern Chile, archaeological evidence of chewing coca leaves with an alkali has been found in graves as old as 4,000 years, and cocaine has been identified in mummified remains up to 1,500 years of age.[147]

Today in Canada, CNS stimulants are available over the counter or by prescription for a variety of clinical indications like congestion, narcolepsy, attention deficit disorders, depression, and as a smoking cessation aid. Given the stimulating and euphoric effects of stimulant drugs, abuse potential for CNS stimulant drugs are high, resulting in the diversion of therapeutic stimulants and clandestine production of illicit stimulants for nonmedical and recreational use.[147,149–151] The use of stimulants is a global problem. In 2016, 34 million individuals used amphetamines and prescription stimulants, 21 million used MDMA/“ecstasy”, and 18 million individuals used cocaine.[152] Nonmedical use of stimulant effects includes improving cognition and focus by increasing neurotransmitter concentrations in synaptic clefts within the brain in academic settings, and in athletics via stimulant agonist action on β-andrenergic receptors that increases cardiovascular output.[141,149,153–156] Recreational use of stimulants typically sees higher than therapeutic doses to elicit desired CNS stimulation effects that include euphoria, increased self-confidence, sexual stimulation, hallucinations, and increased energy; however, acute and chronic recreational use of stimulants comes with increased risks for hypertension, serotonin syndrome,

hepatotoxicity, neurological degradation, transmission of infectious diseases, seizures, stroke, and heart failure.[157–163]

In this study, commonly encountered stimulants and selected metabolites were selected for analysis in DBS samples. The panel of analytes are: cocaine (CN), benzoylecgonine (BZE), cocaethylene (CE), ecgonine methyl ester (EME), anhydroecgonine methyl ester (AME), amphetamine (AMP), methamphetamine (MA), phentermine (PH), 3,4-methylenedioxy-methamphetamine (MDMA), 3,4-methylenedioxyethylamphetamine (MDEA), 3,4-methylenedioxyamphetamine (MDA), bupropion (BUP), hydroxybupropion (OH-BUP), and methylphenidate (MP). Analytes were selected for their prevalence and forensic relevance.

1.5.1 Cocaine and Selected Metabolites

Cocaine is an alkaloid present in the leaves of *Erythoxylum coca*, used for thousands of years by the peoples of northern South America.[148] Cocaine was first extracted from coca leaves by Albert Niemann in 1860 and its chemical structure was determined in 1894 by Richard Willstätter.[34,164] The stimulant and anesthetic effects of cocaine were characterized following the extraction and purification of the drug, and recreational use worldwide began soon after.[34,148] Today in Canada, there are no legitimate clinical uses for CN, however, it is still used in certain jurisdictions as a vasoconstrictor and local anesthetic in certain nasal and ophthalmological procedures.[148,165] The UN Office on Drug and Crime (UNODC) estimated the 2017 global production of pure cocaine was 1,976 tons, while 1,275 tons of adulterated cocaine was seized.[166] From the UNODC's figures, it is clear that cocaine is significantly cut with adulterants. Adulterants in cocaine, like fentanyl and fentanyl-analogues, can have significant, even fatal, toxicologic effects.[167,168] However, cocaine adulterants are not within the scope of this study and are excluded from this work. *E. coca* leaves and derivatives, including cocaine, are Schedule I drugs in Canada.

Cocaine is typically administered by insufflation of the powdered hydrochloride salt, or smoking a freebase derivative, or IV injection of a cocaine hydrochloride solution.[2] The RoA for cocaine has been shown to influence the onset of subjective effects of cocaine, with the fastest onset from SM, then IV, and intranasal IN having the slowest onset of effects.[61,169] Bioavailability (the fraction of drug entering systemic circulation) of cocaine varies by RoA. Cocaine IN bioavailability range from 25% to 94%, and varies due to propensity to swallow some of the dose, and vasoconstriction of nasal capillaries by blocking NE reuptake as the drug crosses sinus mucosal membranes.[2,170] Cocaine is highly permeable across mucosal membranes, permeating by simple diffusion without competition.[170] Smoking cocaine gives an intense and rapid onset of action as the drug readily crosses across alveolar membranes into the blood for circulation, however, bioavailability for SM ranges 57% to 70%, and dependent on the temperature and smoking topography.[2,171] Once in the blood, cocaine readily crosses the BBB by passive diffusion and proton-antiporter transport.[172]

Cocaine anesthesia results from sodium channel blockade in peripheral nerve cells, increasing the threshold required for an action potential.[2] Cocaine vasoconstriction is the result of agonist action on cardiovascular adrenergic receptors.[173,174] Cocaine binds DA transporters (DAT), 5-HT transporters (SET) and NE transporters (NET) with similar affinities, and inhibits neuro-transmitter reuptake by the presynaptic neuron.[159,175] Increased neurotransmitter concentrations in the synapse increases post-synaptic neuronal stimulation. The primary psychostimulant mechanism of CN is its action on dopamine.[159,175] Dopamine buildup in the limbic system in the frontal cortex, especially in the nucleus accumbens, produces euphoric pleasure and acts on the reward pathway, reinforcing cocaine seeking, and is an important mechanism in developing cocaine dependence.[159,176]

Cocaine's primary metabolites benzoylecgonine and ecgonine methyl ester are formed by different mechanisms. Benzoylecgonine is formed by spontaneous hydrolysis at physiological pH (*in vivo* and *in vitro*) and by hepatic methylesterases.[30,177] Ecgonine methyl ester is produced by plasma cholinesterase and hepatic carboxylesterase action on cocaine.[30]

Anhydroecgonine methyl ester and benzoic acid is formed while smoking freebase and sodium bicarbonate derivatives of freebase cocaine.[178] Anhydroecgonine methyl ester is an analytical marker of smoking cocaine in forensic analyses.[171] The degree of AME formation is temperature dependent. Approximately 50% of CN is converted to AME and benzoic acid at 350-400 ℃, and almost all CN was pyrolyzed to AME and benzoic acid at 650 ℃.[171] Anhydroecgonine methyl ester also displays neurotoxic effects on memory and potentiation of CN effects, and because the degree of conversion of CN to AME is highly variable, AME may have greater significance than just as a marker of smoking cocaine, and its toxicology requires further study.[178,179]

Cocaethylene is an equipotent metabolite of cocaine formed by a transesterification reaction catalyzed by hepatic carboxylesterases with cocaine and ethanol.[34] Cocaine and CE show equivalent inhibition of DAT, reinforcing the effects of cocaine in the limbic system when CN and CE are present together.[34] Cocaethylene is metabolized to BZE; however, CE is longer acting than CN. In a human study, mean half-lives ($t_{1/2}$; ± standard error) of CN and CE administered by IV were 1.07 ± 0.09 hr and 1.68 ± 0.11 hr, respectively.[180] Another human study determined mean $t_{1/2}$ of CN and CE were roughly 1.5 hr and 2.5 hr, respectively, for both 0.25 mg/kg and 0.50 mg/kg doses by IV.[181] The longer half-life of CE means it is active longer, and can be detected in blood samples roughly 2.5 to 5 hours longer than CN. Given that CE and CN are equipotent, it has been suggested to add blood concentrations of CE and CN when interpreting the effects of the two drugs on intoxication, task performance, and toxicity.[34] The

chemical structures and pathways of formation for CN, CE, BZE, EME and AME are summarized in Figure 2.

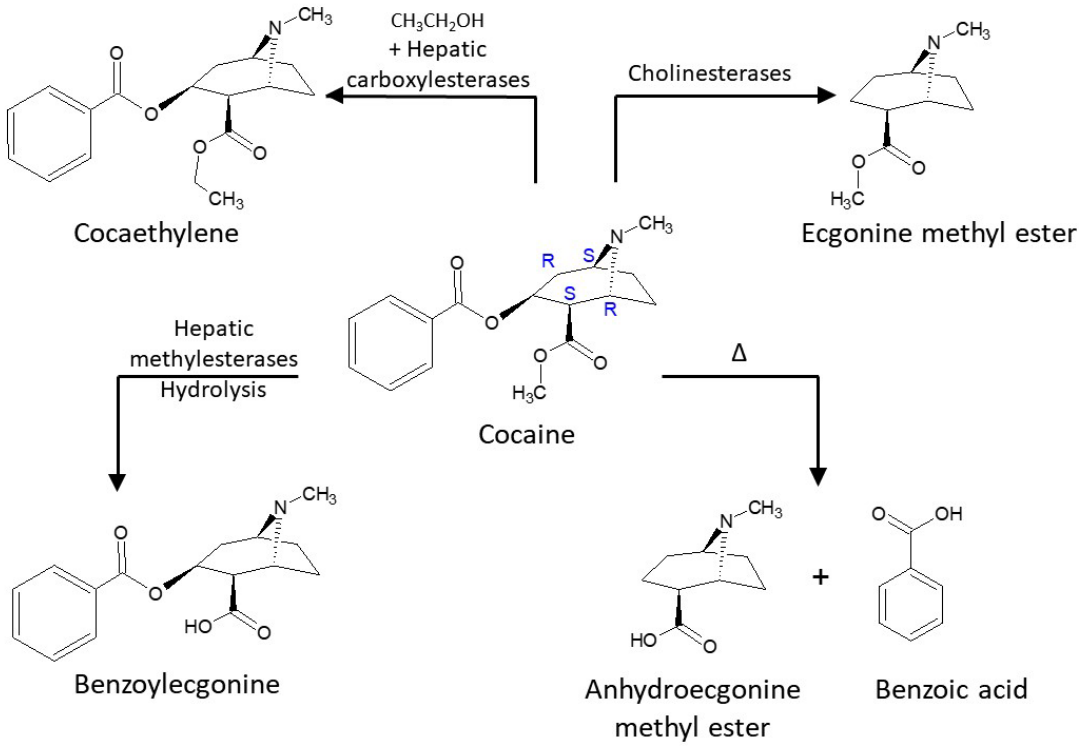

Figure 2: Cocaine related analyte structures and formation pathways.

Cocaine and its metabolites are excreted into urine by simple filtration in the glomeruli of the kidneys.[2] Though the $t_{1/2}$ of CN is relatively short, repeated use of cocaine can prolong the detection of CN in urine, presumably due to release of drug sequestered in tissues.[182]

1.5.2 Amphetamine, Methamphetamine and Phentermine

Amphetamine (AMP), methamphetamine (MA) and phentermine (PHEN) are potent CNS stimulants with similar structures to the endogenous monoamine neurotransmitters serotonin, norepinephrine and dopamine.[183] Amphetamine was first synthesized in Germany by Lazar Edeleano in 1887.[184] The same year, Nagayoshi Nagai isolated ephedrine from *Ephedra sinica*, and later synthesized methamphetamine from ephedrine in 1893.[183] Like coca leaves, alkaloid

derived from *Ephedra* have been used for thousands of years for its stimulant and antitussive effects.[183,185] Considerable attention was given to sympathomimetic compounds following Oliver and Schaefer's 1895 work on adrenal gland extracts, and methamphetamine was synthesized in Germany by E. Schmidt, independently from Nagai's earlier work.[183] In 1929, methamphetamine was separated into its dextro- and levo-isomers, and potent CNS stimulant effects from the *d*-MA and not *l*-MA were soon discovered.[186] Amphetamine and MA are Schedule I drugs in the Controlled Drugs and Substances Act of Canada.

Amphetamine's clinical effects of bronchodilation, pressor action, and reversal of barbiturate coma were reported by George Piness in 1930.[187] Amphetamine was available OTC in inhalers as Benzidrine for treatment of sinus congestion and asthma in 1932.[151,183] Interestingly, CNS stimulant effects of amphetamine were not reported until 1933; amphetamine misuse soon followed.[183,188] Amphetamine was widely available over the counter (OTC) and by prescription throughout most of the 20th century to treat sinus congestion and narcolepsy, as a weight-loss aid and appetite suppressant, and for hyperactive children.[183] Amphetamine has a high potential for abuse given its euphoric effects and reinforcement of dopaminergic reward pathways.[158] Reports of AMP misuse became increasingly frequent from the 1940's onward, reaching "epidemic" levels in the 1950's in Japan and Sweden, and increasingly problematic in the US during the 1960's. Misuse of AMP led to its control and withdrawal from OTC availability in the second half of the 20th century.[183,189] Today in Canada, amphetamine is available as the active component in Adderall® and the active metabolite of the prodrug lisdexamfetamine (Vyvance®) for the treatment of attention deficit disorders. Amphetamine can also arise in the body as a metabolite of methamphetamine.[43] Nonmedical AMP is available by illicit manufacture or diversion of therapeutics. Diversion is most likely in grade school and post-secondary

environments.[149,190] A survey of 826 physician respondents reported the majority of physicians believed one or more patients feigned symptoms to obtain an ADHD diagnosis, pursued prescription stimulants to improve academics, or diverted their prescription stimulants.[191]

Methamphetamine was widely available OTC and by prescription from the 1940's throughout the mid-20th century as aids in weight loss and wakefulness.[151] Methamphetamine was sold OTC as Pervertin in National Socialist Germany starting in 1938, Philopon and Sedrin in Imperial Japan from 1941 on, and as Methedrine or Desoxyn in the United States beginning in 1943.[151,192–194] During the war, MA was recommended or given freely to troops, airmen, laborers and housewives for increased alertness, energy, focus, and lift in mood. Most shockingly, MA was used to fuel the German Blitzkrieg attacks during WWII, and German troops consumed up to 35 million MA tablets in May through June of 1940 alone.[151,192–194] For excellent insight into the use of MA during the WWII, the author recommends reading Blitzed: Drugs in Nazi Germany by Norman Ohler. Negative effects of methamphetamine use were soon apparent to German troop commanders, and military MA use became far more judicious, falling to just 1 million MA tablets per month by December of 1940.[193] Civilian and military use and misuse of MA continued throughout the 1940's. Imperial Japan stockpiled a large amount of MA for military and industrial used during WWII.[151] Following the end of the war, the surplus MA flooded Japan and misuse of the drug became rampant between 1945 and 1957, a period known in Japan as the "first methamphetamine epidemic".[151] The timeline of MA controls follows that for AMP, given the contemporaneous availability and shared effects. Controls on MA began nationally in the 1950's, and became global in the 1970's as the negative effects of MA misuse became apparent.[151,183,186,189] In Canada, there is no therapeutic psychostimulant use for MA, though the drug (Desoxyn) is rarely prescribed in the US for treatments of attention deficit

disorders and obesity.[195] Illicit use and production of MA remains prevalent. Clandestinely manufactured MA was identified in roughly one third of all US drug seizures, and was the most identified drug seized in the US west of the Mississippi River in both 2016 and 2017.[196]

Amphetamine and MA have similar pharmacokinetics and will be described as one. Amphetamine and MA are stereoisomeric and the psychoactive effects are caused by the dextrorotary isomers S(+)-AMP and S(+)-MA.[197] Clinically, S(+)-AMP is the principle ingredient in Adderall® and generic versions, and S(+)-MA is found in Desozyn and generic equivalents.[195,197,198] Currently, R(-)-AMP is found in a 1:3 enantiomeric mixture with S(+)-AMP (Adderall® and equivalents), and *l*-MA is available OTC in decongestant inhalers.[195,197,198] The isomers of amphetamine, methamphetamine, and the chemical structures of phentermine, ephedrine, dopamine, norepinephrine, and serotonin are presented in Figure 3.

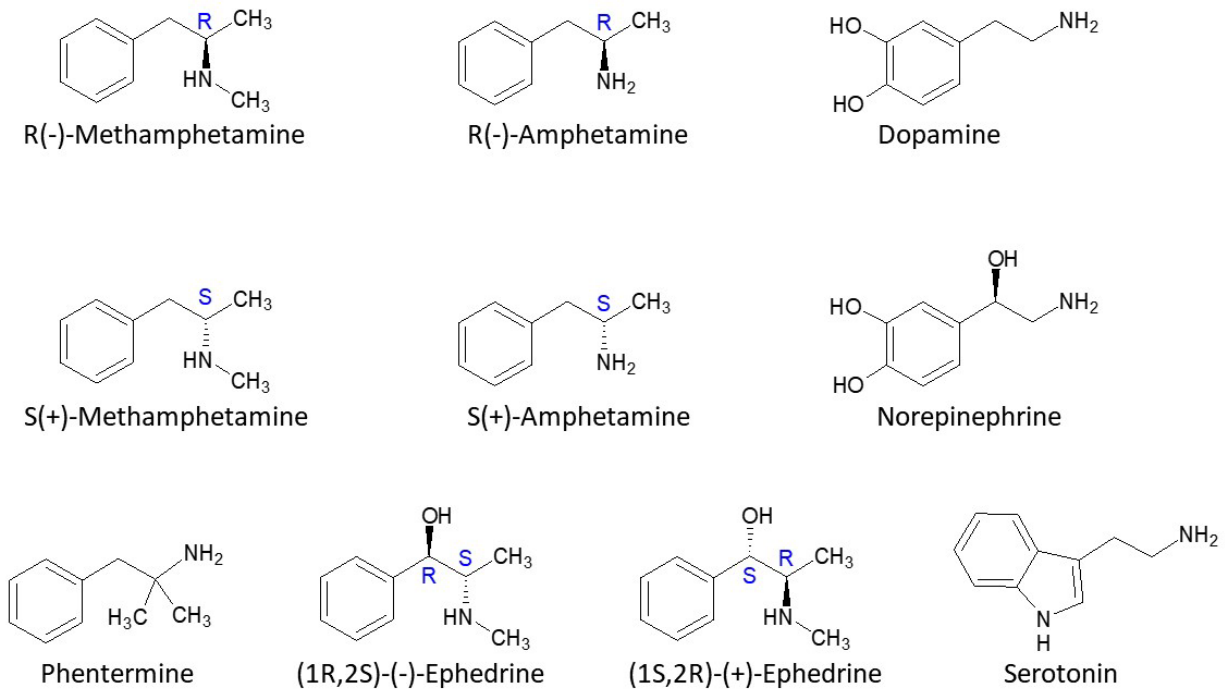

Figure 3: Chemical structure of amphetamine related compounds, serotonin, dopamine and norepinephrine.

Therapeutic formulations of MA and AMP taken orally are readily absorbed, with a bioavailability of 67%.[2] Route of administration for nonmedical and recreational misuse of AMP and MA typically include oral, IV, SM, and IN.[158] IV is associated with higher risks for unwanted effects that include anxiety, psychosis, paranoia and hallucinations.[158,199] Oral ingestion does not elicit amphetamine "rush", and people seeking euphoric high effects from AMP and MA are more likely to follow IV, SM, or IN administration routes.[158] Amphetamine and MA are weakly basic, low molecular weight and highly lipophilic molecules that easily cross epithelial membranes when smoked or snorted.[158] The drugs are volatile, lending to misuse by smoking. Bioavailability for SM can be up to 90% with a rapid and intense onset of the desired effect, which is distinct from the effects following oral or IN administration.[2]

CNS stimulant effects of AMP and MA are the result concerted stereoselective inhibition of SERT, NET, and DAT reuptake transporters, the release of NE, DA, and 5-HT from presynaptic vesicular monoamine transporters (VMATs) and reversal of SERT, NET, and DAT presynaptic transporters that results in efflux of DA, NE, and 5-HT into the synaptic cleft.[200–202] S(-)-isomers of AMP and MA have similar affinities with VMATs as DA, and are readily taken into VMATs.[202] VMAT efflux of neurotransmitters into presynaptic intracellular fluid is caused by collapse of the pH gradient across the vesicular gradient (pH = 5.5 within VMAT, intracellular pH = 7.5) by neutralizing the acidic pH within the VMAT.[202,203] Reversal of SERT, NET, and DAT is caused by S(+)-AMP and S(+)-MA agonism on trace amine-associated receptor-1 (TARR1).[200,201,204] Methamphetamine is shown to bind with TAAR1 and activate protein kinase C phosphorylation, leading to presynaptic DAT internalization and efflux of DA.[204] Amphetamine reversal of DAT was later shown to follow the same mechanism.[201] Amphetamine and MA mediated action on NE transporters is equivalent to DAT, and DA, and NE are both

released into the synaptic cleft in roughly equal proportions in a dose-dependent manner.[201] SERT reversal and presynaptic efflux of 5-HT is also triggered by AMP and MA, though to a lesser extent than for NE and DA.[201] Like cocaine, reward pathway reinforcement by DA stimulation is an important mechanism for developing dependence on AMP or MA.

Phentermine (PH) is a structural isomer of methamphetamine (Fig. 3) and was approved as a short-term weight loss aid by the FDA in 1959.[205] Phentermine has been considered an atypical amphetamine and is not subject to strict controls placed on AMP and MA since PH has a low potential for abuse.[206] Phentermine came to notoriety in the 1990's in "fen-phen", a combination of fenfluramine and phentermine that increased the risk of newly diagnosed and potentially fatal cardiac valve disorders, and "fen-phen" was withdrawn from markets in 1997.[207–209] Phentermine is still available in the US, though it was withdrawn from European markets in 2000 for its poor risk-benefit ratio.[205]

Phentermine is primarily an adrenergic agonist and lacks the psychostimulant action of AMP and MA, nor dependence from dopaminergic reward-reinforcement.[210] Phentermine causes release of NE from presynaptic vesicles in neurons in the lateral hypothalamus.[205] Increasing NE concentration in the synaptic cleft increases the stimulation of β2-adrenergic receptors, causing a reduction in appetite.[205,211]

Amphetamine, methamphetamine, and phentermine are excreted in urine by simple filtration in a pH dependent manner. Approximately 45% of dose of MA is excreted in urine at normal pH within 24 hours. Excretion of AMP, MA, and PH can be accelerated by making the urine acidic, creating an ion trap for the drugs.[2,212] Roughly 7% of a given dose of methamphetamine is excreted as amphetamine following N-dealkylation by hepatic CYP2D6.[43]

1.5.3 3,4-Methylenedioxymethamphetamine Related Drugs

The ring-substituted phenethylamine compounds 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyethylamphetamine (MDEA), and 3,4-methylenedioxy-amphetamine (MDA) are potent CNS stimulant empathogens known as "ecstasy"-group drugs, or designer drugs. Ecstasy group drugs are popular for their euphoric effects, lifted mood and energy, changes in perception, enhanced empathy and emotional connectedness, and increased pleasure and sensation from intimate and sexual stimulation.[213–215] In Canada, MDA, MDMA, and MDEA are listed on Schedule I of the Controlled Drugs and Substances Act.

MDA was first synthesized in 1910 by German chemists Mannich and Jacobsohn.[216] MDA was marketed in the US for antitussive, ataractic, and anorexigenic uses in the 1950's and 1960's.[217] Abuse of MDA for its psychostimulant properties was reported in the 1960's, and MDA was listed on the original Schedule I table in the American Controlled Substances Act of 1970.[218] MDMA was first described after a two-step synthesis from safrole in an E Merck patent written in 1912.[214,219] Pre-clinical trials of MDMA to investigate NE-like substances were conducted in 1927 at Merck; however, psychotropic effects in humans was first reported by Alexander Shulgin and David Nicols in 1978.[219,220] The role of Shulgin in the development and popularity of ecstasy-group drugs should be stated. Shulgin synthesized and tested over 200 designer drugs himself and with a close cohort, and introduced a number of colleagues to the drugs themselves and their syntheses.[221,222] Recreational use of MDMA is reported in the early 1970's in the Chicago area, purportedly after Shulgin's synthesis of MDMA was provided to a "psychology student in the Midwest" in 1970.[221] Shulgin as the origin of modern MDMA use is speculative; however, the popularity of the drug skyrocketed world-wide in the 1980's and was added to controlled drug schedules that decade.[219,221] MDEA was first synthesized by Shulgin in 1967, but

wasn't reported in literature until 1978.[221,223,224] MDEA effects are similar to MDMA, though shorter lived.[223] MDEA is an example of an early designer drug, with a molecular structure different from a scheduled drug but maintains similar psychotropic effects. MDEA was scheduled as a Class I drug in the US in 1987, but remained uncontrolled in Germany and The Netherlands until the early 1990's.[145] Ecstasy-group drugs are manufactured clandestinely in tablet, powder and crystal forms of varying ecstasy content.[166,225] Adulterants found in ecstasy include caffeine, methamphetamine, mephedrone, and novel psychoactive substances that can elicit undesired or toxic effects.[166,225–227]

Ecstasy-group drugs are typically taken orally, though insufflation and smoking is also reported.[228] Typical oral doses for MDEA, MDMA and MDA are 100-200 mg, 100-200 mg, and 60-120 mg, respectively.[145,158] Like amphetamines, ecstasy-group drugs are isomeric, weakly basic, are highly bioavailable orally, and can readily cross cellular membranes and distribute into tissues.[158,229] The chemical structures of MDMA, MDEA and MDA are presented in Figure 4.

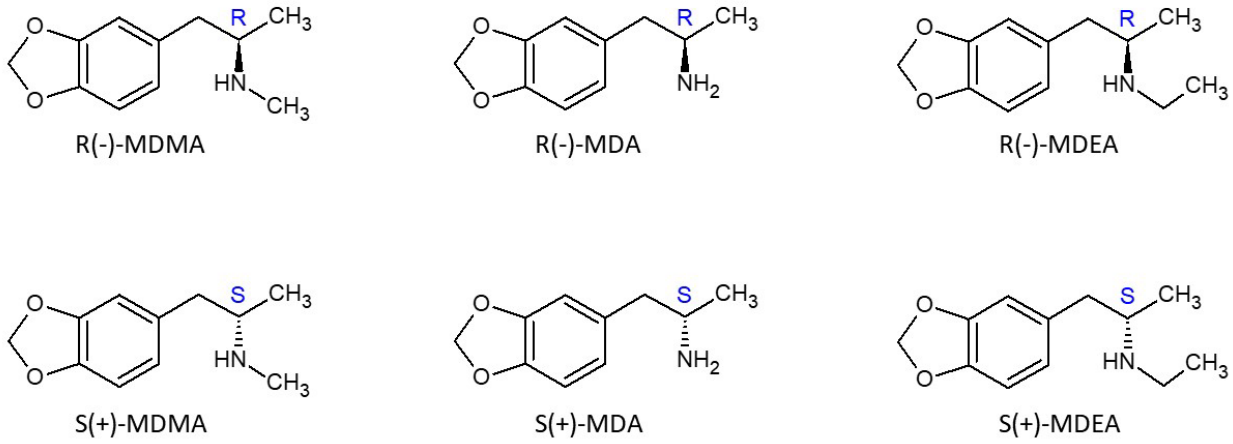

Figure 4: Chemical structure of MDMA, MDA and MDEA.

Ecstasy-group drugs elicit psychostimulant effects by primarily acting on SERT, as well as DAT and NET in an enantiomeric mechanism similar to those of MA and AMP.[145] MDMA, MDEA

and MDA affinities for 5-HT presynaptic transporters are stereoselective, and the empathogenic effects are primarily due to reuptake inhibition and efflux of 5-HT from presynaptic neurons.[145,158,230] TAAR1 activation is stereospecific to (*S*)-MDMA, leading to presynaptic internalization and reversal of SERT, DAT, and NET.[230] Observations from animal experiments and human studies comparing (S) to (R)-isomers of MDA, MDMA and MDEA support the stereospecific action of (S)-isomers on TAAR1.[145,158,217,230] Some studies have shown ecstasy-group drugs release intracellular stores of 5-HT, DA, and NE; however, amphetamine-like action on VMATs in animal models is not observed; SERT downregulation is seen following repeated MDMA administration, indicating that the primary action of MDMA is on SERT and not on VMATs.[231,232] R(-)-MDMA has higher affinity for post-synaptic 5-HT_{2A} receptors and potentiates secondary signaling, which some studies, though not all, indicate R(-)-MDMA elicits hallucinogenic effects at recreational doses.[233,234] Recent investigations have shown R,S(±)-MDMA to be a useful post-traumatic stress disorder therapy. Preclinical studies of R(-)-MDMA has been shown to have equal efficacy with R,S(±)-MDMA on social and emotional behaviors in patients with post-traumatic stress disorder, without the psychostimulant effects and abuse risks that come with S(+)-MDMA, though more research is needed.[233]

MDMA, MDEA, and MDA are metabolized extensively by hepatic enzymes, and metabolic clearance of MDMA ranges from 50-75% of a given dose.[43,234] Methylenedioxy-ring demethylation follows CYP2D1, 2D6, 3A2, and 3A4 action, and N-dealkylation of the amine side chain is due to CYP1A2, 2D1, 3A4, and 3A2 metabolism.[145] Roughly 20% of a given MDMA dose is excreted unchanged in urine.[234] MDA is produced from N-dealkylation of MDMA.[145,158]

1.5.4 Bupropion and Hydroxybupropion

Bupropion (BUP) is a substituted cathinone CNS stimulant with selective inhibition of NE and DA reuptake and noncompetitive inhibition of nicotinic acetylcholine receptors (nAChR).[235,236] Bupropion was first described in a patent filed by Glaxo Wellcome Inc in 1970 (claims priority Dec. 4, 1969, granted June 25, 1974) with noted antidepressant effects in mammals without inhibition of monoamine oxidase.[237] Stimulant effects were observed in animals at doses well above those that elicited antidepressant effects.[237] Bupropion is available by prescription in Canada, the US, and much of Europe for the treatment of major depression disorder (MDD) and seasonal affective disorder (Wellbutrin®), as a smoking cessation aid (Zyban®), and in combination with naltrexone (Contrave®) for chronic weight management.[235,238,239].

Bupropion is available in 3 oral formulations of racemic R,S-BUP for immediate, sustained, and extended slow release.[235,240] Bupropion is often categorized as an "atypical antidepressant", shown to have equivalent efficacy with some tricyclic antidepressants (TCAs) and selective serotonin reuptake inhibitors (SSRIs), without the unwanted weight gain, somnolence, and sexual dysfunction effects of some antidepressants.[235] Unlike TCAs, BUP does not inhibit monoamine oxidase, nor elicit sympathomimetic or anticholinergic effects.[241] Pharmacologic effects are due to BUP and its active metabolite hydroxybupropion (OH-BUP).[236,242] Antidepressant action is due to relatively weak NET and DAT inhibition by BUP and OH-BUP with zero serotonergic activity, though the mechanism of transporter inhibition is not fully understood.[235,243] PET imaging has confirmed BUP and OH-BUP's clinical effect by inhibiting striatal uptake of a radiolabeled DAT-selective ligand up to 24 hr after BUP administration.[244] Higher blood OH-BUP concentrations are more predictive of smoking cessation outcomes than BUP concentrations, and OH-BUP may significantly contribute to smoking cessation.[242,245]

Bupropion's efficacy as a smoking cessation aid may be due to the simultaneous noncompetitive antagonist of BUP and OH-BUP on nAChRs and dopaminergic alleviation of withdrawal symptoms by inhibiting DAT.[236,246]

Pharmacologic activity of OH-BUP is stereoselective. Racemic BUP is metabolized to (2S,3S)-OH-BUP and (2R,3R)-OH-BUP, and drug activity is due to BUP and (2S,3S)-OH-BUP.[236,241,246] The stereochemistry of BUP and OH-BUP is presented in Figure 5. The (2S,2S)-OH-BUP isomer has a higher affinity for DAT and preferential blockade of nAChR subtypes associated with nicotine's behavioral effects than racemic BUP, but not for NET.[236]

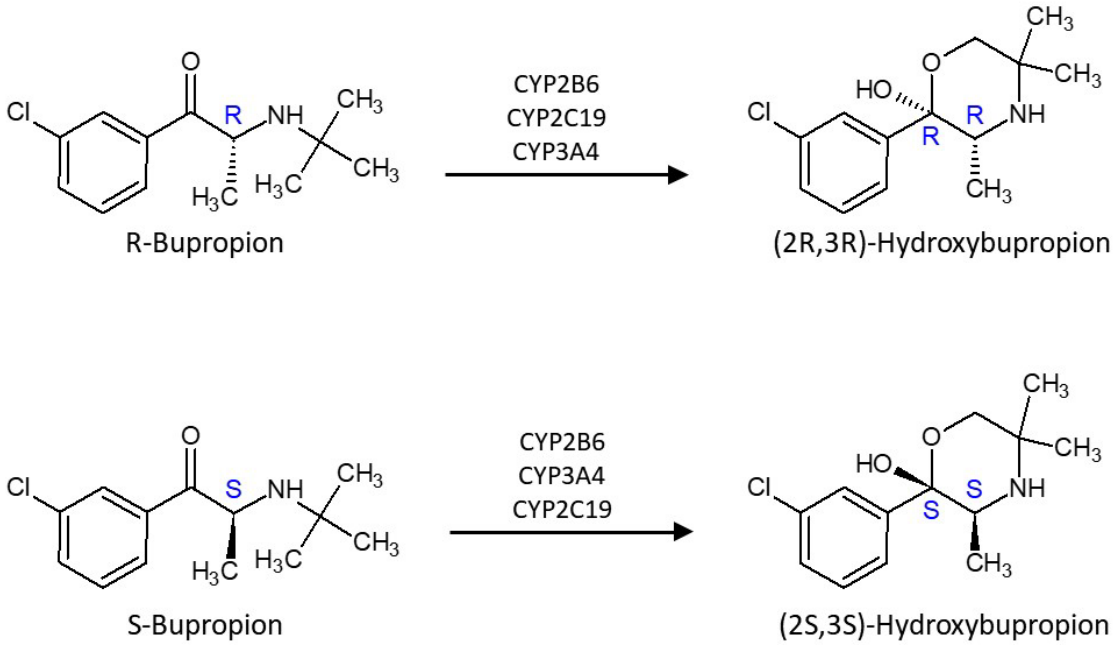

Figure 5 Chemical structure of bupropion and hydroxybupropion.

Metabolism of BUP to OH-BUP is primarily mediated by CYP2B6.[240,247,248] Stereoselective metabolism of BUP in human liver microsomes is reported. Formation of (2S,3S)-OH-BUP is more favorable in CYP2B6, and observed elevated hepatic excretion of S-BUP relative to R-BUP is a product of stereoselective CYP2B6 activity.[240,248] A fraction of OH-BUP is produced

by CYP2C19 and CYP3A4.[240] CYP2D6 polymorphisms have equivalent effects on R,S-BUP hydroxylation rates without effect on plasma BUP concentrations or elimination of BUP.[249] CYP2B6*6 homozygotes displayed decreased hydroxylation and CYP2D6*4 types had increased OH-BUP formation rates.[249] CYP2C19 variants did not affect the disposition of OH-BUP.[249]

Less than 1% of a given BUP dose is eliminated unchanged in urine.[250] Glucuronidation of BUP metabolites is extensive; only 10% of a BUP dose is excreted as unchanged drug or free metabolites.[250] Glucuronidation of BUP metabolites is stereoselective, with (R,R)-isomers of OH-BUP and hydro-bupropion are the most abundant metabolites of BUP in urine.[250] Stereospecific metabolism and elimination pathways may have important clinical effects on BUP and its active metabolite (2S,3S)-OH-BUP; however, the lack of polymorphism effect on BUP plasma concentrations and clearance speaks to complex metabolism for BUP. CYP2D6, CYP3A4, and CYP2C19 are relatively minor contributors to BUP metabolism and therefore clinical outcomes are relatively unchanged across polymorphisms in OH-BUP metabolism.[240,249]

Bupropion is a safe and effective treatment for MDD and tobacco dependence, however, seizures, though rare, have been reported at high therapeutic doses.[251] Misuse of BUP for "cocaine-like high" has been reported via IN and IV injection of BUP.[252–254] Effects of BUP misuse include tachycardia, hallucinations, tremors, and agitation.[255] Seizures are a common indication of BUP overdose and occur regardless of RoA.[255] Severe cases of BUP poisonings are survivable with supportive care. Of 975 BUP poisonings captured by US poison centres between 2000 to 2013, four fatalities were reported.[255]

1.5.5 Methylphenidate

Methylphenidate (MP) is a CNS stimulant prescribed for attention deficit disorders and narcolepsy.[143] Methylphenidate was first synthesized in 1944 and saw clinical use in the 1950's and 1960's to reverse barbiturate-induced coma; today it is better known by its trade name, Ritalin® and its wide use for the treatment of attention deficit disorders in children and adults.[256] Methylphenidate is a piperidine-derived molecule with two chiral centers and 4 enantiomers.[143] The first MP formulations were a mix of all 4 enantiomers. Present MP formulations are made from d,l-*threo*-methyphenidate, with pharmacological activity coming from the d-*threo* isomer.[143,257] The structure of methylphenidate is presented in Figure 6.

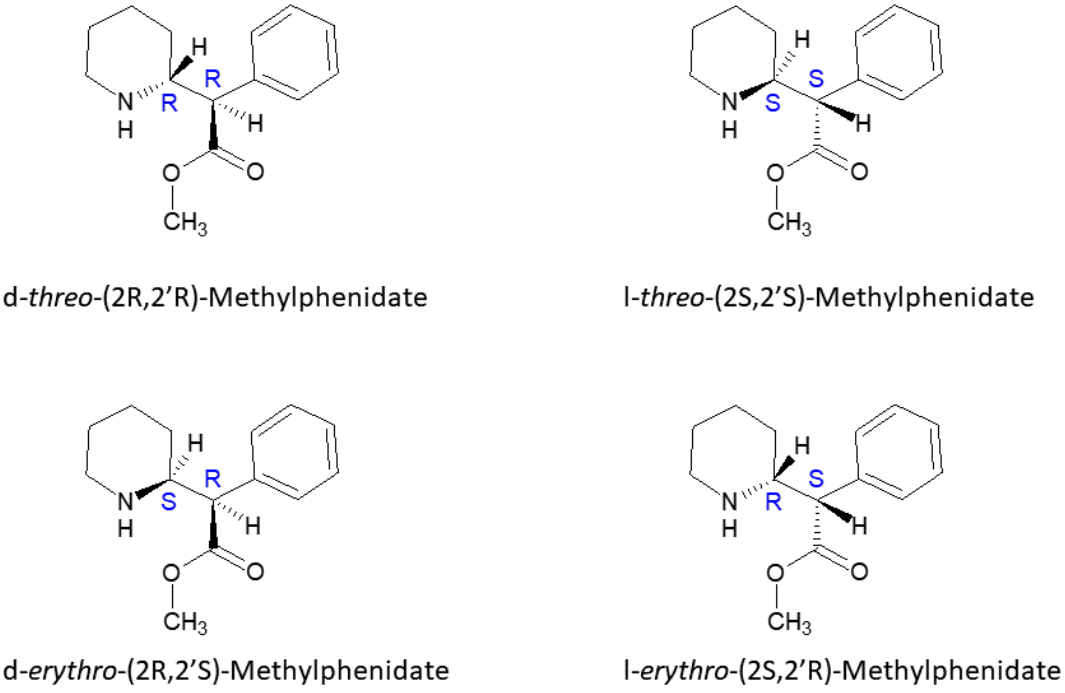

Figure 6: Chemical structure of methylphenidate isomers.

Methylphenidate and dopamine compete for binding in lieu of their shared phenethylamine structure.[258] The clinical effects of MP are due to blockade of presynaptic DAT by d-*threo*-MP; l-*threo*-MP binds non-specifically in humans and does not increase synaptic concentrations of

DA.[257] Inhibition of DA reuptake leads to neurotransmission amplification by prolonged presence of DA in the synaptic cleft.[258] *In vivo* microdialysis and photon emission computed tomography imaging with labeled MP has shown MP uptake is highest in the striatum and nucleus accumbens.[143] Methylphenidate mediated improvements in accuracy and reaction time for go/no-go sustained attention tasks studied with functional MRI imaging have shown MP promotes activity in brain networks associated with sustained attention and improved task performance, and may be a mechanism for the cognitive improvements observed with MP treatment.[259,260] Improvements in cognition and focus are the primary reason for diversion of MP for nonmedical use in secondary school students, though snorting and IV misuse of crushed MP for recreational euphoric effects is also reported.[261,262]

Methylphenidate is readily absorbed by oral administration, with a relatively short plasma $t_{1/2}$ of 1.5 to 2.5 hr.[143] Extended release formulations of MP are available (e.g., Concerta®) for once-daily dosing, with $t_{1/2}$ of roughly 4 hours in extended release tablets.[143] Most of MP is hydrolyzed to the inactive metabolite ritalinic acid by carboxylesterases.[143,258] Major metabolism by carboxyesterase-1 isoform may show enatioselection of l-*threo*-MP, resulting in prolonged half-life and higher circulating concentrations of active d-*threo*-MP.[258] Less than 1% of a MP dose is excreted unchanged in urine, and 60% to 80% of the dose is eliminated in urine as ritalinic acid.[258] Minor metabolites excreted in the urine are lactam and an active metabolite, hydroxymethylphenidate.[258]

1.5.6 Task and Driving Impairment by Stimulant Drugs

Drugs impair task performance, including driving, by acting on the brain in proportion to the brain drug concentration. In the EU, between 28 to 53% of drivers seriously injured in automotive accidents test positive for at least one psychoactive substance.[263] Compared to sober

drivers, polydrug use increases the risk of serious injury or death 5 to 30-fold.[264] Polydrug use is prevalent in medical and nonmedical users of therapeutic stimulants and ecstasy users, increasing the risk for negative driving outcomes in stimulant users.[153,213,264,265] Polydrug use also happens unwittingly as adulteration of illicit stimulants is common.[166–168,225–227] Stimulant drugs are often the second most commonly detected class of drugs in drivers involved in accidents, random roadside surveys studies, and driving violations.[266–270] Regionally and in commercial drivers, stimulants have been reported as the most common drug detected in drivers following traffic violation stops.[155,270] Truck drivers that tested positive for stimulants have been reported to have a greater proportion of driving infractions compared to drivers free of stimulant drugs.[271] Both illicit and therapeutic stimulants may improve certain tasks at lower blood concentrations, however, stimulants can impair driving tasks in the intoxication or "high" phase, and post-intoxication in the withdrawal, or "crash" phase, and have contributed to impaired driving accidents and deaths.[142,155,213,272–279]

Low doses of stimulants are shown to improve simple task performance, but the observed improvements can reduce or disappear as the complexity of task increases.[2] Therapeutic doses of methylphenidate show improvement in driving, especially in subjects with attention deficit disorders.[142,280] Driving is a vigilance task, and lapses of attention can lead to accidents.[281] Enhanced vigilance task performance and improved performance over time are seen with administration of AMP and MP, and the benefits are not limited to fatigued subjects.[144] Driving task improvements are also observed following administration of recreational stimulants. Methamphetamine showed dose dependent improvements (0, 20, or 40-60 mg) in driving simulation tasks in both narcoleptics and controls.[282] Cocaine can improve reaction time and task

vigilance. In a study of non-simulated driving and ecstasy, road tracking performance (lateral lane position) improved in individuals following a single 75 mg dose of MDMA.[272]

However, in the same MDMA study, accuracy of speed adaptation during car-following was poorer following MDMA administration.[272] The authors highlighted some important limitations of this study. 75 mg is a relatively low dose for recreational MDMA use, and multiple doses of MDMA are taken over successive days, and this study does not capture impairments following real-world use of MDMA.[272] Another study of MDMA showed drivers were prone to accepting higher levels of risk, though MDMA doses in this study were also lower (mean 56 mg) than typical recreational use.[283] Speeding, jumping red lights, and other reckless driving is reported in injuries and serious accidents that resulted from MDMA, MDA and MDEA impairment, and speak to the potential role that risk taking behavior has in stimulant impaired driving.[213,275,284,285]

Methamphetamine impairments of driving tasks are similar to those seen with ecstasy-group drugs. Behavioral manifestations of MA and AMP intoxication are expressed while driving, and are seen as erratic driving, drifting both out of the lane of travel and off the road, and speeding, and MA-related traffic fatalities are consistent with these driving behaviors.[273,286,287] Drivers impaired by MA also have shorter following distances between vehicles and left less distance between oncoming traffic when making turns in traffic.[288]

Drivers impaired by cocaine exhibit risky driving behaviors common with other stimulant impaired driving: speeding, deviation from lane of travel, and running stop signals.[278,289] Further, positive physical effects and overconfidence following cocaine and other stimulant misuse can impair decision making when driving, and may make driving under the influence of cocaine more likely than under the influence of cannabis.[279,289]

In addition to impairment during stimulant highs, the pattern of binge-use for stimulants can cause significant fatigue, or "crash" following extended sessions of stimulant use.[155,287] The crash phase can come on sharply, sometimes within 15 to 30 minutes after binge use. Crash-phase related lethargy, psychomotor retardation, cognitive impairments, and dysphoria can persist for several days.[290] Stimulant crash and withdrawal symptoms are consistent with dopamine and serotonin depletion and can impair driving tasks.[157,232,291,292]

1.6 Interpretive Challenges

Blood delivers drugs to the brain, making blood drug concentration a surrogate estimate of brain drug concentration. Although oral fluid or urine analyses for drugs are practiced forensically, blood, and only blood, provides interpretive value to assess allegations of impairment. Jurisdictions worldwide have established drug in blood concentration *per se* limits for impaired driving, thus requiring methods for convenient sampling and accurate quantitation of drugs in blood. Current methods of liquid blood collection are invasive, require trained staff or medical professionals, and often hours have passed between an incident and sample collection. Such time delays often render interpretation of drug concentrations in blood at the time of the incident impossible. A simple and timely method for sampling blood that can be performed in a number of environments is required.

Another challenge is the lack of correlation between intoxication and drug concentrations in blood, be it from wet whole blood or DBS sample measurements. Case studies have shown significant variation in drug in blood levels following traffic accidents and observed driving behaviors, and intoxicated drivers have a wide range of drug in blood concentrations. Once source of variability in drug in blood concentrations is tolerance. Tolerance is the reduction of a drug's effect with repeated administration of that drug. It follows that tolerant individuals will

have higher stimulant blood concentrations than non-tolerant individuals for a given level of intoxication. Another confound is crash phase intoxication, in which a person is impaired by effects typically associated with CNS depressants, potentially at low stimulant in blood concentrations. Yet another significant challenge is the propensity for polydrug use among recreational drug users. Drugs with synergistic impairment may present serious effects at lower blood concentrations. The disposition and effects of polydrug use may be mitigated or enhanced by genetic factors, especially for prodrugs or drugs with active metabolites.

1.7 Goals of This Study

The aim of this study was to optimize and develop a validated method for the simultaneous screening and quantitation of the panel of stimulants at forensically relevant blood concentrations in microvolume DBS samples using sonication and SPE drug isolation with UPLC-QTOF-HRMS instrumentation, and to investigate room temperature stability of the analytes in DBS samples over 8 weeks.

Chapter 2

2 Optimization of Sample Preparation for Analysis of Stimulants in DBS Samples

DBS sampling is a stable, cost effective, and easy method for analyses of endogenous compounds and xenobiotics in blood. Recent developments in analytical instrumentation have made accurate and reliable detection and quantitation of analytes at low concentrations in microvolume samples possible. The optimization of DBS sample preparation for the analysis of stimulants in DBS samples is described in this chapter.

2.1 Considerations in DBS Sample Preparation

The two main challenges of drug analyses in DBS are the sample matrix and low masses of analytes. Blood is a complex matrix of cells, proteins, lipids, and cellular residues that can suppress analyte signal in LC-MS analyses. Components in a sample that alter the signal of an analyte are known as “matrix interferences”. Analytes in blood have to be isolated from matrix interferences using extraction methods like SPE. Drug concentrations in blood are often forensically relevant at low concentrations ($< 10^1$ to 10^2 ng/mL), and drugs may only be present in picogram amounts in a 20 µL DBS sample. Even with ultrasensitive instrumentation, it is necessary to recover as much of the analyte as possible for reliable analyses of DBS samples. Drug extraction from DBS samples ideally take as few steps as possible as each preparation step can introduce potential for result variability and analyte loss. The combination of matrix interferences and low levels of analytes require a reliable, efficient, and high recovery sample extraction method for DBS drug analyses.

2.2 Experimental

Sample preparation optimization followed Scientific Working Group for Forensic Toxicology (SWGTOX) method validation criteria for matrix effects (±25%). Matrix effects were calculated from the ratio of drug response in post extraction spiked samples to drug response in neat standards at 10 ng/mL, 100 ng/mL, and 1000 ng/mL concentrations. Drug recovery was calculated from the ratio of analyte response in drug positive samples to drug response in post extraction spiked samples.

2.3 Chemicals and Reagents

All solvents used were LC/MS grade. Methanol (MeOH), acetonitrile (ACN), water, and 2-propanol (IPA) were obtained from EMD Millipore (Billerica, Massachusetts). All other chemicals used were reagent grade. Ammonium hydroxide (NH_4OH), ammonium formate, and formic acid were purchased from Fisher Chemicals (Bridgewater, New Jersey). Phosphoric acid (H_3PO_4) and sodium hydroxide (NaOH) were purchased from BDH (Radnor, Pennsylvania). Leucine encephalin (LeuEnk) was provided by Waters (Medford, Pennsylvania). Drug standards (CN, BZE, CE, EME, AMP, MA, MDMA, MDEA, MDA, PH, BUP, OH-BUP and MP) and deuterated internal standards (CN, BZE, CE, EME,-d3; MDA, MDEA, MDMA,-d5; OH-BUP-d6, AMP-d8, BUP-d9, and MA-d11) were obtained from Cerilliant Corporation (Round Rock, Texas). Drug-free sheep blood was obtained from the Canadian Food Inspection Agency (Ottawa, Ontario).

2.4 Sample Preparation

Drug-free sheep blood was serially diluted for extraction optimization at drug concentrations of 10, 100, and 1000 ng/mL. Drug positive samples, negative controls, and post-extraction spike

(PES) samples (20 µL, n = 3) were spotted on Whatman® 903 Protein Saver Cards (GE Healthcare, Chicago, Illinois) and dried overnight at ambient conditions. Whole DBS samples were cut from sample cards using a ½″ punch. DBS samples were extracted in 1 mL of extraction solvent within fresh test tubes immersed in an ultrasonic bath using an FS20D Ultrasonic Cleaner (Fisher Scientific, Waltham, Massachusetts). Extractions using water and solutions of 10%, 20%, 30%, 40%, and 50% MeOH in water as extraction solvents were assessed.

2.5 Solid Phase Extraction

Three different wettable Oasis® SPE products (Waters Corporation) were assessed during method optimization: PRiME HLB 10 mg sorbent 96 well plates, PRiME MCX 10 mg sorbent 96 well plates, and a product introduced after the onset of this work, PRiME MCX µElution 2 mg sorbent 96 well plates.

2.5.1 Hydrophilic-Lipophilic Balance Solid Phase Extraction

Oasis® PRiME HLB (HLB) is a wettable "Hydrophilic-Lipophilic Balance" polymeric based sorbent material. The surface chemistry of sorbent material is a proprietary blend of hydrophilic N-vinylpyrrolidone groups and lipophilic divinylbenzenes that allow for simultaneous extraction of neutral, basic, and acidic analytes and removal of phospholipid matrix interferences from biological samples. SPE methods in this study followed a generic protocol from the vendor. Sample extracts from DBS spots were acidified to 2% H_3PO_4 and loaded onto 10 mg sorbent wells by gravity. Loaded wells were washed with 0.5 mL 5% MeOH. Analytes were eluted by gravity into clean collection plates with 300 µL 9:1 ACN:MeOH. Elutants were acidified with 30 µL 0.5M HCl, dried under vacuum at 30°C and reconstituted in 200 µL MPA and centrifuged for

14 min. at 13,000 RPM and 4°C. Supernatants were transferred into microinserts in autosampler vials for analysis.

2.5.2 Mixed-Mode Cation Exchange Solid Phase Extaction

Oasis® PRiME MCX (MCX) is a wettable mixed mode cation exchange SPE product. MCX sorbent material is made of a proprietary ratio of a polymeric reverse phase and sulfonic acid ion exchange functionalities that are ideal for basic analyte extraction. The protocol used in this work followed a generic method from the vendor. Sample extracts were acidified to 2% H_3PO_4 and loaded onto 10 mg sorbent wells by gravity. Loaded wells were washed sequentially with 0.5 mL portions of 100 mM ammonium formate + 2% formic acid (100 mM AmF + 2% FA). Samples were eluted with 300 µL 5% NH_4OH in MeOH by gravity. Elutants were acidified with 55 µL 0.5M HCl and then evaporated to dryness under vacuum at 30°C. Dried samples were reconstituted in 200 µL MPA, centrifuged and 14 min. at 13,000 RPM at 4°C, and the supernatants were transferred into microinserts in autosampler vials for analysis.

2.5.3 µElution Mixed Mode Cation Exchange SPE Chemistry

Waters µElution (µMCX) plates used in this work were made from the same Oasis® PRiME MCX sorbent surface chemistry but in 2 mg beds of 30 µm particle diameter. Sorbent material in µElution plates is packed with smaller pore space and required a vacuum manifold for sample loading and well washing. Samples (1 mL) were acidified to 2% H_3PO_4 and loaded onto 2 mg sorbent beds under 10 to 15 kPa vacuum. Wells were washed with sequential 200 µL aliquots of 100 mM AmmF + 2% FA and MeOH under 5 to 10 kPA vacuum. Samples were eluted with 2 25 µL portions of 1:1 ACN-MeOH + 5% NH_4OH. Elutants were diluted with 150 µL 2% FA and transferred to microinserts in autosampler vials for analysis.

2.6 UPLC-QTOF-HRMS Analysis

Analyses were performed using a Waters Acquity® UPLC with a Waters Acquity® HSS-C18 1.8 µm, 2.1 mm x 150 mm column, equipped to a Waters Xevo® G2-XS QTOF-HRMS with electrospray ionization (ESI).

2.6.1 UPLC Instrument Conditions

UPLC analysis was controlled using MassLynx 4.1 software (Waters). Analytes in neat standards (100 ng/mL) were run over various mobile phase gradient conditions to determine optimal column retention, analyte resolution, and run time. Injection volumes of 2, 5 and 10 µL were assessed. Mobile phase A was 5 mM ammonium formate + 0.1% formic acid and mobile phase B was ACN + 0.1% formic acid. Optimized UPLC conditions are presented in Table 3.

Table 3: UPLC instrument conditions.

UPLC Parameters:		**Mobile Phase Ramp:**	
Sample chamber temperature:	5°C	100% MPA	0 - 1.1 min.
Column temperature:	45°C	15% MPB	at 3.0 min.
Flow rate:	0.4 mL/min	30% MPB	at 4.8 min.
5 mM ammonium formate + 0.1% formic acid:	MPA	50% MPB	at 5.9 min.
Acetonitrile + 0.1% formic acid:	MPB	100% MPB	7.9 - 9.0 min.
Run time:	11 minutes	100% MPA	9.0 - 11.0 min.

2.6.2 QTOF-HRMS Instrument Conditions

Mass spectral analysis was performed using Xevo® G2-XS QTOF-HRMS instrumentation with electrospray ionization (ESI) in positive ion mode. Full scan MS^E from 40 Da to 601 Da was acquired using MassLynx 4.1 software. LeuEnk LockSpray™ mass correction at 278.1141 Da was acquired every 20 seconds (0.1 second per acquisition) and applied throughout every

analysis. QTOF instrument calibration and LockSpray setup was performed prior to every analysis. A retention time standard was run in triplicate prior to samples to insure analyte stable retention times for sample analysis. Optimized ESI and QTOF settings are presented in Table 4.

Table 4: ESI and QTOF Instrument Settings.

QTOF Instrument Settings:			
Cone voltage:	25 V	Cone gas flow rate:	25 L/Hr
Capillary voltage:	1.0 kV	Acquisition time:	0 - 10 min.
Source offset:	40 V	MSE mass acquisition range:	40 - 601 Da
Source temperature:	140 C°	Low collision energy:	4.0 V
Desolvation gas temperature:	250 Cº	High collision energy ramp:	10 - 40 V
Desolvation gas flow rate:	900 L/Hr	LockSpray reference mass:	278.1141 Da

2.7 Data Processing

Drugs were identified from MS^E data in UNIFI 1.8.2 (Waters), and peak areas were integrated for quantitative comparison. Analyte identification was assigned in in UNIFI from monoisotopic mass spectra match generated by UNIFI from analyte entries in the Waters Scientific Library and Waters Forensic Toxicology Solution Library. Mass spectra matches were assigned in UNIFI from high and low collision energy mass spectra information. Identification criteria were retention time error <0.1 minutes and accurate mass error < 10 mDa. Accurate mass error was calculated in UNIFI from the ratio of the difference between measured mass and monoisotopic mass to monoisotopic mass.

2.8 Results

Retention times and the accurate masses of quantitation ions and diagnostic fragment ions for the analytes and internal standards used in this work are presented in Table 5, with parent ion masses in bold. UNIFI determined analyte responses using the peak area of parent ions. Later quantitative comparisons for AMP and PH used responses of the most abundant diagnostic fragments.

Table 5: Analyte Retention Times and Ions Accurate Mass and Internal Standards.

Analytes:	Retention time (minutes):	Quantitation ion:	Diagnostic ions:	Internal standard:
CN	5.87	**304.1593**	182.1187, 150.0930	CN-d3
CE	6.25	**318.1731**	196.1340, 150.0930	CE-d3
BZE	4.96	**290.1405**	168.1028, 105.0330	BZE-d3
EME	1.41	**200.1304**	182.1187, 150.0930	EME-d3
AME	3.54	**182.1187**	150.0930, 91.05423	-
AMP	4.55	91.0543	**136.1117**, 119.0863	AMP-d8
MA	4.75	**150.1281**	91.0543, 119.0863	MA-d11
PHEN	4.9	91.0543	**150.1281**, 65.0377	-
MDMA	4.78	**194.1195**	163.0753, 135.0456	MDMA-d5
MDEA	5.06	**208.1349**	163.0753, 135.0456	MDEA-d5
MDA	4.61	**163.0753**	180.1037, 135.0456	MDA-d5
BUP	6.1	**240.1159**	131.0746, 184.0534	BUP-d9
OH-BUP	5.55	**256.1114**	131.0746, 167.0489	OH-BUP-d6
MP	5.58	**234.1532**	84.0804, 174.1284	-

The UPLC conditions were used throughout optimization and method development. A total ion chromatogram (TIC) of the analytes is presented in Figure 7.

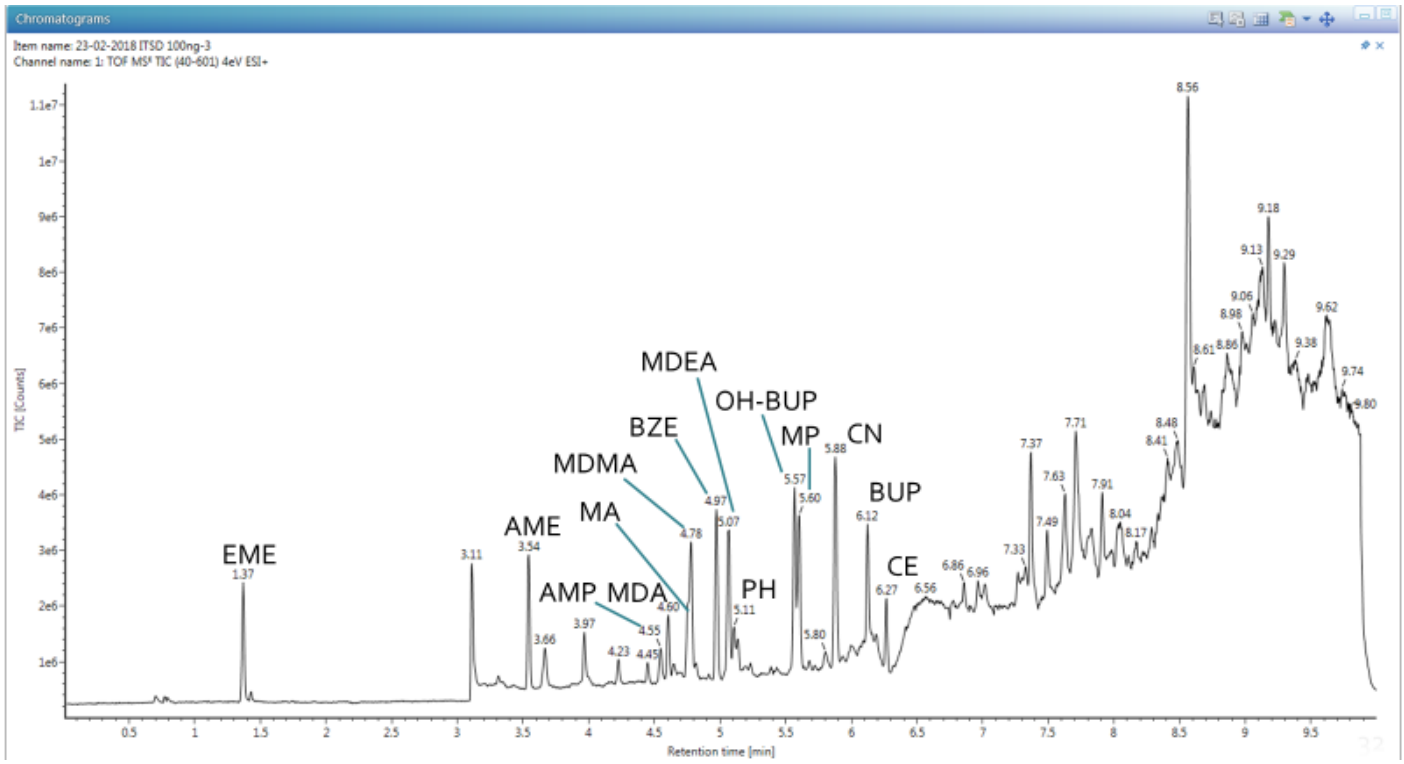

Figure 7: UPLC-QTOF-HRMS total ion chromatogram for analytes in a standard solution.

2.8.1 Expression of Drug Levels

Drug responses were calculated in UNIFI from the peak area of the parent ion of analytes identified by the software using the identification criteria of < 10 mDa mass error and < 0.1 minute retention time error.

2.8.2 Extraction Solvent and SPE Method Characterization

Extraction solvents of 0%, 10%, 20%, 30%, 40%, and 50% MeOH were assessed for recovery and matrix effects using HLB, MCX, and µMCX. Matrix effect acceptance criteria was set at ± 25%. Average 10 ng/mL DBS recoveries (n = 3) in 40% and 50% MeOH extraction solvents using HLB ranged from 60% (MDA) to 0% (PH and MDMA); 40% and 50% extraction

solvents were removed from the study because of poor analyte recovery. Data presented in this section is from 2 µL injections.

Mean recoveries for 10 ng/mL and 100 ng/mL DBS samples (n = 3) using 0%, 10%, 20%, and 30% MeOH solvents with HLB are presented in Figure 8.

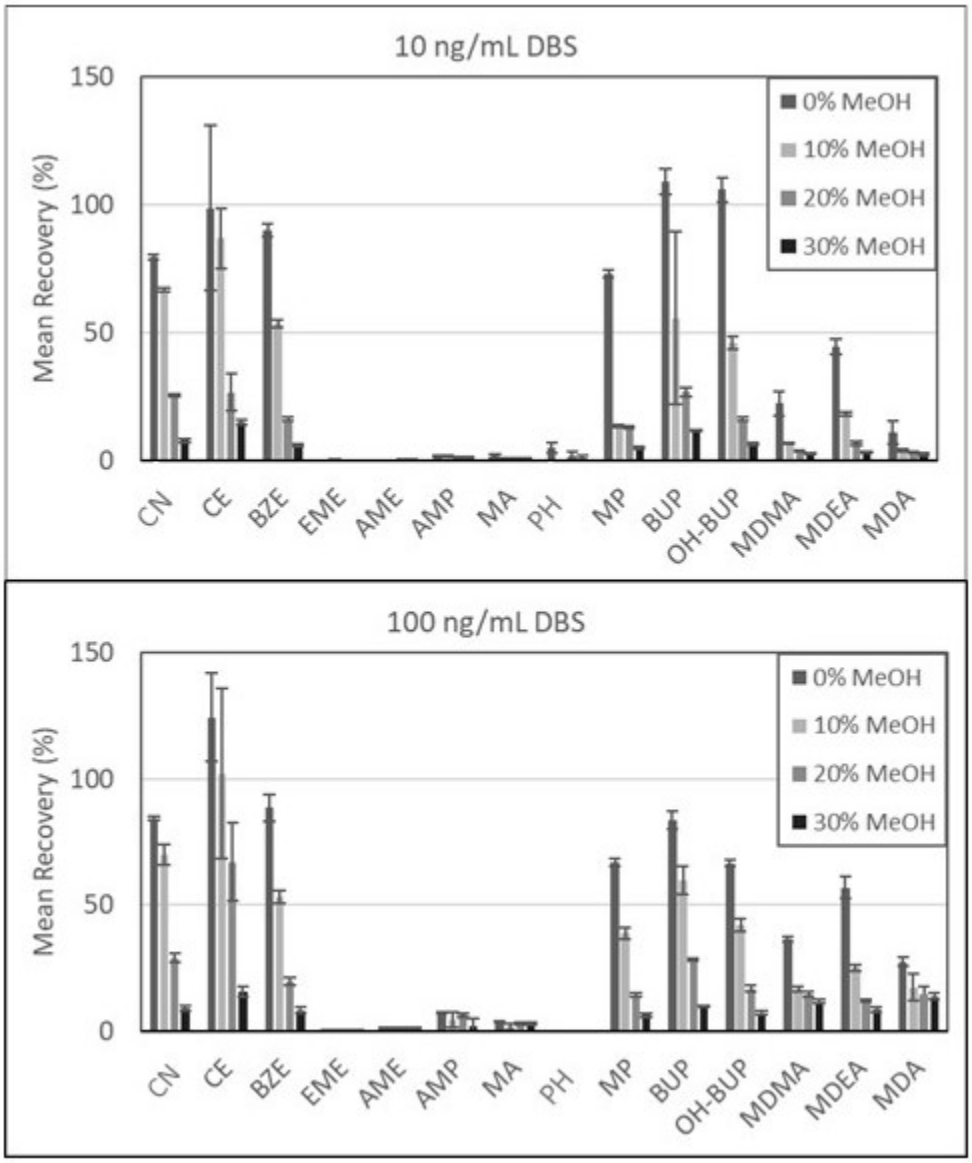

Figure 8: Mean drug recoveries for 10 ng/mL and 100 ng/mL DBS samples from 0%, 10%, 20%, and 30% MeOH extraction solvents with 10 mg PRiME HLB SPE plates (0% MeOH = 100% H_2O).

Mean matrix effects for 10 ng/mL and 100 ng/mL DBS samples (n = 3) using 0%, 10%, 20% and 30% MeOH extraction solvents with HLB PRiME are presented in Figure 9.

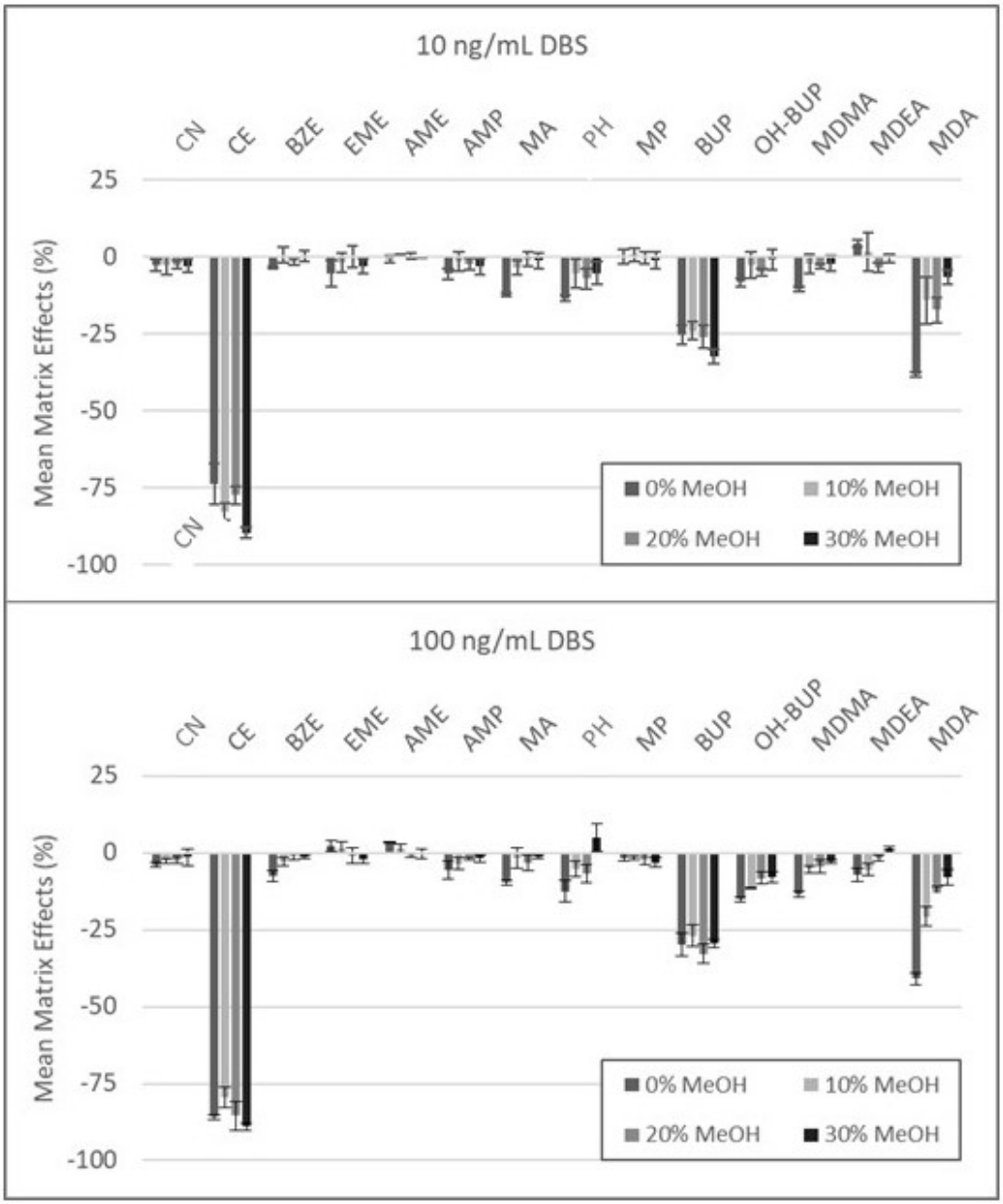

Figure 9: Mean matrix effects of 10 ng/mL and 100 ng/mL DBS samples from 0%, 10%, 20%, and 30% MeOH extraction solvents with 10 mg PRiME HLB SPE plates (0% MeOH = 100% H_2O).

Mean drug recoveries from 10 ng/mL, 100 ng/mL, and 1000 ng/mL (n = 3) using 0%, 10%, 20%, and 30% MeOH extraction solvents with PRiME MCX are presented in Figure 10.

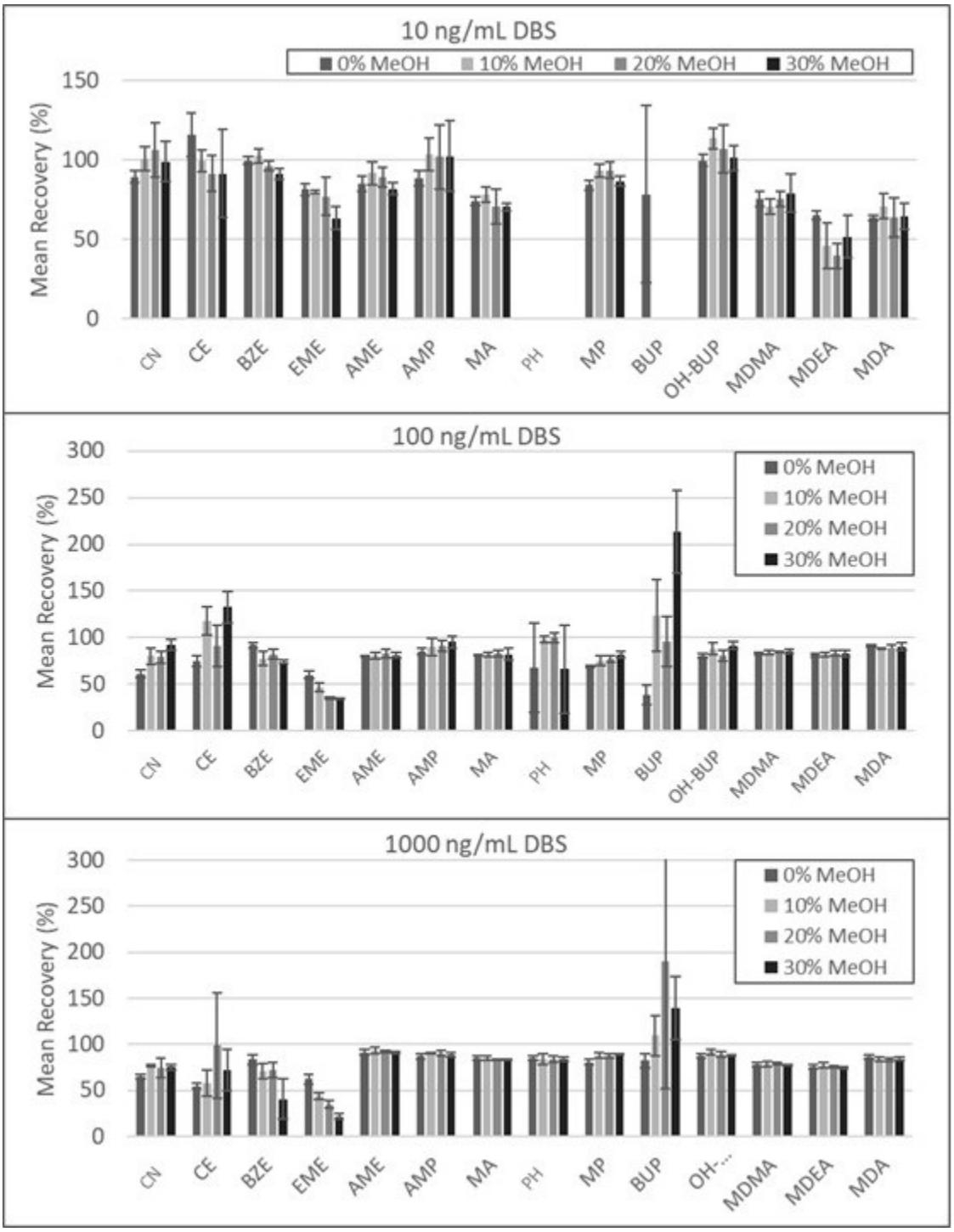

Figure 10: Mean drug recoveries for 10 ng/mL, 100 ng/mL, and 1000 ng/mL DBS samples from 0%, 10%, 20%, and 30% MeOH extraction solvents with 10 mg PRiME MCX SPE plates (0% MeOH = 100% H_2O).

Mean matrix effects for 10 ng/mL, 100 ng/mL, and 1000 ng/mL DBS samples (n = 3) using 0%, 10%, 20%, and 30% MeOH extraction solvents with MCX is presented in Figure 11.

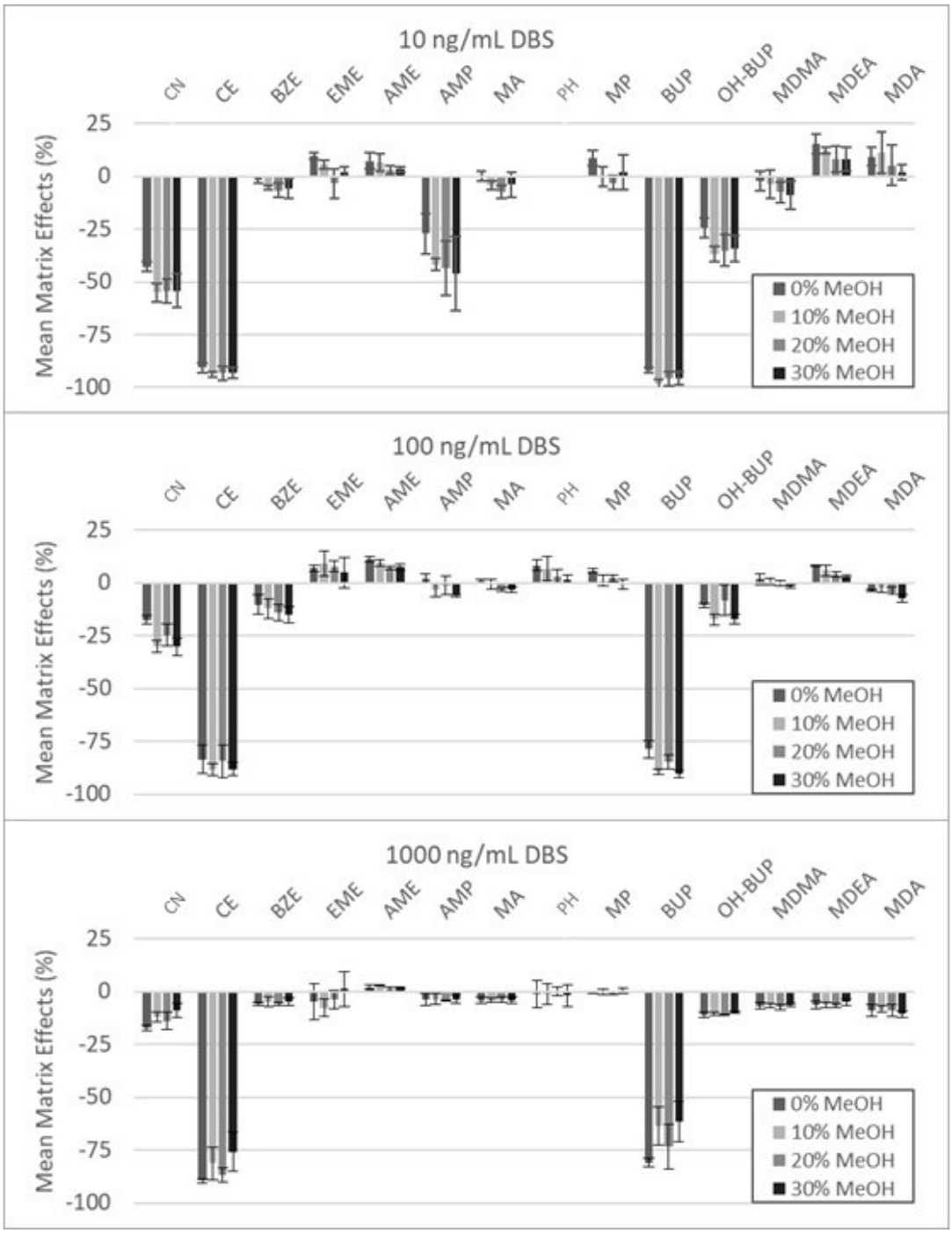

Figure 11: Mean matrix effects of 10 ng/mL, 100 ng/mL, and 1000 ng/mL DBS samples from 0%, 10%, 20%, and 30% MeOH extraction solvents with 10 mg PRiME MCX SPE plates (0% MeOH = 100% H_2O).

Mean µMCX recovery and matrix effects are presented in Figures 12 and 13 respectively, from 10, 100, and 1000 ng/mL DBS (n = 3) extracted in 1 mL 0%, 10%, 20%, and 30% MeOH.

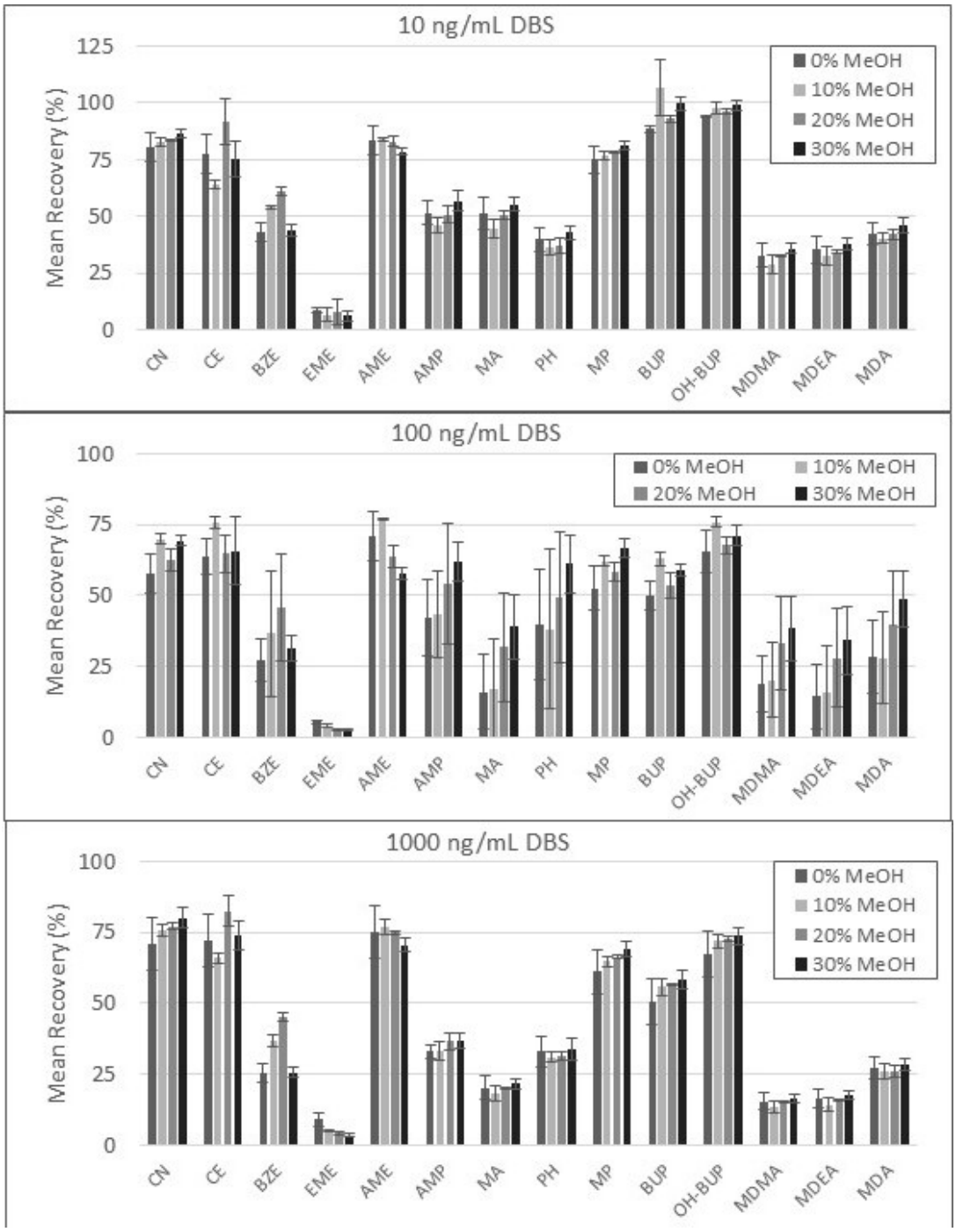

Figure 12: Mean drug recoveries for 10 ng/mL, 100 ng/mL, and 1000 ng/mL DBS samples from 0%, 10%, 20%, and 30% MeOH extraction solvents with 2 mg µMCX SPE plates (0% MeOH = 100% H_2O).

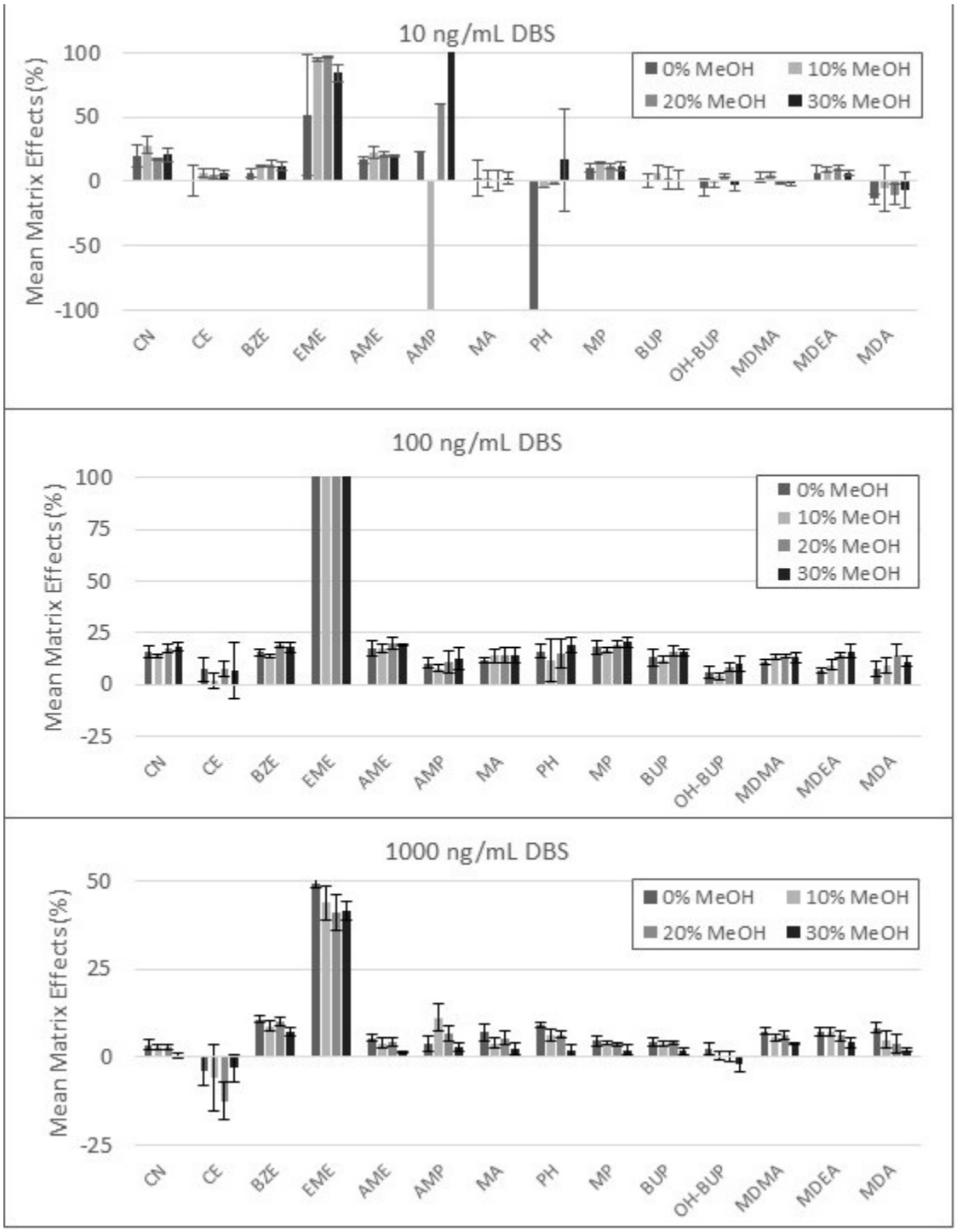

Figure 13: Mean matrix effects of 10 ng/mL, 100 ng/mL, and 1000 ng/mL DBS samples from 0%, 10%, 20%, and 30% MeOH extraction solvents with 2 mg µMCX SPE plates (0% MeOH = 100% H_2O).

2.8.3 Injection Volume Characterization

Because of the potentially picogram amounts of analytes in DBS samples, injection volumes of 2 µL, 5 µL, and 10 µL were assessed. It was expected that increasing the injection volume would improve analyte signal due to correspondingly larger amount of analyte on column. Injection volumes were assessed concordantly with the matrix effect and recovery studies across HLB, MCX, and µMCX plates using 0%, 10%, 20%, and 30% MeOH solvents.

Matrix effects were magnified across all extraction solvents and SPE methods with increasing injection volumes. Mean matrix effects from 10 ng/mL DBS (n = 3) using MCX with 0%, 10%, 20%, and 30% MeOH for 2 µL, 5 µL, and 10 µL injection volumes is presented in Figure 14, and is typical of the observed changes in matrix effects with increased injection volumes.

2.9 Discussion

Extraction solvents and the SPE products evaluated in this work were assessed by the recoveries and matrix effects observed for each SPE plate type and extraction solvent composition. Accepted matrix effect criteria were set at ± 25%. An acceptance criterion for analyte recovery was not set; however, recovery values approaching 100% are ideal, especially given the picogram levels of drugs in microvolume DBS samples at forensically relevant concentrations.

Initial work compared HLB and MCX SPE protocols. MCX offered comparable recovery and matrix effects to HLB for all methanolic extractions following generic vendor protocols. Matrix effects for HLB (2 µL injections) were within ± 25% for 11 of 14 analytes at all concentrations. Matrix effects for MCX (2 µL injections) were within accepted criteria for 11/14 analytes at 100 ng/mL, 12/14 analytes 1000 ng/mL, and 8/14 analytes at 10 ng/mL, typically as ion suppression.

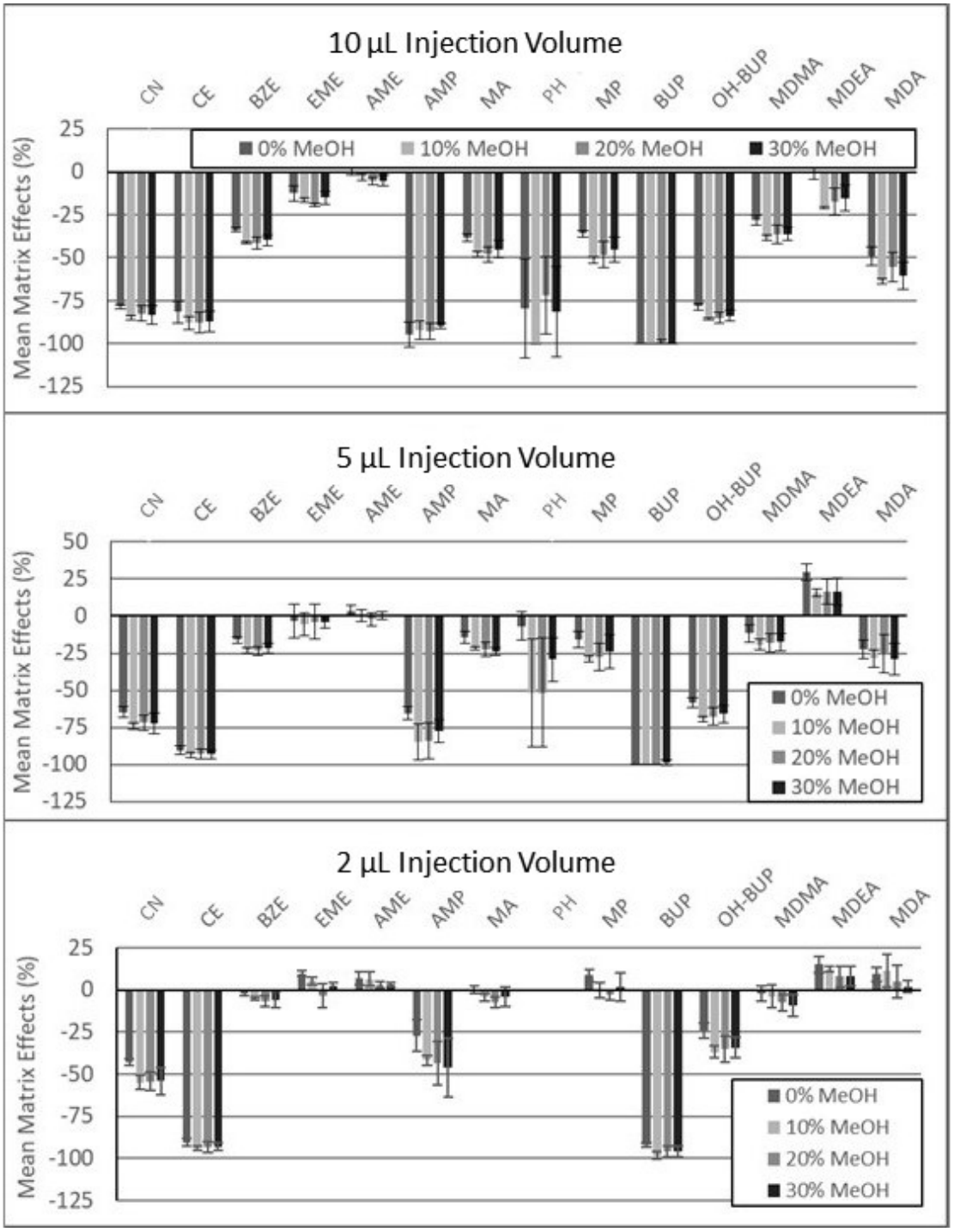

Figure 14: Increasing mean matrix effects with increasing injection volumes of 2 µL, 5 µL, and 10 µL, from 10 ng/mL DBS samples using MCX SPE with 0%, 10%, 20%, and 30% MeOH extraction solvents (0% MeOH = 100% H_2O).

In general, analyte recovery decreased with increasing MeOH content in the loaded extraction solvent. This trend was strongest in HLB extractions. Using HLB, mean cocaine recovery in 10 ng/mL DBS samples fell from 79.5% in 0% MeOH to 7.7% in 30% MeOH, a typical trend for HLB recoveries. HLB also suffered poor recoveries for AMP, MA, PH, EME, and AME. Poor recoveries in HLB were due to analyte loss during SPE wash. To test this, wash fractions were collected and analyzed by the UPLC-QTOF-HRMS methods in this study, and significant drug responses were detected in HLB wash fractions. Of HLB and MCX, 0% MeOH with MCX clean-up gave the best recovery across analytes, ranging from 38.9% to 91.8%.

The overall performance of MCX was superior to HLB for the analytes and SPE protocols used in this work, however, anomalously high recoveries were observed for BUP (> 125%) in both MCX and HLB. The cause of the abnormal BUP recoveries is unknown, however, analyte reduction-oxidation has been observed in our lab during evaporation under vacuum.[293] Both MCX and HLB sample preparation methods required sample evaporation and reconstitution. A hypothesis of this mechanism is reduction of OH-BUP to BUP during evaporation. BUP and a component in the elutant undergo redox reactions, with the reaction driven to the right as the concentration of these components increase with solvent evaporation. Further assessment of this phenomena will be left for future work.

PRiME µMCX plates were included for assessment after the onset of this work. The small elution volumes (50 µL) for µMCX permits simple dilution of samples prior to injection. Recovery from µMCX were lower than for MCX, ranging from 9% to 75%; however, the anomalous BUP recoveries were not seen for µMCX extractions. Matrix effects for µMCX using 0% MeOH were within ± 25% for all analytes except EME in 100 ng/mL and 1000 ng/mL DBS samples, and for all analytes except EME (51.3%) and PH (-100%).

Results from the injection volume assessment showed improved matrix effects for 2 µL injections over 5 µL and 10 µL volumes. Larger injection volumes were tested to see if improved analyte signal was seen with greater injection volumes; however, matrix effects were magnified with increasing injection volume.

2.9.1 Comparison of SPE Methods

Following generic vendor protocols, the µMCX SPE plates performed the best, analyte recovery with µMCX was suitable. Overall, µMCX displayed the least matrix effects of all sample preparation methods compared in this study. In particular, matrix effects were improved for MA, AMP, CN, and MDMA. Matrix effects for EME were outside accepted criteria; however, EME is an inactive metabolite of CN, and accurate quantitation of EME concentrations may not be forensically or clinically relevant.

The evaporation step is eliminated using µMCX. Removing the evaporation step reduced sample preparation time from 10-11 hours to 4 hours. Anomalous BUP recoveries observed in HLB and MCX were absent in µMCX, supporting the notion that some redox conversion between OH-BUP and BUP may be occurring during evaporation.

One need for evaporation is the removal of organic solvents that can impact chromatography. Analytes solvated in organic solvents have a more complex partition from the organic solvent to the aqueous mobile phase, and again to the solid phase of the column. This can result in bimodal or broadened peaks. The impact of organic solvent content in samples on chromatography with the UPLC conditions used in this study is presented in Figure 15. Increasing organic solvent content in samples resulted in poor chromatography. Though µMCX extracts are 50 µL, the final sample composition is 25% organic solvent, enough to cause peak broadening (Figure 15).

However, using µMCX, peak broadening of analytes was only seen at 10 µL injections. Thus, 2 µL injections were chosen for their improved matrix effects and mitigation of chromatography problems due to organic solvent content in samples.

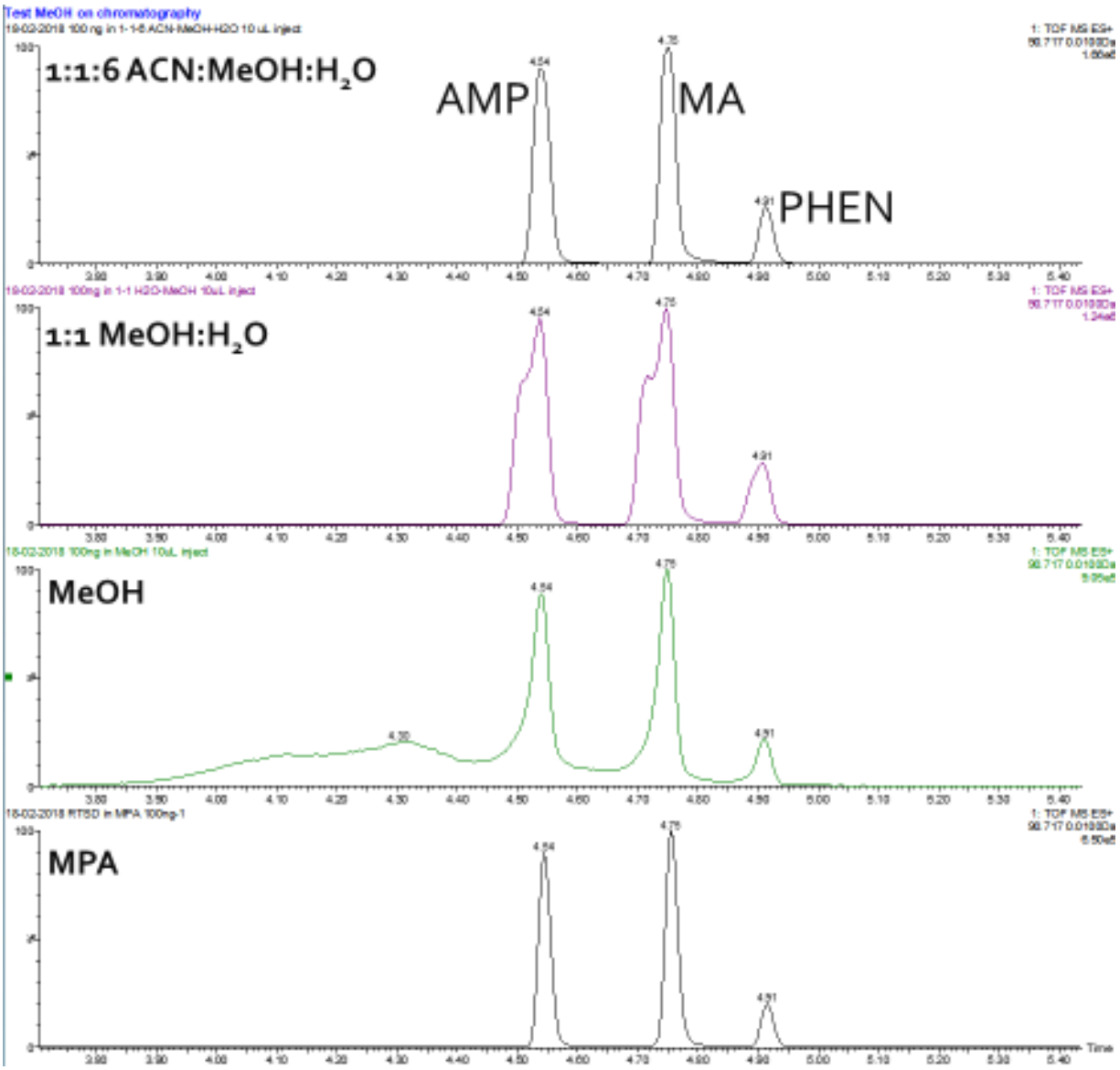

Figure 15: Effects of organic solvents in samples on chromatography of amphetamine, methamphetamine and phentermine.

From the results of sample preparation optimization studies, µMCX was chosen for method validation. Water (0% MeOH) was chosen as the extraction solvent for simplicity, solvent consumption considerations, and lack of clear improvement in sample clean-up in the MeOH compositions tested in this work. Injection volume was set at 2 µL due to the observed improvements in matrix effects and chromatography.

2.9.2 Sonication Extraction Time Characterization

Sonication extraction time was characterized after a sample preparation method was selected. Drug-positive DBS samples at 1000 ng/mL and drug-free DBS samples (n = 3) were extracted in 1 mL of H_2O for 60 minutes. The extraction solvent was removed after 10, 20, 30, and 40 minutes of extraction time and replaced with fresh 1 mL volumes of H_2O. The final 1 mL was removed after 60 minutes. Extracts were prepared following the µMCX sample preparation method and UPLC-QTOF analysis developed in this work. Extraction time was evaluated from the cumulative analyte responses for time points compared to total cumulative analyte response after 60 minutes. Mean cumulative responses for analytes are presented in Table 6. The majority of all analytes are extracted within the first 10 minutes of sonication. Almost all of the analytes are extracted within 30 minutes (range 93.3% to 100%; mean 98.5%), and 30 minutes was the chosen period for ultrasonic extraction.

Table 6: Cumulative mean analyte response of sonication extraction time intervals.

	Cumulative mean analyte response:		
Analyte:	**10 min (%):**	**20 min (%):**	**30 min (%):**
CN	88.2	98.5	99.7
CE	85.1	97.2	98.9
BZE	89.9	99.5	99.9
EME	83.0	97.7	99.2
AME	89.9	98.3	99.3
AMP	83.5	100.0	100.0
MA	85.8	96.3	98.0
PH	77.4	95.8	100.0
MDMA	78.7	90.4	93.8
MDEA	87.1	97.3	98.6
MDA	78.1	89.6	93.3
BUP	84.7	97.9	99.8
OH-BUP	84.4	96.9	99.0
MP	87.6	97.8	99.2

2.10 Conclusion

The data presented shows that ultrasonic extraction with PRiME MCX µElution SPE is an efficient sample preparation method for toxicological analyses of DBS samples. Sample clean-up using µMCX gives suitable analyte recovery and improved matrix effects, while also reducing sample preparation time by several hours. Sample preparation parameters may have to be optimized for each compound of interest. The sample preparation developed in this work was suitable for 13 of 14 analytes, EME was the only analyte that did not meet acceptance criteria for matrix effects. However, accurate quantitation of EME is generally not of forensic or clinical interest, and its omission from validation does not diminish the general scope of this work.

Chapter 3

3 Simultaneous Screening and Quantitation of Stimulants and Selected Metabolites in Dried Blood Spot Samples Using UPLC-QTOF-HRMS

3.1 Introduction

Drugs impair task performance, including driving, by acting on the brain in proportion to the brain drug concentration. Forensic investigations of impairment require blood samples reflective of the time of the investigated incident for appropriate interpretation of drug concentrations in blood. Several hours can pass between an incident in question and the drawing of whole blood in drug impaired investigations and this time delay can render forensic interpretations of drug concentrations at the time of an incident impossible. Further, the establishment of *per se* limits of stimulant drugs in blood require timely sample collection and accurate quantitation of stimulants to accurately and fairly assess allegations of drug impairment. A blood sampling method that addresses the time course problem of current blood sampling protocols is required for drug impaired investigations. Dried blood spot sampling is a simple and cost-effective sampling method that can be performed in almost any environment and with minimal training. Not only do DBS samples display equivalent or superior analyte recovery and stability, the sampling method permits accurate quantitation of drug concentrations.

Stimulant drugs, including cocaine, amphetamines, and methamphetamines, are a commonly encountered class of drugs in drivers involved in accidents, random roadside surveys studies, and driving violations.[266–270] Driving while impaired by stimulant drugs has contributed to accidents and deaths.[142,155,213,272–279] The goal of this work is to develop a validated method for the simultaneous screening and quantitation of a panel of forensically relevant stimulant drugs in

DBS samples using UPLC-QTOF-HRMS instrumentation and to assess the room temperature stability of the analytes in DBS samples over 8 weeks.

The development of high resolution and sensitive mass spectrometry has made quantitation of ng/mL-range drug concentrations of microvolume (i.e. 10-20 µL) DBS samples possible. Commonly encountered stimulants and selected metabolites [cocaine (CN), benzoylecgonine (BZE), cocaethylene (CE), ecgonine methyl ester (EME), anhydroecgonine methyl ester (AME), amphetamine (AMP), methamphetamine (MA), phentermine (PH), bupropion (BUP), hydroxybupropion (OH-BUP), methylphenidate (MP), 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyethylamphetamine (MDEA), 3,4-methylenedioxyamphetamine (MDA)] were analyzed in 20 µL DBS samples prepared from blood spiked with anlaytes at concentrations ranging from 10 to 1000 ng/mL. The chosen concentration range encompass established drug driving *per se* limits, therapeutic, and toxic to fatal drug blood concentrations.[295]

3.2 Materials and Methods

3.2.1 Chemicals and Reagents

Chemicals and materials used in method validation were the same used in sample preparation optimization, presented in Chapter 2.3 and 2.4, unless otherwise noted. Standards used in drug interference assessment (codeine, fentanyl, hydrocodone, hydromorphone, mephedrone, methadone, morphine, norcodeine, noroxycodone, oxycodone, oxymorphone, pseudoephedrine, Δ9-THC, 11-nor-9-carboxy-Δ9-THC, 11-hydroxy-Δ9-THC) were purchased from Cerilliant; (R,S)-(±)-ephedrine, (R)-(+)-β-methylphenethylamine (BMPEA), and norephedrine were obtained from Sigma Aldrich (Oakville, Ontario); cathine was purchased from LGC Standards

(Manchester, New Hampshire). Drug-free human whole blood was purchased from Utak laboratories Inc (Valencia, California).

3.2.2 Preparation of Solutions

All DBS samples and standard solutions were prepared using fixed-volume and digital positive displacement microdispensers from Drummond Scientific (Broomall, Pennsylvania). Drug standards were diluted in methanol (MeOH) and acetonitrile (ACN) stocks from the following 1 mL ampules: AMP, MA, MDMA, MDEA, MDA, PHEN; "Amine Mix-6", 250 µg/mL in MeOH. CN, BZE, CE, EME; "Cocaine Mix", 250 µg/mL in ACN. AME, 1 mg/mL in ACN. BUP, 1 mg/mL in MeOH. OH-BUP, 1 mg/mL in ACN. MP, 1 mg/mL in MeOH. Deuterated internal standards (CN, BZE, CE, EME: d3; MDA, MDEA, MDMA: d5; OH-BUP-d6, AMP-d8, BUP-d9, and MA-d11) were similarly diluted from 100 µg/mL and 1 mg/mL (MA-d11 only) solutions. Instrument calibration (0.5 mM ammonium formate in 90:10 ISA:H_2O) and LeuEnk LockSpray™ solutions were prepared per vendor instructions (Waters, Meford, Pennsylvania).

3.2.3 Sample Preparation

Drug-free sheep blood was serially diluted for calibration curves at drug concentrations of 0, 10, 50, 100, 250, 500 and 1000 ng/mL. Bias was assessed in each calibration curve from low, mid and high bias samples at 25, 400 and 800 ng/mL, respectively. Drug positive calibrators, negative controls and post-extraction spike (PES) samples (20 µL, n = 3) were spotted on Whatman® 903 Protein Saver Cards (GE Healthcare, Chicago, Illinois) and allowed to dry overnight. Samples for analyte stability analysis at 0, 10, 100 and 1000 ng/mL (n = 3, stability time points of 0, 1, 4 and 8 weeks) were kept in a paper envelope and stored in the dark at room temperature prior to extraction; however, temperature or humidity conditions were not recorded.

Whole DBS samples were punched from sample cards using a ½″ punch and extracted with 2 ng of each internal standard in 1 mL of water for 30 minutes by sonication in clean test tubes. DBS card punches were removed from test tubes and extracts were acidified to 2% concentrated H_3PO_4 and vortexed. Acidified extracts were loaded via a vacuum manifold onto Oasis® PRiME MCX µElution 96 well plates. Loaded wells were sequentially washed with 200 µL of 100 mM ammonium formate + 2% formic acid and 200 µL MeOH. Analytes were eluted from wells using 2 × 25 µL aliquots of 1:1 ACN:MeOH + 10% NH_4OH into clean collection plates. Calibrators and negative controls were diluted with 150 µL 2% formic acid. PES sample elutants were spiked to calibrator levels with drug positive aliquots of 150 µL of 2% formic acid. Neat standards at calibrator levels were prepared by adding drug positive aliquots of 150 µL of 2% formic acid to 50 µL of blank elution solvent in empty collection plate wells. Diluted elutants and neat standards were mixed on a shaker plate at 500 RPM for 30 seconds, then transferred to microcentrifuge tubes. Samples were centrifuged at 13,000 RPM for 14 minutes at 4 °C. Supernatants were transferred to glass inserts in autosampler vials for analysis.

3.2.4 Instrumentation

Samples were loaded into the autosampler of an Acquity® UPLC with an Acquity® HSS-C18 1.8 µm, 2.1 mm × 150 mm column, equipped with Xevo® G2-XS QTOF-HRMS instrumentation with electrospray ionization (ESI) in positive ion mode. Full scan MS^E data was acquired using MassLynx 4.1 software. UPLC and QTOF instrument conditions are presented in Chapter 2.4.1 and 2.4.2, respectively.

3.2.5 Identification and Quantitation

Drug identifications were made in UNIFI from MS^E data following the methods described in Chapter 2. UNIFI does not allow the user to edit identified peaks or peak areas. Invalid areas from erroneous peak shapes generated by the software led to poor quantification data. Because of this, UNIFI was abandoned for quantitation.

Analyte and internal standard peak areas were calculated in QuanLynx 4.1 software using a mass window of 0.01 Da and retention time window of 0.05 minutes. Analytes for quantitation were identified in MassLynx by retention time and the diagnostic ions presented in Table 5 (Chapter 2.8). Deuterated internal standards were not readily available for AME, PH or MP at the time of this study. Internal standards used for PH and MP were MA-d11 and OH-BUP-d6, respectively; response ratios (analyte peak area/internal standard peak area) for AME were not calculated.

Peak areas calculated in QuanLynx were manually reviewed prior to export as .xml files into Excel 2013 (Microsoft Corporation, Redmond, Washington). Quadratic calibration curves were calculated from peak area ratios of analyte to internal standard at each calibrator level using $1/x^2$ weighting with XLSTAT 2019.1.2 Build 56803 add-in for Excel (Addinsoft, Paris, France).

3.2.6 Method Validation

Method validation followed standard practices for forensic toxicology applications.[296] LOD, LOQ, recovery, bias, precision, and carryover were assessed in 5 calibration curve analyses at 6 analyte levels (n = 3) on 5 different days. Drug interferences, matrix interferences, internal standard interferences, matrix effects, and DBS card stability were assessed in separate experiments at analyte levels of 0, 10, 100 and 1000 ng/mL (n = 3). Statistical analysis was performed using Excel 2013 and XLSTAT.

3.2.7 Limit of Detection and Limit of Quantitation

LOD was administratively defined as the lowest non-zero calibrator identified by UNIFI in all 5 standard curves. LOD was manually confirmed in QuanLynx. Analyte ion signal to noise ratios (S/N) of 5 was set as an identification criteria. LOQ was determined at the lowest non-zero calibrator in which detection, precision, and bias criteria were met in all 5 standard curves. LOQ was not determined for AME due to the lack of an appropriate internal standard.

3.2.8 Calibration Model

Calibration curves were calculated using quadratic nonlinear regression with $1/x^2$ weighting in XLSTAT from triplicate DBS samples at 10, 50, 100, 250, 500 and 1000 ng/mL in 5 different standard curves on 5 different days. Fit to the calibration model is reported as the coefficient of determination, R^2. The standard deviation (σ) and variance (σ^2) for each analyte response ratio were plotted against concentration across the calibration range for each standard curve to determine the weighting factor. Appropriate weighting was assessed qualitatively; the σ of relative responses were proportional to concentration and $1/x^2$ weighting was selected.[297]

3.2.9 Recovery and Matrix Effects

Recovery at calibrator levels was calculated as [analyte peak area of drug positive sample/analyte peak area in blank sample spiked after extraction] × 100%. An acceptance for recovery was not set. Matrix effects were determined by comparing analyte peak areas in blank sample extracts spiked to concentrations of 10, 100 and 1000 ng/mL (n = 3) to neat standards at identical concentrations using the formula [(mean peak area of analyte in blank sample spiked after extraction/mean peak area of analyte in neat standard) – 1] × 100%. A matrix effect value

less than zero reflects ion suppression and a matrix effect value greater than zero indicates ion enhancement. Matrix effects within ± 25% were deemed acceptable for quantitation.

3.2.10 Precision

Precision expressed as the coefficient of variation (%CV) was determined for each analyte at every calibrator level (n = 3) for each of the 5 standard curves as [analyte response ratio σ/mean response ratio] × 100%. The criteria for %CV acceptance was set at 15%.

3.2.11 Bias

The standard curve data used to determine precision was used in the bias studies. Bias was assessed at low, mid and high concentration levels (n=3) in each of the 5 standard curves. Concentrations for bias samples were calculated from their relative responses and calibration curve regression equations. Bias was calculated using [100% × (mean of calculated concentration – nominal concentration)/nominal concentration]. The maximum acceptable bias was set at ±20%.

3.2.12 Selectivity

Blank samples from 5 different blood matrices were analyzed for peaks interfering with analytes or internal standards. Blank samples fortified with internal standards at 400 ng/mL (n = 3) were extracted to assess interferences with analytes of interest. Blank samples spiked to 1000 ng/mL (n = 3) without internal standard were extracted to assess interference with internal standard ions. DBS samples prepared from blood spiked with cannabinoids (50 ng/mL, n = 3), opioids (various concentrations; n = 3) and other amphetamine-related compounds (300 ng/mL, n = 3), were extracted to assess interferences from other commonly encountered analytes.

3.2.13 Analyte DBS Stability

Drug positive DBS samples (10, 100 and 1000 ng/mL; n = 3 for all time points) were prepared and analyzed at time points of 0, 1, 4 and 8 weeks after sample spotting. Dried DBS samples were kept in a paper envelope and stored in the dark at room temperature prior to extraction. Analyte stability was assessed by comparing response ratios of analytes at time points of 1, 4 and 8 weeks to time zero response ratios. Acceptance criteria was not set for card stability.

3.2.14 Analyte Stability on Autosampler

Mean analyte responses at time zero for samples at 10, 100 and 1000 ng/mL (n = 3) were compared to mean analyte responses after 44 hrs on the instrument to assess analyte stability using [(mean time zero analyte response/analyte response after 44 hr on instrument −1) × 100%]. Acceptable stability criteria was set at ±15%.

3.2.15 Carryover

Blank samples were analyzed immediately following high concentration samples and neat standards to assess carryover with every analysis. The concentration deemed free of carryover was the highest concentration at which no analyte carryover was observed.

3.2.16 Hematocrit Effects

Hematocrit effects on matrix effects and recovery was assessed. Human whole blood was separated into plasma and erythrocyte fractions by centrifugation at 2200 rpm for 15 minutes. HCT levels were prepared from plasma and erythrocyte fractions at HCT levels of 0.25, 0.45 and 0.75. DBS samples were spotted from the HCT blood prepared at analyte concentrations of 0 ng/mL, 10 ng/mL, 100 ng/mL and 1000 ng/mL (n = 3) and dried overnight prior to analysis.

3.3 Results

A quantitative UPLC-QTOF-HRMS method for DBS samples between 10 and 1000 ng/mL was achieved for cocaine, cocaethylene, amphetamine, methamphetamine, MDMA, MDEA, MDA, bupropion, hydroxy-bupropion and methylphenidate. An LOD of 10 ng/mL was achieved for all analytes. Recovery and DBS card stability were analyte dependent.

3.3.1 Method Validation

Quantitative method validation criteria was met for 10/14 analytes and semi-quantitative for BZE, EME, and PH. Though AME responses were concentration dependent, this analyte was not quantitated.

3.3.2 Limit of Detection and Limit of Quantitation

The lowest calibrator concentration that met identification criteria in UNIFI was administratively defined as the LOD. All detected analytes were confirmed manually in QuanLynx. All analytes had an LOD of 10 ng/mL in DBS samples. Average S/N in 10 ng/mL DBS samples ranged from 16.3 (PH) to 686.3 (CN).

The LOQ was determined at the lowest concentration with acceptable bias, precision and detection criteria in all 5 standard curves. A 10 ng/mL LOQ was achieved for all analytes except PH. Phentermine quantitation criteria to 10 ng/mL was in met in 4 of 5 standard curves, however the lowest PH concentration with acceptable quantitation criteria in all 5 standard curves was 50 ng/mL. AME was not quantitated in this work. Analyte LOD and LOQ values are presented in Table 7.

3.3.3 Calibration Model

Calibration with quadratic regression and $1/x^2$ weighting were used to model standard curves from analyte response ratios (n = 3) at 6 calibrator levels in 5 different standard curves analyzed on different days. R^2 values of the calibration curves for the 10 analytes with acceptable quantitation criteria ranged from 0.980 to 0.999. The range of R^2 values for each analyte across the 5 standard curves is presented in Table 8.

3.3.4 Recovery

Recovery was analyte dependent, average recoveries are presented in Table 4. Mean analyte recoveries ranged from 6.1% to 77.5%. Average recoveries for the 10 quantitative analytes ranged from 66.3% to 76.6%. Mean recoveries for BZE, EME and PH were 29.9%, 6.1% and 77.5%, respectively.

3.3.5 Matrix Effects

Matrix effects were within ± 25% at all levels for all analytes except EME and PH. Mean matrix effects for EME was 89.3% (range -13.9 to 173.0%). Mean PH matrix effects at 10 ng/mL were -31.8%. Average PH matrix effects at 100 ng/mL and 1000 ng/mL were 16.1% and 9.0%, respectively. For quantitative analytes, mean matrix effects ranged from 0.5% to 13.1%. In general, ion enhancement was noted for all analytes, however, every matrix effect value for the quantitative drugs were within ± 25% acceptance criteria (range from -18.7% to 23.4%). Matrix effect values are presented in Table 9.

Table 7: Validation results for quantitative and semi-quantitative analytes.

														Bias (%)					
	LOD	LOQ	Recovery (%)			Matrix effects (%)			Precision (%CV)			Low (25 ng/mL)			Mid (400 ng/mL)			High (800 ng/mL)		
Analytes	(ng/mL)	(ng/mL)	mean	min	max	mean	min	max	mean	min	max	mean	min	max	mean	min	max	mean	min	max
CN	10	10	73.4	35.3	115.7	12.3	1.9	23.2	2.7	0.2	8.2	-6.1	-11.7	-1.0	0.3	-7.6	12.2	-3.9	-5.8	1.3
CE	10	10	74.4	33.4	110.9	1.2	-16.3	13.8	4.0	0.3	8.3	-10.5	-12.2	-5.1	2.0	-5.2	12.8	-5.5	-13.8	-0.6
AMP	10	10	74.5	31.3	113.9	11.6	3.9	23.4	2.5	0.6	6.5	-4.0	-5.9	0.4	-3.2	-8.4	8.4	-5.1	-7.0	0.0
MA	10	10	76.6	22.8	118.7	6.9	-15.8	16.8	4.3	0.2	10.5	-6.8	-10.8	-2.5	-1.5	-10.7	10.9	-5.9	-15.8	5.1
MDMA	10	10	73.4	17.2	117.0	10.9	3.1	21.9	5.1	0.2	14.7	-6.8	-8.7	-3.2	1.2	-5.9	10.2	-5.8	-10.4	2.4
MDEA	10	10	66.9	24.5	102.6	6.1	-3.4	16.7	5.9	0.8	13.3	-12.3	-15.0	-7.2	7.6	-0.2	13.9	-6.5	-12.6	2.0
MDA	10	10	71.4	22.4	112.0	1.0	-12.7	8.8	3.1	0.9	6.4	-6.2	-8.9	-3.1	-1.3	-7.0	6.1	-3.2	-6.0	1.5
BUP	10	10	66.3	31.1	101.7	7.0	-2.4	12.1	2.8	0.6	6.5	-12.8	-14.9	-11.1	0.1	-4.6	11.2	-1.9	-5.0	1.4
OH-BUP	10	10	76.3	35.4	115.8	6.6	-1.8	10.9	3.7	0.2	8.1	-7.0	-11.9	-3.7	-1.9	-6.9	6.8	-2.6	-6.4	1.5
MP	10	10	72.2	32.0	107.0	0.5	-18.7	10.7	5.4	0.7	10.9	-9.9	-17.2	-1.8	-2.2	-5.5	5.8	-3.2	-8.0	1.2
BZE	10	10	29.9	11.2	61.8	10.9	1.5	16.6	2.7	0.2	9.5	-4.9	-9.7	-1.0	-0.3	-4.1	5.8	-4.2	-5.6	-1.8
EME	10	10	6.1	2.3	13.1	89.3	-13.9	173.0	3.6	0.4	8.4	-6.1	-7.7	-4.7	2.6	-4.2	8.4	-2.4	-7.8	3.6
PH	10	50	77.5	24.2	119.1	1.5	-100.0	20.2	7.6	1.2	37.8	-13.9	-27.9	-8.7	-2.5	-12.3	10.5	-4.1	-12.8	6.8

Table 8: Analyte retention times, ion masses, and coefficient of determinations from calibration curves.

Analytes	Retention time (minutes)	Quantitation ion	Identifying ions	Internal standard	R^2 values min	R^2 values max
CN	5.87	**304.1593**	182.1187, 150.0930	COC-d3	0.986	0.997
CE	6.25	**318.1731**	196.1340, 150.0930	CE-d3	0.988	0.992
BZE	4.96	**290.1405**	168.1028, 105.0330	BZE-d3	0.989	0.997
EME	1.41	**200.1304**	182.1187, 150.0930	EME-d3	0.990	0.995
AME*	3.54	**182.1187**	150.0930, 91.05423	-	-	-
AMP	4.55	91.0543	**136.1117**, 119.0863	AMP-d8	0.996	0.999
MA	4.75	**150.1281**	91.0543, 119.0863	MA-d11	0.985	0.997
PH	4.9	91.0543	**150.1281**, 65.0377	MA-d11	0.872	0.994
MDMA	4.78	**194.1195**	163.0753, 135.0456	MDMA-d5	0.980	0.997
MDEA	5.06	**208.1349**	163.0753, 135.0456	MDEA-d5	0.983	0.991
MDA	4.61	**163.0753**	180.1037, 135.0456	MDA-d5	0.991	0.999
BUP	6.1	**240.1159**	131.0746, 184.0534	BUP-d9	0.988	0.994
OH-BUP	5.55	**256.1114**	131.0746, 167.0489	OH-BUP-d6	0.990	0.995
MP	5.58	**234.1532**	84.0804, 174.1284	OH-BUP-d6	0.982	0.993

**An internal standard for AME was unavailable at the time of this study, therefore calibration curves were not generated.*

3.3.6 Precision

Intra-curve precision acceptance criteria (15 %CV) was met for all analytes at every concentration in all 5 standard curves except for PH, which had satisfactory precision at all concentrations in 4 of 5 standard curves. Excluding PH, values for %CV ranged from 0.2% to 14.7%. Precision was assessed for bias samples, and with the exception of PH, precision was acceptable for all analytes at low, mid and high bias levels in all standard curves, and ranged from 0.1% to 12.5%. Precision values are presented in Table 7.

3.3.7 Bias

With the exception of PH, bias was acceptable (within ± 20%) for all low, mid, and high concentration bias samples in 5 standard curves, and ranged from -15.8% to 15.0%. Bias samples for PH were acceptable in all standard curves at mid and high levels only; PH bias at low concentrations was acceptable in 4 of 5 standard curves. Bias for PH ranged from -27.9% to 10.5%. For low levels, bias is generally negative but within acceptable ranges, indicating the calibration model is elevated at lower concentration regions on the calibration curve. Bias is presented in Table 7.

3.3.8 Selectivity

False identifications were not detected in blank DBS samples and in DBS samples positive for other drugs. Other drugs analyzed were commonly encountered opioids, cannabinoids, and other stimulants. All analytes were fully resolved from other drugs with common ions, with the exception of AMP and BMPEA. Retention times (RT) for AMP and BMPEA were 4.55 and 4.59 respectively, with a resolution score of 0.727, calculated as [$2 \times (RT_{BMPEA} - RT_{AMP})$/(Peak

$Width_{BMPEA}$ + Peak $Width_{AMP}$)]. Resolution scores > 1 are fully resolved. In the absence of BMPEA, the method is quantitative for AMP.

3.3.9 Analyte DBS Stability

Analyte stability within DBS cards during storage was analyte dependent. Stability was assessed from the ratio of analyte relative ratios to time zero analyte relative ratios and is presented in Figures 16 through 19. A stability value less than 100% indicates analyte loss in the sample. In general, low concentration DBS samples (10 ng/mL) were less stable than higher concentration samples (100 ng/mL and 1000 ng/mL) over all time points (1, 4 and 8 weeks). In general, relative response to time zero fell in week one and were steady through weeks 1 to 8 with the exception of EME and BUP, which showed a steady decrease in time zero relative response. In week 8, relative responses for MDEA, MDA, and MP were greater than 150% in 1000 ng/mL samples, and greater than 200% for BZE in 100 ng/mL and 1000 ng/mL samples. Excluding these unexpectedly high stability values, average stability was 70.4% for all analytes, and ranged from 0% to 124%.

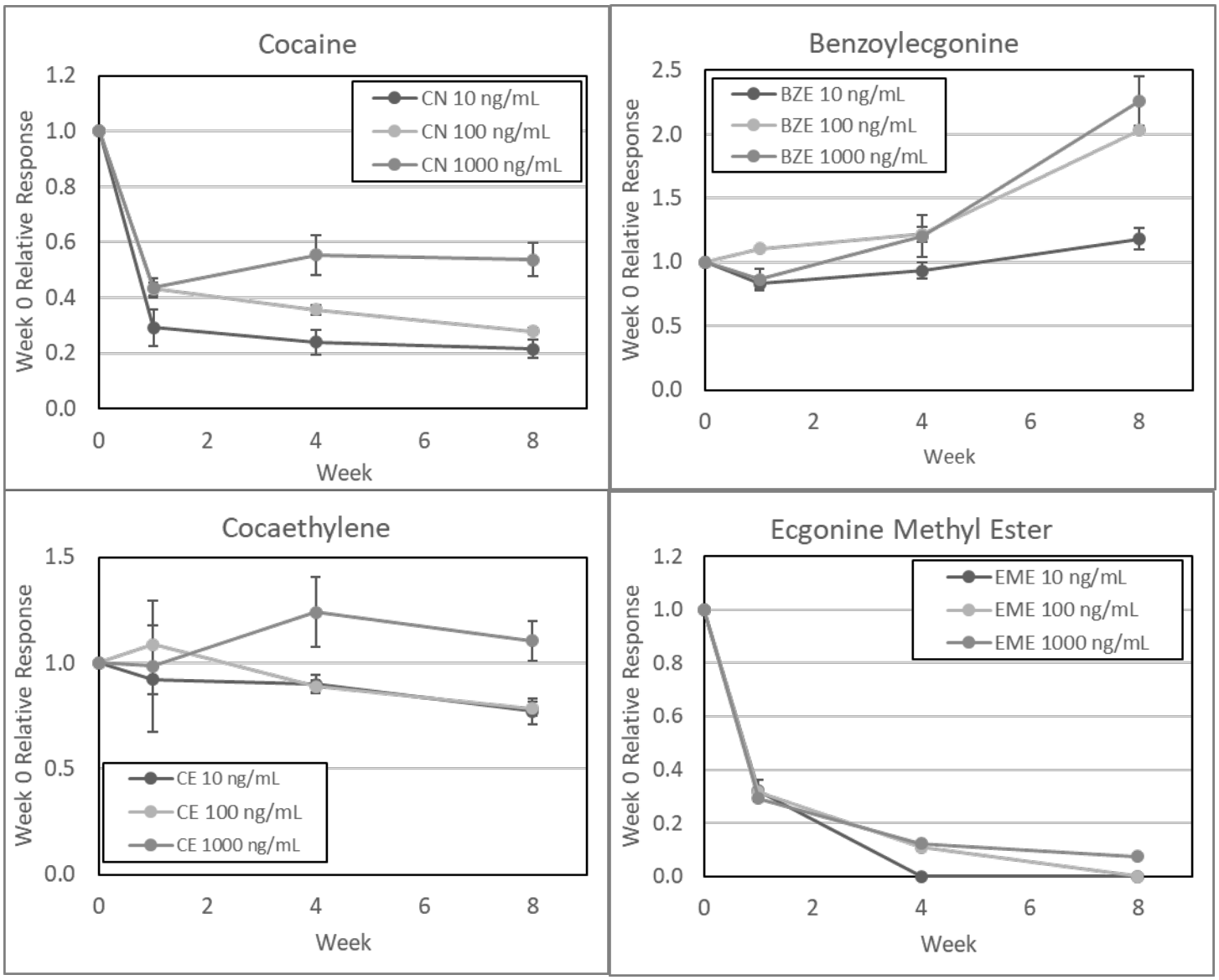

Figure 16: Eight-week stability of cocaine and selected metabolites in DBS samples.

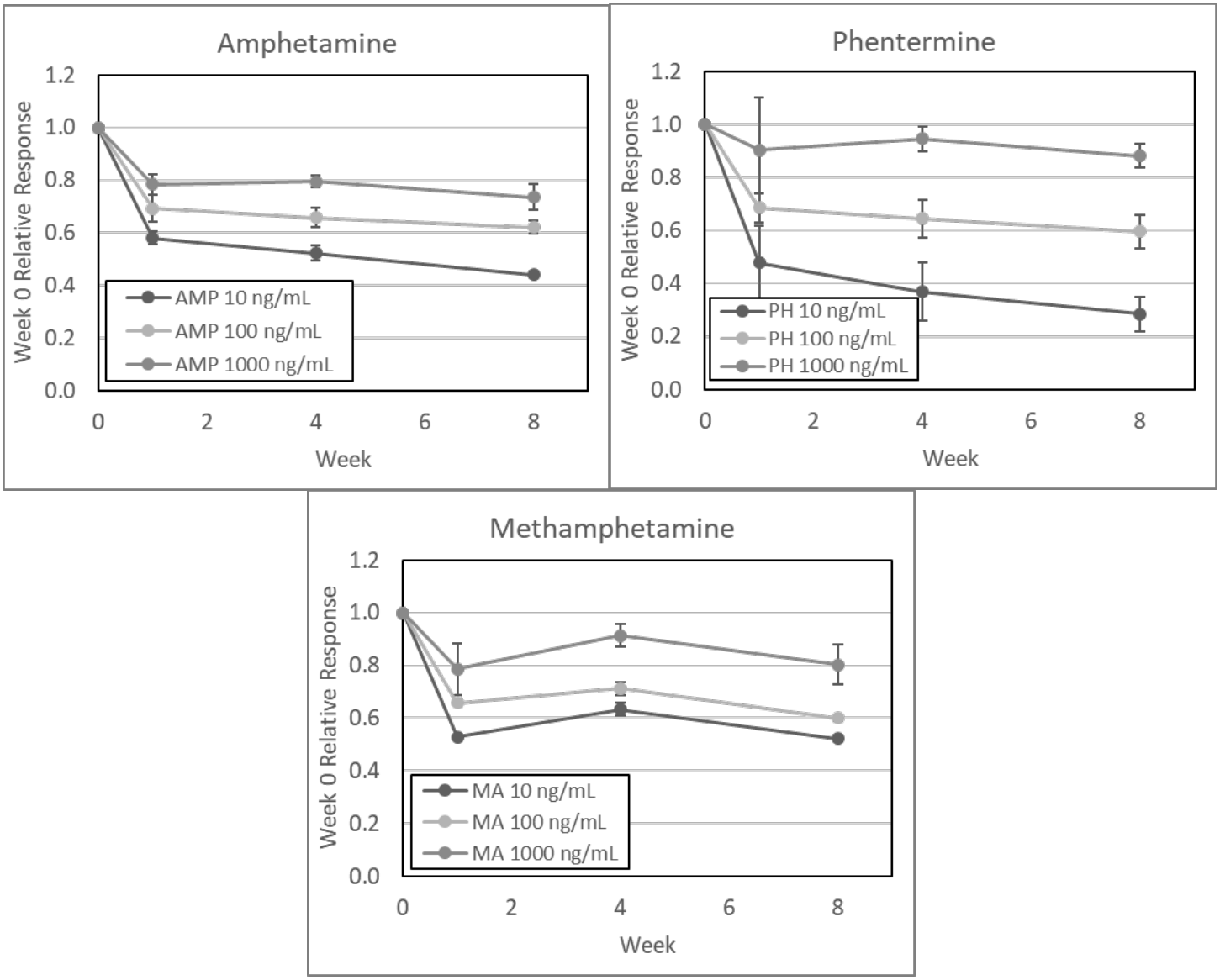

Figure 17: Eight-week stability of amphetamine related compounds in DBS samples.

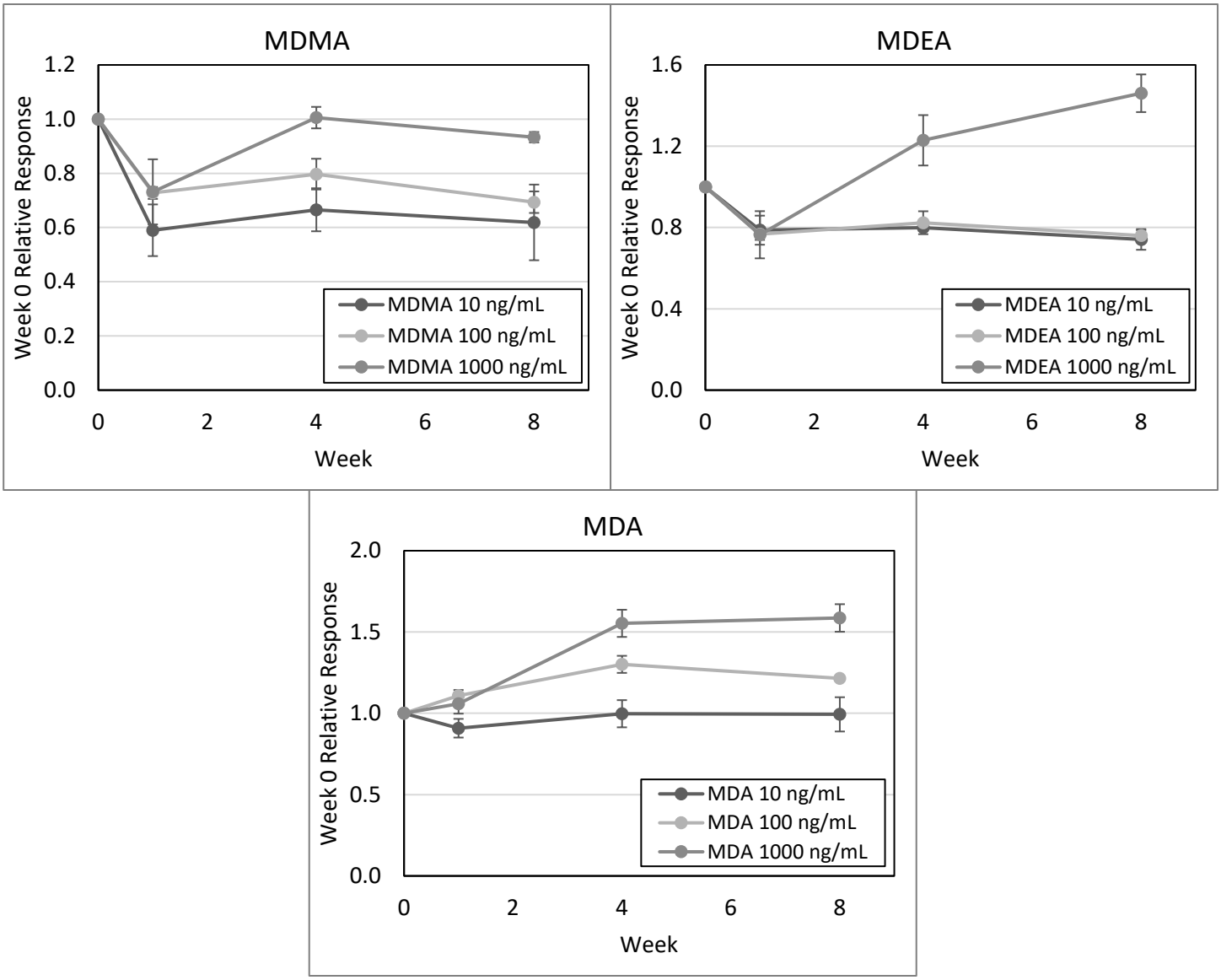

Figure 18: Eight-week stability of MDMA, MDEA, and MDA in DBS samples.

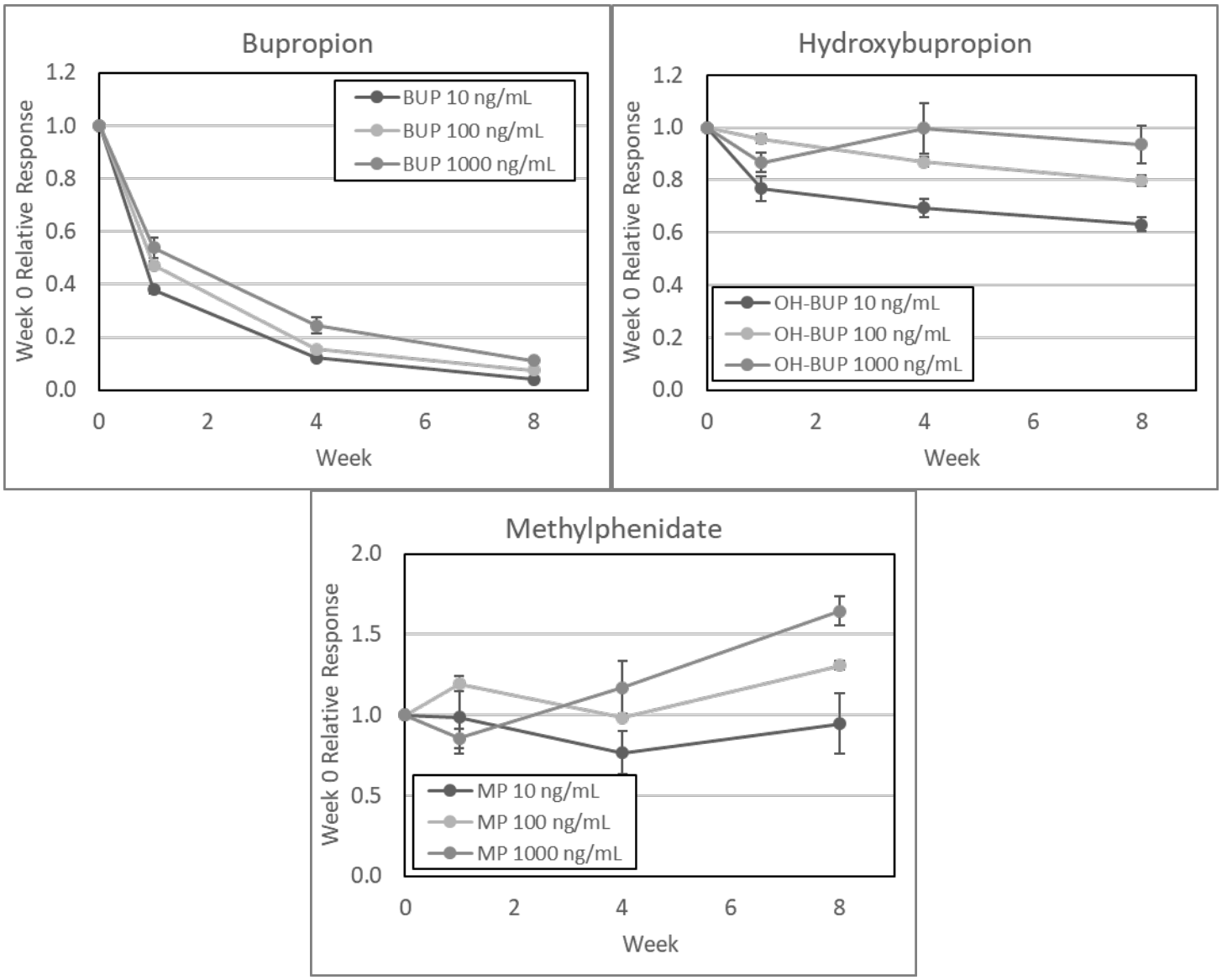

Figure 19: Eight-week stability of bupropion, hydroxybupropion and methylphenidate in DBS samples.

3.3.10 Analyte Stability on Autosampler

Instrument drug stability was assessed by comparing analyte peak areas after 44 hours on the instrument autosampler to time zero analyte peak areas. All analytes met instrument stability criteria (within ± 15%) except BZE, EME, and PH. For analytes that met stability criteria, mean instrument stability was 0.9% and ranged from -14.2% to 14.9%.

3.3.11 Carryover

Mobile phase negative controls and blank samples were injected immediately following high concentration samples and neat standards to assess UPLC carryover. No carryover was observed.

3.3.12 Hematocrit Effects

Matrix effects across HCT ranges are presented in Table 9, and ranged from -24.8% to 33.8%, and generally showed ion enhancement. Mean analyte recoveries ranged from 5.0% to 91.8% across all HCT levels. Mean analyte recovery and matrix effects for analytes at 10 ng/mL, 100 ng/mL and 1000 ng/mL, across HCT levels of 0.25, 0.45 and 0.75 are presented in Table 9. Matrix effects were < 20% for all concentrations and all HCTs for CN, CE, BZE, EME, AME, AMP, MA, MDMA, MDEA, BUP and OH-BUP. Matrix effects for PH were 33.8% for 0.25 HCT at 10 ng/mL and acceptable for all other HCTs and concentrations. Matrix effects for MP were suitable for all HCTs at 100 ng/mL and 1000 ng/mL, and for 0.75 HCT in 10 ng/mL DBS. Methylphenidate was detected in blank DBS samples in the hematocrit study. Mean MP response in blank DBS was 31% of 10 ng/mL MP responses and is likely why matrix effects for MP were outside acceptance criteria for 10 ng/mL DBS samples at 0.25 and 0.45 HCT levels. No other analytes were detected in blank samples in the HCT study. A general improvement in analyte recovery and matrix effects were observed with increasing drug concentration for all HCT levels.

Table 9: Matrix effects of DBS samples of hematocrits of 0.25, 0.45 and 0.75 at 10, 100, and 1000 ng/mL.

CN		Mean Recovery (%)	SD	Mean Matrix Effects (%)	SD
10 ng/mL	25% HCT	60.3	±2.3	21.1	±2.5
	45% HCT	65.8	±6.6	14.4	±8.4
	75% HCT	59.8	±4.8	10.8	±3.3
100 ng/mL	25% HCT	64.5	±11.2	22.8	±9.0
	45% HCT	52.2	±15.1	21.8	±12.3
	75% HCT	61.8	±17.3	10.6	±9.0
1000 ng/mL	25% HCT	84.6	±0.1	6.0	±1.0
	45% HCT	70.9	±16.1	6.2	±1.8
	75% HCT	80.4	±0.4	7.4	±1.6

CE		Mean Recovery (%)	SD	Mean Matrix Effects (%)	SD
10 ng/mL	25% HCT	53.3	±10.2	2.5	±5.8
	45% HCT	44.6	±17.5	-21.6	±3.2
	75% HCT	52.3	±22.0	-24.8	±9.1
100 ng/mL	25% HCT	57.2	±4.0	4.4	±16.0
	45% HCT	56.3	±27.5	-8.5	±28.3
	75% HCT	57.8	±20.9	-17.2	±12.2
1000 ng/mL	25% HCT	88.4	±3.5	6.6	±3.5
	45% HCT	81.1	±14.5	-2.6	±4.7
	75% HCT	80.2	±2.7	-1.1	±6.7

BZE		Mean Recovery (%)	SD	Mean Matrix Effects (%)	SD
10 ng/mL	25% HCT	36.2	±3.6	18.3	±3.5
	45% HCT	20.1	±8.1	16.4	±0.5
	75% HCT	31.6	±15.4	15.0	±1.6
100 ng/mL	25% HCT	43.6	±9.9	19.7	±2.0
	45% HCT	20.2	±0.9	18.3	±0.7
	75% HCT	46.4	±15.4	13.6	±3.3
1000 ng/mL	25% HCT	57.3	±5.2	19.1	±3.8
	45% HCT	54.8	±20.9	17.2	±0.3
	75% HCT	31.9	±29.5	13.9	±1.3

EME		Mean Recovery (%)	SD	Mean Matrix Effects (%)	SD
10 ng/mL	25% HCT	5.5	±0.6	24.4	±2.9
	45% HCT	5.0	±1.4	18.8	±1.3
	75% HCT	6.1	±2.2	15.7	±4.6
100 ng/mL	25% HCT	5.7	±0.7	22.5	±2.7
	45% HCT	5.8	±1.7	17.8	±1.5
	75% HCT	5.9	±1.4	15.2	±1.3
1000 ng/mL	25% HCT	14.4	±1.7	19.0	±1.4
	45% HCT	11.3	±4.1	15.4	±0.6
	75% HCT	10.3	±3.7	13.3	±1.1

Table 9 Continued: Matrix effects of DBS samples of hematocrits of 0.25, 0.45 and 0.75 at 10, 100, and 1000 ng/mL.

MDMA		Mean Recovery (%)	SD	Mean Matrix Effects (%)	SD
10 ng/mL	25% HCT	62.7	±0.6	12.4	±6.3
	45% HCT	43.4	±1.4	15.3	±10.0
	75% HCT	54.8	±2.2	6.8	±5.5
100 ng/mL	25% HCT	60.2	±0.7	24.1	±2.2
	45% HCT	35.8	±1.7	18.6	±1.8
	75% HCT	52.9	±1.4	15.0	±3.3
1000 ng/mL	25% HCT	85.8	±1.7	12.4	±1.0
	45% HCT	73.0	±4.1	12.4	±0.8
	75% HCT	86.5	±3.7	10.7	±1.2

MDA		Mean Recovery (%)	SD	Mean Matrix Effects (%)	SD
10 ng/mL	25% HCT	61.3	±6.8	28.6	±4.1
	45% HCT	42.9	±8.6	18.2	±7.9
	75% HCT	54.7	±19.7	15.7	±8.6
100 ng/mL	25% HCT	59.8	±5.4	19.3	±1.8
	45% HCT	35.3	±10.6	15.0	±1.3
	75% HCT	52.0	±13.5	12.7	±3.2
1000 ng/mL	25% HCT	76.5	±2.3	23.4	±1.2
	45% HCT	59.7	±17.6	21.6	±1.0
	75% HCT	77.4	±1.3	19.8	±0.9

AME		Mean Recovery (%)	SD	Mean Matrix Effects (%)	SD
10 ng/mL	25% HCT	67.3	±2.7	23.4	±2.5
	45% HCT	44.5	±14.8	16.6	±1.6
	75% HCT	52.6	±20.0	15.4	±2.0
100 ng/mL	25% HCT	66.8	±4.3	19.5	±3.0
	45% HCT	51.8	±10.0	13.6	±3.0
	75% HCT	60.4	±18.4	8.6	±4.1
1000 ng/mL	25% HCT	91.8	±4.0	8.6	±4.1
	45% HCT	79.3	±11.0	7.4	±4.1
	75% HCT	85.4	±2.3	12.4	±5.1

MDEA		Mean Recovery (%)	SD	Mean Matrix Effects (%)	SD
10 ng/mL	25% HCT	56.5	±2.7	22.5	±2.8
	45% HCT	35.5	±14.8	17.1	±5.9
	75% HCT	50.8	±20.0	9.9	±5.5
100 ng/mL	25% HCT	59.2	±4.3	23.3	±2.2
	45% HCT	34.2	±10.0	18.5	±3.6
	75% HCT	53.5	±18.4	15.7	±3.8
1000 ng/mL	25% HCT	87.5	±4.0	14.1	±3.6
	45% HCT	78.3	±11.0	10.4	±2.3
	75% HCT	88.8	±2.3	7.8	±3.2

Table 9 Continued: Matrix effects of DBS samples of hematocrits of 0.25, 0.45 and 0.75 at 10, 100, and 1000 ng/mL.

AMP		Mean Recovery (%)	SD	Mean Matrix Effects (%)	SD
10 ng/mL	25% HCT	63.5	±2.3	16.1	±1.0
	45% HCT	42.8	±6.6	10.6	±2.9
	75% HCT	59.7	±4.8	3.5	±1.8
100 ng/mL	25% HCT	61.7	±11.2	18.4	±1.1
	45% HCT	38.6	±15.1	12.8	±1.1
	75% HCT	55.8	±17.3	9.2	±4.6
1000 ng/mL	25% HCT	78.9	±0.1	22.3	±0.6
	45% HCT	62.8	±16.1	19.6	±1.4
	75% HCT	80.0	±0.4	16.8	±0.8

MA		Mean Recovery (%)	SD	Mean Matrix Effects (%)	SD
10 ng/mL	25% HCT	58.8	±3.65	19.9	±2.08
	45% HCT	42.3	±8.12	15.4	±1.80
	75% HCT	57.5	±15.44	13.3	±1.71
100 ng/mL	25% HCT	59.1	±9.92	18.2	±2.73
	45% HCT	36.0	±0.94	13.0	±1.06
	75% HCT	56.1	±15.45	8.4	±2.94
1000 ng/mL	25% HCT	90.7	±5.23	6.6	±2.21
	45% HCT	78.1	±20.95	6.2	±1.07
	75% HCT	91.5	±29.47	6.3	±3.55

PH		Mean Recovery (%)	SD	Mean Matrix Effects (%)	SD
10 ng/mL	25% HCT	66.7	±10.2	33.8	±6.4
	45% HCT	54.3	±17.5	17.7	±7.3
	75% HCT	59.9	±22.0	24.7	±5.5
100 ng/mL	25% HCT	66.7	±4.0	24.2	±4.1
	45% HCT	43.8	±27.5	19.6	±3.1
	75% HCT	61.3	±20.9	15.9	±4.5
1000 ng/mL	25% HCT	73.9	±3.5	22.2	±1.0
	45% HCT	56.1	±14.5	17.5	±3.7
	75% HCT	72.1	±2.7	14.1	±1.3

MP		Mean Recovery (%)	SD	Mean Matrix Effects (%)	SD
10 ng/mL	25% HCT	66.1	±1.9	35.4	±5.9
	45% HCT	44.6	±10.0	32.9	±6.1
	75% HCT	58.6	±19.5	23.7	±6.3
100 ng/mL	25% HCT	62.7	±2.5	22.0	±3.4
	45% HCT	54.4	±11.9	19.6	±7.4
	75% HCT	60.7	±17.8	12.5	±4.8
1000 ng/mL	25% HCT	81.9	±1.4	17.9	±0.4
	45% HCT	71.6	±13.2	13.9	±2.5
	75% HCT	83.4	±1.9	10.0	±1.7

Table 9 Continued: Matrix effects of DBS samples of hematocrits of 0.25, 0.45 and 0.75 at 10, 100, and 1000 ng/mL.

BUP		Mean Recovery (%)	SD	Mean Matrix Effects (%)	SD
10 ng/mL	25% HCT	57.0	±4.9	17.2	±2.7
	45% HCT	41.0	±12.5	-1.8	±1.1
	75% HCT	47.4	±14.9	-1.4	±8.8
100 ng/mL	25% HCT	58.5	±2.8	23.8	±4.2
	45% HCT	49.7	±14.6	13.5	±12.5
	75% HCT	56.5	±13.7	5.7	±16.7
1000 ng/mL	25% HCT	90.8	±4.8	2.4	±5.3
	45% HCT	74.8	±14.2	4.1	±2.6
	75% HCT	81.7	±3.1	4.7	±5.7

OH-BUP		Mean Recovery (%)	SD	Mean Matrix Effects (%)	SD
10 ng/mL	25% HCT	78.7	±24.5	-11.7	±4.1
	45% HCT	43.9	±9.1	-23.3	±13.5
	75% HCT	43.9	±9.5	-24.0	±2.9
100 ng/mL	25% HCT	64.0	±3.5	6.2	±12.9
	45% HCT	68.6	±31.9	-15.1	±22.2
	75% HCT	61.6	±19.3	-20.7	±19.2
1000 ng/mL	25% HCT	83.1	±3.3	-8.0	±5.5
	45% HCT	65.3	±21.8	-14.8	±14.1
	75% HCT	52.2	±2.9	-10.0	±2.2

3.4 Discussion

A method for screening and quantitation of selected stimulant drugs in microvolume dried blood spot samples using full-scan QTOF-HRMS in MS^E mode has been developed. Method validation demonstrated acceptable bias, accuracy, instrument stability and matrix effects for the quantitation of CN, CE, AMP, MA, MDMA, MDEA, MDA, BUP, OH-BUP, and MP from 10 ng/mL to 1000 ng/mL in DBS samples. The method was validated over a wide range of concentrations that captures therapeutic, recreational, and toxic blood concentrations for these analytes. Semi-quantitative results were obtained for BZE, EME, and PH. Anhydroecgonine methyl ester was included as a marker of cocaine pyrolysis and was not assessed for validation. The LOQ of 10 ng/mL was obtained at the lowest calibrator concentration. The results in this work show lower LOQ and LOD may be possible, even in 20 µL DBS samples. Though analyte stability in DBS samples was analyte dependent, CE and OH-BUP displayed good DBS stability across all time points and concentrations. Mean CE response relative to time zero was 96.5% and ranged from 77.2% to 124.0%, and the mean OH-BUP response relative to time zero was 83.5% with a range of 63.2% to 99.7%. Amphetamine, MA, PH, and MDMA showed a decrease in week 1 responses relative to time zero but remained generally stable through week 8 after the initial decrease in response. Week 4 and 8 responses for AMP, MA, and MDMA ranged from 44.1% to 100.6%. The results here show promise for the use of DBS samples as a matrix for drug testing in forensic toxicology and other applications.

Matrix effects were suitable for the majority of analytes at all concentration levels. Only EME and PH displayed matrix effects outside of accepted criteria. Mean EME matrix effects were 51.3%, 167.2% and 49.3% for 10 ng/mL, 100 ng/mL and 1000 ng/mL DBS samples, respectively. For PH, matrix effects were outside acceptable criteria only for 10 ng/mL spots,

at -31.8%. Matrix effects for PH were 16.1% and 9.0% for 100 ng/mL and 1000 ng/mL samples, respectively. Average matrix effects for all acceptable values, including those for PH, was 7.9%, and ranged from -18.7% to 23.4%. Accepted mean analyte matrix effects at all concentrations ranged from 0.5% to 13.1%. Though matrix effects determined here generally indicate matrix enhancement, the results of this study show that Oasis® PRiME MCX µElution sufficiently removed matrix interferents for all but EME. Because the elution volume of MCX µElution wells is only 50 µL, simply diluting the samples prior to injection was possible. Using µElution well plates eliminated up to 7 hours of sample prep time required for evaporation of larger volume elutants used in typical SPE methods.

Good linearity through the calibration concentration range was observed for most analytes. However, some compounds, methamphetamine in particular, displayed nonlinear responses 1000 ng/mL samples. Due to the observed nonlinear responses, all calibration models used quadratic regression with $1/x^2$ weighting for all analytes. Bias and precision were acceptable for all analytes in all standard curves except for PH. Bias and precision for PH was suitable in 4 of 5 standard curves. For low concentration bias samples, mean bias values for all analytes and standard curves were generally below 0% (range 0.4% to -17.2 %). The consistent negative bias at low levels indicate that drug concentrations at low levels would be routinely underestimated, beneficial to those accused of drug-impaired driving. The persistent negative bias at low concentrations in the models calculated in this work indicate care should be taken to establish detection limits lower than 10 ng/mL and may require a narrower calibration concentration range to satisfy bias criteria at low levels. Mid and high bias samples for all analytes did not display a positive or negative bias.

Though validation for the analysis of 10 stimulant drugs in DBS samples was achieved, a number of limitations are present. DBS analyte stability was poor for some of the analytes. analyte stability was especially poor for EME and BUP. The minimum EME responses relative to time zero of 0.0% were seen at week 4, and minimum BUP response was 4.0% at week 8. Methylphenidate, MDEA, MDA, and benzoylecgonine displayed increased responses relative to time zero for 100 ng/mL and/or 1000 ng/mL samples to varying degrees in week 4 and 8 time points. Mean responses relative to time zero for MP, MDEA, MDA and BZE ranged from 130.1% to 225.9% for these analytes. Previous studies have proposed hydrolysis of CN to BZE is inhibited on DBS due to the loss of water, and CN breakdown to BZE seen in wet blood samples is limited in DBS samples.[86,88] The observed increase in BZE responses over time was not expected, and the cause of increased analyte responses over time in this work is not known at this time.

Other studies have shown analyte levels in DBS samples retain original concentrations after prolonged storage, even at air temperature. [85,88,298] A recent study by Moretti *et al.* showed negligible degradation over 10 weeks for cocaine and selected metabolites in DBS samples using the same DBS cards and stored at room temperature.[88] In our work, cocaine week 1 responses relative to time zero responses displayed degradation ranging from 70.8% to 56.4%, compared to a 20% median degradation for CN reported in Moretti.[88] However, our results showed that CN was generally stable after the initial drop in response, with degradation relative to time zero responses ranging from 78.5% to 44.5% after 8 weeks of storage at room temperature. BZE and EME in our work also displayed poor DBS stability compared to the results published in Moretti. Cocaethylene stability in our results showed better stability that in reported in other work.[88] CE

displayed good stability (mean degradation of 13.4% after 8 weeks) in all our samples vs. similar stability in just 2 of 5 cases reported by Moretti.

A significant challenge in using microvolume DBS samples for quantitation is determining sample volume. For applications that require measuring accurate concentrations, as in measuring drugs in blood for legal *per se* limits, volumes of blood must be known. Attempts have been made to correlate DBS spot area to sample volume for drug analysis.[299] Though DBS volume and area may be related, spot area is influenced by both hematocrit and DBS card type, making volume estimations from spot areas unreliable.[20,85,300–303] Being able to reliably obtain a fixed volume of blood for DBS samples would allow precise concentration calculations from drug responses in DBS samples. A number of volumetric absorptive microsampling devices (VAMS) have been developed to sample fixed microvolumes of blood. VAMS devices range from simple pads on the ends of small strips to shaped tips of porous media attached to plastic handles designed for easy use for sample collection by unskilled persons. Though VAMS were not used in this study, the method developed in this work has shown accurate quantitation of drug concentrations is possible in microvolume DBS samples even at low concentrations in samples of known volumes.

Though VAMS can assist in solving challenges in DBS sampling, correlations with venous whole blood and the capillary blood collected in DBS samples needs to be determined before VAMS/DBS samples can be used in the field.[90,302] The ratio of whole blood/DBS sample drug concentrations has been shown to be close to unity for 12 drugs that influence driving performance, including amphetamine, MDMA and MDA.[91] Regardless, the difference between capillary blood and venous blood cannot be taken for granted. A recent study highlighted this issue and demonstrated differences in CN, BZE and EME concentrations for paired samples of

venous whole blood and capillary blood DBS samples. However, this study used 6 mm punches of DBS samples, and some of the differences in paired samples may be due to bias introduced in partial punching. Partial punching of DBS samples is problematic as hematocrit levels and radial chromatography of blood spreading across DBS cards have been shown to impart uneven distributions of analytes across DBS sample areas.[300,304,305] Regardless of accurate volumes collected with VAMS, the concentrations of drugs in microsamples cannot be accurately measured and interpreted until the relationship between venous and capillary blood can be established for analytes of interest.

3.5 Conclusion

A quantitative method was validated for 10 commonly encountered stimulants and selected stimulant metabolites in 20 µL DBS samples using UPLC-QTOF-HRMS analyses. The method is semi-quantitative for an additional 4 analytes. The LOD was 10 ng/mL for all analytes, and LOQ was 10 ng/mL for all fully validated analytes. The method shows accurate quantitation of concentrations that range from the 10's of ng/mL to toxic levels is possible in DBS samples. Matrix effects, precision and bias was acceptable for all analytes except ecgonine methyl ester and phentermine. Though the use of microsampling is promising, a number of challenges prevent widespread use of dried blood spot samples. The results of this study highlight some of those challenges. Extended DBS sample stability for some analytes was poor, though modified DBS media have been shown to impart drug stability in DBS samples as good as, or better than preserved wet whole blood sampling[84,85]. The requirement of accurate sample volumes for quantitation of drug concentrations is also acknowledged. The development of volumetric absorptive microsampling devices may be able to address volumetric and stability requirements, but the relationship between capillary blood and venous blood drug concentrations needs to be

understood before the use of microsampling can be used for quantitation and interpretation in forensic casework. Despite these microsampling challenges, the method presented in this work demonstrates DBS sampling is an effective technique for the screening and quantitation of drugs and may be an approach useful to overcome a number of challenges in determining relevant blood-drug concentrations in cases of drug impaired driving.

Chapter 4

4 Conclusion

4.1 General Conclusions

A validated method for screening and quantitation of selected stimulant drugs in microvolume DBS samples using UPLC-QTOF-HRMS analyses has been developed. Sample preparation was optimized using ultrasonic extraction and MCX µElution SPE. The µMCX plates proved to be an efficient and effective sample cleanup method, and the absence of an evaporation step in µMCX methods reduced sample preparation time by several hours. Method validation following SWGTOX criteria was performed using the optimized sample preparation protocol developed in this work. Method validation demonstrated acceptable bias, accuracy, instrument stability and matrix effects for the quantitation of CN, CE, AMP, MA, MDMA, MDEA, MDA, BUP, OH-BUP, and MP from 10 ng/mL to 1000 ng/mL in DBS samples. Semiquantitative analysis was achieved for BZE, EME, AME and PH. Drug stability on DBS cards was drug dependent. The results of this work show DBS can be an effective technique for the screening and quantitation of commonly encountered stimulants in forensic analyses and may be a technique that can overcome a number of challenges present in current blood sampling techniques employed in drug impaired driving investigations.

4.2 Future Work

Microvolume DBS samples show tremendous potential for pharmaceutical and toxicological analyses and future research is warranted. A number of challenges remain before widespread use of microvolume sampling is employed in drug testing, and the results of this study highlight some of those challenges. Stability for some analytes in the DBS samples in this work was poor, though other studies have shown DBS imparts equivalent or superior drug stabilities relative to

preserved wet blood samples. The primary challenge facing DBS samples is collection of accurate sample volumes. A number of volumetric absorptive microsampling devices have been developed that may address drug stability and volume accuracy challenges, and these products should be assessed. Further work should develop validated methods for analysis of multiple classes of commonly encountered drugs. The simultaneous detection and quantitation of stimulants, opioids, cannabinoids, benzodiazepines, and other drugs in DBS samples would be of great value in toxicological and pharmacological analyses. Finally, ultra-sensitive analytical methods like triple quadrupole MS instrumentation should be employed to push detection limits of drugs in DBS samples to the ng/mL or pg/mL levels required by *per se* limits in drug impaired driving legislation, and for high potency drugs like carfentanil seen in impaired driving casework today.

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