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The Development and Optimization of a Two-Dimensional LC-QqQ-MS-Method for the Combined Analysis of Synthetic Cannabinoids and Designer Cathinones in Urine

Holly Penefsky

THE DEVELOPMENT AND OPTIMIZATION OF A TWO-DIMENSIONAL LC-QQQ-MS-METHOD FOR THE COMBINED ANALYSIS OF SYNTHETIC CANNABINOIDS AND DESIGNER CATHINONES IN URINE

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By

Holly S. Penefsky

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Approved June 11, 2021

_____ Stephanie J. Wetzel, Ph.D. Assistant Professor of Chemistry and Biochemistry (Committee Chair)	_____ Frederick Fochtman, Ph.D. Director of Forensic Science and Law, Retired (Committee Member)
_____ H.M. Skip Kingston, Ph.D. Professor of Chemistry and Biochemistry (Committee Member)	_____ Michael Cascio, Ph.D. Associate Professor of Chemistry and Biochemistry (Committee Member)
_____ Philip Reeder, Ph.D. Dean, Bayer School of Natural and Environmental Sciences	_____ Ellen S. Gawalt, Ph.D. Chair, Department of Chemistry and Biochemistry Professor of Chemistry and Biochemistry

ABSTRACT

THE DEVELOPMENT AND OPTIMIZATION OF A TWO-DIMENSIONAL LC-QQQ-MS METHOD FOR THE COMBINED ANALYSIS OF SYNTHETIC CANNABINOIDS AND DESIGNER CATHINONES IN URINE

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Dissertation supervised by Stephanie Wetzel

The compounds and chemical compositions of synthetic cannabinoids and designer cathinones are designed to mimic the intoxicating effects of Δ^9-tetrahydrocannabinol and amphetamines, respectively. In order to skirt existing drug laws, non-controlled ingredients are used, and the original chemical structures of current drugs are being modified using analogs or derivatives. These continually changing chemical compositions pose a problem for policymakers, and forensic and analytical scientists, as users are able to attain a "legal high" and avoid detection in standard drug screens. Commonly, toxicology laboratories utilize a screening method, such as immunoassay, for the presumptive identification of designer drugs. When a screening method yields a positive result, a confirmatory method, such as liquid chromatography (LC) or gas chromatography (GC) coupled with mass spectrometry (MS), is applied to quantify the compound present

more sensitively and specifically. Current analytical methods analyze synthetic cannabinoids and designer cathinones are using separate methods. The first major task of this research was to utilize a liquid-liquid extraction (LLE) process to move both synthetic cannabinoids and designer cathinones into the same sample for a single method of analysis on the LC-triple quadrupole-MS (LC-QqQ-MS). In order to increase peak capacity and eliminate a sample cleanup step, the second major task was to develop and optimize a two-dimensional (2D) LC-QqQ-MS method. Once an effective method of separation using 2D-LC-QqQ-MS was developed and optimized, a standard drug panel was applied to evaluate the efficiency and proposed application to real urine samples. The 2D-LC-QqQ-MS method was successful in separating synthetic cannabinoids, designer cathinones and a standard drug panel from one another. The method was spike validated using the cutoff concentration for methamphetamine in urine, demonstrating acceptable recoveries. A significant impact of this work is the elimination of a sample cleanup step and application to real urine samples. This method standardization permits universal applicability to urine samples and eliminates potential loss of analytes due to processing.

DEDICATION

This dissertation is dedicated to my family for challenging me, supporting me, believing in me and for loving me through my entire academic career.

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I don't think there will ever be enough words to express how grateful I am to my advisor, Dr. Stephanie Wetzel. Your patience, understanding, support and encouragement has meant the most to me over the last 12 years. Thank you for believing in me, despite my circumstances, and for always opening your door to me. Thank you for affording me the opportunity to work and teach in your lab with the undergraduate Forensics students, and then concomitantly supporting my decision to leave the university early to go and work in the industry for my daughter's benefit. From undergraduate to graduate school, you have made me a much stronger person and allowed me to accomplish my dreams.

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To my sweet girls, Olivia, Madilyn, and Adriana, I love you more than you will ever know. The hugs, smiles, and boundless joy you have given to me has pushed me to the finish line. I'm so thankful to be your mommy. Olivia, I did this especially for you. The strength you had at two days old to undergo neurosurgery and then how I've been blessed to watch you continue to grow as a resilient, young lady, inspires me and drives me to always want to make you proud. Mom, thank you for being the mother and the father

in my life, for teaching me responsibility at a young age, for always demonstrating a hard work ethic, and for championing me through my entire academic endeavor.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

LC	liquid chromatography
GC	gas chromatography
MS	mass spectrometry
LLE	liquid-liquid extraction
LC-QqQ-MS	liquid chromatography-triple quadrupole-mass spectrometry
2D	two-dimensional
SAMHSA	Substance Abuse and Mental Health Services Administration
LSD	lysergic acid diethylamide
COVID-19	Coronavirus
THC	Δ^9-tetrahydrocannabinol
CB1	cannabinoid receptor 1
CB2	cannabinoid receptor 2
MDPV	methylenedioxypyrovalerone
HPLC	high performance liquid chromatography
TCD	thermal conductivity detector
FID	flame ionization detector
ECD	electron capture detector
EI	electron impact ionization
CI	chemical ionization
m/z	mass-to-charge ratio
MS/MS	tandem mass spectrometry

KOH	potassium hydroxide
BSTFA	bis(trimethylsilyl)trifluoroacetamide
SPE	solid phase extraction
SEC	size-exclusion chromatography
GPC	gel-permeation chromatography
UHPLC	ultra-high performance liquid chromatography
UV-Vis	ultraviolet-visible
DAD	diode array detector
PMT	photomultiplier tube
ELSD	evaporative light scattering detection
ESI	electrospray ionization
APCI	atmospheric pressure chemical ionization
MALDI	matrix-assisted laser desorption/ionization
AP	atmospheric pressure
TOF	time-of-flight
DC	direct current
RF	radiofrequency
QqQ-MS	triple quadrupole mass spectrometer
Q_1	first quadrupole
Q_2	second quadrupole
Q_3	third quadrupole
SRM	selected reaction monitoring
MRM	multiple reaction monitoring

SALLE	salting-out assisted liquid-liquid extraction
SIM	selected ion monitoring
HCl	hydrochloric acid
WSI	working solution I
WSII	working solution II
WSIII	working solution III
WSIV	working solution IV
rpm	rotations per minute
N_2	nitrogen
HILIC	hydrophilic interaction liquid chromatography
RPLC	reverse phase liquid chromatography
AJS	Agilent jet stream
CE	collision energy
EDTA	ethylenediaminetetraacetic acid
1D	one-dimensional
AF	ammonium formate
IDMS	isotope dilution mass spectrometry
SIDMS	speciated isotope dilution mass spectrometry

1. INTRODUCTION

1.1 *Introduction to drugs of abuse*

Street drugs are commonly abused, as people use them to enhance their current emotional state, to cope with stresses, both physical and mental, to enhance their performance, to satiate curiosity, as a result of social pressure, or as a response to current and underlying psychological stressors.[1] Some of the most commonly abused drugs include heroin, cocaine, methamphetamine, dimethyltryptamine, gamma-hydroxybutyric acid, ketamine, khat, lysergic acid diethylamide, marijuana, phencyclidine, synthetic marijuana and synthetic cathinones.[2]

Stimulants, such as cocaine, produce an initial euphoria that permits the user to experience intense sensations of pleasure followed by feelings of power, self-confidence, and increased energy.[3] Starkly contrasting, opioids such as heroin create feelings of relaxation and satisfaction after the initial euphoria.[3] A person who is abusing street drugs may be attempting to feel less anxious, stressed or depressed, and begins using drugs to escape reality.[3] Others may feel pressure to perform better at work, school or in extracurricular activities, and therefore turn to drugs to aid them in the process. A person may also feel pressure from one's peers and engage in abusing drugs.[4] Another important consideration includes those who have been prescribed drugs, legally, especially opioids, for pain management and they become addicted.[5] Regardless of the reason or reasons, drugs are seemingly everywhere, and are constantly being abused.[3]

1.1.1 *Commonly abused drugs*

Alcohol, illicit drugs, tobacco, and prescription drugs continue to be misused and abused by millions of Americans every year. According to the Substance Abuse and

Mental Health Services Administration's (SAMHSA) 2019 National Survey on Drug Use and Health, almost 20.4 million people aged 12 years or older had a substance use disorder in the past year.[6] Some of the most commonly abused street drugs typically fall into several broadly defined categories: narcotic analgesics, psychomotor stimulants, central nervous system depressants, anti-anxiety agents, sedative/hypnotics, hallucinogens/psychedelics and cannabis.[7]

Marijuana use in the past year has significantly increased in adults aged 26 years and older, while additionally, marijuana use disorder has significantly increased in adolescents.[6] There has been no change in cocaine use in all age groups, while there has been an upward trend in methamphetamine use and a significant increase over 2016 - 2017 in adults 26 years and older.[6] Prescription stimulant misuse appears to be trending downwards in those 18 - 25 years old, while lysergic acid diethylamide (LSD) use has significantly increased in adolescents, with a slight increase in young adults and adults.[6]

There are approximately 10.1 million people that have an opioid misuse disorder, which can be defined as heroin use or prescription pain reliever misuse.[6] In people aged 12 years or older, prescription opioid misuse includes hydrocodone (5.1 million users), oxycodone (3.2 million), codeine (2.4 million), tramadol (1.3 million), buprenorphine (686,000), morphine (455,000), methadone (240,000) and fentanyl (269,000), while heroin users account for another 745,000.[6] Not surprisingly, the most prevalent sources from which prescription pain relievers were obtained were either given by, bought from, or taken from a friend or relative.[6] Opioid use disorder has decreased from 2 million to 1.6 million users, as efforts to increase access to medication-assisted treatment, psychosocial and community recovery supports appear to have had a positive effect.[6] However, overdose

deaths have increased in 2019 by approximately 4.6%, emphasizing the risks of potent illicit synthetic opioids and the need to continue to engage people in treatment and recovery services.[6]

It is important to note that all of the 2019 data from the SAMHSA report, as referenced above, was all collected before the SARS-Cov-19 (Coronavirus or COVID-19) pandemic. COVID-19 has brought upon a great fear of illness and/or death from the virus, including the fear of resuming normal life activities and neglecting one's own health or mental health needs for fear of infection.[8] The coronavirus has also inflicted isolation, an overall loss of familiar daily structure, financial stress, being unemployed or trying to find employment, new expectations (i.e. children at home instead of in daycare/school and parents are expected to become at home teachers), an inability to get medical care and follow up because such care may be deemed "non-essential", and increases in domestic violence and child abuse or neglect.[9] Most importantly, the coronavirus is expected to create substantial increases in substance use disorders, mental illness and suicides in all age groups, validating the continuing importance of this research project.[10]

1.2 *Prevalence of synthetic analogues*

A cause for concern in the United States is the rising availability of synthetic cannabinoids and designer cathinones. Both types of substances are deemed "illegal", but it has not dissuaded their use.[11] Each year, new synthetic cannabinoids and cathinones appear, simply varying by the addition or removal of a substituent group.[12]

Historically, khat shrubs have been grown in parts of Eastern Africa and the southwestern Arabian Peninsula.[13] Here, members of the Muslim community chew up the khat leaves as part of a deep-rooted practice.[14] The availability of khat has increased

significantly over the last several decades and has expanded to other regions of the world.[13] The World Health Organization has estimated that more than 20 million people chew khat on a regular basis worldwide.[15] This has been facilitated by a rise in the number of immigrants from khat-producing areas and by enhanced methods of khat transportation and distribution.[16]

Khat's cathinone analogues have been synthesized since the 1920s, following the lead of Europe.[17] Because of the strict regulations in the United States, synthetic cathinones are mostly synthesized in underground laboratories and made available to users in illicit markets.[18] Typically, they are synthesized in the form of a white or brown crystal-like powder, tablets or capsules and sold to consumers in small plastic or foil packages, labeled as "bath salt" accompanied with the phrase "not for human use."[19] In other cases, these products are sold using illusory labels, such as "plant food," or "insect repellant," in conjunction with likeable brand names to make them even more appealing.[18] With improved synthesis and marketing efficiencies, there appears to be a recent trend toward a rapid increase in availability of synthetic cathinones in many parts of the world, including North America, Europe and Asia.[20]

1.2.1 *Synthetic cannabinoids and designer cathinones*

In recent years, synthetic cannabinoids and cathinones have been designed to mimic the intoxicating effects of Δ^9-tetrahydrocannabinol (THC) and amphetamines, respectively.[21] In order to skirt existing drug laws, non-controlled ingredients are used, and the original chemical structures of current drugs are being modified using analogs or derivatives.[21] These continually changing chemical compositions pose a problem for policymakers, and

forensic and analytical scientists, as users are able to attain a "legal high" and avoid detection in standard drug screens.[21]

Synthetic cannabinoids are mainly categorized into two groups: the classical structures related to THC, and the non-classical structures such as aminoalkylindole, 1,5-diarylpyrazole, quinolones, arylsulfonamides, and eicosanoids.[22] Those that are being used as drugs of abuse include JWH-018, JWH-073, and MAM-2201. They exert their effects by acting as full agonists on cannabinoid receptors (CB1 and CB2) in the body, which are part of a complex endocannabinoid system that is not yet completely understood.[22] These compounds undergo metabolism through the body and are excreted in urine as metabolites. The detection period of synthetic cannabinoid metabolites in urine is between 24 and 72 hours, as can be seen in Figure 1.1.[23]

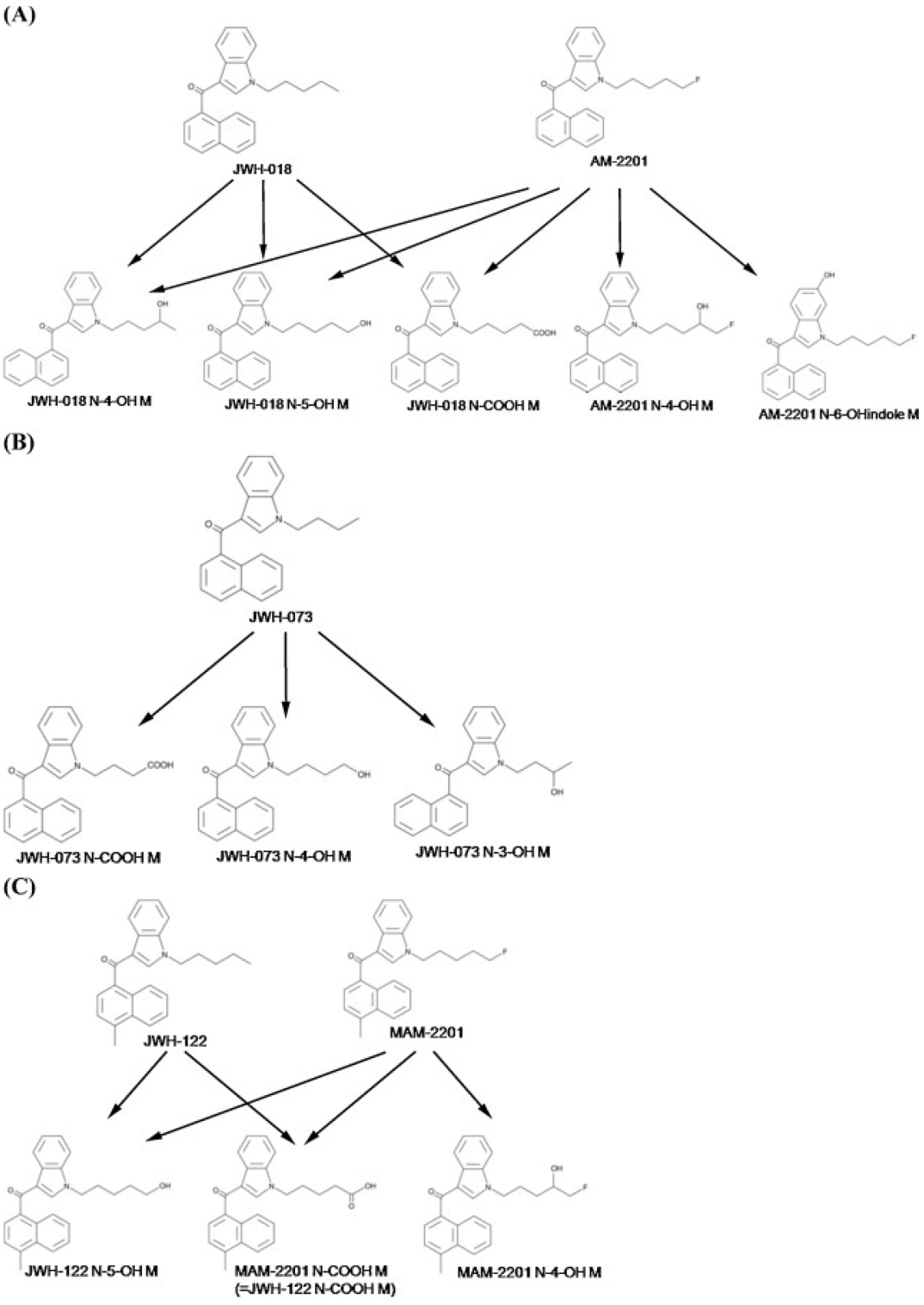

Figure 1.1 Proposed phase 1-metabolic pathways of five naphthoylindole-based synthetic cannabinoids: (A) JWH-018, AM-2201 and their metabolites, (B) JWH-073 and its metabolites, and (C) JWH-122, MAM-2201 and their metabolites.[23]

Cathinone, the principal active ingredient in the leaves of the khat plant, can be considered the prototype from which a range of synthetic cathinones have been developed.[22] Some synthetic cathinones that are being used as drugs of abuse include mephedrone, methylenedioxypyrovalerone (MDPV) and methylone.[24] They are phenylalkylamine derivatives and are termed "bk-amphetamines" for the beta-ketone moiety.[24] In addition, they may possess both amphetamine-like properties and the ability to modulate serotonin, causing distinct psychoactive effects.[24] In the body, MDPV, mephedrone, and methylone act as dopamine reuptake inhibitors, leading to euphoria, excitement and hallucinations.[25] The detection period of unchanged MDPV, and mephedrone and methylone metabolites in urine is between 24 and 36 hours.[24] See Figure 1.2 for the structure of cathinone and the structurally similar analogs.

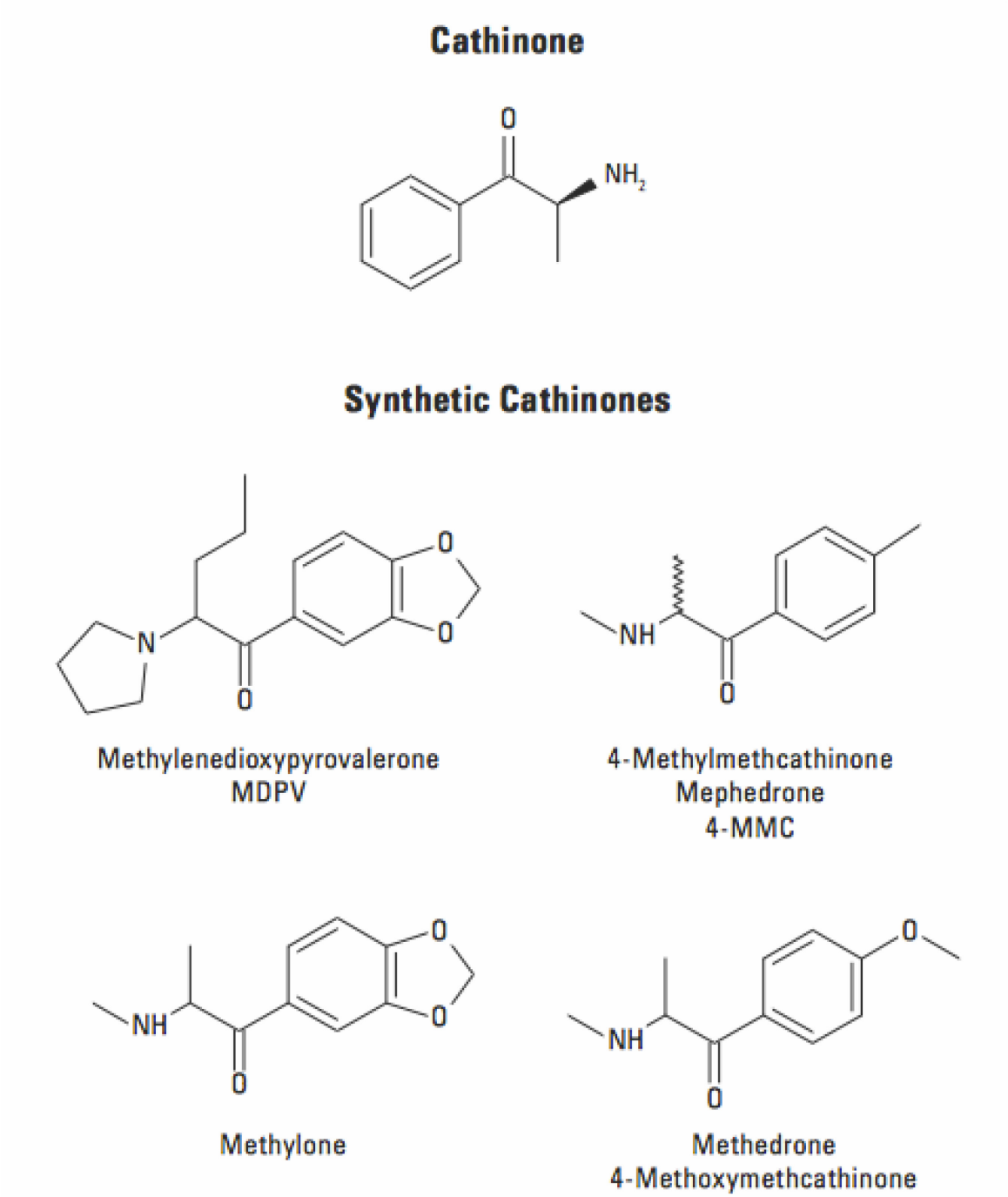

Figure 1.2 The structures of synthetic cathinones and how they are chemically similar to cathinone.[26]

1.3 *Current analytical instrumentation for detecting and quantifying drugs of abuse*

Analyzing samples that could contain multiple compounds can be performed using a variety of related techniques. In forensic toxicology, the screening tests are usually carried out using immunological methods that are typically designed for single compounds or a class of compounds.[27] Traditional drug screening methods would likely be unable to detect designer drugs, as they are constantly being manipulated in structure. A confirmation analysis is also necessary in conjunction with the immunoassay test, due to the high occurrence of false positive results.[27] These confirmation analyses are usually carried out by chromatographic/spectrometric analyses.[28] In clinical and forensic toxicology, screening by immunoassay and confirmation by Gas Chromatography-Mass Spectrometry (GC-MS) has been the standard used in laboratories for the detection of illicit drugs.[28] While GC-MS demonstrates the necessary sensitivity and specificity, samples must be derivatized prior to analysis.[29] Liquid Chromatography-Mass Spectrometry (LC-MS) eliminates the need for time-consuming immunoassay and derivatization steps, and is easily adaptable to detecting the wide range of emerging drugs of abuse.[27] Currently, there are separate analytical methods for analyzing cannabinoids and cathinones, with LC-MS being the most common analytical technique.[30,31] Therefore, there was a need to develop a single method of analysis that is capable of detecting both groups of drugs. High performance liquid chromatography (HPLC) coupled with tandem MS can be used to quantify multiple cannabinoids and cathinones within a single sample. The method employed is dependent on the nature of the sample and will be detailed in the following sections.

1.3.1 *Current GC-MS techniques*

Gas chromatography (GC) is a separation technique that is able to isolate volatile analytes of interest from the matrix, or components of a mixture. This is achieved by partitioning compounds between a mobile phase, which is an inert gas, and a stationary phase, a chemical coating inside of a column that can selectively attract components in a sample mixture.[32] High purity helium, hydrogen or nitrogen is used as the mobile phase in GC analyses. Columns can be either packed or capillary, depending on the application. Packed columns are "packed" with liquid-coated particles that act as the stationary phase.[32] Capillary columns have the liquid stationary phase coated on the inside walls of the tubing.[32] The stronger the analyte interaction is with the stationary phase, the longer the analyte will interact and therefore the more time it will take to elute from the GC column.[32] An important aspect to consider is the boiling point of a particular analyte, as this directly implicates the retention time due to its vapor pressure.[32] The column is attached to an inlet and a detector, within an oven. The oven is used to heat the column, typically using temperature programming, to elute the analytes of interest so they can be detected and quantified. The inlet contains a liner that holds and heats the injected sample until it vaporizes and then moves it onto the beginning of the GC column. The detector detects the components that are eluted from the column by converting it to an electronic signal that is sent to a data system and the signal is plotted versus time.[32]

There are a variety of detectors that can be integrated with the GC system, including but not limited to thermal conductivity (TCD), flame ionization (FID), electron-capture (ECD) and MS. The TCD operates under the principle that there is a thermal conductivity difference between the inert gas and the analyte of interest, and can thereby measure that

difference and generate a signal.[32] An important feature of the TCD is that it can detect water in addition to organic compounds. The FID burns the effluent in a hydrogen flame and measures the ions that are created from the analyte of interest.[32] In order to be detected using FID, the organic compounds must be able to undergo oxidation.[32] The ECD measures the changes in an electron current when organic compounds react with electrons generated from a radioactive beta emitter.[32] In order to detect compounds using the ECD, they must be able to react with electrons.[32] MS measures the mass to charge ratio of ions generated from the effluent, using either electron impact ionization (EI) or chemical ionization (CI).[33] In EI, once the sample elutes from the column, it enters the ion source where electrons are accelerated at 70 volts and impart sufficient energy to remove outer shell electrons from the analytes, producing positive ions.[33] The high energy generated from EI causes the ions to fragment into smaller ions.[33] In CI, the reagent gas becomes ionized first and is present in a much larger abundance than the analytes of interest.[34] The reagent gas ions react with the analyte molecules either by protonation or proton abstraction, causing the analyte to become charged.[34] These ions move into the mass analyzer where they are separated based on their mass to charge ratios, become detected and are then converted to a digital output.[35]

GC-MS has long been the gold standard for forensic analyses.[28] In GC-MS, a mixture of compounds are separated by volatility while traveling through a GC column, become ionized when hit by electrons, and are filtered through a quadrupole before the mass to charge ratio (m/z) of each component is determined by the detector.[28] The mass spectrum produced can then be compared to a spectral library of known compounds for identification. While mass spectrometry (MS) yields the m/z of a compound, tandem mass

spectrometry (MS/MS) involves fragmenting the compound after initial MS and then determining the m/z of each fragment.[28] This is especially beneficial when precursor ions have identical masses. However, there are also challenges with GC and GC-MS analyses, as the samples may contain nonvolatile components, such as the matrix (urine in this case).[28] In this instance, the sample must be processed or "cleaned up" before the analysis can be carried out. GC-MS analysis usually requires time-consuming derivatization and extraction processes in order to attain selectivity of certain compounds.[28]

Lehmann et al. carried out GC-MS quantification of THC-COOH in urine by adding potassium hydroxide (KOH), allowing the urine to hydrolyze in a water bath, adjusting the pH to 4.5 with glacial acetic acid and then applying the modified urine to a C18 SPEC microcolumn disc.[60] The disc was then washed with diluted acetic acid and the contents were derivatized with bis(trimethylsilyl)trifluoroacetamide (BSTFA) before drying under a gentle stream of nitrogen and then extracting with n-hexane.[60] In another study, Hong et al. was able to identify designer cathinones in urine utilizing GC-MS by first extracting the samples using solid phase extraction (SPE) and then subjecting them to derivatization using heptafluorobutyric anhydride and ethyl acetate.[61] Both Lehman et al. and Hong et al. were able to quantify cannabinoids or cathinones at levels that would be considered near the cutoff concentrations in urine, but the samples required extensive sample preparation prior to the analyses.[60, 61]

1.3.2 Current HPLC and LC-MS techniques

Column liquid chromatography (LC) is a technique that is used to separate solvent-soluble species, non-volatiles or thermally labile compounds that are unable to be analyzed by GC.[36] Like GC, the analytes of interest are separated from the matrix, or a mixture of

compounds, by partitioning between a stationary phase and mobile phase. However, the mobile phase is a liquid, instead of a gas, in LC. Separation is based on the polarity, electrical charge, or molecular size of the compound.[37]

When considering polarity, normal phase and reversed-phase chromatography are the modes for separation.[37] Normal phase chromatography utilizes a polar stationary phase, such as silica particles, and a nonpolar mobile phase, such as acetonitrile, to increase the retention of polar compounds on the column.[37] Reversed-phase chromatography is just the opposite of normal phase, whereby the stationary phase is composed of nonpolar particles, such as octadecylsilane, and a polar mobile phase, such as water, to increase the retention of nonpolar compounds on the column.[37]

For separations based on charge, commonly called ion-exchange chromatography, there are two types: cation exchange and anion exchange.[37] The nature and strength of acids or bases that make up the stationary phase surface dictate the type of ions that are attracted and can be retained.[37] A cation exchange involves the attraction of a positively charged analyte with a negatively charged stationary phase particle. In anion exchange, a negatively charged analyte is attracted to a positively charged stationary phase particle.[37]

Separations based on size are often referred to as size-exclusion chromatography (SEC) or gel-permeation chromatography (GPC) as they utilize stationary phases with a distribution of pore sizes to elute larger analytes first and smaller analytes last.[37] Mobile phases are employed for elution purposes, but also to prevent the analytes from interacting with the stationary phase based on charge or polarity.[37] These techniques are typically used to determine the molecular weight distributions of polymers and oligomers, instead of small molecules.[37] Like GC, LC can also be coupled to a mass spectrometer for the

detection of analytes. However, unlike GC, there are a multitude of sources that are able to ionize analytes in many different ways.

Subcategories of LC include HPLC and ultra-high-performance liquid chromatography (UHPLC), with the difference between the two depending on the pressure applied to the chromatographic system. HPLC is capable of reaching pressures up to 6000 psi, while UHPLC can reach up to 15,000 psi, allowing for the use of smaller particle-sized columns and concomitantly increased resolution, speed, and sensitivity in chromatographic analyses.[37]

Once the analytes of interest have been separated from the matrix they need to be identified and quantified. Depending on the nature of the compound, different detectors can be used. Ultraviolet-visible (UV-Vis) detectors contain a deuterium and tungsten lamp to cover a range of wavelengths at which conjugated analytes can absorb light and be detected.[38] Similar to UV-Vis, a diode array detector (DAD) also generates a spectrum based on the analyte's absorption of light, but contains multiple photodiode arrays to obtain information over a wide range of wavelengths at one time.[38] In contrast, the UV-Vis detector measures absorption at a fixed wavelength. Fluorescence detection typically uses a xenon lamp and specific excitation and emission wavelengths for each analyte.[39] In this instance, when light energy, a photon, is absorbed by the analyte it moves some of the electrons from the ground state to a high-energy, excited state (excitation).[39] In this higher energy and vibrationally excited state, the electrons undergo a loss of some vibrational energy and "relax" or move back into the ground state while emitting light in the form of fluorescence (emission).[39] Photons that are generated during fluorescence are measured using a photomultiplier tube (PMT).[39] If an analyte does not contain a chromophore or a

fluorophore, and therefore cannot be measured using UV-Vis/DAD or fluorescence, then evaporative light scattering detection (ELSD) is an alternative.[40] When the analyte and more volatile mobile phase elute from the column, they become nebulized and are then heated with gas to evaporate the solvent from the analyte.[41] The analyte moves through an optical chamber causing incident light scattering which is then measured using a PMT.[41] MS is a detector that can also be used in tandem with LC as a means to measure analytes of interest that have been separated on a column. As with GC-MS, the analytes need to be ionized in order to be detected.

There are a variety of sources that can be used to ionize the effluent from the column, before passing into the mass spectrometer. Some of the most common sources include, but are not limited to, electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and matrix-assisted laser desorption/ionization (MALDI). ESI is considered a "soft" ionization technique in which the eluent from the chromatography column moves through a charged capillary tube, becomes nebulized once exiting the capillary and forms a thin spray of charged droplets.[42] The solvent associated with the droplets begins to evaporate under a steady stream of heated nitrogen gas, transferring the residual charge to the analytes which are then drawn into the mass spectrometer by a series of small apertures and focusing voltages.[42] In contrast to ESI, where ionization occurs in the liquid phase, ionization takes place in the gas phase with APCI.[43] At atmospheric pressure, the effluent traverses a capillary, becomes nebulized, and is heated in the source housing before passing by a Corona needle, where ionization occurs.[43] In positive ionization, the relative proton affinities of the reactant ions and the gaseous analytes molecules allow either proton transfer or adduction of reactant gas ions to produce the ions of the molecular species.[43]

The evaporated mobile phase acts as the ionization gas and the reactant ions are formed due to the effect of the Corona needle on the nebulized solvent.[43] The primary ions formed by the Corona discharge include N^+ or $O^\bullet$ that can then go on to form secondary reactant gas ions through collisions with vaporized solvent molecules.[43] From here, the ions are then drawn into the mass spectrometer and focused by a series of voltages and lenses.[43] MALDI can be utilized under atmospheric pressure (AP) or vacuum, depending on the sample type. AP-MALDI is advantageous in that it can analyze volatiles, since it does not need to be pumped down to vacuum.[44,45] An aliquot of the sample is mixed together with matrix and spotted on a target plate.[43,44] Once dried, the target plate is placed into the source housing and a high voltage is applied on the surface of the plate.[43,45] A stream of dry nitrogen diffuses around the area surrounding the plate to assist in the transport of ions towards the mass spectrometer.[43] In positive mode, analytes are protonated in the hot plume of ablated gas through analyte-matrix collisions.[43] These ions travel through a transfer capillary from the MALDI interface into the mass spectrometer.[43] In order to achieve high analytical sensitivity with this source, the matrix absorption wavelength must correlate with the laser wavelength, and the m/z of the matrix must not overlap with the m/z of the analytes of interest in the mass spectrum.[43] Regardless of the ionization source, the mass analyzer is responsible for ion separation and determines the sensitivity of the mass spectrometer.[45]

There are a multitude of mass spectrometers, but the most common include magnetic sector, quadrupole, time-of-flight (TOF), ion trap, and quadrupole ion trap.[46] It is important to note that there is not a single mass analyzer that is best suited for all applications as each mass analyzer has its own advancements and limitations.[46] A

magnetic sector uses a magnetic field to isolate ions of specific m/z values from others.[46] Initially a high voltage is applied, permitting the ions to accelerate into the magnetic sector where they are exposed to a magnetic field.[46] The magnetic field is applied perpendicularly, causing the ions to arc (more or less depending on their mass) towards the detector.[46]

A quadrupole mass analyzer is composed of four parallel hyperbolic metal rods that have either a direct current (DC) or radiofrequency (RF) voltage applied to them.[46] The quadrupole can be used as an ion guide along the z-axis when RF only voltages are applied and act as an ion guide, thereby allowing transmission of all entering ions.[47] DC operates to defocus the ions, but when used in conjunction with RF voltages, the quadrupole now acts as a mass filter by providing a stable trajectory for ions with a specific m/z, while other ions experience an unstable oscillation, collide with the rods and are not detected.[48] The ions begin to oscillate within the quadrupole according to the Mathieu equation, as seen in Figure 1.3.[48]

$$\frac{m}{z} = K\frac{V}{r^2\omega^2}$$

m = mass of the ion

z = charge of the ion

K = constant

V = voltage applied

r = effective distance between the electrodes

ω = oscillation frequency

Figure 1.3 The Mathieu Equation[48]

A TOF mass spectrometer operates differently than the aforementioned mass analyzers as it is considered a pulsed, non-scanning MS that separates and detects ions of various m/z by measuring the time it takes for ions to travel through a flight tube with a known distance.[48] Upon ionization, ions become packetized before being accelerated into the flight tube by an ion acceleration electrode.[48] The packetized ions experience unique velocities in the flight tube, when acceleration and kinetic energy are held constant.[48] As a result, the m/z values are able to be determined by calculating the time it takes for the ions to move through the flight tube from the ion source, into an ion optic device called a reflectron, and then back to the detector.[48] Ions that have a smaller m/z will traverse the fastest, penetrating only slightly into the reflectron before reversing direction back towards the detector.[47,48] Conversely, the larger m/z ions move the slowest, penetrating deeper into the reflectron, before reversing direction and arriving at the detector last.[48]

Ion trap mass analyzers can be grouped into two main categories: "dynamic" and "static".[48,49] Both types of ion traps store the ions in the trap and use RF and DC fields to manipulate them in a series of precisely timed events.[49] These become advantageous mass analyzers when high resolution and sensitivity are necessary, or extensive MS/MS experiments are desired for further fragmentation and identification.[48,49] The caveat to trapping the ions for an extended period of time, is that the ions can begin to undergo unimolecular decomposition, experience space charge effects from other ions or neutral molecules or endure changes in motion due to inadequate electric fields.[49]

Dynamic "quadrupole" ion traps store ions that are either formed within or injected into in a three-dimensional quadrupole storage device, and then RF potentials are ramped along one of the ion trap electrodes to eject ions in ascending order from the trap into the

detector.[49] Static ion traps are typically used in conjunction with sector instruments as the field is kept at a constant value for transmission of an ion.[49] Ion cyclotron resonance mass spectrometers are an example of static traps that utilize the concept that ions move in a circular path when in a fixed magnetic field.[49] The cyclotron frequency of the ion's circular motion is dependent on the mass.[49] Thereby, measuring the cyclotron frequency allows for the determination of the ion's mass.[49] Packets of ions are detected simultaneously by passing near detection plates and inducing image currents that can be amplified and digitized, instead of hitting a detector like other mass analyzers.[49]

In this work, a tandem mass spectrometer, specifically, a triple quadrupole mass spectrometer, was coupled to a high-performance liquid chromatography system to carry out the separation, ionization, and detection of the synthetic cannabinoids and designer cathinones. As mentioned before, a quadrupole is made up of four parallel rods that are approximately 10-20 mm in diameter and 15 - 25 cm in length.[43] The four rods are electrodes with electric fields around them, allowing ions to travel down through the rods.[43] There are both DC and RF voltages in the range of 102 - 103 V applied to the electrodes.[43] Each pair of rods is connected so that the rods have exactly the same voltage as the one directly opposite it.[43] As an ion enters the quadrupole assembly in the z-direction, an attractive force is exerted on it by one of the rods with its charge actually opposite to the ionic charge.[43] If the voltage applied to the rods is periodic, attraction and repulsion in both the x- and y-directions will alternate in time.[43] The ion may also travel down the quadrupole in the z-direction without touching any rods, provided its motion around the z-axis is stable.[43] Christie G Enke and Richard A. Yost were credited with developing the first triple quadrupole system while trying to avoid chromatography to separate a sample

mixture in one stage, fragment it and identify the compounds in another stage.[50] R. Graham Cooks was already doing "double-focusing" tandem mass spectrometry at Purdue University at the time, using an electric sector and a magnetic sector to select a "parent" ion from the sample, and then analyze the fragment ions that were produced from the parent ions colliding with neutral gas molecules.[51] However, there was low fragmentation efficiency and thus low signal in the electric sector with this process, as the ions typically only experienced a single collision with the helium gas.[51] Enke and Yost added a third quadrupole to their system to trap the ions inside of a quadrupole collision chamber where they would undergo multiple collisions to increase signal intensity.[50] In the design, the first quadrupole was used to select a specific ion, which is then passed into the second quadrupole where the specific ions underwent collisions to form fragments.[50] These fragments were then allowed to pass into the third quadrupole, where all or only specified "daughter" ions (fragments) were permitted to pass through for detection.[50].

An Agilent 6460 triple quadrupole mass spectrometer was used in this research project (Figure 1.4).

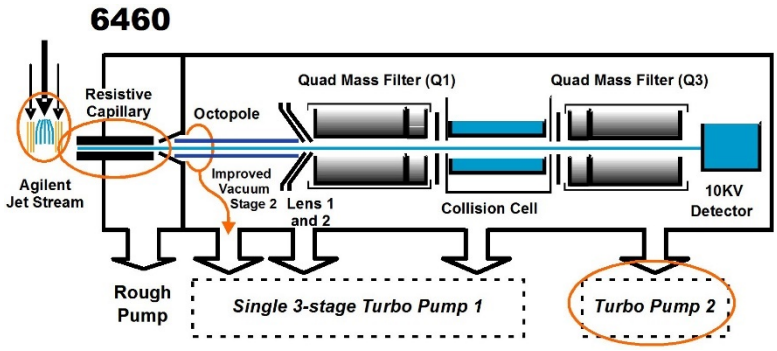

Figure 1.4 Agilent 6460 Triple Quadrupole Mass Spectrometer Schematic[52]

The triple quadrupole system actually consists of two quadrupoles (Q_1 and Q_3), that can act as ion guides or mass filters, and a hexapole collision cell, typically called the

second quadrupole (Q_2), which also acts as an ion guide or to carry out fragmentation.[43, 53] The collision cell utilizes nitrogen gas as it is an inert, non-reactive gas to collide with ions from Q1 to yield fragment ions and neutral molecules.[52] There are a few scan modes available to choose from, including precursor ion, product ion, neutral loss and selected reaction monitoring (SRM).[43, 53] Precursor ion scan is used to scan all of the ions in the first quadrupole (Q_1) before undergoing collision with nitrogen gas in the collision cell, and then setting the third quadrupole (Q_3) at fixed potentials so as to only allow specific fragment ions, daughter ions, to pass through.[43, 53] This type of scan is useful when the daughter ions are known, but the parent ion is unknown, yielding more information about the overall structure of the parent ion from the pieces of the parent ion.[53] Product ion scan involves fixing the potentials in Q_1 so that only a specific parent ion can pass through and into the collision cell.[43, 53] The selected m/z is fragmented in the collision cell and then all daughter ions are scanned in Q_3.[53] This type of scan is helpful in determining the unique daughter ions that are associated with the parent ion of interest.[53] Neutral loss scan is carried out by scanning Q_1 and Q_3, but with a constant mass offset to allow for the selective recognition of all ions after fragmentation in the collision cell, resulting in the loss of a given neutral fragment.[43, 53] This scan type is useful when you suspect that the neutral loss mass is characteristic of a class of compounds or closely related compounds, and there is interest in identifying the mixture components that belong to that class.[53, 54] SRM conditions have the Q_1 and Q_3 potentials fixed for a specific parent ion that undergoes fragmentation and then produces a specified daughter ion.[43, 53] Selected reaction monitoring is also referred to as multiple reaction monitoring (MRM) as multiple precursor to product ion transitions are able to be isolated and examined during an analysis.[55] An

important thing to note is that the LC-QqQ-MS does not necessitate derivatization of samples prior to analysis, as GC-MS does, and yields very specific and sensitive results. It is advantageous to be able to simplify the process and decrease analysis time, all while producing exceptionally reliable results.

Zaitsu et al. was able to determine and quantify the metabolites of newer designer cathinones in urine utilizing GC-MS and LC-MS, however, sample cleanup was still necessary in order to reduce matrix effects from the urine.[62] In another study, Glicksberg and Kerrigan were able to isolate cathinones from urine samples over a period of months using SPE columns, evaporating the eluent to dryness under nitrogen and then reconstituting in the appropriate mobile phase for LC-MS identification and quantification.[63]

1.3.3 *Current sample cleanup techniques for drugs in urine*

In forensic laboratories, sample cleanup is necessary prior to instrumental analysis to ensure the successful identification and quantification of analytes in biological samples.[62-64] Often times, the traditional SPE and liquid-liquid extraction (LLE) cleanup methods are unfitting for designer drugs because they show a lack of class selectivity and pose a greater risk of decomposing the analyte of interest during the extraction process.[64] Murakami et al. was able to introduce a new sample cleanup technique that utilizes molecularly imprinted polymer-based SPE to extract designer cathinones from urine and blood matrices without sacrificing recoveries and concomitantly reducing matrix effects when analyzed using tandem LC-MS.[64] Yanes and Lovett utilized a salting-out assisted liquid-liquid extraction (SALLE) technique instead of traditional LLE to detect and quantify four urinary metabolites of JWH-018 and JWH-073.[65] In doing so, they saved on

processing time and solvent waste was reduced, while also shortening the UHPLC analysis time by converting a typical gradient method to an isocratic elution.[65]

1.4 *Major limitations of preexisting methods*

There is a myriad of limitations to preexisting methods in the literature, including those mentioned in the previous sections of this chapter. Sample cleanup is necessary to reduce matrix effects from blood, urine and other biological matrices, and this consumes additional laboratory time before the samples can be analyzed by mass spectrometric detection methods.[56, 57, 62-65] In order to analyze multiple drug groups including cathinones and cannabinoids, multiple extraction techniques need to be employed, as the different drug classes are composed of either acidic or basic properties.[56-58] The most important aspect to consider is that sample cleanup is unavoidable and the detection of cannabinoids and cathinones is carried out separately, no matter the analytical technique used.[56-58, 62-65] Kemp et al. developed a method to quantitatively analyze Δ^9-tetrahydrocannabinol and six metabolites in plasma and urine.[56] They carried out the analysis and quantification using GC-MS coupled with selected ion monitoring (SIM) to detect the trimethylsilyl derivatives of the cannabinoids.[56] In doing so, they were able to enhance the chromatographic separation and mass spectral characteristics, but also introduced an additional sample preparation step into the process. Gerace et al. was able to determine several synthetic cathinones and an amphetamine-like compound in urine.[57] The sample preparation included a liquid-liquid extraction under alkaline conditions, followed by derivatization with trifluoroacetic anhydride before detection using GC-MS.[57] Montesano et al. (2014) employed a micro-solid phase extraction (μ-SPE) procedure for the determination of cannabinoids and their metabolites in urine.[58] While they were able to minimize the cost

of the analysis by using a μ-SPE technique with C_{18} tips that only required a 2 hour enzymatic hydrolysis using 90 microliters (μL) of urine, sample preparation was still necessary, thereby decreasing overall throughput time.[58]

1.5 *Motivation*

The objective of this project was to develop and optimize a two dimensional-liquid chromatography-triple quadrupole-mass spectrometry (2D-LC-QQQ-MS) method capable of effectively separating and quantitating designer drugs in a single urine sample, using one method of analysis. This method can then be applied to a panel of drugs to demonstrate its efficiency and used in forensic laboratories to increase sample throughput. In order to complete this project, a specified set of objectives needs to be fulfilled.

1.5.1 *Research Objectives*

Specific Aim 1: Extract and separate synthetic cannabinoids and designer cathinones using one method of analysis.

Specific Aim 2: Develop and optimize a 2D-LC-QqQ-MS method for synthetic cannabinoids and designer cathinones.

Specific Aim 3: Expand the 2D-LC-QqQ-MS method to include all drug classes.

1.6 *Proposed development of new sampling techniques*

Because synthetic cannabinoids and designer cathinones are easily manipulated in structure, they still remain a public health and safety issue when considering drug testing.[59] Most cannabinoids and cathinones are unable to be detected in routine drugs of abuse screening tests, thereby creating an uptick in the popularity of use.[59] The overarching goal of this work is to develop and optimize a 2D-LC-QqQ-MS method capable of effectively separating and quantitating designer drugs in a single urine sample, using one method of

analysis. This will be accomplished through liquid-liquid extraction (Chapter 2), optimizing the ionization source and chromatography of cannabinoids and cathinones using two different LC columns (Chapter 3), and then collecting fractions from the first column and injecting them onto the second column to form a two dimensional LC method that can effectively separate cannabinoids and cathinones from each other (Chapter 4). This method can then be applied to a standard urine panel of drugs (Chapter 5) to demonstrate its efficiency and be used in forensic laboratories to eliminate sample prep, decrease costs, and increase sample throughput.

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2. SAMPLE CLEANUP UTILIZING A LIQUID-LIQUID EXTRACTION TECHNIQUE

2.1 *Introduction to liquid-liquid extraction*

LLE is a technique that has been used to clean up and enrich samples prior to an analysis.[1] In principle, an analyte of interest is able to partition itself between two immiscible solvents, an aqueous layer and an organic layer.[1] Depending on the nature of the analyte, it can be forced into either layer by changing the pH of the solution or by salting out. Kneisel and Auwärter used a carbonate buffer solution with a pH of 10 to force synthetic cannabinoids present in human serum, into the organic phase so that they could be dried down under nitrogen and reconstituted in mobile phase for tandem LC-MS (LC-MS/MS) analysis.[2] In another experiment, cannabinoids were extracted from wastewater samples using sodium chloride, hydrochloric acid (HCl), and a 1:1 hexane:ethyl acetate (v/v) solution followed by centrifugation, and were then analyzed using ultra-high performance supercritical fluid chromatography coupled to a triple quadrupole mass spectrometer.[3]

In this work, a sample cleanup step is necessary prior to analysis, as the matrix at hand is urine. Based on numerous searches in the literature, SPE or LLE are the most appropriate cleanup methods for this type of analysis.[1-3] Therefore, a LLE method was developed to remove salts, lipids and non-volatile materials from biological samples and to separate a substance selectively from a mixture.

2.2 *Materials and methods*

2.2.1 *Reagents and supplies*

JWH-018 N-5-COOH, JWH-073 N-5-COOH, JWH-073 N-4-COOH-D_5, and MAM-2201 N-4-OH, methylone, MDPV, MDPV-D_8 and mephedrone were purchased from Cayman Chemical (Ann Arbor, MI, USA). Certified synthetic urine, HPLC grade methanol, HPLC grade acetonitrile, HPLC grade water, potassium hydroxide, concentrated hydrochloric

acid, glacial acetic acid, 1-chlorobutane, and formic acid (99.0+% Optima LC-MS grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Disodium phosphate, and monosodium phosphate were purchased from Sigma-Aldrich (Milwaukee, WI, USA). 15 mL polypropylene centrifuge tubes with conical bottoms were purchased from VWR (Atlanta, GA, USA).

2.3 *Procedure*

2.3.1 *Standards*

Working Solution I (WSI) for cathinones was prepared at 0.01 mg/mL in methanol by adding 500 µL of 1 mg/mL of each designer cathinone analyte to a 50 mL volumetric flask and bringing to volume with methanol. Working Solution II (WSII) for cathinones was prepared at 0.001 mg/mL in methanol by adding 1 mL of WSI to a 10 mL volumetric flask and bringing to volume with methanol. WSII for cannabinoids was prepared at 0.001 mg/mL in methanol by adding 500 µL of 0.1 mg/mL of each synthetic cannabinoid analyte to a 50 mL volumetric flask and bringing to volume with methanol. Working Solution III (WSIII) for cannabinoids was prepared at 0.0001 mg/mL in methanol by adding 1 mL of WSII (cannabinoids) to a 10 mL volumetric flask and bringing to volume with methanol. Working Solution IV (WSIV) for cannabinoids was prepared at 0.00001 mg/mL in methanol by adding 1 mL of WSIII (cannabinoids) to a 10 mL volumetric flask and bringing to volume with methanol. All working solutions were stored frozen at < 10 °C. Internal standard solution for cathinones and cannabinoids were prepared separately at 0.0001 mg/mL in methanol by adding 10 µL of 0.1 mg/mL of MDPV-D_8 or JWH-073 N-4-COOH-D_5 to a 10 mL volumetric flask and bringing to volume with methanol. Both internal standard solutions were stored frozen at < 10 °C.

2.3.2 *Calibrators*

Six calibrators were prepared in 15 mL polypropylene tubes using synthetic urine. See Table 2.1 for the preparation of each sample.

Table 2.1 Calibrators prepared using cathinone and cannabinoid working solutions and synthetic urine.

Sample	Cannabinoid Working Solution	Amount (uL)	Cannabinoid Concentration (ng/mL)	Cathinone Working Solution	Amount (uL)	Cathinone Concentration (ng/mL)
Calibrator 1	IV	25	0.025	II	50	5
Calibrator 2	IV	50	0.05	II	100	10
Calibrator 3	IV	100	0.1	I	25	25
Calibrator 4	III	50	0.5	I	50	50
Calibrator 5	III	100	1	I	100	100
Calibrator 6	II	25	2.5	I	200	200

2.3.3 *LLE procedure*

A negative control was prepared by adding 200 µL of synthetic urine to a clean polypropylene tube. 50 µL of the cannabinoid and cathinone internal standard solutions were added to all six calibrators in the polypropylene tubes. Next, 1 mL of 0.5 M phosphate buffer (pH = 6.8, +/- 0.1) was added to each tube. 150 µL of concentrated HCl was added to all samples and then vortexed for 10 seconds. 3 mL of chlorobutane were added to each sample and then vortexed for 30 seconds. All samples were centrifuged at 3500 rotations

per minute (rpm) for 5 minutes and then the top, organic layer was transferred to a clean, polypropylene tube. The samples were evaporated at 40 °C under a steady stream of nitrogen (N_2) gas until dry (approximately 20 minutes). Next, 150 µL of potassium hydroxide (KOH) were added, followed by 3 mL of chlorobutane and then all samples were vortexed for 30 seconds. Each tube was centrifuged for 5 minutes at 3500 rpm and then the top organic layer was transferred to a clean, polypropylene tube. Next, 25 µL of 10% HCl were added before the samples were evaporated at 40 °C under a gentle stream of N_2 gas until dry. The samples were reconstituted in 50 µL of mobile phase (0.1% formic acid in water:0.1% formic acid in acetonitrile, 50:50, (v/v)), vortexed for 10 – 15 seconds and transferred to autosampler vials that contained inserts.

2.4 *Results and discussion*

The metabolites of a group of cannabinoids and the parent compounds for a group of cathinones were used for the initial method development, as the analyses were carried out using synthetic urine and ultimately what was available in the laboratory. The cannabinoids included JWH-018 N-5-COOH, JWH-073 N-5-COOH, and MAM-2201 N-4-OH, and the cathinones included methylone, MDPV, and mephedrone. Six calibrators were prepared in synthetic urine to bracket cutoff concentrations for marijuana derivatives and amphetamine derivatives.[4] The calibrators were analyzed using an LC-QqQ-MS system that was able to confirm the presence of both the cannabinoids and cathinones in each sample, except for the negative control.

See Tables 2.2 and 2.3 for details regarding the structure, molecular formula and molecular weight for each cannabinoid and cathinone, respectively.

Table 2.2 Structure, molecular formula, and molecular weight of synthetic cannabinoids.[5-8]

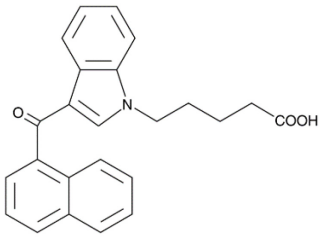
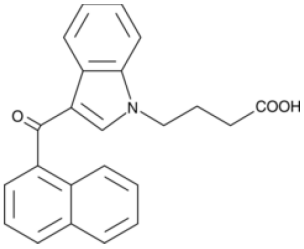
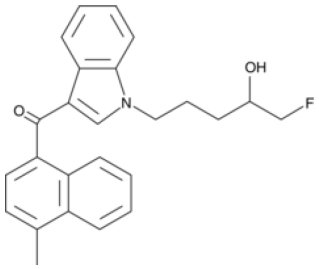
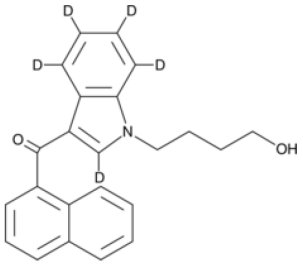
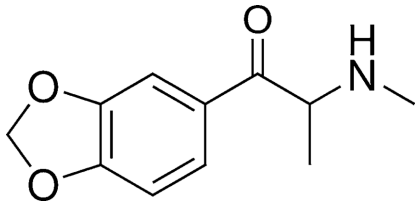
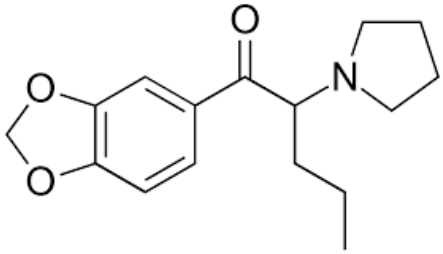
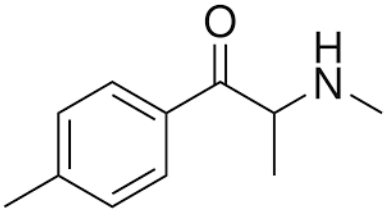
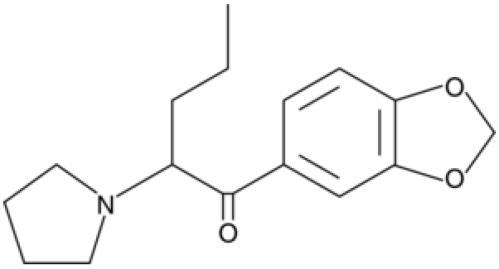
Cannabinoids			
Analyte	Structure	Molecular Formula	Molecular Weight (g/mol)
JWH-018 N-5-COOH		$C_{24}H_{21}NO_3$	371.4
JWH-073 N-4-COOH		$C_{23}H_{19}NO_3$	357.4
MAM-2201 N-4-OH		$C_{25}H_{24}FNO_2$	389.5
JWH-073 N-4-COOH-D_5		$C_{23}H_{16}D_5NO_2$	348.5

Table 2.3 Structure, molecular formula, and molecular weight of designer cathinones.[9-12]

Cathinones			
Analyte	Structure	Molecular Formula	Molecular Weight (g/mol)
Methylone		$C_{11}H_{13}NO_3$	207.2
MDPV		$C_{16}H_{21}NO_3$	275.3
Mephedrone		$C_{11}H_{15}NO$	177.2
MDPV-D_8		$C_{16}H_{21}D_8NO_3$	283.3

2.5 *Conclusions*

Using LLE, the urine sample was cleaned up to reduce matrix effects and the cannabinoids and cathinones were present in the same sample. The different drug groups were forced into the organic phase at different pH's since the synthetic cannabinoids are acidic compounds and the cathinones are basic. By adding concentrated HCl to the aqueous phase before the extraction solvent was added, the acidic conditions caused the synthetic cannabinoids to move into the organic phase. Conversely, adding concentrated potassium hydroxide created a basic pH, causing the cathinones to move into the organic phase with the cannabinoids. This was advantageous because a single method of analysis could now be developed to analyze for and quantify both cannabinoids and cathinones in the same sample.

The sample cleanup step was successful, but time-consuming overall. In a forensic laboratory setting, this would hinder the throughput of samples and cost more money to keep the LLE reagents in stock. The next steps of the project involved eliminating the sample cleanup step and instead focusing on a different approach for analyzing both groups of drugs in a single sample using one method.

2.6 *References*

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6. JWH 073 N-Butanoic Acid metabolite (cas 1307803-52-6). (n.d.). Retrieved April 19, 2021, from https://www.caymanchem.com/product/9000866/jwh-073-n-butanoic-acid-metabolite

7. MAM2201 N-(4-Hydroxypentyl) Metabolite (cas 1537889-05-6). (n.d.). Retrieved April 19, 2021, from https://www.caymanchem.com/product/11778/mam2201-n-(4-hydroxypentyl)-metabolite

8. JWH 073 N-(4-Hydroxybutyl) Metabolite-d5. (n.d.). Retrieved April 19, 2021, from https://www.caymanchem.com/product/10934/jwh-073-n-(4-hydroxybutyl)-metabolite-d5

9. Methylone (Hydrochloride) (cas 186028-80-8). (n.d.). Retrieved April 19, 2021, from https://www.caymanchem.com/product/10986/methylone-(hydrochloride)

10. 3,4-Methylenedioxy Pyrovalerone (CAS 687603-66-3). (n.d.). Retrieved April 19, 2021, from https://www.caymanchem.com/product/10624/3%2C4-methylenedioxy-pyrovalerone

11. Mephedrone (Hydrochloride) (cas 1189726-22-4). (n.d.). Retrieved April 19, 2021, from https://www.caymanchem.com/product/10801/mephedrone-(hydrochloride)

12. 3,4-Methylenedioxy Pyrovalerone-D8 (hydrochloride) (CAS 1246820-09-6). (n.d.). Retrieved April 19, 2021, from https://www.caymanchem.com/product/10679/3%2C4-methylenedioxy-pyrovalerone-d8-(hydrochloride)

3. SOURCE OPTIMIZATION AND METHOD DEVELOPMENT FOR THE SECOND DIMENSION OF 2D-LC-QQQ-MS

3.1 *Introduction*

The objective of this project was to develop and optimize a 2D-LC-QqQ-MS method capable of effectively separating and quantitating designer drugs in a single urine sample, using one method of analysis. A LLE method was developed in Chapter 2 to have both the cannabinoids and cathinones present in a single urine sample for analysis, but it was quite laborious and time-consuming. The next stage of this project is to avoid a sample cleanup procedure and instead use a more robust column for the first dimension of the 2D-LC-QqQ-MS method that can handle the salts and other impurities that would come from the urine. This chapter does not focus on the method optimization of the first dimension, but instead efforts are made to compare APCI and ESI sources for optimization of the signal intensities of the cathinones and cannabinoids in the second dimension.

The first LC column will be a polar Hydrophilic Interaction Liquid Chromatography (HILIC) column. Ideally, it will be robust enough to handle the salts and other impurities from a urine sample and will eliminate the need for pre-treatment/sample preparation. A HILIC column is ideal for the chromatography of very polar compounds that are challenging to retain in Reverse Phase Liquid Chromatography (RPLC).[1] A water layer forms on the surface of the stationary phase when mobile phase passes through, allowing for the partitioning of polar compounds into it.[1] The nonpolar cannabinoids should elute first, while the polar cathinones will elute last. A gradient elution will be developed and optimized to sufficiently separate the cathinones from the cannabinoids, with minimal emphasis on baseline separation of the peaks within the groups.

It is important to consider solvent compatibility between the two columns used for 2D-LC. HILIC solvent strengths: Acetonitrile < Methanol < Water. RPLC solvent strengths: Water < Methanol < Acetonitrile. A high acetonitrile concentration in the HILIC fractions creates a decreased retention of analytes in RPLC, causing band broadening. There are a few solutions to the solvent compatibility problem, including transferring a small volume of the fraction to the second column, diluting the fraction with a weak eluent (in this case water), or trapping the solvent and removing the solvent (by evaporation). Using methanol and water, instead of acetonitrile and water for the mobile phase could also help to dampen the effect of a strong eluent being transferred to the second column.

The second column needs to have a completely different separation mechanism than the first column. Optimum occupancy of the two-dimensional space occurs when the separation mechanisms of the two dimensions have distinct retention profiles.[2] The higher the orthogonality, the closer the system gets to theoretical peak capacity. Therefore, a system with high orthogonality that meets the needs of the experiment is HILIC x RPLC.[2]

3.2 *Materials and methods*

3.2.1 *Reagents and supplies*

JWH-018 N-5-COOH, JWH-073 N-5-COOH, JWH-073 N-4-COOH-D_5, and MAM-2201 N-4-OH, methylone, MDPV, MDPV-D_8 and mephedrone were purchased from Cayman Chemical (Ann Arbor, MI, USA). Certified synthetic urine, HPLC grade acetonitrile, HPLC grade water, and formic acid (99.0+% Optima LC-MS grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). A ZORBAX Eclipse Plus C18 Rapid Resolution High Throughput column with the dimensions 2.1 mm internal diameter

(ID) x 50 mm length, 1.8 µm particle size was purchased from Agilent Technologies (Santa Clara, CA, USA).

3.2.2 *Instrumentation*

3.2.2.1 *ESI-QqQ-MS*

An Agilent 6460 Triple Quadrupole (Santa Clara, CA, USA) Mass Spectrometer with an orthogonal Agilent Jet Stream (AJS) ESI source, equipped with Mass Hunter, Qualitative Analysis and Source Optimizer software was optimized for the analysis of designer cathinones. All analyses were completed in positive ionization mode with specific MRM transitions and collision energies (CE) selected for the quantifier and qualifier ions of the cathinones: mephedrone m/z 178 -> 160, 145 (CE 14, 18 V); methylone m/z 208 -> 160, 132 (CE 16, 28 V); MDPV m/z 276 -> 126, 135 (CE 30, 26 V); and MDPV-D_8 m/z 284 -> 134, 175 (CE 28, 24 V).[3-5] The optimized source conditions were generated from the Source Optimizer software and manually evaluated to yield: gas temperature 230 °C; gas flow 10 L/min; nebulizer 35 psi; sheath gas temperature 300 °C; sheath gas flow 11 L/min; capillary voltage 2500 V; nozzle voltage 2000 V.

3.2.2.2 *APCI-QqQ-MS*

An Agilent 6460 Triple Quadrupole (Santa Clara, CA, USA) Mass Spectrometer with an APCI source, equipped with Mass Hunter and Qualitative Analysis software was optimized for the analysis of synthetic cannabinoids. All analyses were completed in positive ionization mode with specific MRM transitions and CE's selected for the quantifier and qualifier ions of the cannabinoids: JWH-018 N-5-COOH m/z 372 -> 155, 127 (CE 28, 52 V); JWH-073 N-4-COOH m/z 358 -> 155, 127 (CE 28, 52 V); JWH-073 N-4-COOH-D_5 m/z 363 -> 127, 155 (CE 52, 28 V) and MAM-2201 N-4-OH m/z 390 ->

169, 141 (CE 28, 32 V).[3-5] The optimized source conditions were manually determined as: gas temperature 325 °C; gas flow 4 L/min; nebulizer 20 psi; capillary voltage 4500 V; corona current 4 µA.

3.2.2.3 *Multimode-QqQ-MS*

An Agilent 6460 Triple Quadrupole (Santa Clara, CA, USA) Mass Spectrometer with a multimode ionization source, equipped with Mass Hunter and Qualitative Analysis software was optimized for the analysis of designer cathinones and synthetic cannabinoids. All analyses were completed in positive ionization mode with the specified MRM transitions and CE's selected for the quantifier and qualifier ions of the cathinones and cannabinoids, as well as the source parameters defined in Section's 3.2.2.1 and 3.2.2.2.

3.3 *Results and Discussion*

Initial method development was carried out using consumables that were readily available in the laboratory. Combined standards were made from 0.025 ng/mL – 1 ng/mL in synthetic urine, containing all of the cannabinoids and cathinones described in Tables 2.2 and 2.3. Each sample was loaded into the Agilent 6460 LC-QqQ-MS auto sampler tray from least to most concentrated. The chromatographic separation was performed using a C18 column described in Section 3.2.1, which was maintained at 50 °C, with an injection volume of 5 µL. As shown in Table 3.1, a gradient elution was developed using 0.1% formic acid in water (Mobile Phase A) and 0.1% formic acid in acetonitrile (Mobile Phase B) for a total run-time of 5.1 minutes. The source parameters can be seen in Section 3.2.2.1 and the MRM transitions are shown in Section's 3.2.2.1 and 3.2.2.2.

Table 3.1 Gradient elution timetable for cannabinoids and cathinones using LC-QqQ-MS equipped with an AJS ESI source.

Time (min)	Flow Rate (mL/min)	A (%)	B (%)
0.00	0.35	90	10
1.50	0.35	80	20
2.00	0.35	45	55
3.50	0.35	35	65
3.75	0.35	35	65
4.25	0.35	25	75
4.35	0.35	90	10
5.00	0.35	90	10
5.10	0.35	10	90

Originally, ESI was the source chosen for this project, as it is suitable for polar compounds and is the leading method of choice for LC-MS coupling. However, the internal standards were more preferentially ionized than the natural compound, causing a smaller signal to be produced for the natural compound versus a larger signal for the internal standard. An alternative source for this project was APCI, as it accomplishes the same task of ionizing molecules. In contrast to ESI, where ionization occurs in the liquid phase, APCI has ionization happening in the gas phase through chemical reactions. Owing to a different ionization mechanism, APCI should not be susceptible to the same ion suppression as ESI.[6] Although APCI methods can typically be less sensitive than ESI methods, APCI methods were also examined as they produce fewer matrix effects. In a study by Grauwiler et al., a tandem LC-MS method was developed for the analysis of cannabinoids in human ethylenediaminetetraacetic acid (EDTA)-plasma and urine after small doses of Cannabis sativa extracts were given and the limits of detection and limits of quantification were found to be acceptable even with APCI methods.[7] The source parameters were optimized for ESI using Agilent's Source Optimizer software, and then

manual analysis of the data. The source parameters for APCI were manually optimized based on the signal intensity for each cannabinoid. When the run was completed, the source was changed to APCI in positive mode and the same samples were analyzed using the parameters specified in Section 3.2.2.2.

To begin data analysis, chromatograms with the MRM transitions from Section's 3.2.2.1 and 3.2.2.2 were extracted and integrated from the total ion chromatogram of each sample. Calibration curves for cannabinoids and cathinones, using ESI and APCI, were constructed by plotting the corrected peak area of each fragment (peak area/internal standard peak area) versus the concentration (Figures 3.1 – 3.4).

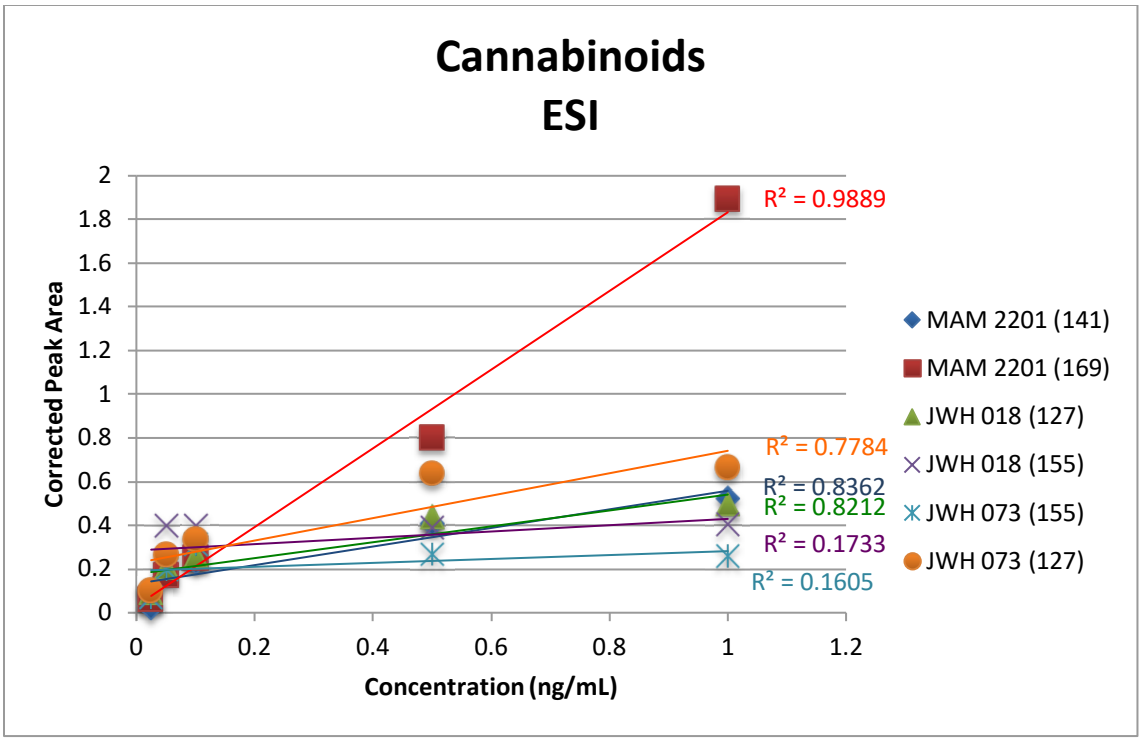

Figure 3.1 Calibration curves with internal standards for cannabinoids using LC-ESI-QqQ-MS.

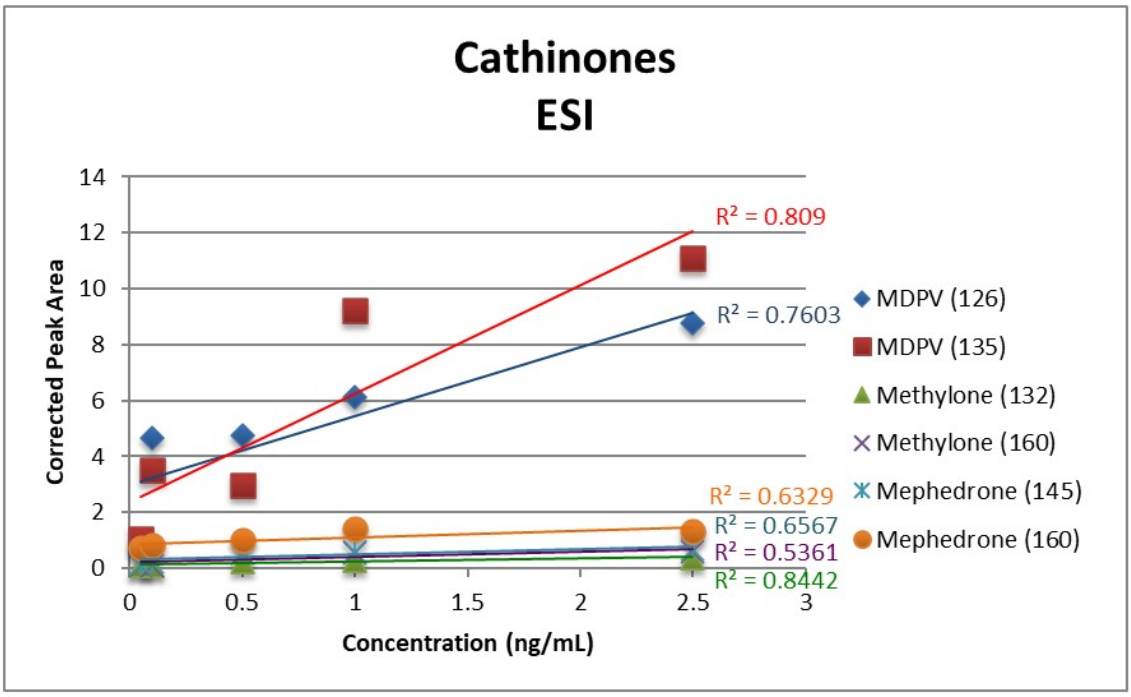

Figure 3.2 Calibration curves with internal standards for cathinones using LC-ESI-QqQ-MS.

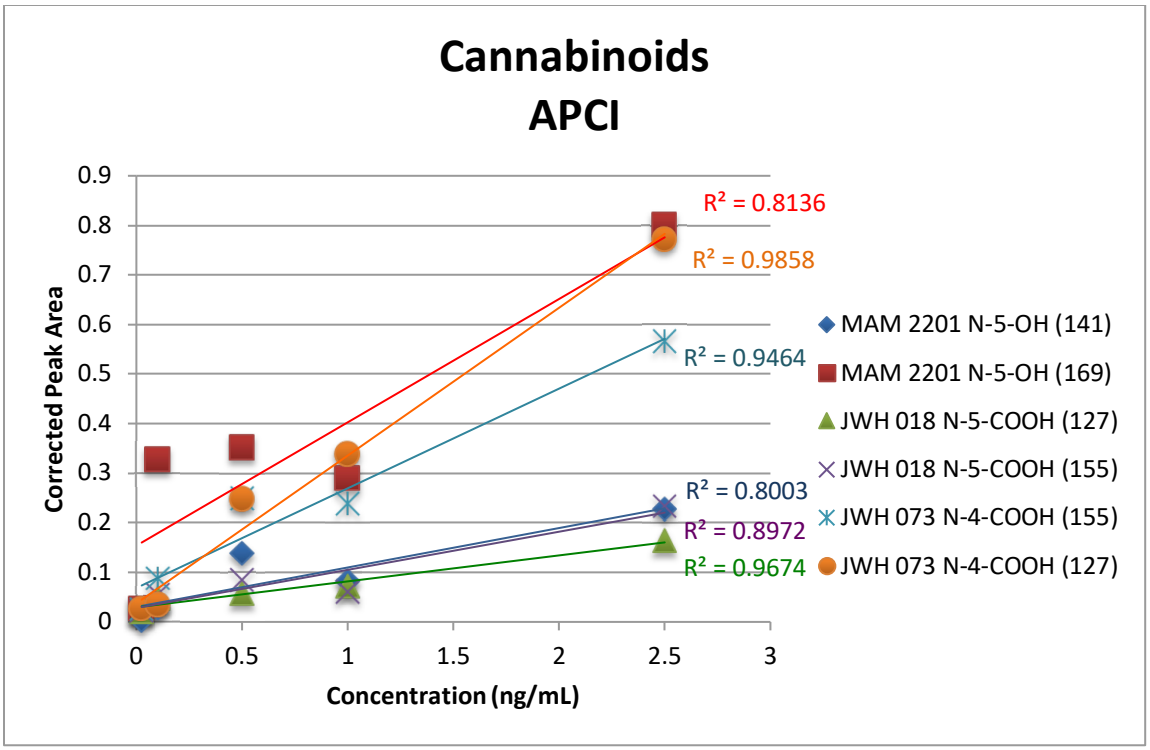

Figure 3.3 Calibration curves with internal standards for cannabinoids using LC-APCI-QqQ-MS.

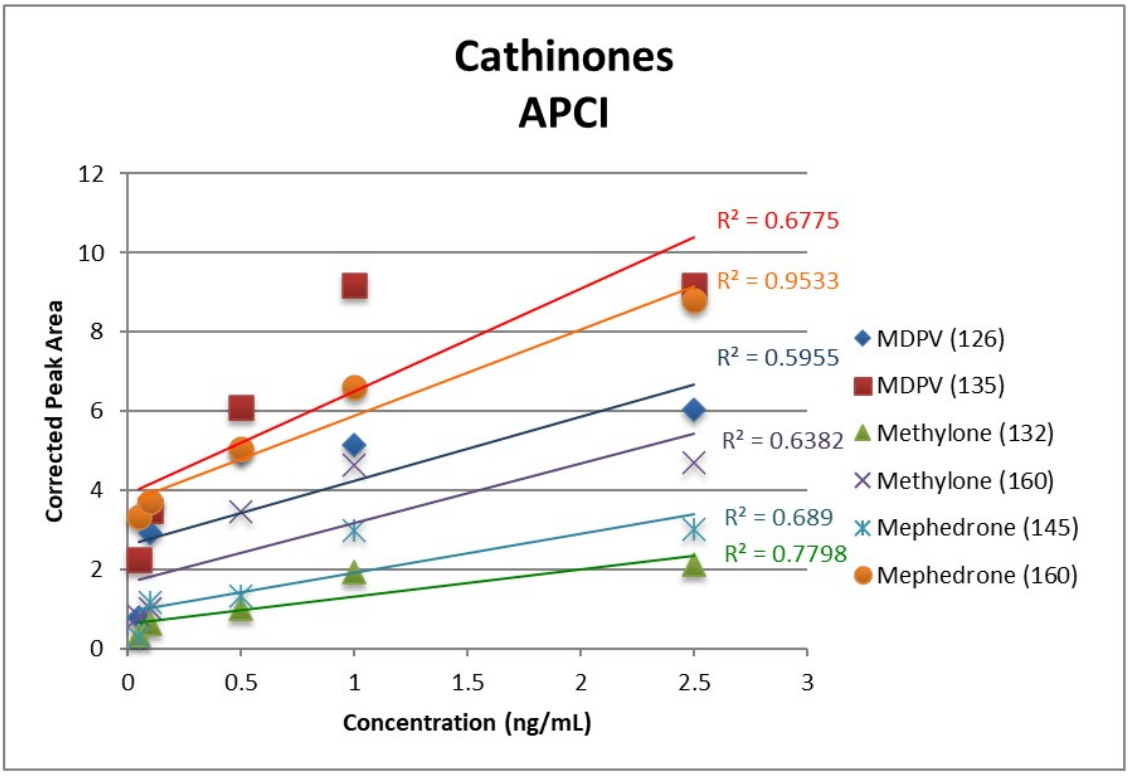

Figure 3.4 Calibration curves with internal standards for cathinones using LC-APCI-QqQ-MS.

The R^2 values for cannabinoids using ESI ranged from 0.1605 - 0.98891, indicating that the sensitivity was overall poor, and the values were not linear. Uncertainty values for the corrected peak areas of MAM-2201 (141, 169 m/z), JWH-018 (127, 155 m/z), and JWH-073 (155, 127 m/z) were 0.0911, 0.0923, 0.0821, 0.1525, 0.0992, and 0.1326, respectively. The R^2 values for cathinones using ESI ranged from 0.53605 - 0.84421, again indicating that the sensitivity was generally poor, and the values were not linear. Uncertainty values for the corrected peak areas of MDPV (126, 135 m/z), methylone (132, 160 m/z), and mephedrone (145, 160 m/z) were 1.611, 2.192, 0.0554, 0.1911, 0.1590, and 0.2171, respectively.

The R^2 values for cannabinoids using APCI ranged from 0.80028 - 0.9858, indicating that the sensitivity was improved from ESI and the values were mostly linear. Uncertainty values for the corrected peak areas of MAM-2201 (141, 169 m/z), JWH-018 (127, 155 m/z), and JWH-073 (155, 127 m/z) were 0.0465, 0.1394, 0.0113, 0.0305, 0.0560, and 0.0419, respectively. The R^2 values for cathinones using APCI ranged from 0.59552 - 0.95328, again indicating that the sensitivity was improved from ESI and the values were more linear. Uncertainty values for the corrected peak areas of MDPV (126, 135 m/z), methylone (132, 160 m/z), and mephedrone (145, 160 m/z) were 1.551, 2.087, 0.4253, 1.320, 0.7761, and 0.5613, respectively.

While calibration curves for cannabinoids and cathinones using ESI and APCI were constructed and compared, quantification was only reliable for JWH-073 (cannabinoids) and MDPV (cathinones), as only their corresponding internal standards were used. R^2 values for JWH-073 using ESI and APCI were 0.1605, 0.7784, and 0.9464, 0.9858, respectively. R^2 values for MDPV using ESI and APCI were 0.7603, 0.809, and 0.5955, 0.6775, respectively. Uncertainty values were comparable across sources for JWH-073 and MDPV. APCI Cannabinoids (JWH-073) had much higher R^2 values than ESI Cannabinoids, while ESI Cathinones (MDPV) had higher R^2 values than APCI Cathinones. Chromatograms of cannabinoids and cathinones using ESI and APCI are shown in Figures 3.5 and 3.6.

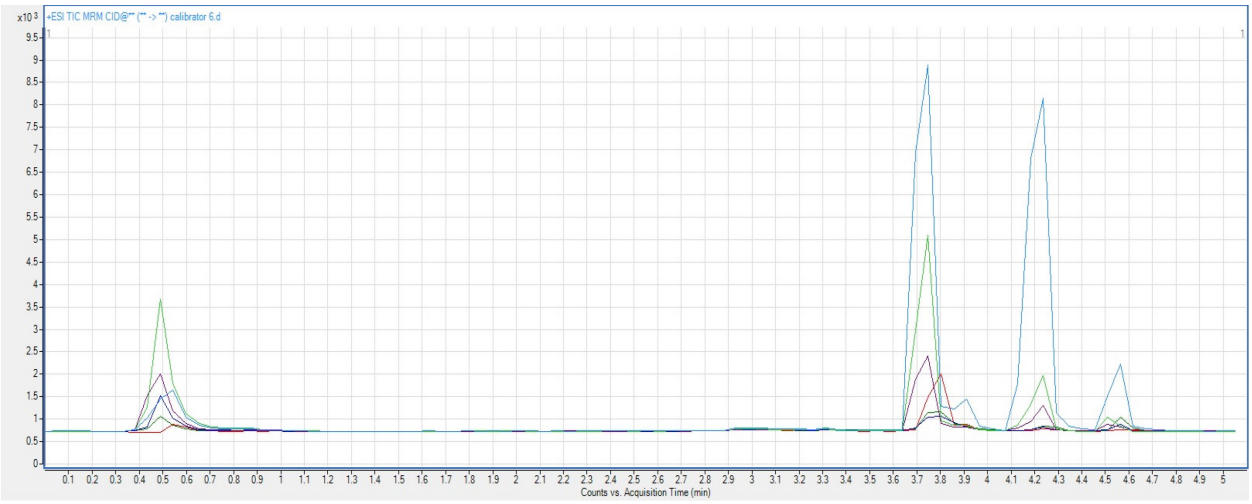

Figure 3.5 Chromatogram of cannabinoids and cathinones using ESI.

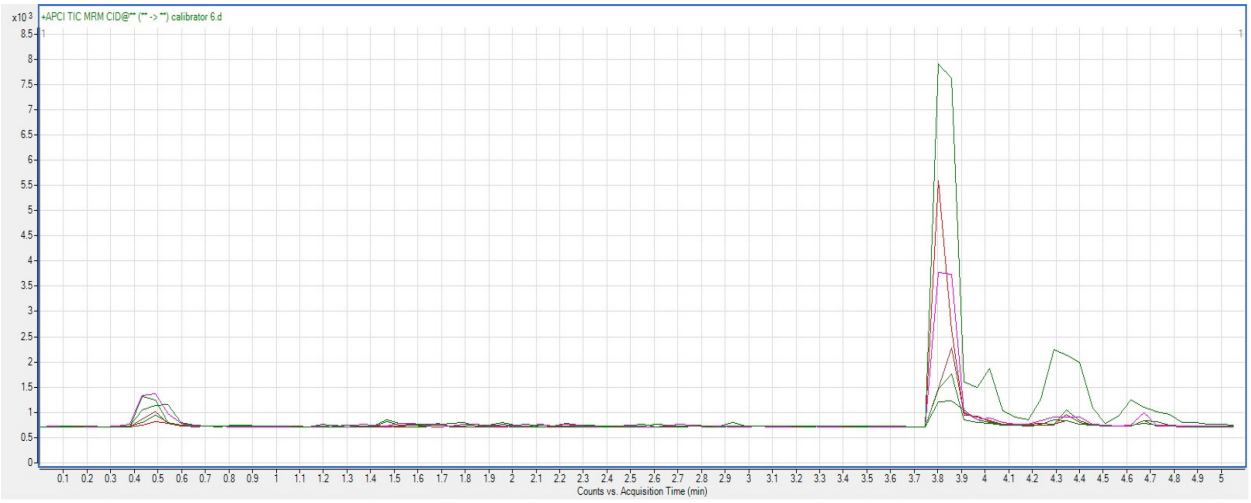

Figure 3.6 Chromatogram of cannabinoids and cathinones using APCI.

3.3.1 *Instrument method optimization*

To compromise for differences in signal intensity between the two groups and two sources, a Multimode source was used. As seen in Figure 3.7, the Multimode source incorporates both ESI and APCI into a single ion source.[8] It can simultaneously generate ions by both ESI and APCI, eliminating the need and time to run samples twice and switch ion sources on the instrument.[8]

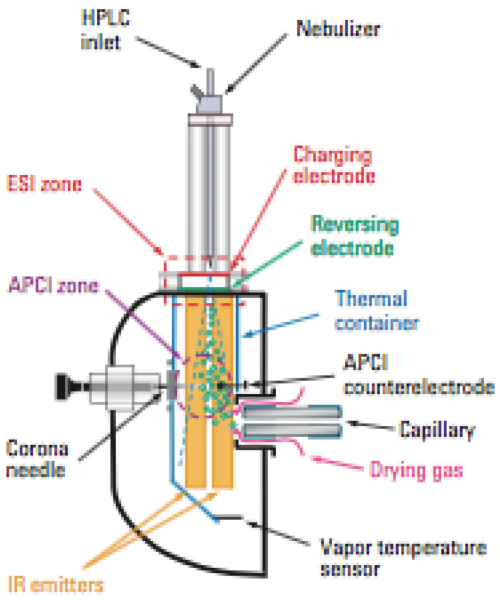

Figure 3.7 Overview of the Agilent Technologies Multimode Source.[8]

A new gradient elution, seen in Table 3.2, was developed to increase retention of the cathinones and to extend the final hold for proper re-equilibration of the column before the next injection, but was not completely optimized for the separation of designer drugs using the column described in Section 3.2.1.

Table 3.2 Gradient elution developed to separate cannabinoids and cathinones using LC-QqQ-MS.

Gradient Elution			
Time (min)	**A (%)**	**B (%)**	**Flow (mL/min)**
0.00	90	10	0.30
0.40	90	10	0.30
1.50	80	20	0.30
2.00	45	55	0.30
3.50	35	65	0.30
3.75	35	65	0.30
4.25	25	75	0.30
4.35	90	10	0.30
7.00	90	10	0.30

The column was heated to 50 °C, with a 5 uL injection volume, utilizing 0.1% formic acid in water, and 0.1% formic acid in methanol as mobile phases A and B, respectively.

Using the gradient elution developed in Table 3.2, and the multimode source on the front end of the QqQ-MS, the chromatogram in Figure 3.8 was produced.

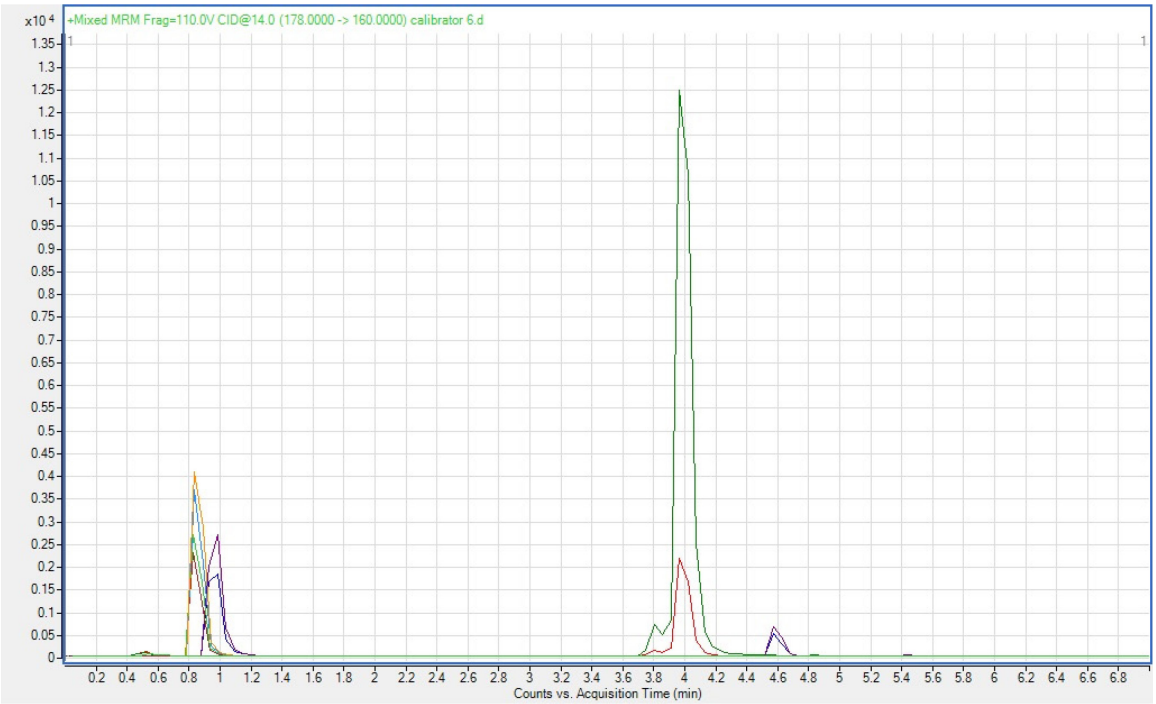

Figure 3.8 Chromatogram of cannabinoids and cathinones using the multimode source.

3.4 *Conclusions*

It can be seen that the signal intensity for both groups of drugs has greatly improved from using ESI or APCI alone. The cathinones eluted between 0.5 - 1 minute, and the cannabinoids eluted between 3.8 - 4.8 minutes. While the cathinones and cannabinoids were successfully separated, the compounds within the two groups were not efficiently separated. A new core-shell column will advance this stage of the project, as the gradient and resolution can be improved upon without back pressure issues.

The resolution between two peaks within the groups was 0.61, indicating the components were co-eluting and the peaks were not baseline separated.[9] In addition, the number of theoretical plates for the column was only 460, demonstrating a low column efficiency producing broader peaks at a given retention time.[9] It is important to note that the column had been damaged, and the standards used were stored improperly over the course of a year. Due to the high back pressures (in excess of 500 bar), it is believed that particles from the mobile phase or sample passed through the inlet frit and clogged the packing bed, or initially accumulated on the frit creating a blockage.[9]

In conclusion, the multimode source was more sensitive to both cannabinoids and cathinones, however more work needs to be done on the chromatography portion of the method. The resolution and peak shape of each component was not optimal, and there appeared to be contamination in the samples. In addition, new standards and the correct internal standards needed to be purchased and used for cathinones (methylone-D_3, mephedrone-D_3, MDPV-D_8) and cannabinoids, as available. The standards used in this project were beyond expiration dates and had not been stored properly. Considering this, the quantitation results should be more reliable when the experiment is repeated.

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4. TWO-DIMENSIONAL LIQUID CHROMATOGRAPHY METHOD DEVELOPMENT

4.1 *Introduction*

With one-dimensional (1D) LC, complex samples may result in un-resolvable, overlapping peaks, as was seen in the results from Chapter 3.[1-3] In 1967, J. Calvin Giddings introduced peak capacity as an alternative measurement of performance to plate count when looking at the maximum number of components that were resolvable by gel filtration chromatography under isocratic conditions.[4] Peak capacity has now been widely accepted as a performance measurement and describes the maximum number of resolvable peaks in a given elution timeframe.[4] The peak capacity must appreciably exceed the number of components in the sample to avoid compromises in resolution.[5] An additional drawback of 1D-LC occurs when compounds that coelute and cannot be differentiated, offering only limited information on affected samples.[1] In 2D-LC, the first dimension offers conventional separation.[2] The eluent from the first separation is then applied to a second-dimension column with separation selectively different than the first column.[2] Consequently, 2D-LC improves the ability to resolve closely related peaks greatly.[1-3] Compounds that coelute in the first dimension can also be separated out in the second dimension.[3]

Utilizing a 2D-LC approach increases the resolving power and peak capacity of the system with a multiplicative effect.[1-3] The first dimension utilizes a polar HILIC column that is composed of silica particles with cross-linked diol groups. A water layer forms on the surface of the stationary phase when mobile phase passes through, allowing for the partitioning of polar compounds into it. The HILIC column is robust enough to handle

removing salts and other impurities from the sample, ultimately eliminating the need for a sample pretreatment and extraction step.[6] The optimal occupancy of the 2D space occurs when the separation mechanisms of both dimensions have distinct retention profiles.[7] Additionally, the higher the orthogonality, the closer the system is able to reach a theoretical peak capacity.[7] Various phases were evaluated, including C18, phenyl and biphenyl. C18 stationary phases contain 18 carbons in a chain and offers one of the simplest hydrophobic interaction with analytes.[7,8] Phenyl stationary phases undergo π-π interactions with analytes and can generate alternate selectivity to an alkyl phase column.[7,8] Biphenyl stationary phases incorporate an aryl linker, making the phase both more hydrophobic than conventional phenyls and providing a larger electron cloud than single phenyl ring phases.[7,8] The second column was specifically chosen and utilizes a "core-shell" technology that reduces backpressure and band broadening, while increasing mass transport and the effective separation of analytes.[7] "Core-shell" refers to a solid silica core that has a porous shell around it that is capable of reducing Eddy Diffusion, versus a traditional completely porous particle.[7,9] The advantageousness of this technology can be explained using the Van Deemter Equation, as seen in Figure 4.1, which describes the three sources of band broadening.

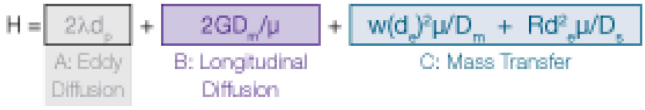

Figure 4.1 The Van Deemter equation.[9]

As seen in Figure 4.2, 2D-LC can be carried out in a comprehensive manner, or by utilizing a "heart-cutting" technique to increase the separation power for complex samples,

to ascertain complementary information, or to make the first dimension of separation compatible with the detection mechanism.[10]

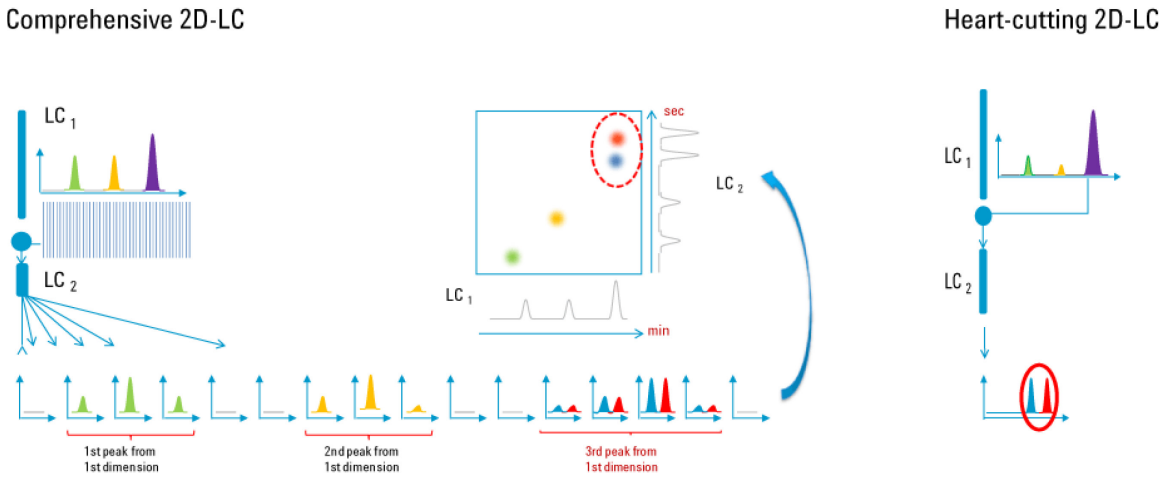

Figure 4.2 The two major types of operation of two-dimensional LC includes comprehensive and heart-cutting techniques.[11]

In comprehensive 2D-LC, all of the effluent is collected from the first dimension and then transferred to the second dimension via manual collection and injection, or by moving into available loops in a multiple-port switching valve before being transferred to the second column.[10] Heart-cutting only collects select peaks of interest from the first dimension and subjects them to the second dimension.[10] This can be advantageous when time is constrained, as the transfer and subsequent separation on the second column is not time dependent on the entire separation from the first dimension.[10]

4.2 *Materials and methods*

4.2.1 *Reagents and supplies*

New synthetic cannabinoid standards were purchased from Cayman Chemical (Ann Arbor, MI, USA), used for the remainder of the project and include: ± JWH 018 N-(4-hydroxypentyl) metabolite, ± JWH 018 N-(4-hydroxypentyl) metabolite-D_5, JWH 073 N-(4-hydroxybutyl) metabolite, JWH 073 N-(4-hydroxybutyl) metabolite-D_5, AM2201 N–(4-hydroxypentyl) metabolite, AM2201 N-(4-hydroxypentyl) metabolite-D_5. New designer cathinone standards were purchased from Cerilliant Corporation (Round Rock, TX, USA), used for the remainder of the project and include: ±-4-methylephedrine HCl (mephedrone metabolite), ±-4-methylephedrine-D_3 HCl (mephedrone metabolite), methylone HCl, methylone-D_3 HCl, 3,4-methylenedioxy pyrovalerone, and 3,4-methylenedioxy pyrovalerone-D_8 (hydrochloride). A Luna® HILIC column with a 150 mm length x 4.6 mm ID and 5 µm particle size and a Kinetex C18 column with a 2.1 mm ID x 50 mm length and 1.8 µm particle size were purchased from Phenomenex (Torrance, CA, USA). Ammonium formate, HPLC grade water, HPLC grade acetonitrile, certified synthetic urine, and formic acid (99.0+% Optima LC-MS grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA).

4.2.2 *Instrumentation*

4.2.2.1 *2D-LC-QqQ-MS coupled with a multimode source*

An Agilent 6460 Triple Quadrupole (Santa Clara, CA, USA) Mass Spectrometer with a multimode ionization source, equipped with Mass Hunter and Qualitative Analysis software was optimized for the analysis of designer cathinone and synthetic cannabinoid metabolites in synthetic urine. All analyses were completed in positive ionization mode

with the source parameters specified in Section's 3.2.2.1 and 3.2.2.2. The optimized MRM transitions and CE's selected for the quantifier and qualifier ions of the cathinones and cannabinoids, include: 4-Methylephedrine HCl (mephedrone metabolite) m/z 180.1 -> 162.0, 117.0 (CE 5, 6); 4-Methylephedrine-D_3 HCl (mephedrone metabolite) m/z 183.1 -> 162.0, 117.0 (CE 5, 6); Methylone HCl m/z 208.2 -> 160.0, 132.0 (CE 14, 26); Methylone-D_3 HCl m/z 211.2 -> 160.0, 132.0 (CE 14, 26); MDPV m/z 276.1 -> 126.1, 135.0 (CE 30, 26); MDPV-D_8 m/z 284.1 -> 126.1, 135.0 (CE 28, 24); JWH-018 N-(4-hydroxypentyl) metabolite m/z 358.2 -> 155.1, 127.2 (CE 30, 52); JWH-018 N-(4-hydroxypentyl) metabolite-D_5 m/z 363.1 -> 155.0, 127.0 (CE 30, 52); JWH-073 N-(4-hydroxybutyl) metabolite m/z 344.1 -> 155.0, 127.0 (CE 30, 52); JWH-073 N-(4-hydroxybutyl) metabolite-D_5 m/z 349.1 -> 155.0, 127.0 (CE 30, 52); AM2201 N-(4-hydroxypentyl) metabolite m/z 376.1 -> 155.1, 127.1 (CE 32, 55); and AM2201 N-(4-hydroxypentyl) metabolite-D_5 m/z 381.1 -> 155.1, 127.1 (CE 32, 55).

4.3 *Data and Results*

New standards were purchased, as described in Section 4.2.1, and used for the remainder of the project, as the previous standards were expired and not the appropriate urinary metabolites that would be detected in a drug screen. These standards include the major urinary metabolites and corresponding internal standards of the cannabinoids and cathinones analyzed previously.[12-22]

The first dimension of the 2D-LC setup included a HILIC column, as described in Section 4.2.1, and contains a silica stationary phase with cross-linked diol groups for greater polar selectivity.[23] HILIC column guidelines recommend not using 100% organic conditions, recognizing that the stationary phase is pH stable from 1.5 - 8, and buffering

the mobile phase to a pH that converts analytes to their ionic form.[24] The addition of ammonium formate into the mobile phase was explored for the HILIC separation, as it tends to produce more stable retention over repetitive injections, whereas formic acid may result in loss of retention over repetitive injections.[24] A buffer solution with a pH of approximately 3, consisting of 150 mM ammonium formate in 98:2 acetonitrile:water (v/v) was optimal for this analysis, as the pKa for each analyte needed to be considered for proper retention and separation in the first dimension. The pKa values for MDPV, methylone and mephedrone metabolites are 8.41, 7.74 and 7.41, respectively.[25] The predicted pKa values for the cannabinoids are around 2.9, near the pH of the mobile phase, so the acid groups of the cannabinoids should be 50% ionized and more polar as a result.[26] While this might impact the resolution of the cannabinoids from each other, it was deemed not as important as separating between the groups of cannabinoids and cathinones. A combined sample was prepared at 0.001 mg/mL in synthetic urine, with an 8 µL injection volume, and underwent an isocratic elution with 150 mM ammonium formate (AF) in 98:2 acetonitrile:water (v/v) at 1 mL/min for 6 minutes. The eluent was diverted to waste at 4.1 minutes to eliminate the synthetic urine components from reaching the mass spectrometer. By changing the pH of the mobile phase to ~3, the analytes were retained on the column, converted to their acidic-form, and were successfully separated from each other and from the cannabinoids, as can be seen in Figure 4.3.[25]

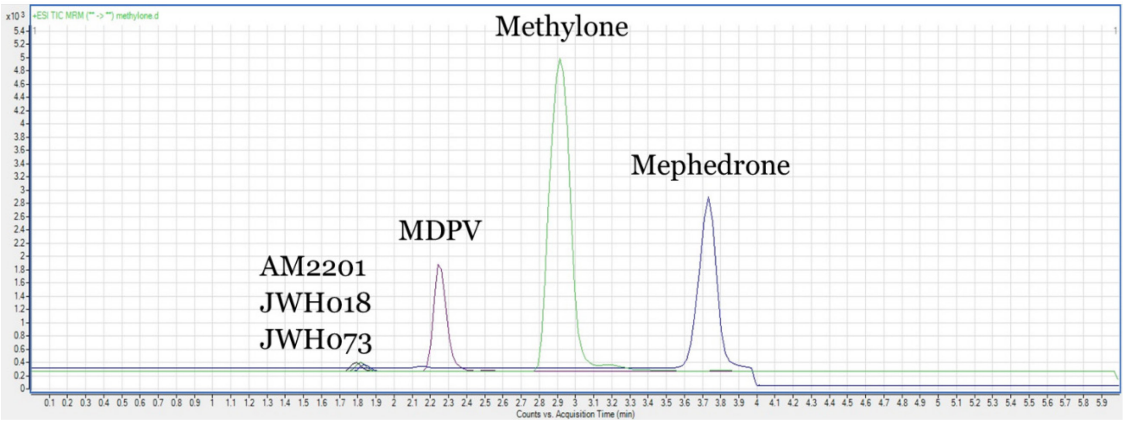

Figure 4.3 Optimized chromatography of cannabinoids and cathinones in synthetic urine for the first dimension of 2D-LC.

Maintaining a mobile phase linear velocity was very important to attempt to replicate the chromatography that was achieved from the first dimension to the second-dimension column specifications, as described in Section 4.2.1. Again, a combined standard was prepared in synthetic urine at 0.001 mg/mL and used to optimize the chromatography in the second dimension. The C18 column was heated to 50 °C with an 8 µL injection volume, utilizing 0.1% formic acid in water and 0.1% formic acid in acetonitrile, as mobile phases A and B, respectively. The optimized gradient conditions can be seen in Table 4.1, with the corresponding chromatogram seen in Figure 4.4. The optimized MRM transitions for each analyte, described in Section 4.2.2.1, can also be seen in Table 4.2.

Table 4.1 Optimized gradient elution conditions for the second dimension of 2D-LC.

Time (min)	A (%)	B (%)	Flow Rate (mL/min)
0	97	3	0.2
8	45	55	0.2
10	30	70	0.2
10.1	97	3	0.2
20	97	3	0.2

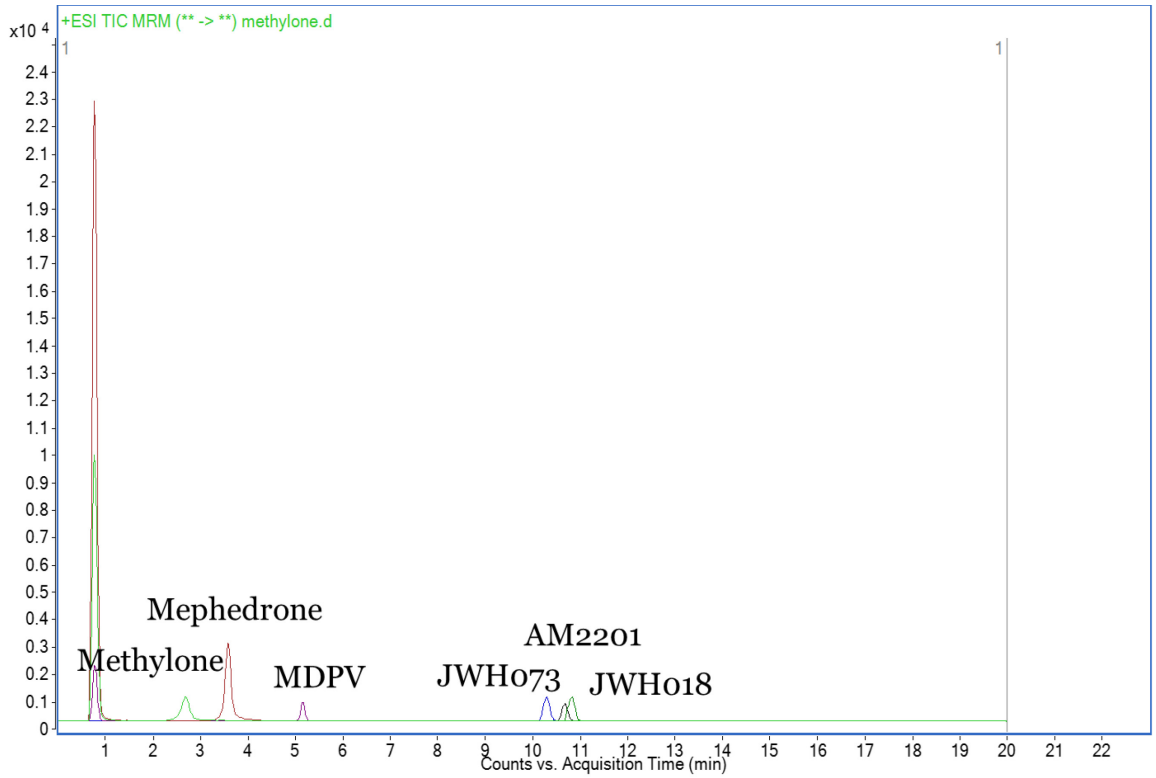

Figure 4.4 Optimized chromatography of cannabinoids and cathinones in synthetic urine for the second dimension of 2D-LC.

Table 4.2 Optimized MRM transitions for the quantifier and qualifier ions, fragmentor voltage and collision energies for each analyte.[27-29]

Analyte	Precursor Ion (m/z)	Quantifier Ion (m/z)	Qualifier Ion (m/z)	Fragmentor (V)	CE (V)
4-Methylephedrine HCl (mephedrone metabolite)	180.1	162.0	117.0	130	5, 6
4-Methylephedrine-D_3 HCl (mephedrone metabolite)	183.1	162.0	117.0	130	5, 6
Methylone HCl	208.2	160.0	132.0	130	14, 26
Methylone-D_3 HCl	211.2	160.0	132.0	130	14, 26
MDPV	276.1	126.1	135.0	130	30, 26
MDPV-D_8	284.1	126.1	135.0	130	28, 24
JWH-018 N-(4-hydroxypentyl) metabolite	358.2	155.1	127.2	130	30, 52
JWH-018 N-(4-hydroxypentyl) metabolite-D_5	363.1	155.0	127.0	130	30, 52
JWH-073 N-(4-hydroxybutyl) metabolite	344.1	155.0	127.0	130	30, 52
JWH-073 N-(4-hydroxybutyl) metabolite-D_5	349.1	155.0	127.0	130	30, 52
AM2201 N-(4-hydroxypentyl) metabolite	376.1	155.1	127.1	130	32, 55
AM2201 N-(4-hydroxypentyl) metabolite-D_5	381.1	155.1	127.1	130	32, 55

4.4 *Conclusions*

New standards were purchased to mimic the most prevalent metabolites of synthetic cannabinoids and designer cathinones that would be detected in urine samples, after oral consumption or inhalation of the drugs. The MRM transitions were optimized for signal intensity and specificity of the compounds, utilizing the multimode source and triple quadrupole mass spectrometer system specified in Section 4.2.2.1.

2D-LC can be carried out using two major separation mechanisms: heart-cutting and comprehensive techniques.[10] Heart-cutting is valuable when the mixture is complex

and certain analytes of interest are isomers and overlapping with other peaks, ultimately inhibiting one's ability to make accurate identifications and quantifications. Comprehensive 2D-LC moves all of the eluent from the first column to the second column for further separation, identification and quantification.[10, 30] A disadvantage to this technique is that only the compounds that are selected to move to the second column are analyzed while all information of the other compounds from the first dimension are lost.[30] 2D-LC is advantageous in that compounds that coelute in the first dimension can be properly resolved in the second dimension, while concomitantly increasing the peak capacity of the system with a multiplicative effect.[1-3] Additionally, it can improve the overall method sensitivity and selectivity for analytes, as was implemented with van de Schans et al. when they were utilizing multiple heart-cutting 2D-LC coupled with a quadrupole ToF-MS analyzing pyrrolizidine alkaloids in tea and food supplements.[30] In the next chapter, heart-cutting will be attempted first, followed by comprehensive 2D-LC separation for the separation, identification and quantification of designer cathinones, synthetic cannabinoids and analytes from a standard 9-panel urine test.

A robust HILIC column was selected for the first dimension to eliminate the need for a sample pretreatment/extraction step for the cannabinoids and cathinones in urine. The HILIC column had a larger internal diameter and particle sizes than those typically seen in fast HPLC/UHPLC methods.[30] It was able to overcome the salts and other impurities from a urine matrix without sacrificing reproducible chromatographic separations. When carrying out method development for the separation of cathinones and cannabinoids from each other on the HILIC column it was important to consider the pKa of each analyte and how changes in pH can affect their ionization states.[25] By modifying the mobile phase

with a 150 mM ammonium formate buffer, the pH was changed to approximately 3, converting the analytes to their acid-forms.[25] This allowed for the retention of early eluting analytes and the successful separation of cathinones and cannabinoids in 4 minutes.

A new core-shell C18 column was purchased for the second dimension to reduce the backpressure and band broadening effects that were being seen in the previous chapters with the ZORBAX C18 column. When considering the van deemter equation, a core-shell column contains particles that contain a solid core and are therefore not completely porous, allowing for a reduction in eddy diffusion while increasing the mass transport and separation of analytes.[7,9] Acceptable separation was attained within the cathinones and cannabinoids utilizing the core-shell column, advancing this project to the next stage: application of analytes from a standard 9-panel urine test to determine the efficacy of this 2D-LC technique for urine samples.

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5. EXPANDING THE TWO-DIMENSIONAL LIQUID CHROMATOGRAPHY METHOD TO INCLUDE ALL DRUG CLASSES FROM A STANDARD 9-PANEL URINE TEST

5.1 *Introduction*

According to NMS labs, a standard 9-panel urine drug panel consists of the following groups of compounds: amphetamines, barbiturates, benzodiazepines, cannabinoids, cocaine, methadone, opiates, oxycodone/oxymorphone and phencyclidine.[1] In order to demonstrate the efficacy of the 2D-LC method that was developed, it was important to ensure that it is robust enough to include the metabolites of the cathinones and cannabinoids that were analyzed in Chapter 4, and to include other drug classes that are present in a standard 9-panel urine drug screen. As discovered and then emphasized in the previous chapter, the pKa of each compound needs to be considered for proper retention and separation on the first and second dimensions of 2D-LC.

5.2 *Materials and methods*

5.2.1 *Reagents and supplies*

± JWH 018 N-(4-hydroxypentyl) metabolite, ± JWH 018 N-(4-hydroxypentyl) metabolite-D_5, JWH 073 N-(4-hydroxybutyl) metabolite, JWH 073 N-(4-hydroxybutyl) metabolite-D_5, AM2201 N–(4-hydroxypentyl) metabolite, AM2201 N-(4-hydroxypentyl) metabolite-D_5 were purchased from Cayman Chemical (Ann Arbor, MI, USA). ±-4-methylephedrine HCl (mephedrone metabolite), ±-4-methylephedrine-D_3 HCl (mephedrone metabolite), methylone HCl, methylone-D_3 HCl, 3,4-methylenedioxy pyrovalerone, and 3,4-methylenedioxy pyrovalerone-D_8 (hydrochloride) were purchased from Cerilliant Corporation (Round Rock, TX, USA). A Luna® HILIC column with a 150

mm length x 4.6 mm ID and 5 µm particle size, a Kinetex C18 column with a 2.1 mm ID x 50 mm length and 1.8 µm particle size, and a Kinetex C18 column with a 2.1 mm ID x 150 mm length and 2.6 µm particle size were purchased from Phenomenex (Torrance, CA, USA). Ammonium formate, HPLC grade water, HPLC grade acetonitrile, certified synthetic urine, formic acid (99.0+% Optima LC-MS grade) and PEEK tubing (1/16" outside diameter) connected with a one-piece PEEK finger-tight fitting were purchased from Fisher Scientific (Pittsburgh, PA, USA).

5.2.2 *Instrumentation*

5.2.2.1 *2D-LC-QqQ-MS coupled with a multimode source*

An Agilent 6460 Triple Quadrupole (Santa Clara, CA, USA) Mass Spectrometer with a multimode ionization source and equipped with Mass Hunter and Qualitative Analysis software was optimized for the analysis of designer cathinone and synthetic cannabinoid metabolites in synthetic urine. All analyses were completed in positive ionization mode with the source parameters specified in Section's 3.2.2.1 and 3.2.2.2. The optimized MRM transitions and CE's selected for the quantifier and qualifier ions of the cathinones and cannabinoids are specified in Section 4.2.2.1. The optimized MRM transitions and CE's selected for the quantifier and qualifier ions of the compounds from the standard 9-panel urine test include: methamphetamine m/z 150.0 -> 91.2, 119.0 (CE 13, 5); phenobarbital m/z 233.0 -> 188.0, 142.0 (CE 4, 16); 2-hydroxyethyl-flurazepam m/z 333.2 -> 246.0, 109.0 (CE 15, 30); THC-COOH m/z 345.0 -> 299.0, 193.0 (CE 25, 35); benzoylecgonine m/z 290.1 -> 168.1, 105.0 (CE 37, 40); methadone m/z 310.2 -> 265.2, 105.0 (CE 25, 33); morphine m/z 286.2 -> 152.0, 128.0 (CE 30, 40); fentanyl m/z 337.2 -> 188.1, 105.1 (CE 31, 50); and oxycodone m/z 316.2 -> 212.1, 241.1 (CE 37, 30).

5.3 *Results and discussion*

The urine testing panel, synthetic cannabinoids and designer cathinones used, and each corresponding pKa can be seen in Table 5.1.

Drug Group	Analyte from Drug Group Selected	pKa
Amphetamines	Methamphetamine	9.9
Barbiturates	Phenobarbital	7.3
Benzodiazepines	2-Hydroxyethyl-Flurazepam	8.2
Cannabinoids	THC-COOH	10.6
Cocaine	Benzoylecgonine	8.6
Methadone	Methadone	9.1
Opiates	Morphine and Fentanyl	8.1 and 9.0
Oxycodone/Oxymorphone	Oxycodone	8.5
Phencyclidine	N/A	8.3

Table 5.1 Drug Groups from a Standard 9-Panel Urine Test, Including Analytes Chosen for Analysis and Corresponding pKa's.[1-9]

Phencyclidine was not chosen, as it was not readily available in the laboratory and would have had to of been purchased. To conserve academic funds, two compounds that were readily available in the laboratory were chosen from the Opiates category to still incorporate 9 compounds from the drug panel. For clarity, optimized MRM transitions for all of the compounds can be seen in Table 5.2.

Table 5.2 Optimized MRM Transitions for Designer Cathinones, Synthetic Cannabinoids and Compounds from a Standard 9-Panel Urine Test.[10-12]

Analyte	Precursor Ion (m/z)	Quantifier Ion (m/z)	Qualifier Ion (m/z)	Fragmentor (V)	CE (V)
4-Methylephedrine HCl (mephedrone metabolite)	180.1	162.0	117.0	130	5, 6
4-Methylephedrine-D_3 HCl (mephedrone metabolite)	183.1	162.0	117.0	130	5, 6
Methylone HCl	208.2	160.0	132.0	130	14, 26
Methylone-D_3 HCl	211.2	160.0	132.0	130	14, 26
MDPV	276.1	126.1	135.0	130	30, 26
MDPV-D_8	284.1	126.1	135.0	130	28, 24
JWH-018 N-(4-hydroxypentyl) metabolite	358.2	155.1	127.2	130	30, 52
JWH-018 N-(4-hydroxypentyl) metabolite-D_5	363.1	155.0	127.0	130	30, 52
JWH-073 N-(4-hydroxybutyl) metabolite	344.1	155.0	127.0	130	30, 52
JWH-073 N-(4-hydroxybutyl) metabolite-D_5	349.1	155.0	127.0	130	30, 52
AM2201 N-(4-hydroxypentyl) metabolite	376.1	155.1	127.1	130	32, 55
AM2201 N-(4-hydroxypentyl) metabolite-D_5	381.1	155.1	127.1	130	32, 55
Methamphetamine	150.0	91.2	119.0	130	13, 5
Phenobarbital	233.0	188.0	142.0	130	4, 16
2-Hydroxyethyl-Flurazepam	333.2	246.0	109.0	130	15, 30
THC-COOH	345.0	299.0	193.0	130	25, 35
Benzoylecgonine	290.1	168.1	105.0	130	37, 40
Methadone	310.2	265.2	105.0	130	25, 33
Morphine	286.2	152.0	128.0	130	30, 40
Fentanyl	337.2	188.1	105.1	130	31, 50
Oxycodone	316.2	212.1	241.1	130	37, 30

5.3.1 *2D-LC-QqQ-MS method optimization to include all drug classes*

Standards were prepared from 80 ng/mL – 0.0001 mg/mL in 98:2 acetonitrile:water (v/v) and synthetic urine and subjected to the 1st dimension of separation utilizing the HILIC column with an isocratic gradient over 6 minutes at 100% of 150 mM AF in 98:2

acetonitrile:water (v/v) with a flow rate of 1 mL/min. While this method was sufficient for the separation of cathinones and cannabinoids, it was not adequate for the separation of compounds from the standard 9-panel urine test. Morphine was not detected at all and the very polar compounds from the standard 9-panel urine test eluted in the dead volume (< 1 minute). The method was modified several times before finally arriving at optimized conditions for the first dimension, including an 8 µL injection volume, utilizing water and 150 mM AF in 98:2 acetonitrile:water (v/v), as mobile phases A and B, respectively. See Table 5.3 for the gradient conditions.

Table 5.3 Optimized Gradient Conditions for First Dimension of Separation of Cathinones, Cannabinoids and Standard 9-Panel Urine Test Compounds.

Time (min)	A (%)	B (%)	Flow Rate (mL/min)
0	3	97	1.5
3.0	3	97	1.5
3.1	80	20	1.5
6.0	80	20	1.5
6.1	3	97	1.5
12.1	3	97	1.5

Now that the first dimension of separation has been optimized to include cathinones, cannabinoids and compounds from a Standard 9-Panel Urine Test, fraction collection for the second dimension was initiated. Initially, the heart-cutting technique was employed and included 4 separate fractions to collect, as can be seen in Table 5.4.

Table 5.4 Heart-Cutting Fraction Collection from First Dimension of 2D-LC.

Fraction	Analyte	Elution Time (min)	Fraction Collection Time (min)
1	THC-COOH	1.252	1-2
	AM2201 Metabolite	1.319	
	JWH 018 Metabolite	1.352	
	JWH 073 Metabolite	1.386	
	Flurazepam	1.419	
	Phenobarbital	1.454	
2	Fentanyl	2.454	2-3
	MDPV	2.638	
	Oxycodone	2.788	
3	Benzoylecgonine	4.591	4-5
	Methylone	4.641	
	Mephedrone Metabolite	4.674	
	Methamphetamine	4.691	
	Morphine	4.774	
4	Methadone	5.762	5-6

In order to collect fractions, the PEEK tubing that runs from port 2 of the solvent selection valve to the source was removed and replaced with a shorter piece of PEEK tubing connected with a one-piece PEEK finger-tight fitting, as can be seen in Figure 5.1.

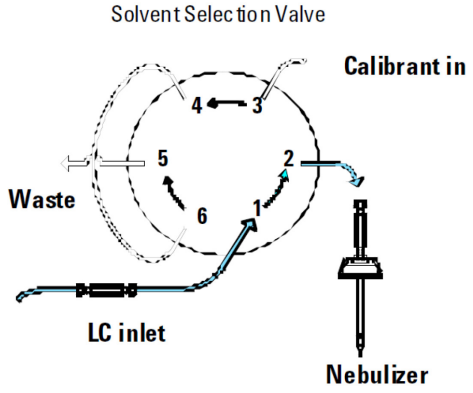

Figure 5.1 Agilent 6460 Triple Quadrupole Solvent Selection Valve Showing LC to LC-MS Flow.[13]

After analyzing the fractions that were collected from the first dimension and then subjected to the second dimension using the parameters in Table 5.3, most of the compounds were not retained on the column or were unable to be detected, and others were

not quantified reliably. A dilution factor of 187.5 was not considered when preparing samples at various concentrations, given that an 8 μL injection was used for the first dimension, and 1.5 mL was collected per fraction. Therefore, a 0.0001 mg/mL standard was analyzed as 5.3×10^{-7} mg/mL in the vial during the second dimension. It is important to note that the fractions were not vortexed prior to an aliquot being transferred to an autosampler vial and subjected to the second dimension.

Next steps included pursuing a comprehensive fraction collection, instead of the heart-cutting technique and also prepping the samples 3 orders of magnitude higher to account for the dilution factor from a 6 mL continuous collection. Improved results for the second dimension can be seen in Figure 5.2.

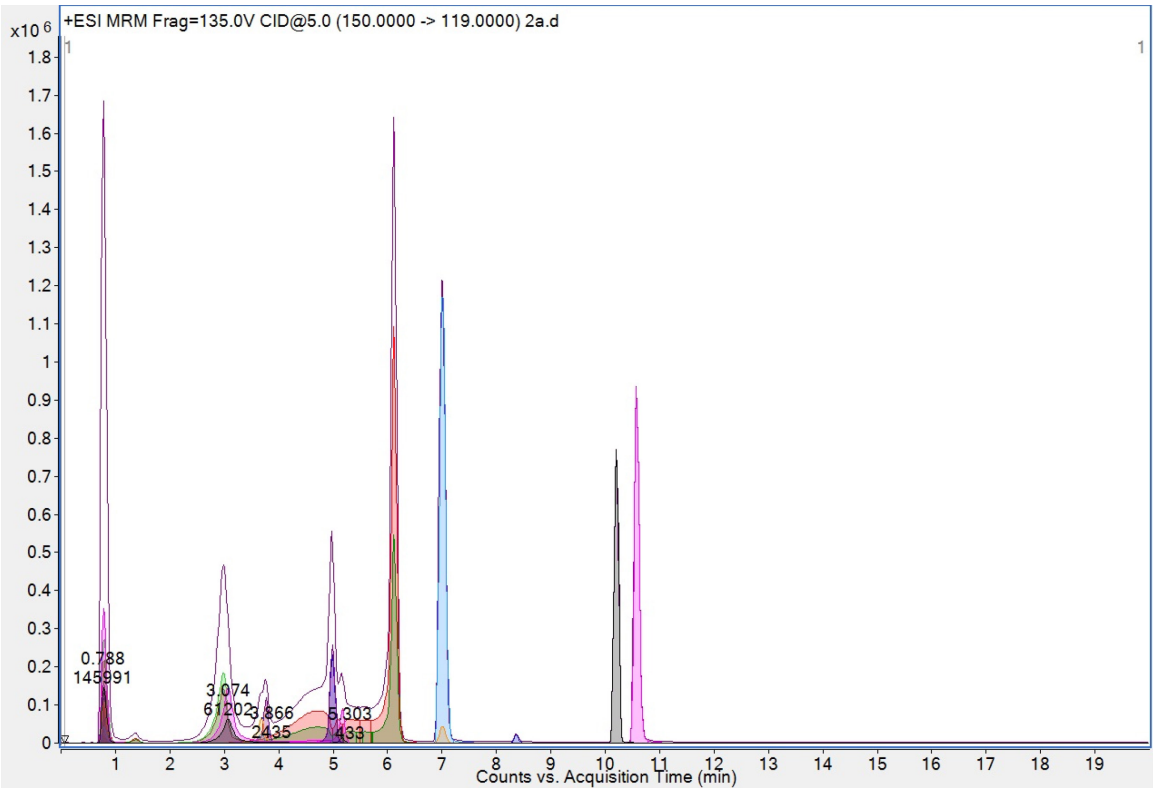

Figure 5.2 The second dimension of separation of standards from 2.0 x 10^{-6} – 0.00013 mg/mL, prepared in synthetic urine.

As can be gleaned from Figure 5.2, some of the compounds are still not being retained on the column, there is poor separation between the analytes, peak fronting/tailing is observed and the backpressure of the column was exceeding the 365 bar maximum limit.[14] Initial troubleshooting steps indicated that the column frit may have been blocked and the injection volume could have been too large.[15] A new column, Kinetex C18 150 mm x 2.1 mm x 2.6 µm, was utilized for the remainder of the project for the second dimension, and the method was converted from 50 mm to 150 mm in length to increase resolution and to overcome the backpressure and injection volume issues. In order to increase retention of the early eluting compounds, mobile phase A was modified from 0.1%

to 0.4% formic acid with an initial hold of 2 minutes, while mobile phase A remained as acetonitrile. Synthetic urine was spiked with all of the compounds from 300 ng/mL – 133 µg/mL. See Table 5.5 and Figure 5.3 for the final gradient parameters and complete elution profile, respectively. Figures 5.4-5.6 show individual extracted MRM chromatograms for the quantifier ion of each compound of interest.

Table 5.5 Final, optimized gradient elution parameters for the second dimension of 2D-LC.

Time (min)	A (%)	B (%)	Flow Rate (mL/min)
0	98	2	0.2
2	98	2	0.2
8	45	55	0.2
11	3	97	0.2
14	3	97	0.2
14.1	98	2	0.2
22.1	98	2	0.2

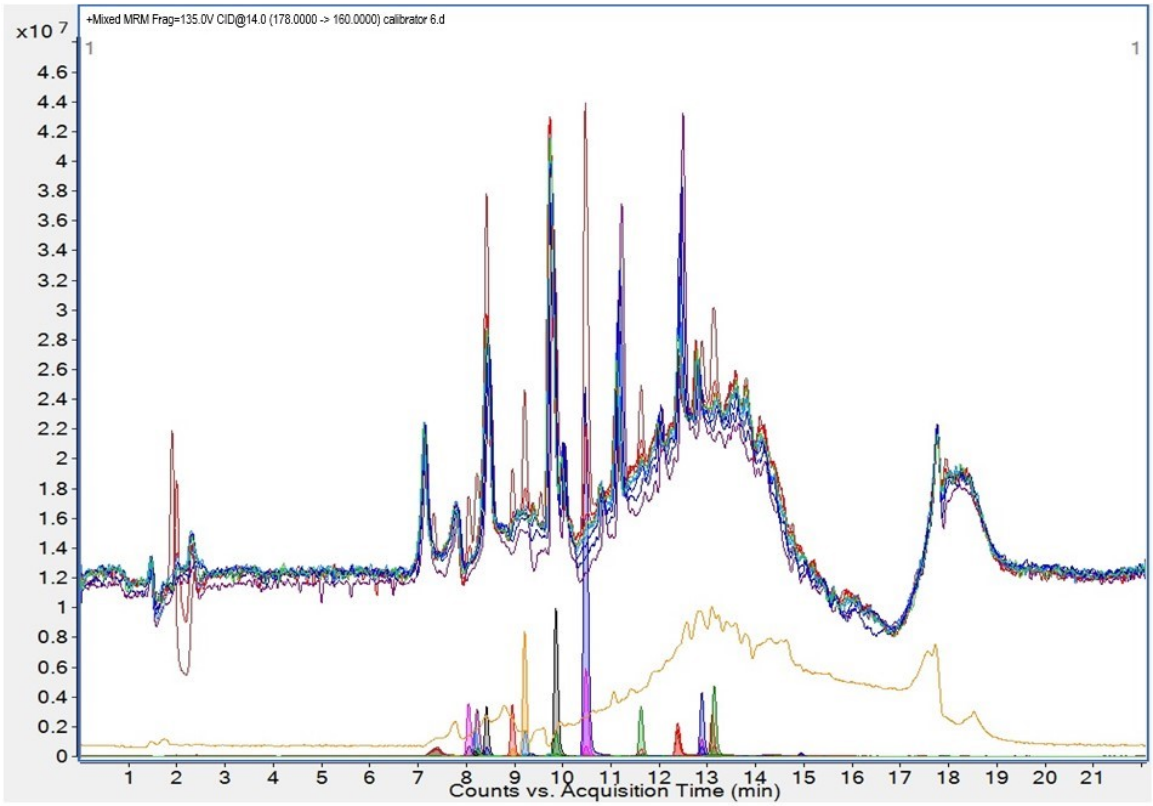

Figure 5.3 Final, optimized chromatogram for designer cathinones, synthetic cannabinoids and compounds from a standard 9-panel urine test for the second dimension of 2D-LC.

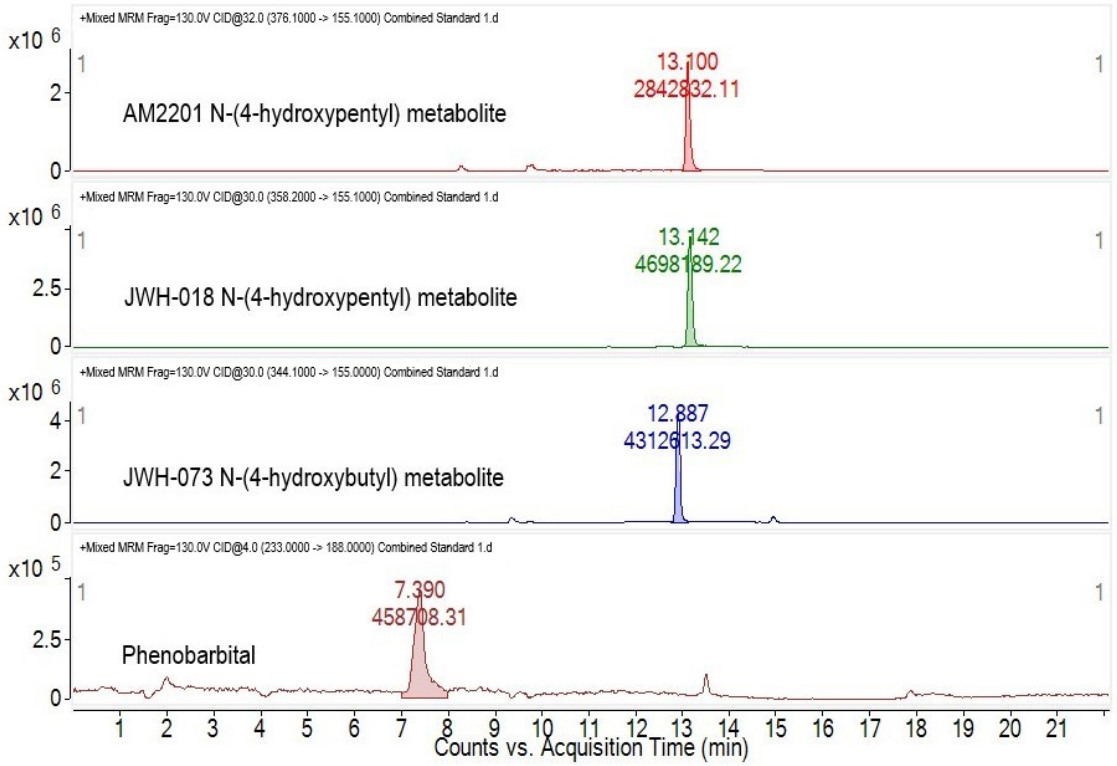

Figure 5.4 Extracted MRM chromatograms of the quantifier ion for AM2201 N-(4-hydroxypentyl) metabolite, JWH-018 N-(4-hydroxypentyl) metabolite, JWH-073 N-(4-hydroxybutyl) metabolite and phenobarbital.

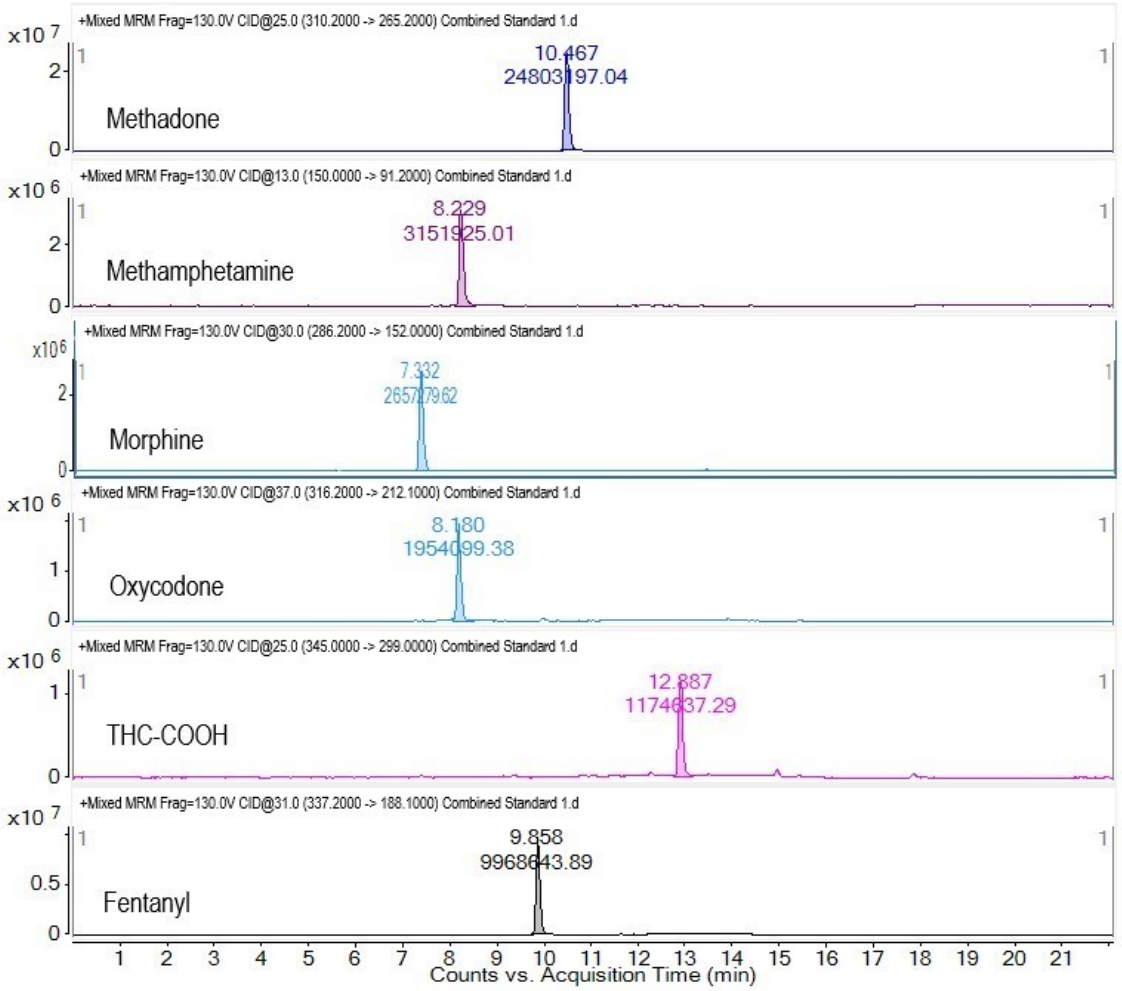

Figure 5.5 Extracted MRM chromatograms of the quantifier ion for methadone, methamphetamine, morphine, oxycodone, THC-COOH and fentanyl.

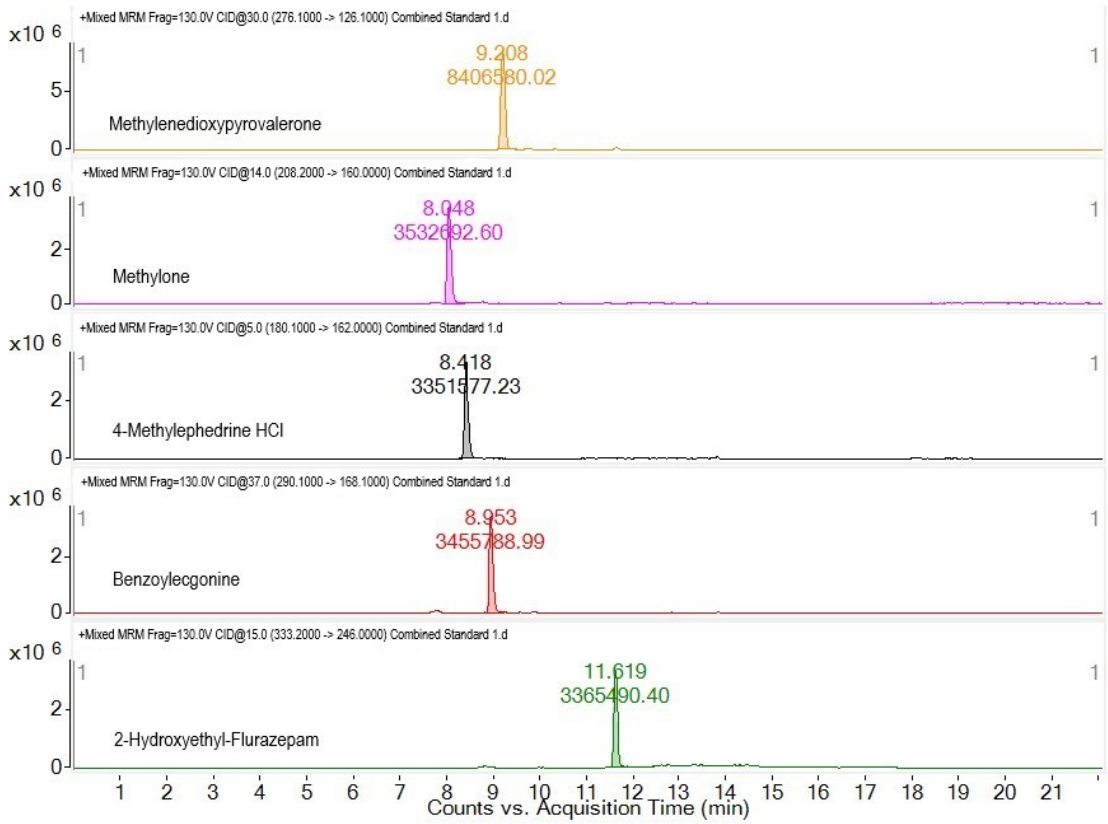

Figure 5.6 Extracted MRM chromatograms of the quantifier ion for MDPV, methylone, 4-methylephedrine HCl, benzoylecgonine and 2-hydroxyethyl-flurazepam.

Internal standards (deuterated analogs) were spiked into the synthetic urine with the analytes at an amount that was < 1% of the analyte of interest. The purpose of using an internal standard for quantification is to correct for any possible variation from the sample preparation to the analysis steps.[17] Calibration curves were constructed by plotting the corrected peak areas versus the concentration of each standard. Corrected peak areas were calculated by dividing the peak area of the quantifier ion for each compound by the peak area of the quantifier ion of the corresponding internal standard.[17] Figure 5.7 shows the calibration curves that were generated for MDPV, methylone, 4-methylephedrine HCl, JWH-018 N-(4-hydroxypentyl) metabolite, AM2201 N-(4-hydroxypentyl) metabolite and

JWH-073 N-(4-hydroxybutyl) metabolite. Trendlines were included to show the coefficient of determination (R^2), a statistical measure of how closely the data fits the regression line.[18] In general, the higher the R^2 value is to 1, the more likely it is that the model explains most of the variability of the response data around its mean.[18,19] All of the R^2 values are greater than or equal to 0.988 with acceptable uncertainty values associated with the data, indicating a linear response between the analytes' signals and concentrations.[19]

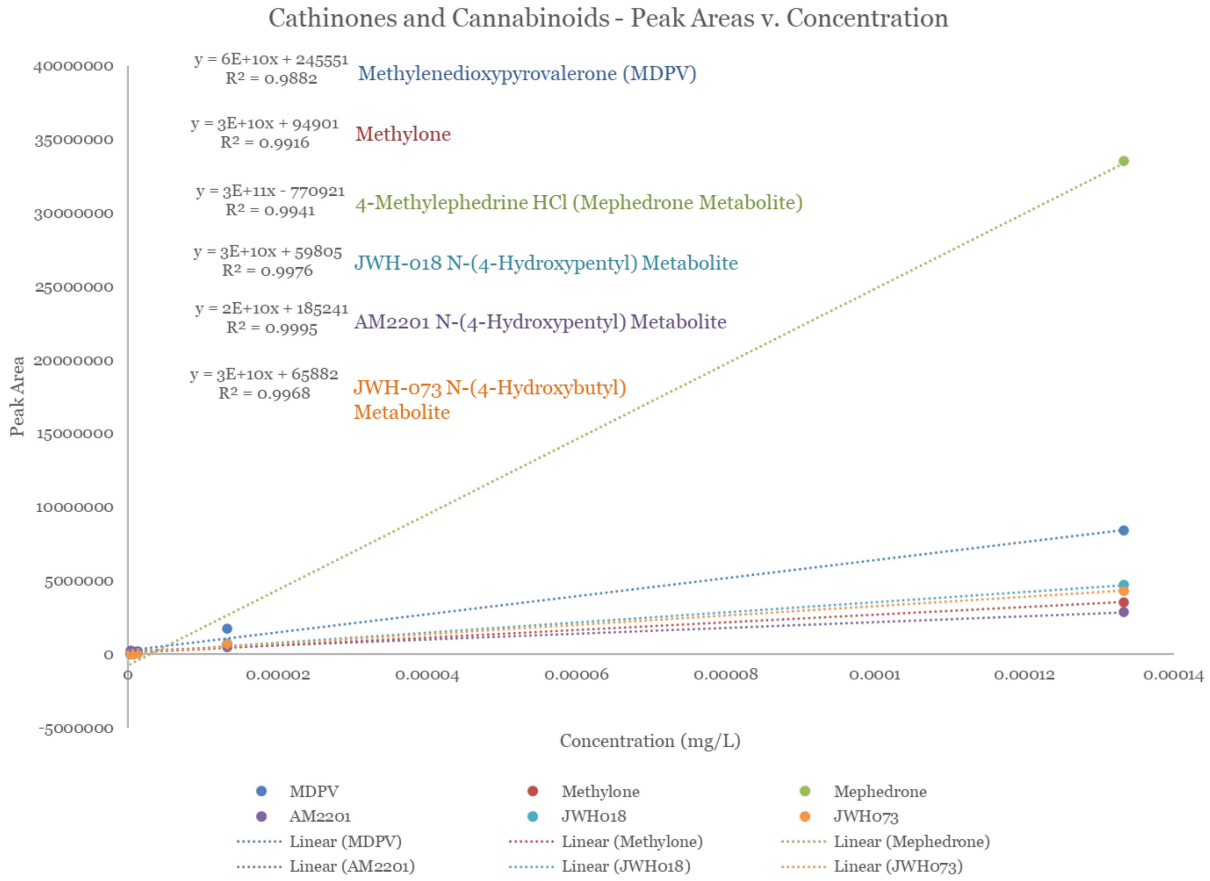

Figure 5.7 Calibration curves of the quantifier ion for designer cathinones and synthetic cannabinoids prepared from 300 ng/mL to 133 µg/mL in synthetic urine.

5.3.2 *Validation of the optimized 2D-LC-QqQ-MS method of analysis*

Validation for the 2D-LC analysis of designer cathinones, synthetic cannabinoids and standard 9-panel urine test was conducted by spiking a known aliquot of synthetic urine with methamphetamine at 500 ng/mL, the Department of Health and Human Services administrative confirmation cutoff level in urine.[16] The recovery for the methamphetamine spike was acceptable at 95.1% in synthetic urine. The chromatogram for the spike validation of methamphetamine in synthetic urine can be seen in Figure 5.8.

Figure 5.8 Extracted MRM chromatogram of the quantifier ion for methamphetamine spiked into synthetic urine at the cutoff concentration of 500 ng/mL.

5.4 *Conclusions*

In conclusion, a single 2D-LC-QqQ-MS method was capable of separating and quantifying synthetic cannabinoids, designer cathinones and compounds from a standard 9-panel urine test, in a single sample of synthetic urine. One drawback that currently exists lies in utilizing deuterated analogs as internal standards, relative to isotopically enriched compounds. While deuterated compounds should theoretically behave similarly to the naturally occurring analyte, they do not. Isotope dilution mass spectrometry (IDMS) can correct for matrix effects and partial analyte loss that may occur during sample preparation and is capable of quantitatively assessing transformations between two species that are unable to be determined by other quantification methods.[20,21] Speciated isotope dilution mass spectrometry (SIDMS), a variation of IDMS, takes it a step further and is able to

account for any transformation of species.[20,21] This becomes very important when considering illicit drug use and the interconversion/metabolism processes that can occur between heroin, 6-acetylmorphine and morphine.[21] While this is beyond the scope of the current project, IDMS/SIDMS is an important consideration for future quantification of toxicological samples to enable a more precise and accurate quantitation compared to a calibration curve and deuterated internal standards.[21]

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6. CONCLUSION

This work focused on developing and optimizing a 2D-LC-QqQ-MS method that was capable of effectively separating and quantifying designer drugs in a single urine sample, using one method of analysis. The greater impact is summarized in the corresponding sections below.

In Chapter 2, the objective involved implementing a sample cleanup step prior to analysis, as the matrix is urine. A LLE method was developed to remove salts, lipids, and non-volatile materials from urine and to separate analytes, selectively, from a mixture. The cannabinoids included JWH-018 N-5-COOH, JWH-073 N-5-COOH, and MAM-2201 N-4-OH, and the cathinones included methylone, MDPV, and mephedrone. Using LLE, the different drug groups were forced into the organic phase at different pH's since the synthetic cannabinoids are acidic and the cathinones are basic. By adding concentrated HCl to the aqueous phase before the extraction solvent was added, the acidic conditions caused the synthetic cannabinoids to move into the organic phase. Conversely, adding concentrated potassium hydroxide created a basic pH, causing the cathinones to move into the organic phase with the cannabinoids. The samples were evaporated to dryness under N_2 gas and reconstituted in 50 µL of mobile phase before analysis by tandem LC-MS

The goal of Chapter 3 was focused on optimizing source parameters and column chromatography for the second dimension of LC. ESI and APCI sources were compared to see which would give the highest signal intensity for the cathinones and cannabinoids. The source parameters were optimized for ESI using Agilent's Source Optimizer software, followed by manual analysis of the data, whereas the parameters for the APCI were manually optimized based on the signal intensity for each cannabinoid. Calibration curves

were constructed and compared for cannabinoids and cathinones using ESI and APCI, but quantification was only reliable for JWH-073 N-5-COOH and MDPV, as their corresponding internal standards were included in the analysis. The cannabinoids had much higher R^2 values when analyzed using APCI, while the cathinones had much higher R^2 values when analyzed using ESI. To compromise for differences in signal intensity between the two groups and sources, Agilent's Multimode source was used for all subsequent analyses. The Multimode source incorporated both ESI and APCI into a single ion source, thereby greatly improving the signal intensity for both the cathinones and cannabinoids. Initially, the second-dimension column used was a reverse phase C18 Zorbax Eclipse column that was readily available in the laboratory. The C18 column was able to successfully separate the cathinones from the cannabinoids in under 5 minutes, but the compounds within the groups were not resolved from one another. The resolution and peak shape of each component was not optimal, and there appeared to be contamination in the samples. Appropriate urinary metabolites, corresponding internal standards and new columns were ordered for the remainder of the project.

In Chapter 4, each dimension of the 2D-LC method was optimized using new standards, internal standards, and new columns. The standards include the major urinary metabolites and corresponding internal standards of the cannabinoids and cathinones analyzed previously: ± JWH 018 N-(4-hydroxypentyl) metabolite, ± JWH 018 N-(4-hydroxypentyl) metabolite-D_5, JWH 073 N-(4-hydroxybutyl) metabolite, JWH 073 N-(4-hydroxybutyl) metabolite-D_5, AM2201 N–(4-hydroxypentyl) metabolite, AM2201 N-(4-hydroxypentyl) metabolite-D_5, ±-4-methylephedrine HCl (mephedrone metabolite), ±-4-methylephedrine-D_3 HCl (mephedrone metabolite), methylone HCl, methylone-D_3 HCl,

3,4-methylenedioxy pyrovalerone, and 3,4-methylenedioxy pyrovalerone-D_8 (hydrochloride). The first dimension of the 2D-LC setup utilized a Luna HILIC column with a larger ID and particle size to be robust enough to handle the salts and other impurities from a urine sample, ultimately eliminating the need for sample pretreatment. A gradient elution was developed and optimized using a buffer solution of 150 mM ammonium formate in 98:2 acetonitrile:water (v/v) to convert the analytes to their ionic form, retain them on the column, and sufficiently separate the cathinones from the cannabinoids, with minimal emphasis on baseline separation of the peaks within the groups. The eluent was diverted to waste after all of the compounds eluted to eliminate the urea from reaching the mass spectrometer. The second column, that contains a C18 "core-shell" stationary phase, utilized a completely different separation mechanism from the HILIC. Optimum occupancy of the two-dimensional space occurs when the separation mechanisms of both dimensions have distinct retention profiles.[1] The higher the orthogonality, the closer the system gets to theoretical peak capacity.[1] Synthetic urine was spiked with the analytes and a gradient method was successfully developed utilizing a 50 mm length C18 "core-shell" column that was able to separate the compounds within the cannabinoids and cathinones from one another.

The goal of Chapter 5 was to also incorporate compounds from a standard 9-panel urine test, collect fractions and test the efficacy of the 2D-LC-QqQ-MS method.[2] Additional compounds that were analyzed included methamphetamine, phenobarbital, 2-hydroxyethyl-flurazepam, THC-COOH, benzoylecgonine, methadone, morphine, fentanyl and oxycodone.[2] Fractions from the HILIC column were collected utilizing both "heart-cutting" and comprehensive 2D-LC techniques, with greater success from the latter.[3, 4]

Dilution factors were an important consideration with both fraction-collecting techniques, ultimately requiring synthetic urine samples to be spiked with higher concentrations of the analytes. There were issues with retaining the compounds beyond the dead volume and exceeding backpressure limits of the 50 mm length C18 column, so a longer 150 mm length C18 column was used to increase resolution and overcome the backpressure issues. Additionally, the mobile phase was modified from 0.1% to 0.4% formic acid in water with an initial hold of 2 minutes. Synthetic urine was spiked with all of the analytes from 300 ng/mL – 133 μg/mL, and corresponding internal standards were added at an amount that was < 1% of the analyte of interest. Quantification was carried out by plotting the corrected peak area of the quantifier ion versus the concentration, generating calibration curves. All of the R^2 values were greater than or equal to 0.988 with acceptable uncertainty values associated with the data, indicating that a linear response was produced between the analytes' signals and concentrations. Validation was carried out by spiking a known aliquot of synthetic urine with methamphetamine at 500 ng/mL, demonstrating the efficacy of the method to detect the analyte at the Department of Health and Human Services administrative confirmation cutoff level in urine.[5] The recovery for the methamphetamine spike was acceptable at 95.1% in synthetic urine.

In the field of toxicology, there are currently sample cleanup/extraction techniques, unique to groups of drugs (acidic/basic) and matrices, that are necessary to carry out before confirmation using mass spectral detection.[6-12] Also, there are separate methods of analysis for cathinones and cannabinoids in urine, so this research project, having eliminated sample cleanup and combined the analysis of cathinones and cannabinoids into one method, would be novel and impactful to the toxicology community.[6-12] Recently, there has been interest

and favor in utilizing mass spectrometry as the screening and confirmation techniques, as there are limitations with immunoassay testing and their ability to screen for a wider variety of compounds at lower detection limits with lower associated costs.[13] The development of this 2D-LC-QqQ-MS method for the identification and quantification of synthetic cannabinoids, designer cathinones and various compounds from a standard 9-panel urine test is expected to be applicable to real-world urine samples and should yield comparable quantitative results to the standards that were evaluated.

The broader impact of this work focuses on if 2D-LC-QqQ-MS were accepted in the forensic science and toxicology communities as a routine test methodology for identifying and quantifying drugs of abuse in urine. Since a comprehensive 2D-LC technique was carried out in this project, it would be feasible to move the methodology online, eliminating human error in transferring eluent from the first to second dimension of separation. This project broadens the current scope of test methodologies, allowing for higher throughput of samples by eliminating sample cleanup/extraction steps from urine, allowing for the quantification of multiple drug types using one method of analysis, decreasing solvent waste, and saving time to promote the progress of science and advancing through the backlog of cases that impact the health and welfare of society.

6.1 *References*

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