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An evaluation of commercially available solid phase extraction cartridges for the isolation of synthetic cannabinoid metabolites from urine

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BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Thesis

**AN EVALUATION OF COMMERCIALY AVAILABLE SOLID PHASE
EXTRACTION CARTRIDGES FOR THE ISOLATION OF SYNTHETIC
CANNABINOID METABOLITES FROM URINE**

by

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B.S., Ohio University, 2011

Submitted in partial fulfillment of the
requirements for the degree of
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ABSTRACT

Synthetic cannabinoids were first created in a pharmaceutical setting where scientists were studying marijuana. Researchers were trying to develop medically beneficial marijuana analogs. The compounds, however, were found to give physiological effects that were more potent than marijuana. Presently, synthetic cannabinoids have become a psychoactive drug of abuse, sold in head shops and over the Internet. New compounds are constantly being synthesized, which makes analysis of the drugs difficult.

Solid phase extraction (SPE) is a well-studied method used in toxicological analysis to extract drugs and their metabolites from biological fluids. This sample preparation method is necessary to isolate the desired components of a sample for analysis by gas chromatography and mass spectrometry (GC/MS). This study sought to compare four brands of commercially available SPE cartridges using a procedure from United Chemical Technologies (UCT) for the simultaneous extraction of the three synthetic cannabinoid metabolites, JWH-018 N-(4-hydroxypentyl), JWH-122 N-(5-hydroxypentyl), and JWH-250 N-(5-hydroxypentyl), from urine. The cartridges from UCT, Thermo Scientific, Agilent Technologies, and SiliCycle were evaluated to

determine how they performed throughout the SPE procedure. A recovery efficiency study was conducted to measure the amount of extracted metabolites from the urine. The responses of the quantification ion of the metabolites from an extracted urine sample were compared to a neat sample and the percent recovery was calculated. A within-run precision study was also utilized to measure the reproducibility of the cartridges, which was determined by the coefficient of variation (CV) of the different brands.

The outcome of this research led to a development of a GC/MS method for detection of the three metabolites, creation of calibration curves for quantification, use of SPE for the extraction of the metabolites from urine, and the quantification of the extracted compounds to determine the efficacy and consistency of four brands of SPE cartridges. Method optimization was able to minimize the interday variations seen in the results of aliquots of the same samples. Optimal parameters include initial validation of the GC/MS method, a clean liner for the analysis of synthetic cannabinoid metabolites, using a GC column with a high temperature limit, and derivatization of the extracts before injection into the GC. While this study shows it is possible to use GC/MS for the analysis of these metabolites, LC/MS does not have the same restrictions because a liner, temperature elution, and derivatization of the analytes are not utilized.

It was determined from the results of these studies that SiliCycle had the most reproducible and efficient cartridges. SiliCycle cartridges had a consistent and fast flow rate with a percent recovery efficiency within $\pm 20\%$ of the actual value. The results from SiliCycle were followed by cartridges from UCT, Thermo Scientific, and Agilent brands, respectively.

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LIST OF ABBREVIATIONS

Δ 9-THC	Delta9-tetrahydrocannabinol
ACN	Acetonitrile
AM	Alexandros Makriyannis
amu	Atomic mass unit
BSTFA	N,O-bis(trimethylsilyl) trifluoroacetamide
°C	Celsius
CB1	Cannabinoid receptor type 1
CB2	Cannabinoid receptor type 2
CV	Coefficient of variation
diH ₂ O	Deionized water
EtOAc	Ethyl acetate
ESI	Electrospray ionization
GC	Gas chromatography
H	Hydrogen
HU	Hebrew University
in	Inch
JWH	John W. Huffman
L	Liter
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LOD	Limit of detection

LOQ	Limit of quantitation
M ⁺	Molecular ion
MeOH	Methanol
mg	Milligram
min	Minute
mL	Milliliter
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
Na ₂ HPO ₄	Di-basic sodium phosphate
NaH ₂ PO ₄	Mono-basic sodium phosphate
ng	Nanogram
SIM	Selected ion monitoring
SPE	Solid phase extraction
SDAPA	Synthetic Drug Abuse Prevention Act
SWGTOX	Scientific Working Group for Forensic Toxicology
TMCS	Trimethylchlorosilane
TOF	Time of flight
UCT	United Chemical Technologies
μg	Microgram
μL	Microliter
U.S.	United States

1. INTRODUCTION

1.1 Synthetic Cannabinoids

1.1.1 Research History

Synthetic cannabinoids are psychoactive drugs of abuse, originally studied in pharmaceutical laboratories beginning in the 1960's. The research scientists in these labs were trying to develop medically useful derivatives of delta9-tetrahydrocannabinol (Δ^9 -THC) that contained its analgesic and anti-inflammatory benefits, without the psychoactive effects (1, 2). With the discovery of the cannabinoid receptors type 1 (CB1) and type 2 (CB2), which are for endogenous neurotransmitters such as anandamide, it was determined that the compounds being developed were agonists to those receptors with a higher binding affinity than Δ^9 -THC (2-11). This resulted in more potent compounds that were not medically useful at the time. The CB2 receptor, located in the peripheral nervous system, involves pain perception in which many therapeutic benefits could be developed, like reducing nausea and intraocular pressure (9, 12-14). The CB1 receptor, located in the central nervous system, includes the psychoactive effects, such as visual and auditory hallucinations (9, 12-14).

Among the first synthetic analogs were the enantiomeric cannabinoid compounds HU-210 and HU-211, which were synthesized in Raphael Mechoulam's laboratory at the Hebrew University (HU) of Jerusalem, and CP-47,497 which was synthesized by Pfizer in the 1980's (15, 16). Alexandros Makriyannis (AM) at Northeastern University and John W. Huffman (JWH) at Clemson University also contributed to the research of synthetic analogues of cannabinoids. Huffman designed many cannabimimetic indoles in

the early 1990's by replacing the aminoalkyl's with n-alkyl chains (9, 17). Specifically, his research allowed for the design of hundreds of JWH compounds, which were among the first synthetic cannabinoids found in herbal incense blends being used as recreational drugs (17). Using Howlett's et al. classification system in *Pharmacological Reviews*, the different structures of synthetic cannabinoids can be separated into four categories: classical cannabinoids, nonclassical cannabinoids, aminoalkylindoles, and eicosanoids (9). The structures of these compounds can be seen in Figure 1.

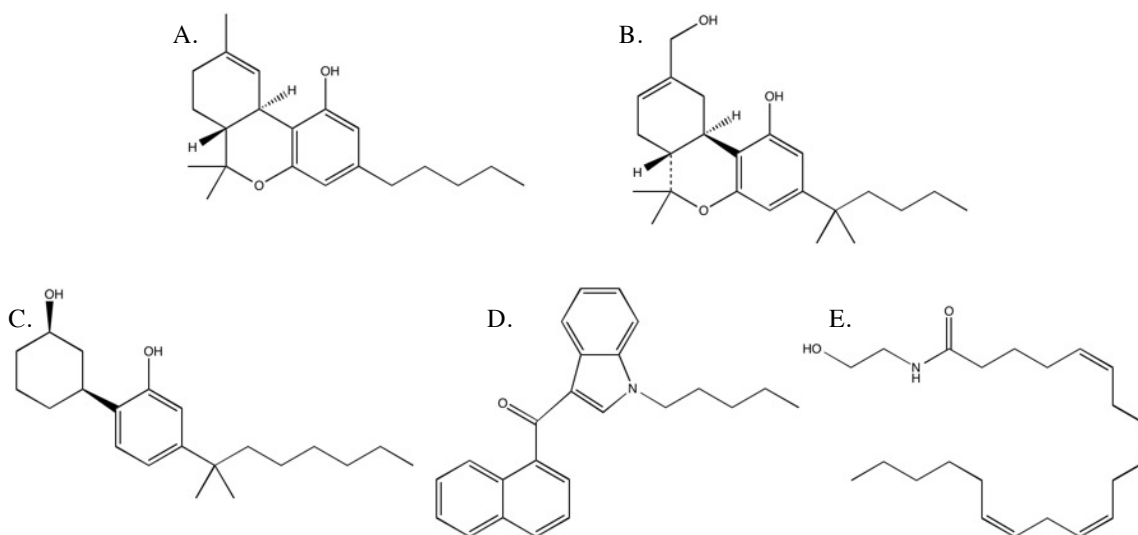


Figure 1. Structures of various synthetic cannabinoids. Classical cannabinoids A.) Δ^9 -THC and B.) HU-210; Nonclassical cannabinoid C.) CP-47,497; Aminoalkylindole D.) JWH-018; Eicosanoid E.) anandamide.

1.1.2 Use and Health Effects of Synthetic Cannabinoids

Herbal marijuana alternatives, smart drugs, and legal highs are all names given to these new psychoactive compounds. The abuse of these drugs is part of a trend to provide consumers with substances advertised as natural, herbal products that give a

marijuana-like high, without its legal ramifications (1, 2, 4, 15). While some of the plant material contains psychoactive properties of their own, like leonurine or nuciferine, the synthetic cannabinoids are sprayed on dried plant material and sold over the Internet disguised as incense (1, 4). These substances are advertised as a legal substitute for marijuana and sold under different brand names, including Tropical Synergy, Spice, K2, and Kronic. After the popularity of these drugs increased, the manufacturers began using cheaper plant materials to gross a higher profit (18).

Synthetic cannabinoids produce similar effects to marijuana, such as euphoria, relaxation, and perception alterations, but due to their increased binding affinities, the effects can be much more intense (3, 19). The adverse side effects of these compounds include anxiety, paranoia, delusions, agitation, and high blood pressure (1, 19, 20). Withdrawal symptoms have been discovered in several accounts of prolonged synthetic cannabinoid use, including tremors, insomnia, diarrhea, headaches, and depression (2). Research has also shown cannabis use can increase the risk of psychosis in individuals with a personal or familial history of mental illness, and the risk can be even more prevalent because of the increased binding affinity of synthetic cannabinoids (21-23). One case report involves a man with a history of cannabis induced psychotic episodes being reactivated after using synthetic cannabinoids (24).

Abuse of synthetic cannabinoids began in Europe in the early 2000's, and it spread to Japan, the United States (U.S.), and Australia throughout the following decade (1, 3, 25, 26). In 2008, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) began to ascertain what was referred to as "Spice" was not herbal, and

German scientists discovered that these new compounds were not detected by drug screens (2, 18). This inconspicuous quality makes synthetic cannabinoids very appealing to a variety of people. They are also easily accessible with cannabis-like effects, and are advertised to be natural and safe. The rapid onset and shorter duration of the compounds are also enticing to users (2-4, 19, 22, 27). According to an anonymous online survey, in 2011, 17% of the almost 15,000 participants worldwide reported trying synthetic cannabinoids (27). These individuals cited several of the previous reasons for their explanation of use (27). Approximately 93% of these users preferred natural cannabis, stating there were more negative effects and longer hangovers associated with their synthetic variants (27).

While these synthetic compounds have similar physiological effects to marijuana, immunoassay screening techniques such as kinetic interaction of microparticles in a solution and enzyme multiplied immunoassay technique do not demonstrate cross reactivity for the compounds (1, 20). With the wide variety of synthetic derivatives constantly changing, the selectivity of a target analyte is hard to identify and then utilize for screening (15). Developing a technique for one subcategory of compounds may not work on others due to the differences in the chemical structures. The availability of the parent drugs and metabolites, limited knowledge of the newer compounds, and a limited spectral library require continuous research to be conducted on these new drugs (15, 28).

1.1.3 Legality and Risks of Synthetic Cannabinoid Use

Synthetic cannabinoids are continuously changing, making the compounds difficult to legislate. Banning began as early as 2008 in Germany, with other European countries, Russia, Australia, and New Zealand following suit in 2010 and 2011 (1, 4, 7, 8, 27). In the U.S., temporary placement of five synthetic cannabinoid compounds as schedule I began in 2010 (29). An extension was placed on the scheduling until Congress passed the Synthetic Drug Abuse Prevention Act (SDAPA) in 2012 (30, 31). This addition to the Controlled Substances Act states that all cannabimimetic agents, including any compound that is a CB1 agonist, are schedule I (31). In the U.S., exposure to synthetic marijuana peaked in 2011 when the American Association of Poison Control Centers logged almost 7,000 reports (32). This number has significantly dropped in the following years as the legislation has taken effect (32). The World Anti-Doping Agency also added natural and synthetic Δ^9 -THC, as well as cannabimimetics to their 2013 prohibited substances list (33). Regulation was difficult due to the rapid production of new compounds once one was banned or scheduled. However, with the specification of any cannabimimetic agents being classified as schedule I in the SDAPA, progress has been made to control these substances.

Constant synthetic modifications to these compounds can lead to health risks for consumers because the metabolism and toxicology of the synthesized cannabinoids remain unknown (34). Serious side effects or possible drug overdose could result from the changes between batches and the unknown toxicity of these synthetic cannabinoids. A German study, conducted in 2008-2009, demonstrates how quickly the banning of

various compounds affects the modifications of the synthetic cannabinoids used as herbal marijuana alternatives (4). Before JWH-018 and CP-47,497-C8 were banned, they were detected in 69% of the samples tested, which decreased to 28% after the banning occurred (4). This led to an increase in JWH-073 being detected, as well as multiple samples containing no synthetic cannabinoids, but including substances like harmine, harmaline, caffeine, nicotine, Δ^9 -THC, and cannabidiol (4). Their study helps to visualize the quick turnaround for these drugs of abuse since a new compound immediately replaces the banned compound.

1.2 Synthetic Cannabinoid Metabolites

1.2.1 Metabolism of Synthetic Cannabinoids

The human body metabolizes drugs in order to excrete them from the body. One way to accomplish this is by aliphatic hydroxylation, in which cytochrome P450 adds a hydroxyl group to the carbon chain to make the compound more polar (35). Figure 2 shows the aliphatic hydroxylation of a JWH compound. In the analysis of urine samples, the metabolites of the synthetic cannabinoids are more prevalent than the parent compounds, which may not be seen at all (1, 7, 12, 14, 34, 36-38). Due to these results, the sample preparation and analysis must be optimized for the metabolites themselves. The synthetic cannabinoids exist in various forms with their own metabolites, all of which have different properties and react with the methods of analysis in diverse ways. This variety is why synthetic cannabinoids are difficult to analyze. Compounds are not always identified because extensive libraries do not exist.

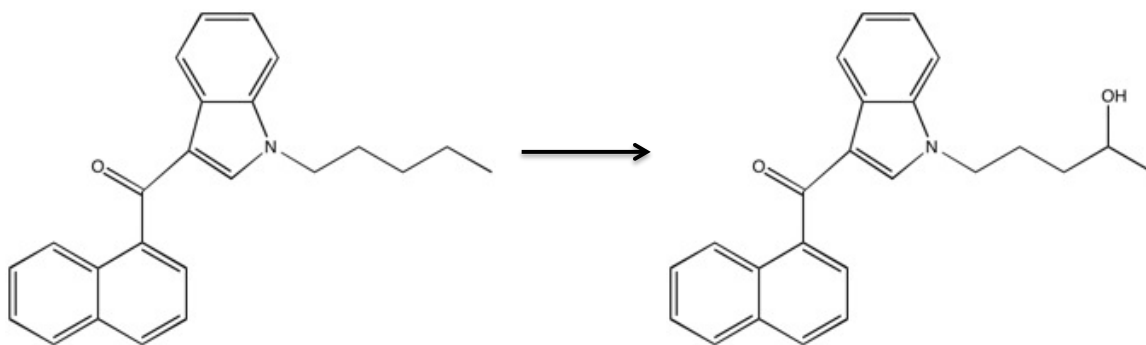
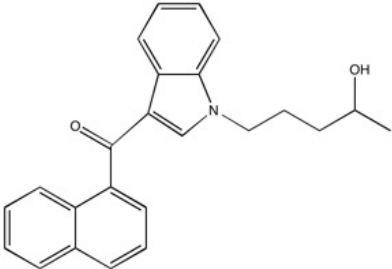
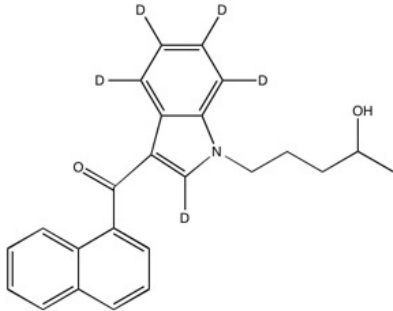
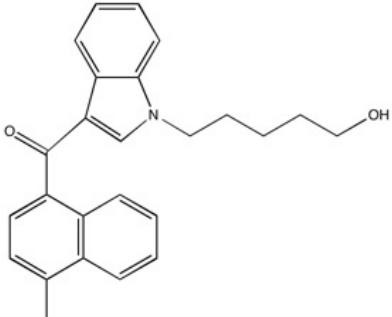
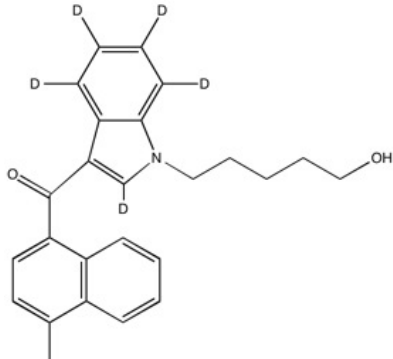
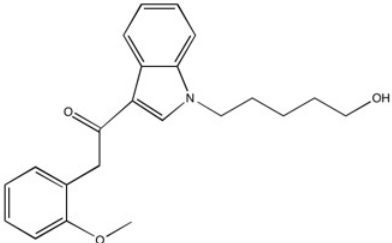
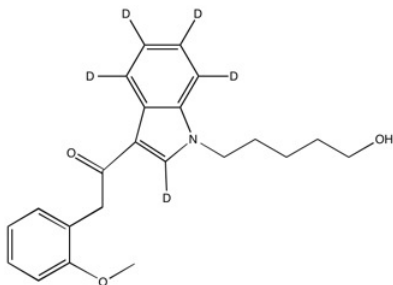


Figure 2: Aliphatic hydroxylation. Synthetic cannabinoid JWH-018 metabolized to JWH-018 N-(4-hydroxypentyl).

1.2.2 Metabolites Utilized in this Study

Three synthetic cannabinoid metabolites were selected to use in this study. Compounds JWH-018 N-(4-hydroxypentyl), JWH-122 N-(5-hydroxypentyl), and JWH-250 N-(5-hydroxypentyl) metabolites were chosen for their availability and appearances in scientific literature. The JWH-018 compound was first identified in smoking mixtures in Germany and Austria in 2008, with JWH-250 identified in 2009, and JWH-122 in 2010 (7, 10, 38). The monohydroxylated forms of the metabolites, with the hydroxyl group on the aliphatic chain, were found to be the most common in urinalysis (7, 38). For these reasons, these metabolites with their deuterated internal standards seen in Table 1, were chosen for this study.

Table 1. Structures of the metabolites and deuterated internal standards utilized in this study.

Synthetic Cannabinoid	Structure	d ₅ Internal Standard
JWH-018 N-(4-hydroxypentyl)	 MW: 357	 MW: 362
JWH-122 N-(5-hydroxypentyl)	 MW: 371	 MW: 376
JWH-250 N-(5-hydroxypentyl)	 MW: 351	 MW: 356

1.2.3 Current Methods of Analysis for Synthetic Cannabinoids and Metabolites

There have been multiple studies done testing methods of analysis on specific parent compounds and metabolites, including several JWH compounds (7, 8, 10, 37, 38).

These studies are typically conducted individually, showing the difficulty in creating a unified method of analysis for all synthetic cannabinoids and their metabolites. With the limited availability of the compounds, research conducted on the variety of synthetic cannabinoids and their metabolites has been difficult. Early research in the analysis of the metabolites required the researchers to synthesize the metabolites themselves (36). In vitro studies using human liver microsomes and in vivo studies using chimeric mice to create the metabolites were performed so analysis of the metabolites could be conducted (12, 14). Companies are now beginning to synthesize the parent compounds and their metabolites at a faster rate, allowing more research to be conducted. However, the recent scheduling recommended by the SDAPA in 2012 hinders research because schedule I licenses are now required to study these compounds.

Methods of analysis for synthetic cannabinoids vary greatly depending on the compounds, whether the metabolites are involved, and the matrix of the sample. Solid phase extraction (SPE) and liquid-liquid extraction (LLE) are used in sample preparation of the synthetic cannabinoid metabolites. Gas chromatography (GC) and liquid chromatography (LC) are used for separation, coupled with mass spectrometry (MS) for detection. Synthetic cannabinoids have been detected on plant material from herbal incense samples purchased online using GC/MS for analysis (20, 26). Methods using GC/MS and LC/MS have been validated to identify synthetic cannabinoids from different matrices like blood, hair, and oral fluid (3, 28, 39, 40). Research studies have been performed comparing solvent extraction and SPE to isolate the metabolites from urine, of which little difference was found (7, 38). Extracted urine samples for synthetic

cannabinoid metabolites have been studied by different forms of both LC/MS and GC/MS. The two methods of analysis have been compared, with LC being able to identify more of the minor metabolites (7, 37).

One study conducted by Dowling and Regan performed a quick method of analysis in which the researchers spiked urine with CP-47,497 and utilized LC-MS/MS for the analysis by diluting the samples with water and acetonitrile without extraction (6). The removal of a sample preparation step before analysis is beneficial, allowing time and money to be saved.

Restek performed a study on extraction efficiency of JWH-018 and -073 metabolites using a C18 SPE cartridge, with analysis by LC-MS/MS (41). They were able to develop a procedure to extract a wide range of metabolites from the samples, including monohydroxylated and carboxylated metabolites (41). This experiment is significant because the range of metabolites produced by the body interact with the sorbent of the cartridges differently. The carboxylated and hydroxylated metabolites are usually extracted separately due to their differences in pKa values. A procedure that can only isolate one type of metabolite is not as useful in a real life setting, since the drugs of abuse can be mixtures of many compounds.

1.3 Solid Phase Extraction

SPE is a sample preparation method that isolates the analytes of interest from biological matrices (35). This is achieved via a sorbent that interacts with the analyte. Solvents are eluted through the cartridge to remove extraneous components according to

the affinity of the compounds with the sorbent (35). The interactions can be manipulated using solubility, adsorption, binding, or electrostatic interactions between the sorbent and analyte. SPE has multiple benefits over LLE, which uses a larger volume of solvents to separate compounds, has limited quantitative recoveries because it may require a three-step extraction procedure, and may undergo incomplete phase separations (35). SPE also minimizes the amount of liquid organic waste and can be automated (35).

The process of SPE includes four main steps: conditioning the cartridge to prepare the sorbent and pack it evenly, applying the sample for the analyte to interact with the sorbent, washing the column to remove the unwanted components, and eluting the analytes with different solvents. Extraction efficiency is not 100% because the analytes interact with the sorbent to varying degrees. The analytes could have a weak interaction with the sorbent and be eluted before the elution step, or they could have a strong interaction and stay adsorbed to the sorbent after elution. For a more efficient extraction, the method development, solvents utilized, and choice of sorbent are critical for the sample preparation.

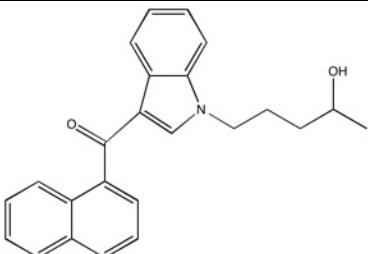
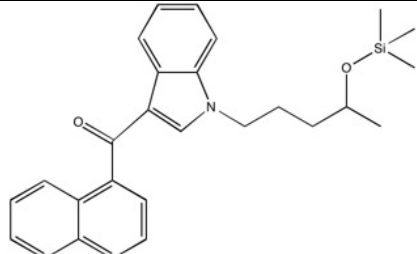
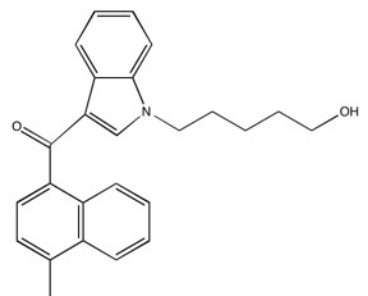
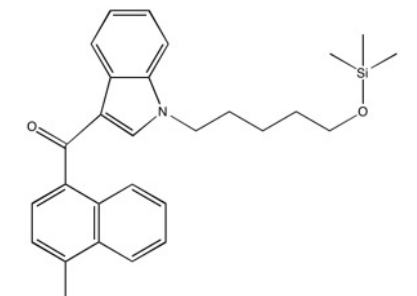
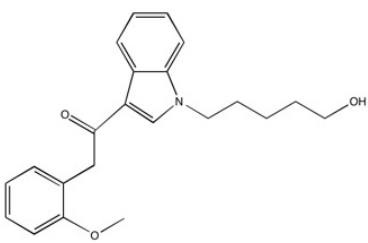
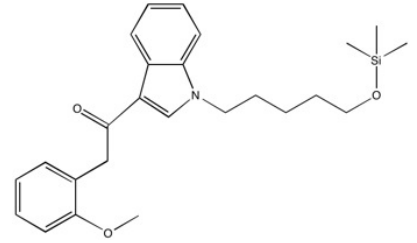
1.4 Derivatization

While GC is an appropriate separation method for many substances, it has specific requirements. The compounds must be volatile because they are vaporized in the injection port and enter the column as a gas. The high injection port temperatures needed for vaporization also require that the compounds be thermally stable so they do not decompose before reaching the column (42). The last consideration for GC is the

polarity of the compounds. Polar groups can interact with a polar column, causing the analytes to elute broadly, resulting in poor peak shape (42). To alleviate these issues, derivatization is utilized. This procedure modifies the compounds to increase thermal stability and volatility, and decrease polarity (43).

Since the body metabolizes synthetic cannabinoids by making them more polar, a derivatizing agent is highly recommended when analyzing toxicological samples by GC/MS. N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) is a silylating reagent that replaces the labile hydrogen of the hydroxyl with a trimethylsilyl group, increasing volatility and thermal stability (42). Trimethylchlorosilane (TMCS) is a catalyst that helps to increase the reactivity of BSTFA (43). The derivatized metabolites used in these experiments can be seen in Table 2.

Table 2. Structures of the metabolites and derivatized metabolites utilized in this study.

Synthetic Cannabinoid	Structure	Derivatized Metabolite
JWH-018 N-(4-hydroxypentyl)	 MW: 357	 MW: 429
JWH-122 N-(5-hydroxypentyl)	 MW: 371	 MW: 443
JWH-250 N-(5-hydroxypentyl)	 MW: 351	 MW: 423

1.5 Objective of this Research

The purpose of this research was to compare the reproducibility and efficacy of four brands of SPE cartridges for the simultaneous extraction of the three JWH metabolites from urine. In order to accomplish this, the instrumental and data analysis methods were optimized for these specific metabolites and their internal standards. An appropriate GC/MS method was selected and optimized for analysis of the compounds.

Next, a SPE procedure from United Chemical Technologies (UCT) for the extraction of “Spice” from urine was utilized to isolate the metabolites (44). Triplicate samples of three concentrations were compared for each cartridge. A multi-point calibration method was created to quantify the metabolites after the extraction from urine.

Since these synthetic cannabinoids have a higher affinity than $\Delta 9$ -THC to the cannabinoid receptors, a lower dose is needed to obtain the same high. This results in a lower concentration located in body fluids, so a change in the reproducibility of the cartridges could be very important. If one cartridge is better suited for lower concentrations, it should be utilized above a cartridge that produces no results. Numerous brands of cartridges and SPE methods were researched in order to determine the best sorbent and procedure to utilize for these analytes. The same SPE procedure was conducted on all of the samples and observations were made to determine the consistency of the elution flow rate through the cartridges. These experiments allowed for a comparison of the cartridges, with the most appropriate brand to be determined.

2. EXPERIMENTAL

2.1 Materials

The metabolites and deuterated internal standards of (\pm)-JWH-018 N-(4-hydroxypentyl), JWH-122 N-(5-hydroxypentyl), JWH-250 N-(5-hydroxypentyl), (\pm)-JWH-018 N-(4-hydroxypentyl)-d₅, JWH-122 N-(5-hydroxypentyl)-d₅, and JWH-250 N-(5-hydroxypentyl)-d₅ were obtained from Cayman Chemical. The chemicals purchased from Fisher Scientific include methanol (MeOH), deionized water (diH₂O), ethyl acetate (EtOAc), and acetonitrile (ACN). Anhydrous mono- and di-basic sodium phosphates, NaH₂PO₄ and Na₂HPO₄, were obtained from Acros Organics. Synthetic urine and BSTFA with 1% TMCS were obtained from Cerilliant. The nitrogen gas was purchased from Airgas East. A TurboVap LV Evaporator from Zymark and an Isotemp 2025 hot water bath from Fisher Scientific were used for solvent evaporation and derivatization. The four, reverse phase, anion exchange solid phase extraction cartridges were purchased from Agilent Technologies, United Chemical Technologies (UCT), Thermo Scientific, and SiliCycle. Specifics about the cartridges names can be seen in Table 3.

Table 3. SPE cartridges compared in these experiments.

Vendor	Cartridge Name
UCT	Clean Screen Extraction Columns CSTHC
SiliCycle	Siliaprep C8/SAX
Agilent	Bond Elut Certify II
Thermo	HyperSep Verify AX

An Agilent 7890A GC combined with an Agilent 5975C inert EI/CI MSD was equipped with an Rxi®-5ms (30 m x 0.25 mm x 0.25 µm) column from Restek with a temperature limit of 350°C. As a result of the nature of the compounds and the GC/MS parameters requiring a higher injection port temperature, the column was changed to an Rxi®-5ht (30 m x 0.25 mm x 0.25 µm) column from Restek with a higher temperature limit of 400°C. Data analysis was conducted using MSD ChemStation E.02.00.493 software from Agilent. GC vials, liners, and caps were obtained from Fisher Scientific.

2.2 GC/MS Method Development

A stock solution of each metabolite was prepared in methanol at a concentration of 1 mg/mL. A working solution containing all metabolites in methanol at a concentration of 50 µg/mL as well as a working solution of the internal standards in methanol at 5 µg/mL were prepared for the GC/MS method development. Mixtures of the three metabolites in a 1:1:1 ratio were added to methanol and six GC/MS methods were tested. A method from Agilent Technologies for the “Confirmation and Quantification of Synthetic Cannabinoids” resulted in the best resolution between peaks, had the fastest run time, and increased sensitivity for the detection of these metabolites (45). This method was selected and changes were made for the specific metabolites. Interday analysis by GC/MS fluctuated greatly. Therefore, multiple parameters of the method were tested in order to reduce the variation. The injection temperature, injection volume, inlet mode, pressure and total flow, oven program, carrier gas flow rate, transfer line temperature, and solvent delay were optimized for the metabolites. A new GC liner

set aside for these experiments was used every time the metabolites were injected into the instrument because it was found to produce more reproducible chromatograms in comparison to a liner used for other samples.

For quantification of the analytes, a multi-point calibration method was created with the ChemStation software, using the optimized GC/MS method. Seven calibration standards of the three metabolites from 30 ng/mL to 10 µg/mL with a deuterated internal standard concentration of 500 ng/mL were diluted and used to make the calibration curves.

The results from the neat compounds were not consistent throughout early analysis conducted on the metabolites. To combat the interday fluctuations of the instrumental analysis, derivatization of the metabolites was performed using BSTFA with 1% TMCS. A new set of six calibration standards from 5 ng/mL to 10 µg/mL of the metabolites and 300 ng/mL of the internal standards were evaporated to dryness, reconstituted in 50 µL BSTFA with 1% TMCS, and immersed in a 65°C water bath for 35 minutes. The previous GC/MS method was optimized for these derivatized compounds by testing parameters focusing on the oven program, injection temperature, and transfer line temperature. The parameters for the final GC/MS method are shown in Table 4.

Table 4. GC/MS parameters for analysis of the derivatized metabolites.

Parameter	
Inlet Mode	Split-less
Injection Temperature	300°C
Injection Volume	2 µL
Carrier Gas	Constant flow, 0.9 mL/min
Oven Program	100°C for 1 min, then 25°C/min to 300°C for 0.5 min, then 5°C/min to 325°C for 3 min
Transfer Line Temperature	280°C
Solvent Delay	5 min
Total Run Time	17.5 min

A SIM method for the metabolites and internal standards was created to increase sensitivity for the detection of these metabolites. A dwell time of 50 milliseconds was utilized. The literature recommends three high mass ions for the analyte and two for the internal standard to be used for quantification (46). The SIM method assesses the selected quantifying and qualifying ions of the metabolites and internal standards in order to reduce the baseline of the chromatographic separation (35). Utilizing two ions allowed the internal standard to be measured by a ratio, instead of the appearance of one ion. The ions chosen for the metabolites and their internal standards are shown in Table 5.

Table 5. Ions selected for SIM method.

Metabolite Group	Ions
Group 1 (JWH-250)	MTB: 144, 302*, 423
	ISTD: 307*, 308
Group 2 (JWH-018)	MTB: 270*, 296, 414
	ISTD: 418, 433*
Group 3 (JWH-122)	MTB: 298, 428, 443*
	ISTD: 433, 448*

Metabolite (MTB) and internal standard (ISTD) ions.

*Starred ions indicate quantifying ions.

2.3 Solid Phase Extraction Procedure

UCT developed a SPE procedure for the extraction of synthetic cannabinoids from urine, which was used in these experiments (44). Sample preparation started with 1 mL of synthetic urine that was spiked with the metabolites and internal standard. The urine was diluted with 2 mL of the pH 6 phosphate buffer solution. The SPE procedure was conducted by gravity elution and the steps can be seen in Figure 3. To concentrate the metabolites, the 6 mL of the 90:10 ethyl acetate and methanol solution was evaporated to dryness. An evaporator was used to emit a constant stream of nitrogen gas over the solution, while the tubes were placed in heated water. The samples were reconstituted in 50 μ L of BSTFA with 1% TMCS, capped, and immersed in a 65°C water bath for 35 minutes. The heat helped to catalyze the derivatization process. The samples were then injected into the GC/MS for instrumental analysis.

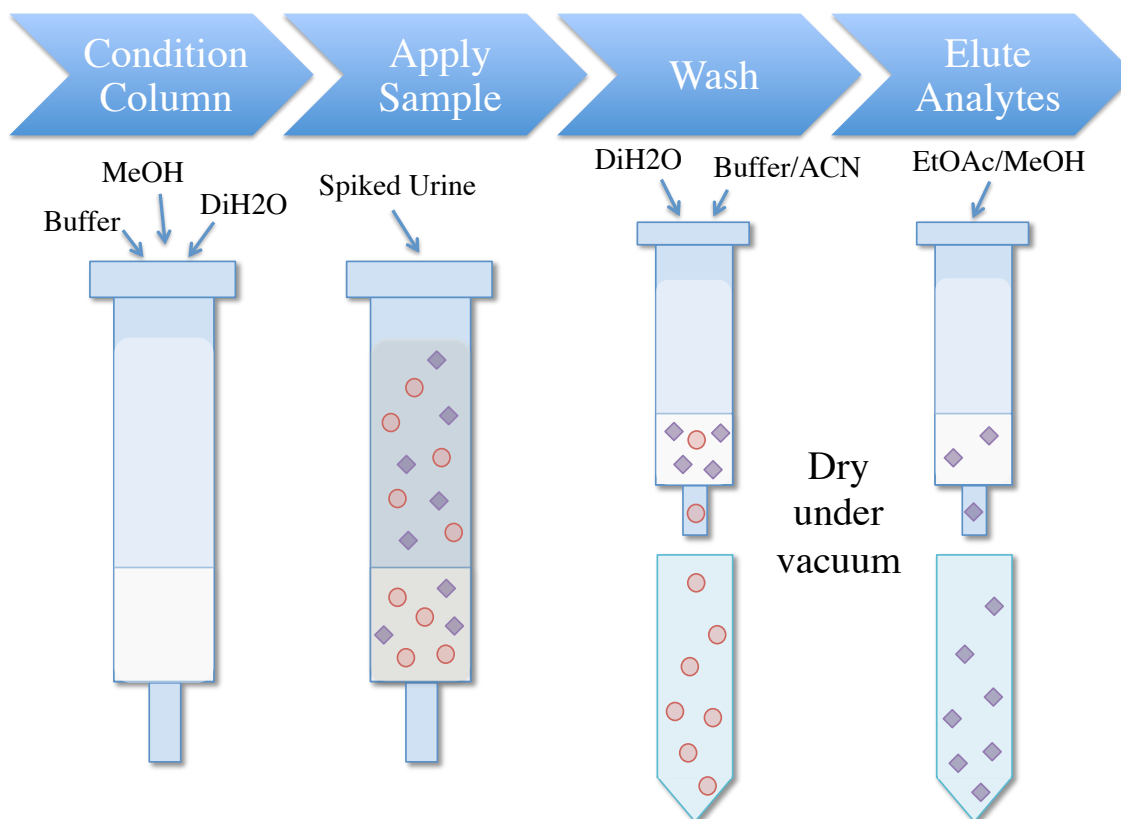


Figure 3: Visualization of the SPE procedure. The SPE cartridges were conditioned with 3 mL methanol followed by 3 mL diH₂O and 1 mL buffer solution. The sample of 3 mL spiked urine was applied to the cartridge and then washed with 3 mL diH₂O and 3 mL of the 80:20 buffer and acetonitrile solution. The cartridges were dried under vacuum for 5 minutes at >10 in mercury. The metabolites were eluted with 6 mL of the 90:10 ethyl acetate and methanol solution into clean vials.

2.4 Data Collection

Working solutions of the metabolite mixture were created in methanol at 10, 1, and 0.05 µg/mL concentrations. A working solution of the deuterated internal standard mixture was created in methanol at a 1 µg/mL concentration. Four sets of calibration standards were spiked into urine samples between 50 and 2000 ng/mL of the metabolites

and 300 ng/mL of the internal standards. The urine samples were used to create calibration curves for each of the four brands of cartridges.

The metabolites were spiked into synthetic urine at three concentrations, 1000, 500, and 250 ng/mL, with 300 ng/mL of the deuterated internal standards. The SPE procedure was performed on the spiked samples in triplicate analysis for each of the four brands. Blank synthetic urine samples were also processed using the SPE procedure in triplicate analysis.

2.5 Data Analysis

Multiple calibration curves for the synthetic cannabinoid metabolites spiked in methanol were created to determine the linear dynamic range of the GC/MS method. A multi-point calibration method for the quantification of the neat standards was made using ChemStation software. Calibration standards were prepared and the response ratio of the metabolite to the internal standard was plotted against the concentration ratio of the metabolite to the internal standard. A least-squares line was applied and the coefficient of determination was used to determine the linearity of the curve. Calibration curves were also created using the extracted spiked urine samples. They were made for each metabolite and brand of cartridges compared in this research. The methods were used for the quantification of the metabolites in the spiked samples.

A recovery efficiency study was performed using the metabolite responses of the quantification ions. The ratio of extracted metabolite responses from the cartridges to the

metabolite responses from neat, un-extracted samples spiked with the same concentrations was determined and a percent recovery was calculated from this ratio.

One study from the Scientific Working Group for Forensic Toxicology (SWGTOX) was also used to analyze the collected data. A within-run precision study measured the coefficient of variation for the triplicate samples of each cartridge brand using the standard deviation and mean response of the replicates (47):

$$\% CV = \frac{s}{\text{mean response}} * 100$$

3. RESULTS AND DISCUSSION

3.1 GC/MS Concerns During Development of the Method

3.1.1 GC/MS Method Optimization

The GC/MS parameters from Agilent Technologies were found to be the most efficient for the detection of the three metabolites with the shortest run time, best resolution, and highest abundance for each metabolite. During method optimization, several parameters were tested in order to create a method that produced consistent results. A GC column with a temperature limit of 350°C was used during GC/MS method development. Column degradation occurred due to the high temperatures above 300°C for the injection port and oven parameters in the GC method. The number of samples analyzed also affected the degree of degradation to the column. This created abundant siloxane peaks in the chromatograms at the lower oven temperatures, as well as an increased baseline from a constant elution of siloxanes at the higher temperatures. To decrease the damage, a column with a higher temperature limit of 400°C was utilized for the experiments conducted after the initial GC/MS method development phase.

Interday analysis of the neat standards showed a great deal of variation in the peak area and ratio of the metabolites to each other. An overlay of chromatograms of aliquots of the same sample injected on different days for three months can be seen in Figure 4. Observed in this figure are the peak heights of JWH-250 decreasing more significantly than the peaks of JWH-122. This indicates that the interday variation affects each metabolite differently.

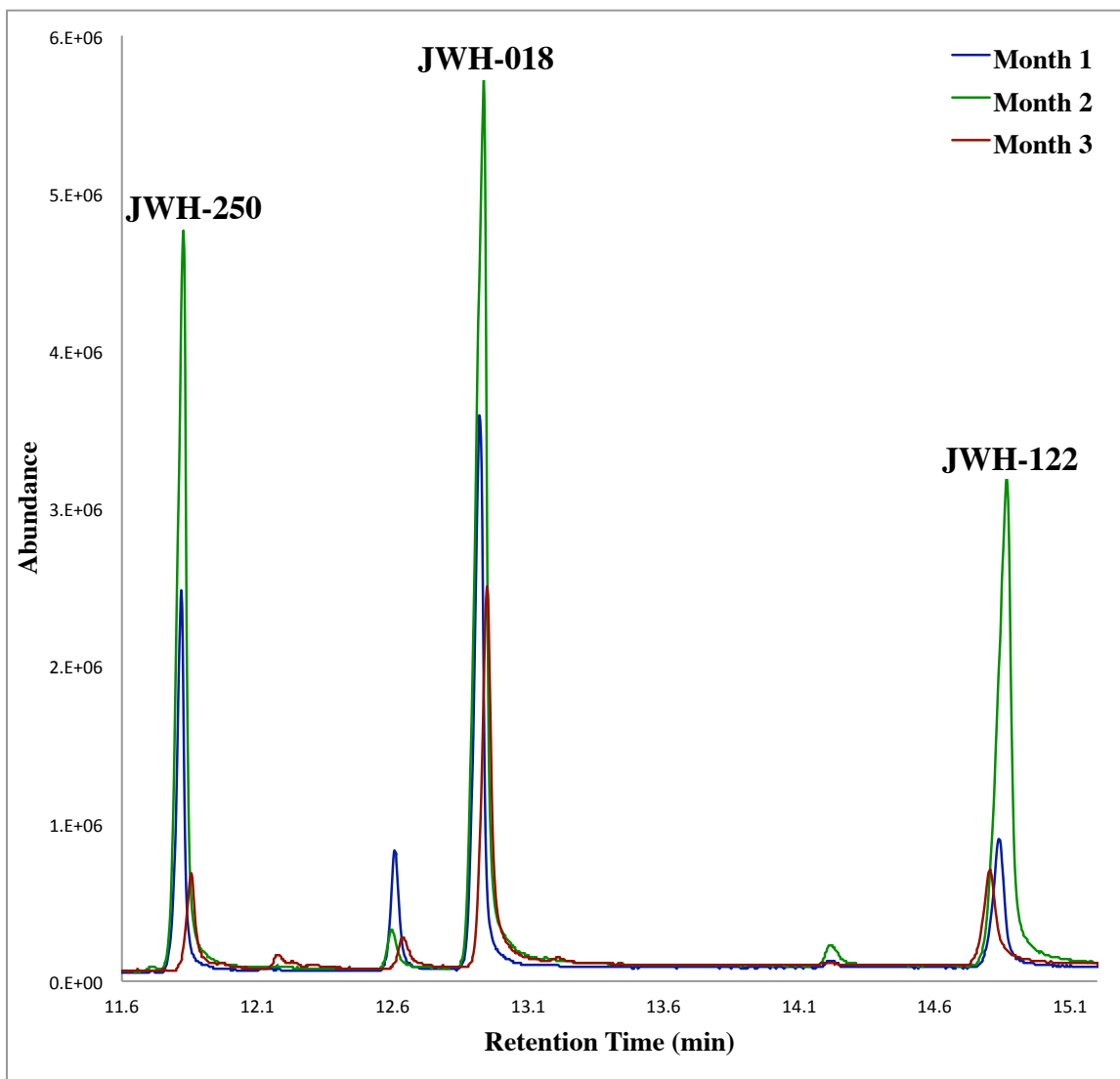


Figure 4: Chromatograms of the interday peak abundance variation of the metabolites. Analysis of the three underivatized metabolites neat in methanol was conducted separately over three months to show the significant differences in peak area using the same method. The ratios of the peak heights between the different metabolites were also affected. Due to the abundance of the metabolites increasing between months 1 and 2, it was determined that the variation was not due to the degradation of the metabolites in methanol.

3.1.2 JWH-018 Internal Standard

The three internal standards in these experiments were chosen because they were the deuterated analogues of the three metabolites. The internal standards are heavier than the metabolites, replacing five hydrogen atoms with deuterium. The deuterated compounds improved the precision of the method because the metabolites and internal standards interact with the column in the same way (35). During GC separation, the compounds have similar retention times, but due to the mass difference, analysis by MS identified them. Each internal standard was a d_5 version of the metabolite; therefore, the molecular ions of the internal standards were expected to be 5 atomic mass units (amu) greater than the metabolites. However, the JWH-018 internal standard had a molecular ion that was only 4 amu higher than the JWH-018 metabolite. The mass spectrum of the JWH-018 internal standard can be seen in Figure 5.

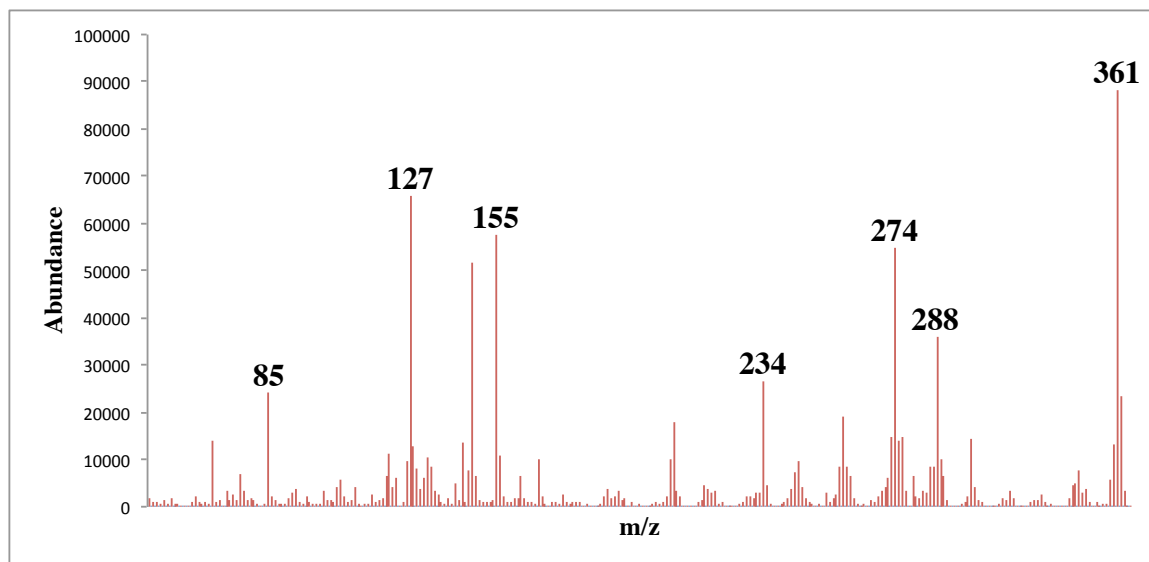


Figure 5: Mass spectrum of JWH-018 deuterated internal standard. The molecular ion is only 4 amu greater than the molecular ion of JWH-018 metabolite of 357 amu.

To determine whether this was a fragmentation effect from the hard ionization method of electron impact, a sample was analyzed by electrospray ionization (ESI) LC time of flight MS at Northeastern University. ESI is a softer method of ionization that results in the protonated molecular ion $(M+H)^+$, so the molecular ion observed is 1 amu higher than the molecular weight of the compound.

The molecular ion by ESI-LC-TOF-MS was 362 amu. Including the addition of the hydrogen, the results were in agreement with the molecular ion observed by electron impact ionization, which was 361 amu. This indicated the ionization method did not cause the fragmentation. The mass spectrum of the JWH-018 internal standard from the softer ionization method of ESI can be seen in Figure 6. Even with derivatization, the molecular ion for the JWH-018 internal standard was 433 amu, which was 4 amu higher than the derivatized JWH-018 metabolite of 429 amu. Since the results were consistent throughout the experiments, the internal standard was still utilized.

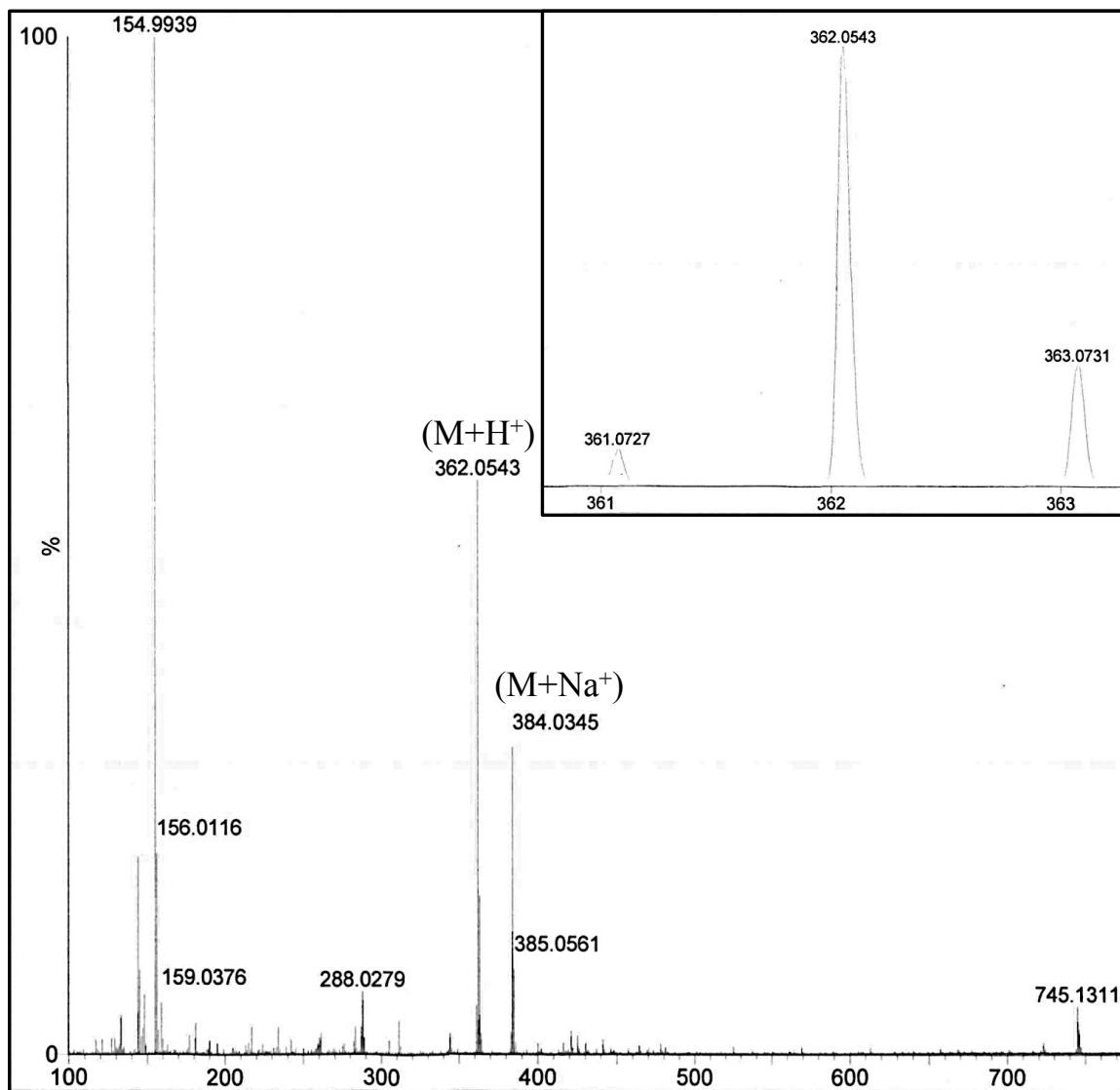


Figure 6: Mass spectrum of JWH-018 deuterated internal standard from electrospray ionization. The spectrum shows the (M+H)⁺ ion at 362. The 384 peak is from the addition of sodium (M+Na)⁺ to the compound. The inset is a close up of the (M+H)⁺ peak.

3.1.3 GC/MS Injection Liner Considerations

Initial tests running neat standards in methanol produced the three metabolite peaks and three additional peaks eluting approximately 0.3-0.6 minutes before their respective metabolites, which is observed in Figure 7. These additional peaks contained

molecular ions that were 2 amu less than the metabolite and deuterated internal standard peaks they preceded, indicating a loss of two hydrogen atoms. The degradation peaks retained similar fragmentation patterns to the metabolites, of which JWH-018 is shown in Figures 8 and 9.

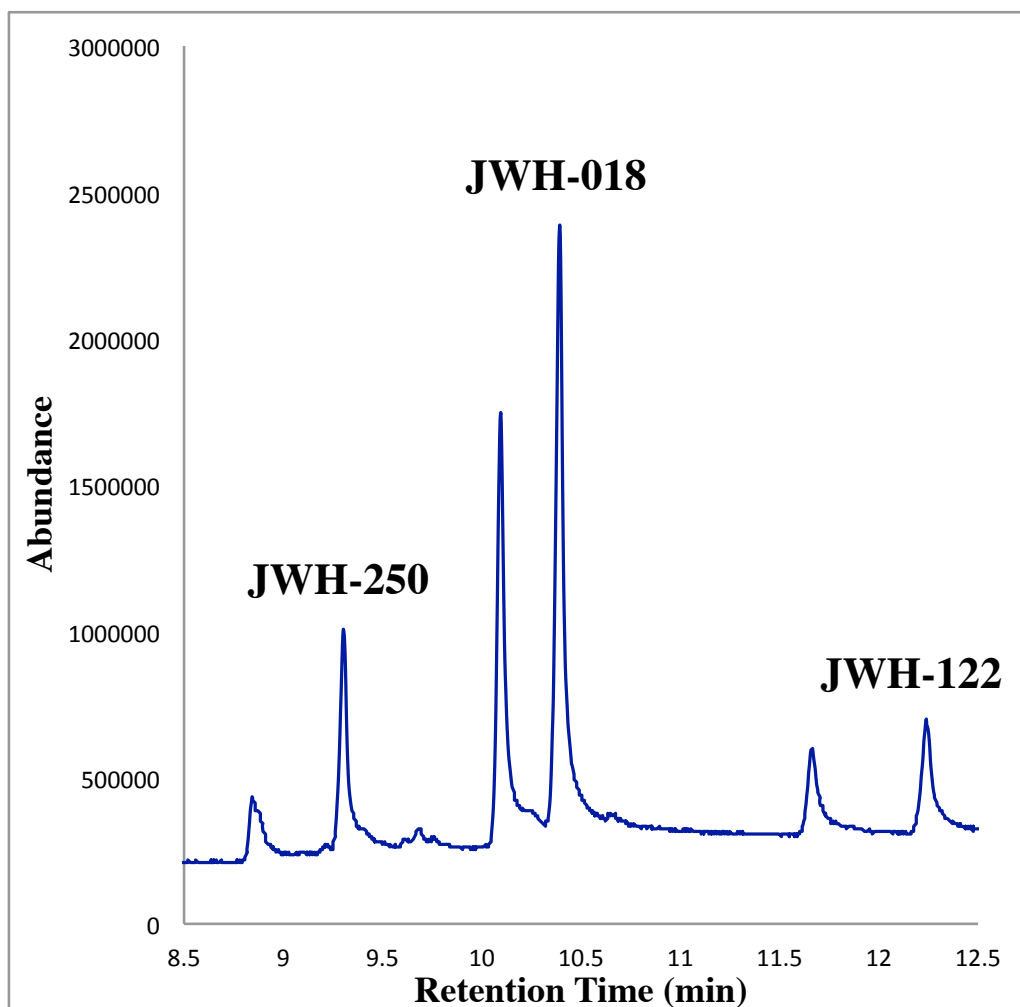


Figure 7: Chromatogram of the degradation observed from the analysis of JWH-250, JWH-018, and JWH-122 metabolites. The peaks observed to the left with a lower retention time are 2 amu less than the metabolites they precede. This chromatogram demonstrates the most extreme degradation seen during this experiment.

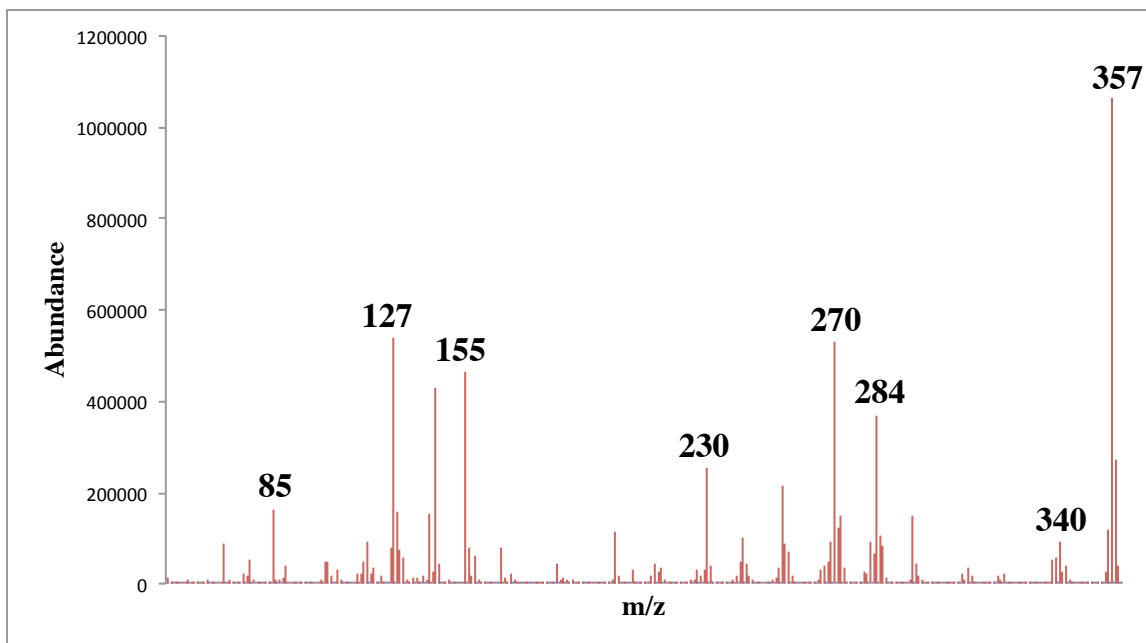


Figure 8: Mass spectrum of JWH-018 metabolite.

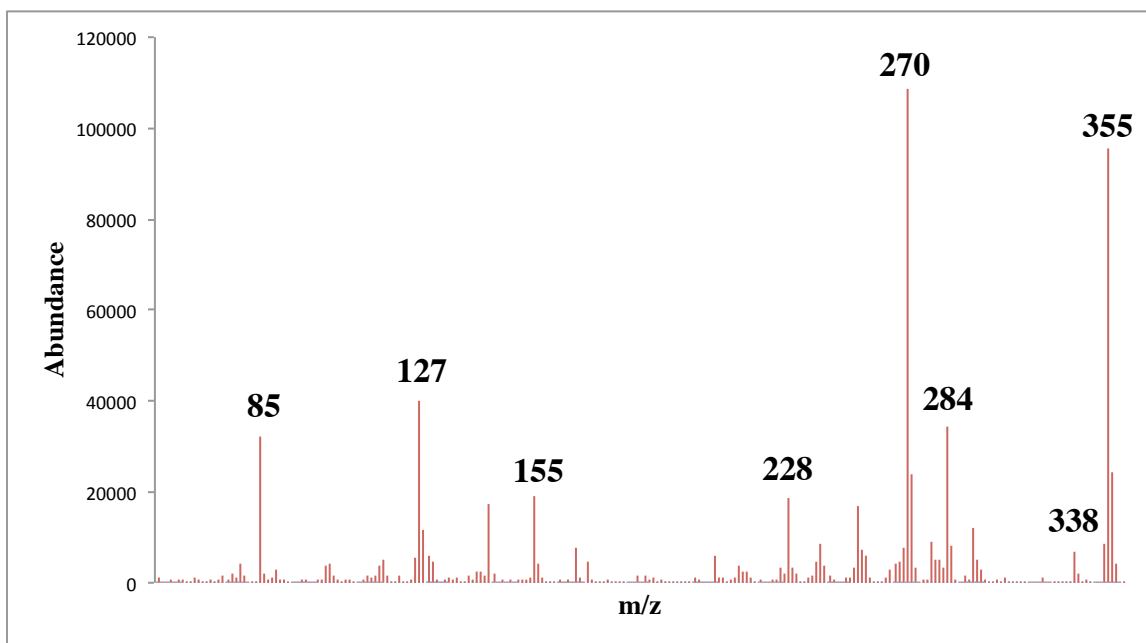


Figure 9: Mass spectrum of peak preceding JWH-018 metabolite.

These peaks were not observed by Caymen Chemical or by LC-TOF-MS analysis. One proposal of the 2 amu difference could be an oxidation of the hydroxyl group,

becoming a ketone in the JWH-018 metabolite and an aldehyde in both JWH-122 and JWH-250 metabolites. To determine whether the oxidation was caused by degradation of the samples, aliquots of the same mixture concentration were tested on different days after being stored for different periods of time. There was no correlation between storage of the standards and peak area of the degraded standards. However, a trend was discovered in the logbook between the instrument maintenance, number of samples injected, and when the metabolites were tested. This information led to the determination that the GC liner affected the analysis of the metabolites. The degraded metabolite peaks persisted until the GC liner was changed.

Table 6 shows the percentage of peak area between the degraded peaks and their corresponding metabolite peaks. The sample in the first trial was the result of a visibly dirty liner. Instrument maintenance which involved changing the liner, septum, and o-ring, and cleaning the ion source was performed on the GC/MS between trials two and three, suggesting a possible solution to the problem, and significantly decreasing the degradation peaks from the JWH-250 and JWH-122 metabolites.

Table 6. Percentage of the degraded peak area to the metabolite peak area.

Trial	JWH-250	JWH-018	JWH-122
1*	50.6%	72.7%	76.6%
2	12.1%	17.6%	19.4%
3	--	19.6%	--
4	--	13.4%	--

*Trial 1 is the chromatogram seen in Figure 6. The dashes represent no percentage differences between the metabolite and degraded peak areas.

Due to the heavy use of the instrument analyzing a wide variety of samples, it was hypothesized that the build up of impurities in the liner caused the oxidation of the metabolites. By using a new liner, there were no impurities to react with the metabolites. An aliquot of a 100 $\mu\text{g}/\text{mL}$ mixture of the metabolites was injected using an older, visibly dirty liner and immediately injected again after the liner had been changed. The chromatogram showing the increased sensitivity and decreased area of the oxidized peaks can be seen in Figure 10. This significant increase in sensitivity allowed for the analysis of smaller concentrations of the standards in the rest of this research

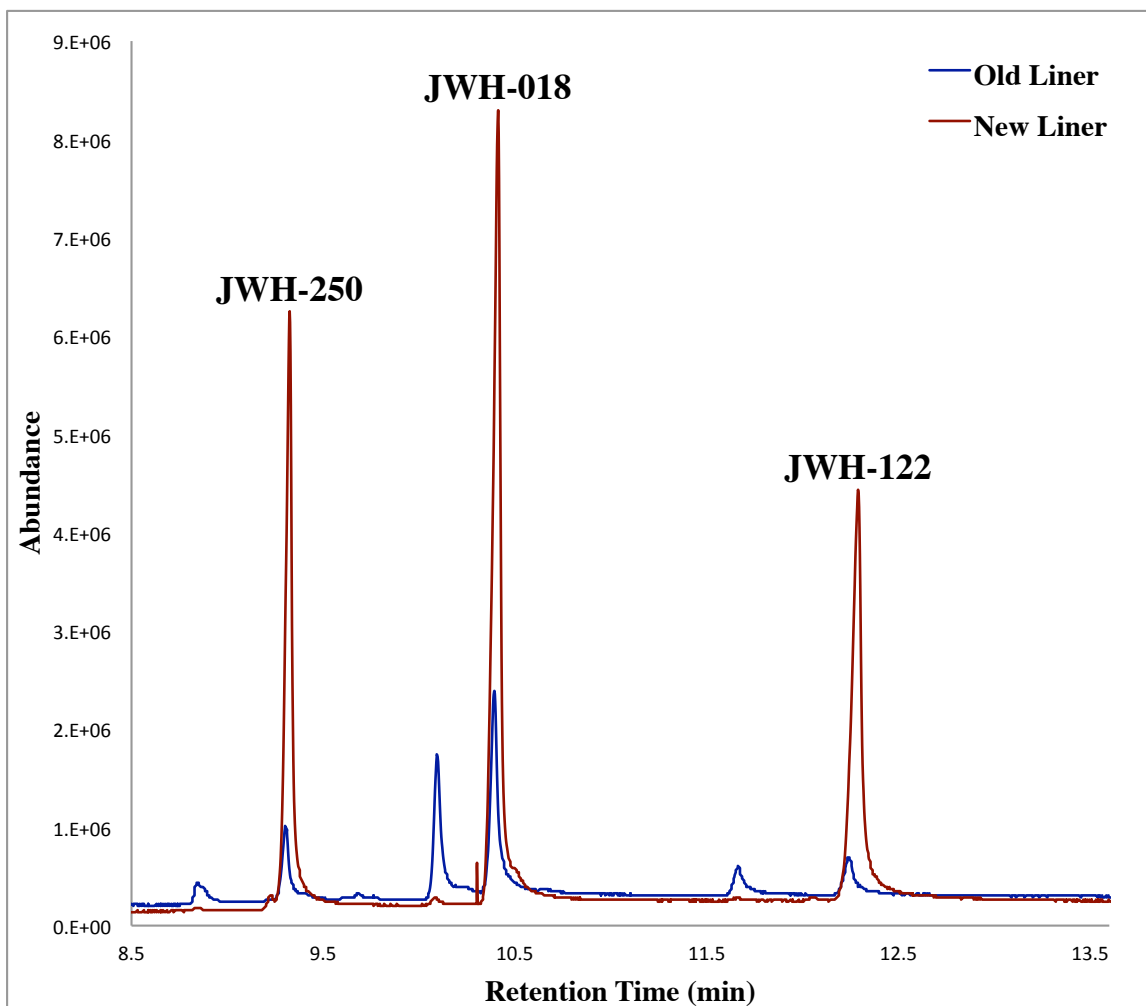


Figure 10: Chromatograms of the metabolites with an old versus a new liner. The overlay of the old liner in blue and new liner in red shows the significant increase in peak area of the metabolites and decrease of the three preceding peaks.

While there was a significant amount of interday variability, samples analyzed on the same day had comparable results. Calibration curves of the neat metabolites spiked in methanol were created. The seven calibration standards were spiked with 30 ng/mL to 10 µg/mL of the metabolites and 500 ng/mL of the internal standards. The coefficient of determination for each metabolite was greater than 0.99 and can be seen in Table 7. In an

effort to determine whether the method maintained linearity at the three lowest concentrations, 30-100 ng/mL, R² values were calculated by ChemStation.

Table 7. Coefficient of determination for the lowest three calibrators and the entire curve.

Metabolite	R² Lowest Concentrations	R² Entire Curve
JWH-250	0.998	0.999
JWH-018	0.999	0.998
JWH-122	0.995	0.999

3.1.4 Derivatization of the Synthetic Cannabinoid Metabolites

The sensitivity of the GC/MS analysis varied from day-to-day, sometimes significantly when the analytes were underivatized. A solution to the sensitivity concerns in the analysis was to derivatize the metabolites using BSTFA with 1% TMCS. Blocking the hydroxyl group of the metabolites with the derivatizing agent decreased the polar interactions with the stationary phase, which decreased the compounds from interacting with the column (43). However, the primary advantage to derivatization is the increased volatility and reproducibility of the flash vaporization process during injection of the samples into the GC. This resulted in a significant increase in peak area of the metabolites, allowing for less of the standards to be spiked into the urine for analysis. Derivatization also helped to increase the thermal stability of the metabolites. Due to the stability and volatility of the derivatized analytes, a more consistent concentration of the metabolites was injected into the column, resulting in more reproducible chromatograms.

The GC/MS method was optimized again for the new derivatized compounds, which focused on oven temperature program, injection port temperature, and transfer line temperature. Since the mass of the compounds was increased by 72 amu from the loss of a hydrogen and an addition of the trimethylsilyl group ($\text{Si}(\text{CH}_3)_3$), a new SIM method was created to account for the differences in mass. A comparison of a 50 $\mu\text{g}/\text{mL}$ metabolite mixture versus a derivatized sample of the same concentration can be seen in Figure 11.

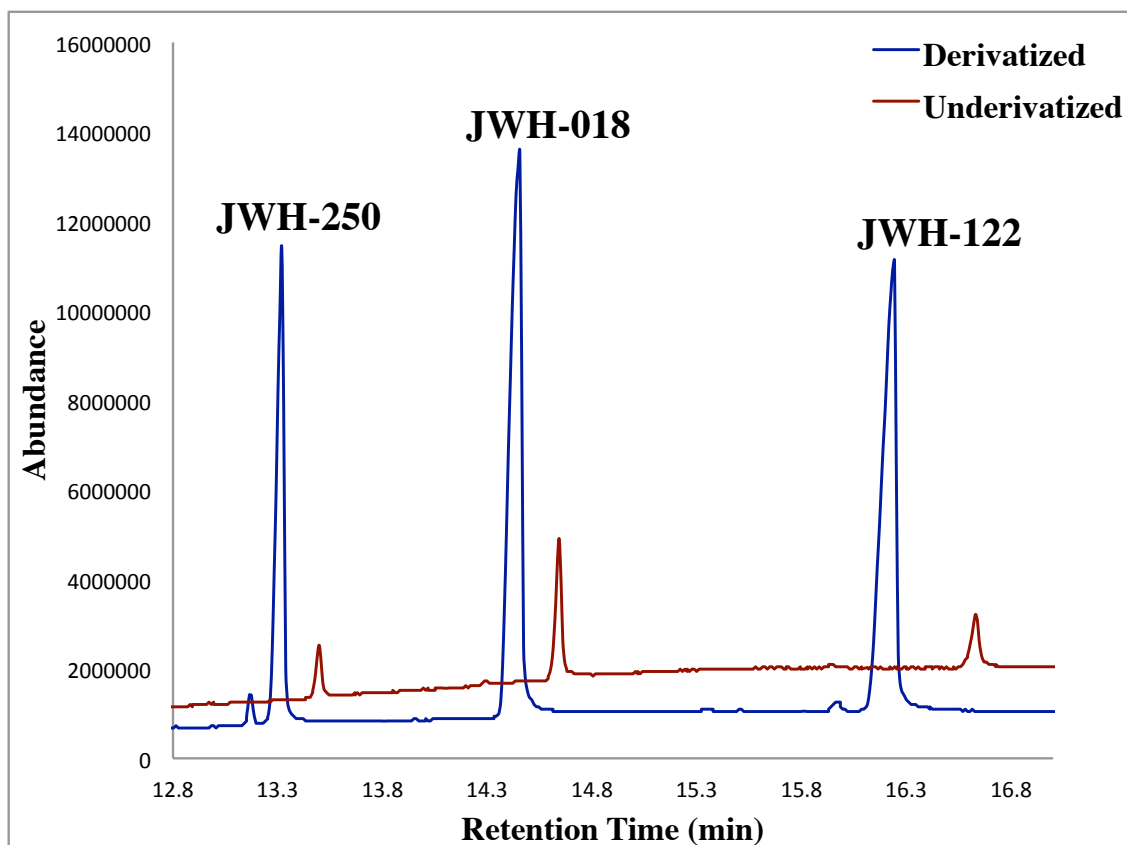


Figure 11: Chromatograms of the metabolites derivatized versus underivatized. Derivatization increased the peak area of the metabolites and decreased the baseline.

Even after all of the considerations and changes to the method, variations in the interday analysis still occurred and can be seen in Figure 12. A 50 $\mu\text{g}/\text{mL}$ mixture of the metabolites was derivatized and injected into the instrument every time samples were

collected and analyzed. The high concentration was analyzed once per every set of samples and was used to compare the results of these experiments with ones conducted previously when a higher concentration was necessary. A separate liner was used for analysis of these compounds; however, it became dirty after a number of samples had been injected. The derivatizing agent caused the liner to become dirtier faster than it had previously. To prevent this from affecting the data, the liner was switched more often. With the increased sensitivity from derivatization, fronting of the peaks was observed, indicating the column was overloaded with the compounds.

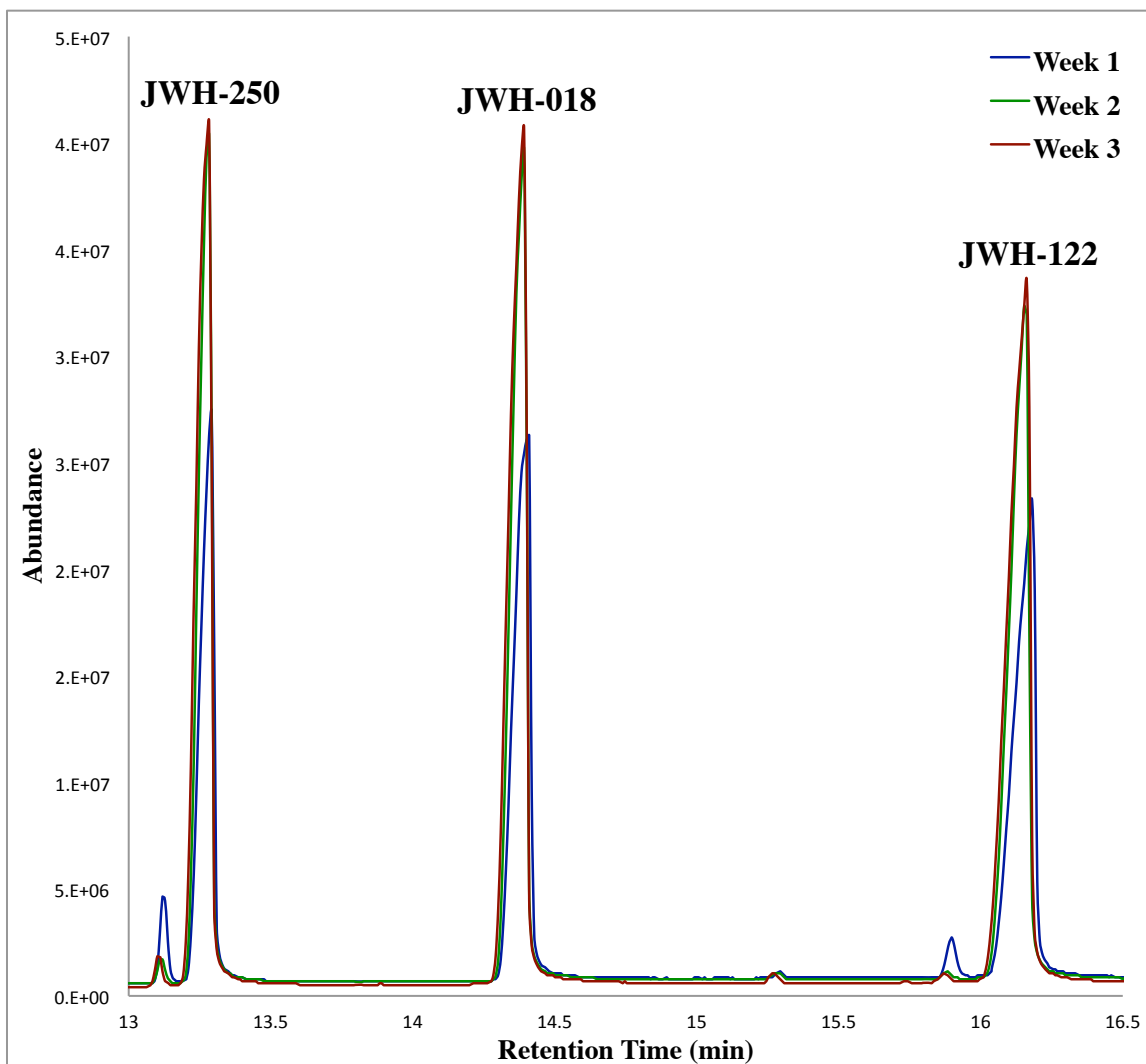


Figure 12: Chromatograms of the interday variation of the derivatized metabolites. Between weeks 1 and 2, a new liner was used. While this showed an increase in abundance of the peaks, the ratios between the metabolites were still similar. The other two samples were also analyzed a week apart; however, they overlapped very well. It indicated that if a clean liner was used with derivatization, the reproducibility increased significantly.

While some interday variability persisted, samples analyzed on the same day had comparable results. A new calibration curve was created including the derivatization step. The seven calibration standards were spiked with 5 ng/mL to 10 µg/mL of the

metabolites and 300 ng/mL of the internal standards. The coefficient of determination for each metabolite was greater than 0.99 and can be seen in Table 8.

Table 8. Coefficient of determination for the lowest three calibrators and the entire curve of the derivatized metabolites.

Metabolite	R ² Lowest Concentrations	R ² Entire Curve
JWH-250	0.999	0.999
JWH-018	0.997	0.999
JWH-122	0.997	0.999

3.2 SPE Procedure Utilized in this Study

3.2.1 Metabolites Interaction with the SPE Cartridges

In the SPE procedure, urine was diluted with a pH 6 phosphate buffer to normalize the samples. Since an individual's urine can have different pH values, adding the buffer regulates the starting pH for all samples. This also kept the metabolites in a neutral state, thus the only interactions were between the nonpolar portions of the cartridges and metabolites. A lower pH would cause the tertiary amine to be protonated and a higher pH would cause the hydroxyl to be deprotonated. Elution by gravity was used for all of the cartridges. This is the slowest form of elution, which allows for the most efficient extraction of the analytes.

The conditioning of the cartridge removed impurities remaining from the manufacturing process and evenly packed the sorbent, while the buffer prepared the cartridge for the buffered urine sample. After the sample was applied, the cartridge was washed with deionized water to remove the aqueous impurities. The 80:20 buffer and

acetonitrile solution removed the other unwanted components of the urine, including the neutral biologicals, polar compounds, and residual organics. The cartridge was dried under a vacuum in order to remove all remaining aqueous solvents because they interfere with derivatization and are difficult to evaporate. After the collection vial was changed, the ethyl acetate and methanol solution eluted the metabolites from the cartridge.

3.2.2 Observations Throughout the SPE Procedure

During the SPE procedure, differences between the brands were observed. SiliCycle had the most consistent flow rate between the cartridges analyzed in triplicate. Within one minute, each of the cartridges were finished eluting the solvents. The procedure from the beginning to the drying step was completed within 22 minutes. Agilent had the most inconsistent flow rate between triplicate samples and could take up to 55 minutes to reach the drying step in the procedure. The results of analyte recovery between the brands also differed. The UCT and SiliCycle cartridges produced results every time the procedure was conducted; however, Thermo and Agilent had several cartridges that gave no results after spiking the urine and conducting the analysis. Table 9 shows the observations made during comparison of the four brands throughout the SPE procedure.

Table 9. Observations made throughout the SPE procedure.

Brand	Elution Rate (min)	Time to Drying Step (min)	Percentage of Cartridges with No Results
SiliCycle	<1	22	0
UCT	3-5	40	0
Thermo	<1	20	10.5%
Agilent	10-15; (27)*	55	13%

Note: The second column reports time values for the range of triplicate elution rates. The consistent elution rates for the triplicate samples demonstrate reproducibility of the cartridges.
*One sample was 27 minutes behind the rest of the Agilent cartridges prepared at the same time.

3.2.3 Calibration Curves for the Brands of Cartridges after Extraction of the Metabolites

Calibration standards that were spiked into urine were made in concentrations between 50 and 2000 ng/mL for each of the cartridges. The linearity for each of the brands of cartridges was different. In this study, data points for the UCT and Thermo brands of cartridges were discarded, which allowed for a focused study over the range in which the cartridges were effective. An example calibration curve for SiliCycle is shown in Figure 13.

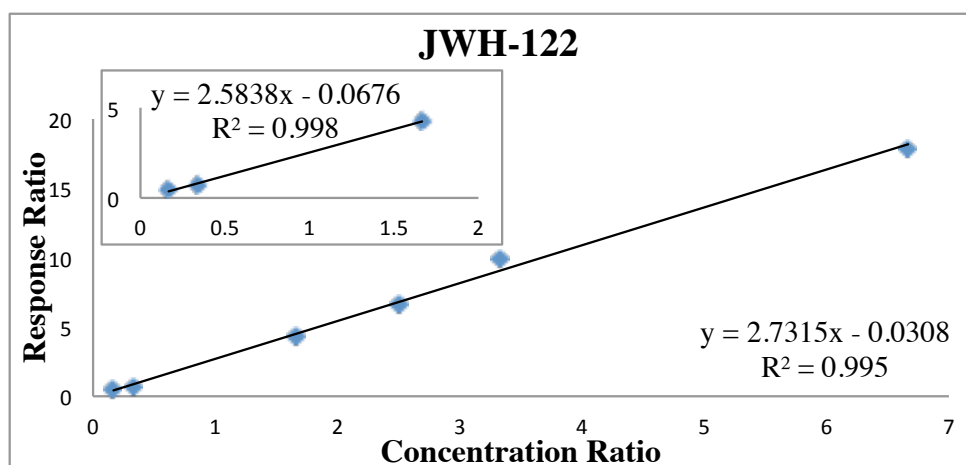
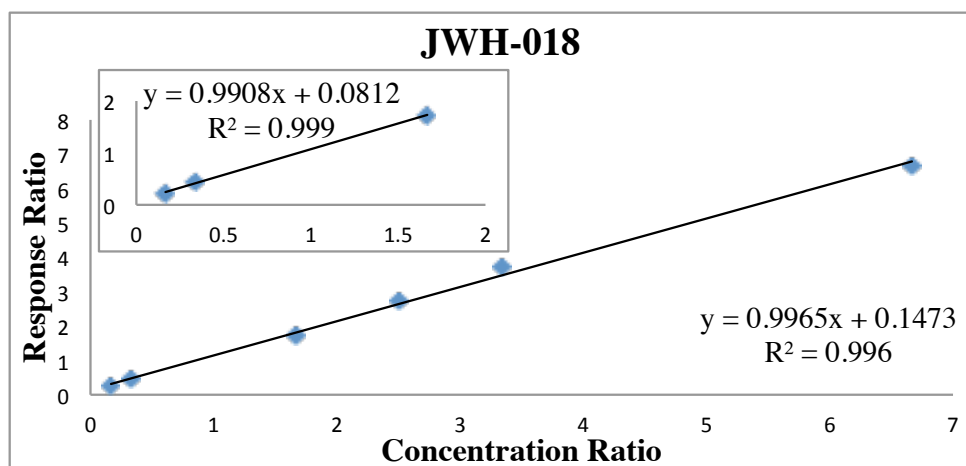
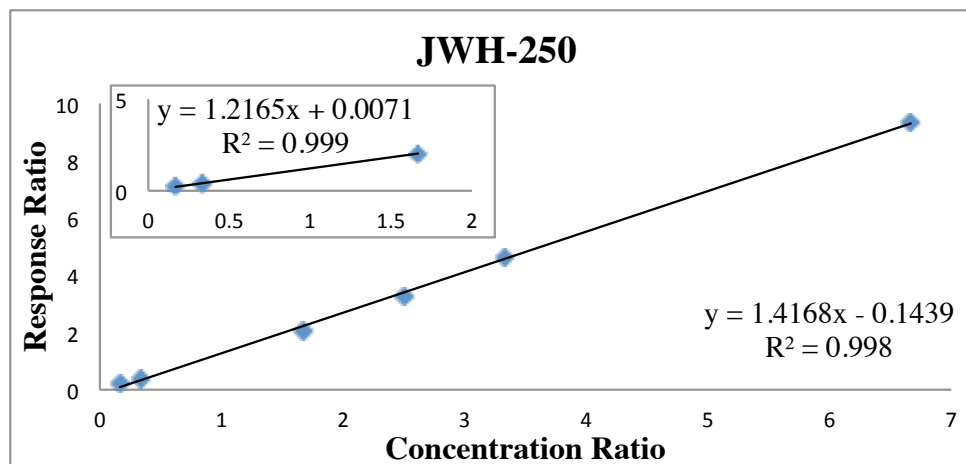


Figure 13: Calibration curves for SiliCycle cartridges. The insets to each curve show the linearity for the three lowest calibrators. The x- and y-axes were calculated by a comparison of the metabolites to the internal standards.

Thermo cartridges had poor linearity at the lower points of the calibration curve; therefore, the lowest data point was discarded. This resulted in an adjusted range of 100-2000 ng/mL, which can be seen in Table 10. While the R^2 values over the entire range was above 0.99, the values at the lowest three calibration points were between 0.96 and 0.99, even after removing the lowest calibration point. This could be due to the stationary phase of the cartridge adsorbing the analytes too strongly or not at all. It could also be from human errors in pipetting and in the SPE procedure.

UCT had poor linearity at the higher points of the calibration curve, which appeared to plateau. The highest data point was discarded, resulting in an adjusted range of 50-1000 ng/mL. This could be due to a saturation of the cartridge at the higher concentrations or from human errors in pipetting and in the SPE procedure.

Agilent and SiliCycle had good linearity throughout the entire concentration range of 50-2000 ng/mL. The linearity for Agilent decreased from JWH-018 to -122 and -250 metabolites, while SiliCycle decreased from JWH-250 to -018 and -122 metabolites. This illustrates the different interactions between the metabolites and the cartridges. The increased linearity could be due to the manufacturing of the cartridges, or from human or instrumental errors throughout the procedure.

Table 10. Coefficient of determination for the brands and metabolites over an adjusted range.

Brand	Metabolite	R ² Lowest Concentrations	R ² Entire Curve
Thermo (100-2000 ng/mL)*	250	0.968	0.996
	018	0.974	0.992
	122	0.993	0.996
UCT (50-1000 ng/mL)*	250	0.999	0.995
	018	0.999	0.997
	122	0.999	0.995
SiliCycle (50-2000 ng/mL)	250	0.999	0.998
	018	0.999	0.996
	122	0.998	0.995
Agilent (50-2000 ng/mL)	250	0.998	0.993
	018	0.999	0.999
	122	0.999	0.999

*Thermo and UCT cartridges required a set of data points to be discarded.

3.2.4 Recovery Efficiency Study for the Extraction of the Metabolites

The recovery efficiency of a cartridge measures the approximate amount of analyte that the cartridge extracts from the matrix. A general recovery efficiency of the metabolites is an important comparison between the cartridge brands. In this experiment, the synthetic urine samples were spiked with 1000 ng/mL of each metabolite and 300 ng/mL of each internal standard. The extracted, derivatized samples were compared to un-extracted, derivatized samples spiked in methanol in order to calculate the recovery efficiency of the quantification ion.

Figure 14 shows the percent recovery for each metabolite and each brand. SWGTOX has a tolerance of $\pm 20\%$ of the actual value, so values between 80-120% are

acceptable (47). Following these guidelines, SiliCycle has the best percent recovery with the triplicate samples and their standard deviations falling within $\pm 20\%$ of 100%. While the averages of the percent recoveries for Thermo and UCT are in the acceptable range, the error bars are outside of the range, which show the variation between the triplicate samples. Agilent had a lower recovery for JWH-250 and -122 metabolites that did not fall within the acceptable range. However, the JWH-018 metabolite had one replicate at 139%, which increased the standard deviation.

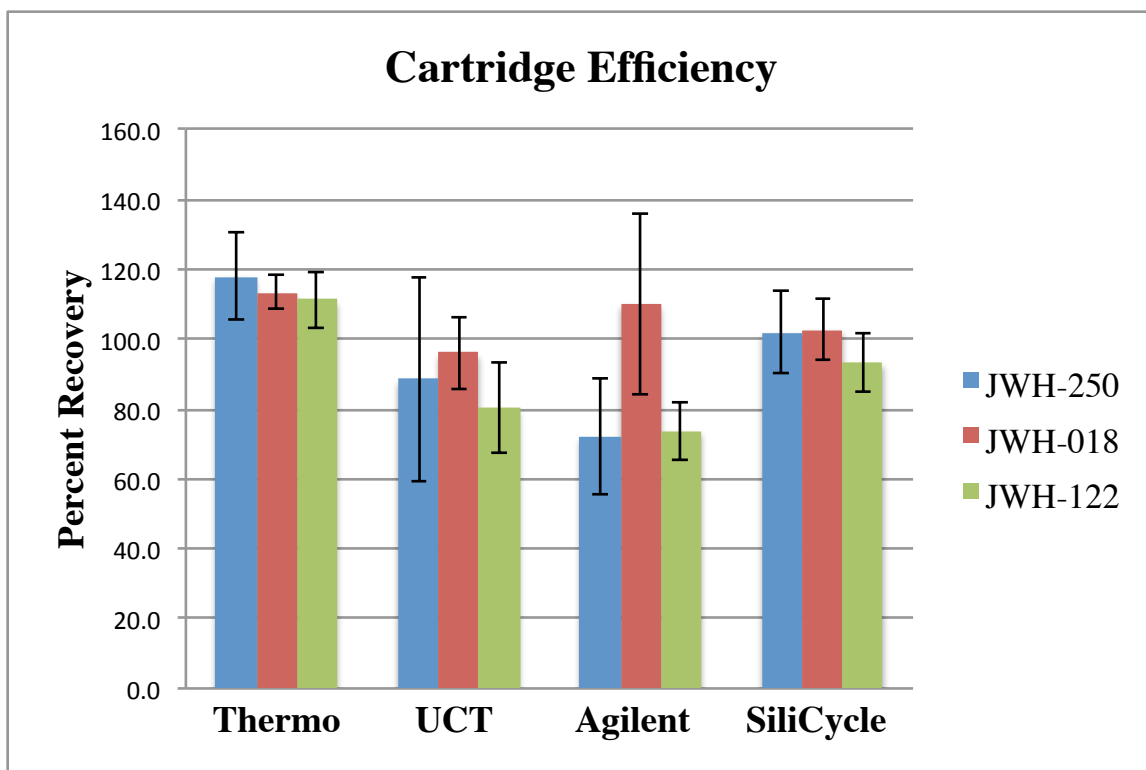


Figure 14: Recovery efficiency percentage of the SPE cartridges for each metabolite. The percent recovery is the average of the triplicate samples and the error bars show the standard deviation for the replicates. SiliCycle has the closest percent recovery to 100%, followed by Thermo, UCT, and Agilent.

A cartridge that has a lower recovery efficiency than 100%, like JWH-250 and -122 for the Agilent cartridges, could indicate that the sorbent does not interact with the

analyte as efficiently as it should. The metabolites could be eluting during the washing step of the procedure. This could also suggest that the sorbent interacts too strongly with the metabolites, with the elution solvent not removing the metabolites from the sorbent. A cartridge that has a recovery efficiency higher than 100%, like the Thermo cartridges, could indicate that other components of the urine adsorb to the sorbent and are then eluted with the metabolites, interfering with the quantitation of the analytes. These differences could also be from human error during the spiking of the samples or from the SPE procedure.

3.2.5 Within-Run Precision Study for Synthetic Cannabinoid Metabolites

The reproducibility of SPE cartridges is very important in a laboratory where half of all samples must be retained when additional testing could be required, or with small samples when the entire sample cannot be consumed. The within-run precision study measures the coefficient of variation to determine the reproducibility of the procedure. In this experiment, the urine samples were spiked with 1000, 500, and 250 ng/mL of the metabolites and 300 ng/mL of the internal standards. The calculation from the SWGTOX validation guidelines was used to compare the results from each brand of SPE cartridges. A higher % CV represented more variation between replicates while a lower value indicated reproducibility. The differences in the % CV for the brands were visible with SiliCycle having the least amount of variation across all three metabolites and Agilent having the most variation. The graphs seen in Figure 15 were created by dividing the standard deviation by the mean for each set of triplicate samples.

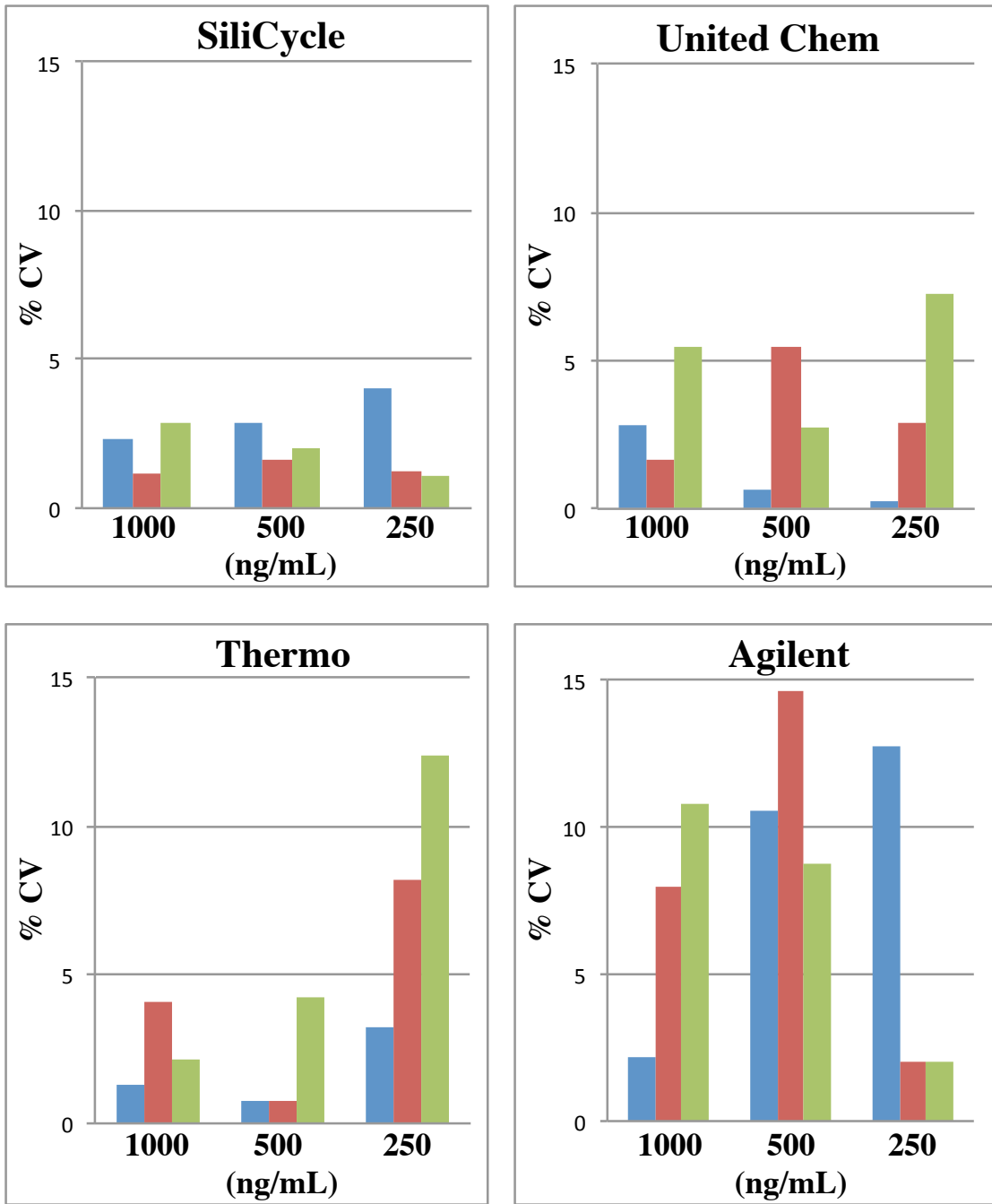


Figure 15: Precision study graphs for each brand of SPE cartridges. The blue columns represent the JWH-250 metabolite, red is JWH-018 metabolite, and green is JWH-122 metabolite.

The SWGTOX acceptable coefficient of variation is <20% (47). According to that requirement, all of the brands fall within the appropriate limits for the within-run precision study. SiliCycle had the best reproducibility because it exhibited the least amount of variation between triplicate samples, followed by UCT, Thermo, and Agilent with the most variation.

Comparison of the observations from section 3.2.2 with the calculated coefficient of variation for each of the samples matched up as well. The consistency of flow rates for UCT and SiliCycle could contribute to the lower variation between triplicate samples. The higher variation seen in Agilent could be due to the inconsistent flow rates observed during the SPE procedure and in the percentage of cartridges that gave no results, which required some samples to be retested. Thermo's increased variability for the lower concentrations could be explained from its calibration curve, where the lower concentrations had the poorest coefficient of determination. While the observations and comparisons of the data are in agreement, more samples must be tested to contribute to these conclusions. Since this experiment was only conducted once, it does not account for the influences from human errors or instrumental variations.

3.3 GC/MS vs. LC/MS for the Analysis of Synthetic Cannabinoid Metabolites

Some of the issues faced during the GC method development could have been avoided by using LC/MS for instrumental analysis. While GC has more theoretical plates for better resolution, LC is a better method of analysis for toxicological samples because volatility, thermal stability, and polarity are not limitations of LC based separations in

comparison to GC (35, 48, 49). In LC, the compounds could be separated based on interactions with the stationary phase of the column as well as a gradient elution rather than the very high temperatures needed to elute the metabolites from the GC column. These temperatures caused a tremendous amount of column degradation, which was observed in the chromatograms as the numerous and abundant siloxane peaks as well as the high baseline. This continuous column bleed made it difficult in choosing unique ions for the SIM method to detect the metabolites.

The condition of the liner was also an important issue to consider. As more samples were injected, the liner became dirtier, resulting in chromatograms with less abundant peaks from samples that were spiked with the same concentration of standards. While derivatization helped to increase the abundances of the peaks, it also appeared to cause the liner to become dirtier, faster. After approximately 90 samples, including blanks, the liner generated a dark area at the depth the samples were injected. Since this was not visible until the end of sample collection, it affected the quantitation of samples that were analyzed on different days than the calibration curves. This indicated a long sequence with many samples should not be run at once due to potential interactions between the metabolites and impurities in the liner.

Using an LC instrument would save the analyst time and the multiple issues associated with interday variations seen in these experiments. LC has improved tremendously over the past few years and has become a robust and reliable technique in toxicological analysis (49). There is no need to derivatize the compounds, making the sample preparation less extensive and less expensive than GC (35). Since there is not a

liner involved with the LC instrumentation, the degradation seen in this study caused by the liner would be eliminated as well. While GC has a better separating power, LC is a more appropriate analytical method for metabolites (48). However, the experiments conducted in this research demonstrate that GC can be utilized to analyze synthetic cannabinoid metabolites if necessary.

4. CONCLUSIONS

The outcome of these experiments led to the development of a GC/MS method for detection of three JWH metabolites, creation of calibration curves for quantification, use of SPE for the extraction of the metabolites from urine, and the quantification of the extracted compounds to determine the efficacy and reproducibility of four brands of SPE cartridges. Method optimization was able to minimize the interday variations seen in the same samples. Optimized parameters include: initial validation of the GC/MS method in each lab, a clean liner for the analysis of synthetic cannabinoid metabolites, use of a GC column with a high temperature limit, and derivatization of the extracts before injection into the GC.

The differences between the types of samples analyzed by a GC can affect the performance of the instrument for these compounds. To produce the best results, the parameters of the GC/MS method must be validated for synthetic cannabinoid metabolites on each instrument utilized. A clean liner must be used and watched closely as the analysis of numerous samples can significantly affect the results of the metabolites. Derivatization must occur in order for the volatility and thermal stability of the metabolites to increase, which heightened the sensitivity for detection of the analytes. A high temperature column must be used, due to the intense temperatures needed for elution of these compounds. This reduces the amount of column degradation and can help to decrease the baseline caused by the elution of siloxanes. Due to the variation of interday analysis, calibration curves must be run at the same time as the samples. Analyzing them at a similar time allows for a more accurate quantification of the metabolites.

A compilation of the observations throughout the SPE procedure and results of the cartridges can be seen in Table 11. SiliCycle cartridges had the most reliable percent recovery, the best reproducibility, and the most consistent and fastest elution rate. UCT had similar results to SiliCycle, except for a larger standard deviation in the recovery efficiency study. Thermo had a higher coefficient of variation at the lower concentrations, seen in the within-run precision study. With the standard deviations taken into account, UCT and Thermo were not within the $\pm 20\%$ accepted by the SWGTOX guidelines for the recovery efficiency study. The Agilent cartridges had the most inconsistent and slowest flow rate. They also had the highest variation between replicates and were not within the accepted tolerance in the recovery efficiency study. However, under the SWGTOX guidelines of $<20\%$ for the coefficient of variation percentage, all of the brands were within the acceptable range.

Table 11. Overall comparison of cartridges throughout these experiments.

		SiliCycle	UCT	Thermo	Agilent
Elution Flow Rate		Fast, consistent	Slow, consistent	Fast, consistent	Very slow, inconsistent
Within $\pm 20\%$ Percent Recovery	Mean	Yes	Yes	Yes	No
	Standard Deviation	Yes	No	No	No
Reproducibility, Highest to Lowest		1	2	3	4
$<20\%$ CV		Yes	Yes	Yes	Yes

The apparent interaction that the metabolites have with impurities in the GC liner may cause degradation peaks in the chromatograms. With this knowledge, measures can be taken to lessen the effects of degradation in a laboratory setting. Derivatization is

usually recommended when analyzing metabolites; however, there are articles that state if a peak is observed to a certain degree, derivatization is not necessary for reliable detection, but may be utilized for considerations related to sensitivity (38). The manufacturer of the metabolites purchased for these experiments stated they did not derivatize the compounds because they could detect them. Based on the significant increase in instrument performance and reproducibility observed in these experiments, derivatization should be done when analyzing these metabolites. This research demonstrates that with constant changes and developments to the instrument and methods, GC/MS can be utilized for this analysis. With the combination of a clean liner and derivatization of the metabolites, reproducible chromatograms can be produced.

From the conclusions drawn from these experiments, LC/MS should be utilized instead of GC/MS for the analysis of synthetic cannabinoid metabolites, especially in a laboratory where the instrument gets a lot of varied use. While this study shows it is possible to use GC/MS for the analysis of these metabolites, theoretically, LC/MS should be more reproducible. The use of GC/MS for the analysis of synthetic cannabinoid metabolites can be done, but if available, LC/MS may be the more appropriate option.

5. FUTURE DIRECTIONS

Future directions of this research would be to conduct the precision study over the course of several days. The method used should be validated over the parameters in the SWGTOX validation document. Optimization of the GC/MS parameters to determine the limit of detection (LOD) and limit of quantitation (LOQ) for the method as well as the four brands of cartridges should be completed. This research would be beneficial for real life applications in the laboratory, as limited samples may be exhausted. Then, the extraction efficiencies of each of the brands could be tested and compared over multiple days with high, medium, and low concentrations. The experiment involving the cartridge comparison could be repeated using LC-MS/MS to test the LOD and LOQ for the cartridges in the same manner and to compare the analysis to the GC/MS results.

This experiment used synthetic urine spiked with the metabolites of interest, but in actual urine samples, the metabolites will be conjugated with glucuronic acid and must undergo hydrolysis before SPE (37). Further studies would need to be done on the hydrolysis of the metabolites and subsequent SPE procedure of the hydrolyzed metabolites to determine if that would affect the efficacy or reproducibility of the cartridges.

The rapid turnover for the abuse of the compounds indicates the applicability of these specific GC/MS and SPE methods may not be relevant when the JWH-018, -250, and -122 compounds are no longer used. The methods optimized in this project may work for other compounds, but they are a starting point for future research of new synthetic compounds. It is imperative that future experiments be conducted utilizing

more metabolites tested of different types of synthetic cannabinoids to determine whether different brands are more useful for certain categories of compounds.

The SPE procedure could be further optimized. The cartridges used in this experiment were mixed mode, reversed phase with anion exchange sites. Since the metabolites were kept in a neutral state, the anion exchange sites were not utilized. After determining the pKa of the metabolites, many experiments could be done by changing the pH of the buffer and ionizing the metabolites to interact with the anion exchange sites. The same experiment could be conducted on nonpolar cartridges as well. The eluent used to elute the analytes from the sorbent could be tested to determine whether a more nonpolar solution would affect the reproducibility or efficiency of the cartridges. Lower volumes of the eluent could also be assessed to decrease the amount of solvents utilized and the evaporation time of the TurboVap. Overall, this area of study has become more popular over the past decade and more research needs to be done to provide toxicology labs with the best methods of analysis for these compounds.

LIST OF JOURNAL ABBREVIATIONS

Anal. Chem.	Analytical Chemistry
Bioorg. Med. Chem.	Bioorganic and Medicinal Chemistry
Bioorg. Med. Chem. Lett.	Bioorganic and Medicinal Chemistry Letters
B. M. J.	British Medical Journal
Clin. Biochem.	Clinical Biochemistry
Drug Alcohol Depend.	Drug and Alcohol Dependence
Forensic Sci. Int.	Forensic Science International
J. Anal. Toxicol.	Journal of Analytical Toxicology
J. Biol. Chem.	Journal of Biological Chemistry
J. Med. Toxicol.	Journal of Medical Toxicology
Neurochem. Res.	Neurochemical Research
Schizophrn. Res.	Schizophrenia Research
Toxicol. Lett.	Toxicology Letters

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CURRICULUM VITAE

