ANALYSIS OF SYNTHETIC CANNABINOIDS IN URINE VIA SOLID PHASE EXTRACTION AND LIQUID CHROMATOGRAPHY WITH TANDEM MASS SPECTROMETRY (LC-MS/MS)

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Abstract:

Synthetic cannabinoids have emerged as a dangerous new trend in illicit drug use. The appointment of many popular synthetic cannabinoids as Schedule I controlled substances has helped to decrease their popularity in recent years, but in order to enforce the new legislation, methods must be developed to detect the drugs in biological samples. The purpose of this study was to improve the detection of synthetic cannabinoids in urine. An LC-MS/MS method was developed to detect 14 synthetic cannabinoid parent compounds and metabolites, and THC. Comparison studies were performed to determine the conditions for the extraction of the target analytes via solid phase extraction (SPE).

The suitability of three different SPE columns and three different eluents were analyzed during two different studies by extracting spiked urine samples and comparing the resulting peak areas. The effect of methanol wash solutions was analyzed by washing the columns with 30% methanol, 70% methanol, and 100% water solutions. A 10% acetonitrile reconstitution solution and 20% methanol reconstitution solution were compared to determine which was better suited for the recovery of the target analytes. Finally, linearity, carryover, and matrix effects validation studies were performed on the final method.

The column and elution comparison studies revealed that the SPEware CEREX® HP SAX 5 mg NBE column paired with a DCM:IPA:NH_4OH elution performed best for the target analytes. The methanol wash study showed that high methanol content in the wash solvent resulted in analyte loss during extraction. The 100% water wash was determined to be most favorable for the extraction of synthetic cannabinoids. The 20% methanol solution greatly improved the recovery of all analytes. The calibration curve included a 1c, 2c, 5c, 10c, 15c, and 25c for all analytes except THC which did not use a 1c calibrator. The linearity study showed that linear calibration models worked for some analytes, while others required a quadratic fit model. No carryover was observed at the upper limit of quantitation. Matrix effects ranged between 64% and 270%. Recovery efficiencies were 12-158%, and process efficiencies were 12-417%.

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CHAPTER I

INTRODUCTION

Synthetic cannabinoids, commonly known as Spice or K2, are compounds originally developed to study the effects of marijuana. The drug class consists of hundreds of structurally similar compounds that are anything but safe. In 2011 the use of synthetic cannabinoids resulted in more than 28,500 emergency room visits in the United States.[1] During the same year, synthetic cannabinoids were the second most abused drugs among 12th graders. As of 2014, the overall use of synthetic cannabinoids has decreased but they still ranks as the third most abused drugs for 8th, 10th, and 12th graders.[2,3]

Despite the alarming number of emergency room visits due to synthetic cannabinoid use, surveys show that the perceived risk of synthetic cannabinoids is low.[2,3] Considering the low perceived risk, health concerns are unlikely to be the cause of decreased use. One likely deterrent is the illegalization of many popular synthetic cannabinoid compounds. Currently, the Drug Enforcement Administration (DEA) has permanently or temporarily added several of the most popular synthetic cannabinoids to the list of Schedule I Controlled Substances. According to the DEA, "Substances in this schedule have no currently accepted medical use in the United States, a lack of accepted safety for use under medical supervision, and a high potential for abuse."[4] Unfortunately

with so many different synthetic cannabinoids available for synthesis, enforcing the ban is difficult.

Currently, few comprehensive drug screens include synthetic cannabinoids. Those that do usually test for only a few of the first synthetic cannabinoids banned by the DEA in 2011. The lack of standard testing for synthetic cannabinoids is a major reason for the continued popularity among adolescents.[5] Thus, improving testing techniques for synthetic cannabinoids is an important area of study. Increased ability to detect synthetic cannabinoids in biological samples is beneficial both clinically and forensically. Clinically, a comprehensive test for popular synthetic cannabinoids would aid doctors in diagnosing synthetic cannabinoid exposure, which then would improve the ability to treat the patient. Forensically, comprehensive testing for illegal synthetic cannabinoids would increase the likelihood that use would result in punishment, which would decrease the popularity of the drug.

Synthetic cannabinoids can be detected in many biological samples; but the sample types most commonly tested are urine,[6–9] serum, or whole blood.[9–12] The synthetic cannabinoid parent drugs are excreted in very low concentrations in the urine. For this reason, urine drug screens rely on the detection of synthetic cannabinoid metabolites rather than the parent drug.[12] The detection of a parent drug is preferable and can be done by testing serum or blood; however, the collection of blood and serum is much more invasive than the collection of urine. The non-invasiveness of urine samples makes it a popular sample for clinical, forensic, and work-place drug testing.

Before a sample can be tested for the presence of synthetic cannabinoid, it must undergo sample preparation. There are two techniques most commonly used to prepare samples for synthetic cannabinoids detection: liquid-liquid extraction (LLE)[6,10–12] and solid-phase extraction (SPE).[7,9] Both techniques are sufficient for sample preparation but also have weaknesses. LLE works well for the extraction of hydrophobic compounds like synthetic cannabinoids but requires large volumes of harmful chemicals. SPE is less favorable for synthetic cannabinoid extraction but requires smaller volumes of both sample and solvent.[13]

A third option for sample preparation is supported-liquid extraction (SLE). SLE combines techniques from LLE and SPE to create a technique that requires small volumes of sample and solvent yet still works well for hydrophobic compounds.[13] One study[7] provides supplementary data that suggest SLE does provide an improved sample preparation for synthetic cannabinoid detection; however, the sample size was small and the difference in extraction efficiencies was not tested for significance.

The purpose of this study was to develop an efficient solid phase extraction (SPE) method for the extraction of several illegal synthetic cannabinoids, Δ9-THC, and THCA and analyze the spiked samples via a liquid chromatography and tandem mass spectrometry (LC-MS/MS) method developed specifically for the analysis of synthetic cannabinoids. The SPE method was developed by analyzing the effects of changing variables such as SPE column type and solvents used for washes, elution, and reconstitution. Finally, the validity of the method was analyzed by performing linearity, carryover, and matrix effects studies.

If, as hypothesized, current SPE methods can be improved significantly for the extraction of synthetic cannabinoids, the newly applied method has potential to improve current testing methods for active drug testing laboratories. Furthermore, the technique may aid in further decreasing the popularity of synthetic cannabinoid by providing information that can be used to enforce the current legislation banning popular synthetic cannabinoids.

CHAPTER II

REVIEW OF LITERATURE

Introduction

Synthetic cannabinoids are compounds that were synthetized due to their structural or functional similarity to the "natural" cannabinoids such as delta-9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive chemical in marijuana. The original use of most synthetic cannabinoids was to research the mechanisms of the cannabinoid system or to study the possible therapeutic uses of both natural and synthetic cannabinoids.[14,15] Cannabinoid research resulted in the development and publication of hundreds of different synthetic cannabinoid compounds. Years later, the publications became guides for the manufacture of recreational synthetic cannabinoids. The emergence of recreational synthetic cannabinoids began as early as 2004 and they steadily gained popularity due to the "legal high" promised to users.[15,16] Since then, new synthetic cannabinoids with various chemical structures have been produced and subsequently outlawed. However, comprehensive detection methods for the illegal synthetic cannabinoids in biological samples still need to be developed and improved.

Production and Marketing

Research suggests that production of recreational synthetic cannabinoids began in

Asian countries, China in particular. The synthetic cannabinoids are synthesized and purified into a powder. The powder is then shipped in bulk to other countries using legal trade routes. The powder can then be used in a few different ways. The most common preparation for synthetic cannabinoids is to add them to a mixture of dried plant material, often labeled as an herbal blend. The combination of the synthetic cannabinoids and plant material is more commonly referred to as Spice; although, Spice is only one of several popular brands of synthetic cannabinoids product.[15–18] The plants used in Spice blends are generally inert, but some may be chosen for their reported psychoactive effects. However, psychoactive effects caused by the plants are weak and outweighed by the much stronger effects of the synthetic cannabinoids.[19]

In the production of Spice, solvents such as methanol or acetone can be used to dissolve the powder before it is sprayed onto a mixture of inert herbs. Spraying the herbs is done on an industrial scale in order to speed the production. The herbs are allowed to dry before they are packaged.[17] Spice is normally packaged in quantities between 0.4g and 3g.[19] The information included on the Spice packaging is often misleading. Packaging may fail to include the synthetic cannabinoids in the ingredients list, may include statements such as "not for human consumption," or by fail to state that the synthetic cannabinoids previously used in the product have changed.[15,16,20] Additionally, the packaging of some Spice products will inform the buyer which illegal synthetic cannabinoids are not included in the product.[15]

Spice is most often burned in rolled cigarette papers, pipes, or water pipes so it can be inhaled. Spice may also be ingested, but ingestion is less common and results in weaker effects than inhalation.

Synthetic cannabinoids can be purchased in forms other than the Spice blend. It is also possible to purchase synthetic cannabinoids in powdered form, but the product may be difficult to find. Because the powder is a more pure form of synthetic cannabinoids, ingestion of the powder produces a stronger effect than ingestion of Spice. The powder can be easily mixed into alcoholic drinks or with other drugs resulting in an increased risk of toxicity due to multiple drug interactions. There is at least one report of a user requiring medical attention after mixing synthetic cannabinoid powder in an alcoholic beverage.[21] Powdered synthetic cannabinoids may also be dissolved in liquid propellants commonly used for electronic cigarettes (e-cigs). The liquid product can then be vaporized and inhaled when used in conjunction with e-cigs.[14]

Legislation

The initial popularity of synthetic cannabinoids as a recreation drug was due to a lack of legislation banning their production, sale, and use. Before synthetic cannabinoids could be made illegal, the structure of the compound had to be determined. The first detection of synthetic cannabinoids in seized samples occurred in late 2008 in Europe[14,17] and the United States.[14] The first synthetic cannabinoid detected was JWH-018, 1-naphthalenyl(1-pentyl-1H-indole-3yl)methanone.[17,19,22] Soon after, JWH-018 and other popular synthetic cannabinoids were made illegal by many legislatures. In the United States JWH-018, JWH-073, JWH-200, CP-47,497, and the CP-47,497 C8 homologue

were temporarily placed on the Schedule I substances list on March 1, 2011.[14,23] On July 9, 2012, those synthetic cannabinoids were permanently added as Schedule I substances.[14]

At the time the first synthetic cannabinoid legislation was passed, most drugs were banned based on their unique chemical structure. This type of legislation does not allow for the automatic scheduling of structurally similar substances. As a result, new structurally similar synthetic cannabinoids gained popularity after the legislation was passed against the first synthetic cannabinoids.[24] There were no legal consequences for using the new structurally similar compounds, so synthetic cannabinoids maintained their popularity as a legal high. Finally, some legislatures enacted "homologue laws." The homologue laws automatically prohibit compounds that are very structurally similar to previously prohibited compounds.[18]

Trends

When synthetic cannabinoids first gained popularity, they were used by a large demographic range. However, several studies show that synthetic cannabinoids are currently used primarily by teens and young adults. According to the 2014 Monitoring the Future Survey,[5] synthetic cannabinoids are the third most highly abused drugs among 8^{th}, 10^{th}, and 12^{th} graders, although marijuana is the most frequently abused substance for all three age groups. The survey also indicates a decrease in the overall frequency of synthetic cannabinoid use for 10^{th} and 12^{th} graders between 2012 and 2014. For 12^{th} graders, the self-reported synthetic cannabinoid use dropped from 11.3% in 2012 to 5.8%

in 2014. For 10th graders, the use dropped from 8.8% in 2012 to 5.4% in 2014. However, the use of synthetic cannabinoids among 8th graders has not significantly decreased.[2,3]

Despite the apparent decrease in synthetic cannabinoid use, new synthetic cannabinoid compounds continue to be produced. Two of the newest class of synthetic cannabinoids to hit the market, AB-PINACA and AB-FUBINACA, are commonly sold in liquid form for use with e-cigarettes. Other new synthetic cannabinoids, such as MAB-CHMINACA and ADB-CHMINACA, reportedly have severe side-effects that send many users to the hospital.[25]

Pharmacodynamics and Pharmacokinetics

Synthetic cannabinoids affect Cannabinoid Receptor 1 (CB1) and Cannabinoid Receptor 2 (CB2), which are both part of the cannabinoid system. CB1 and CB2 are also affected by Δ^9-THC. For this reason, synthetic cannabinoids are reported to produce a "cannabis-like" high. However, many synthetic cannabinoids have the potential to bind much more strongly to the CB receptors. As a result, a stronger positive effects can be achieved with a smaller dose. Likewise, toxic effects are also amplified, even with small doses.[20,22]

Not only are the strength of synthetic cannabinoid effects unpredictable, but the nature of the effects are also unpredictable. For example, one user may present to the hospital with tachycardia (rapid heartbeat) and dilated pupils, while a second user may suffer from bradycardia (slow heartbeat) and constricted pupils.[15,26]

Much research still needs to be completed regarding the absorption, distribution, metabolism, and excretion of synthetic cannabinoids. However, there have been some

studies that provide valuable information about the pharmacokinetics of certain synthetic cannabinoid compounds. A 2012 study[6] administered a 0.15 g of a synthetic cannabinoid product to an individual and monitored the resulting concentration of drug metabolites in individual's urine over a period of 65 hours. The results of this study showed a detection window of the synthetic cannabinoid metabolites for 2-3 days in urine with a limit of quantitation around 0.1 ng/mL, with maximum concentration around 10 ng/mL at 1-3 hours post-administration.

In another study, Gurney, et al[27], collected case reports and quantitative results for synthetic cannabinoids in urine, whole blood and serum. In blood, levels as low as 0.2 ng/mL were detected. In urine, metabolite concentrations were as low as 0.03 ng/mL. The concentration of synthetic cannabinoid detected varied between individuals and different synthetic cannabinoid compounds. A third study[28] included the quantification of 862 synthetic cannabinoid positive cases. In whole blood concentration ranged between about 0.05 ng/mL and about 68 ng/mL.

Chromatography and Detection Methods

Proper chromatographic separation of structurally similar compounds is very important for the detection of synthetic cannabinoids. Chromatography separation of cannabinoids in biological samples is most often completed via either Gas Chromatography (GC) or Liquid Chromatography (LC). The chromatographic separation is then followed by detection via Mass Spectrometry (MS).[29,30] The analysis of synthetic cannabinoids by GC-MS is complicated by the structural similarity of synthetic cannabinoid parent and metabolite structures and fragmentation which makes selective

identification difficult. Finally, the thermal-instability of some synthetic cannabinoids makes their detection by GC-MS impossible in most cases.[30]

Unlike most GC-MS methods, LC-MS methods are able to separate and identify structurally similar synthetic cannabinoids and their metabolites. Detection of metabolites is particularly important for the development of a method to detect synthetic cannabinoids in urine because the parent compounds of many synthetic cannabinoids are not excreted in urine.[31] These advantages of LC-MS over GC-MS methods are why LC-MS is most commonly used for the analysis of synthetic cannabinoids in biological samples.[30]

Sample Extraction

Both natural and synthetic cannabinoids are usually prepared for detection by either a liquid-liquid extraction (LLE) or solid-phase extraction (SPE).[30] LLE is favorable for synthetic cannabinoid extraction due to the hydrophobicity of the compounds. Kacinko et al[11] used a LLE method for the extraction of JWH-018, JWH-073, JWH-019, and JWH-250 from whole blood. The reported recovery efficiency for the method was between 60.6% and 92.8% for all analytes and met all validation requirements. However, the recovery efficiency may be negatively impacted when more synthetic cannabinoids are included in the extraction method. Kneisel and Auwärter[12] used a LLE technique for the detection of 30 synthetic cannabinoids in serum, but the recovery efficiencies ranged between 5.7% - 56%. These recoveries were much lower than the recovery of Kacinko's less inclusive method. Another downfall of LLE is the need for large volumes of odorous or carcinogenic solvents.

SPE is a more complex extraction process than LLE, but the use of extraction columns requires a smaller volume of solvent and the solvents are usually less toxic.[13] SPE methods can also have recovery efficiencies comparable to or better than the recovery efficiencies of LLE methods. Erol Öztürk, et al[9] used a SPE preparation for the LC-MS/MS analysis of JWH-018 and two of its metabolites in blood and urine with recovery efficiencies of 87–98% and 85–96%. Sundström, et al[32] developed a ultra-high performance liquid chromatography/high-resolution time-of-flight mass spectrometry (UHPLC-HR-TOFMS) method to detect several illicit drugs including 54 synthetic cannabinoid analytes and THCA. The recovery efficiency if the method was between 46% and 82% except for HU-210 which had a recovery efficiency of 27%.

Recently there has been a gain in popularity of the supported liquid extraction (SLE) technique. SLE uses techniques from both LLE and SPE. Using SLE can increase the extraction efficiency.[13] Currently there is very little literature available on the use of SLE for urine cannabinoid methods. One study[7] successfully used SLE for the extraction of natural cannabinoids from urine. A 2014 study by Scheidweiler and Huestis[7] successfully used SLE for the extraction of synthetic cannabinoids in urine. In the 2014 synthetic cannabinoids study, preliminary data were gathered on the extraction efficiency of SLE compared to SPE, and showed that SLE was better suited for the extraction of parent synthetic cannabinoids than SPE. The comparison study included a very small sample size of two parent synthetic cannabinoids and two metabolites, but the SLE method was able to successfully extract 53 synthetic cannabinoid analytes with recovery efficiencies between 43.7% and 109.3%. Although SLE shows potential for an efficient

extraction of synthetic cannabinoids, it is not an extraction method that is currently available to most labs.

Conclusion

The literature shows that synthetic cannabinoids pose a significant health risk. Despite the health risks, they continue to be popular drugs of choice for teens. One way to further decrease the popularity of synthetic cannabinoids is to improve the detection methods for biological samples. SPE is an extraction method that is cleaner than simple dilution methods, produces less waste than LLE methods, and is more readily available than SLE methods. Additionally, SPE has been shown to successfully extract synthetic cannabinoids from urine in the past. Therefore, maximizing synthetic cannabinoid recovery from a SPE extraction method is a bases for further research in improving synthetic cannabinoid detection.

CHAPTER III

METHODOLOGY

Overview

The purpose of this study was to develop a liquid chromatography and tandem mass spectrometry (LC-MS/MS) method to detect several synthetic cannabinoids in urine and to use that detection method to evaluate the of effects of altering variables of a solid phase extraction (SPE) method. The variables examined in this study include SPE column type, elution solvent used, solutions used for washing the column, and solution used for reconstitution.

In this study, blank urine samples were spiked with a known concentration of drug standard. The urine used in this study contained no personal identifying information. The spiked blank urine was then extracted via various SPE methods and analyzed by LC-MS/MS. The recovery of drug from urine was determined by comparing peak areas or concentration and used to compare the extraction efficiency of each extraction. The most efficient variable from each method was then combined into a final SPE method that was used for validation studies.

Materials

The materials required for this study include drug standards, drug-free urine, reagents, and solid phase extraction (SPE) columns. The drug standards used in this study were obtained from Cerilliant. The certified drug-free urine was purchased from UTAK. Reagents, laboratory grade or higher, were purchased from VWR International. The SPE columns used were CEREX® HP SAX 5 mg NBE, CEREX® HP SAX 2.5 mg NBE, and CEREX® HP SCX 5 mg NBE extraction columns which were purchased from SPEware Corporation.

Liquid Chromatography and Tandem Mass Spectrometry

The LC-MS/MS Shimadzu 8040 shown in Figure 1 was used to analyze samples extracted via SPE. Lab Solutions software was used to remotely control the LC-MS. The column that was used for analysis is the Raptor™ Biphenyl with 50 mm x 3.0 mm dimensions and 2.7 µm particle size. The guard column used to protect the column was the Raptor™ Biphenyl EXP® with 5 mm x 3.0 mm dimensions and 2.7 µm particle size. Both the column and guard column were obtained from Restek Corporation.

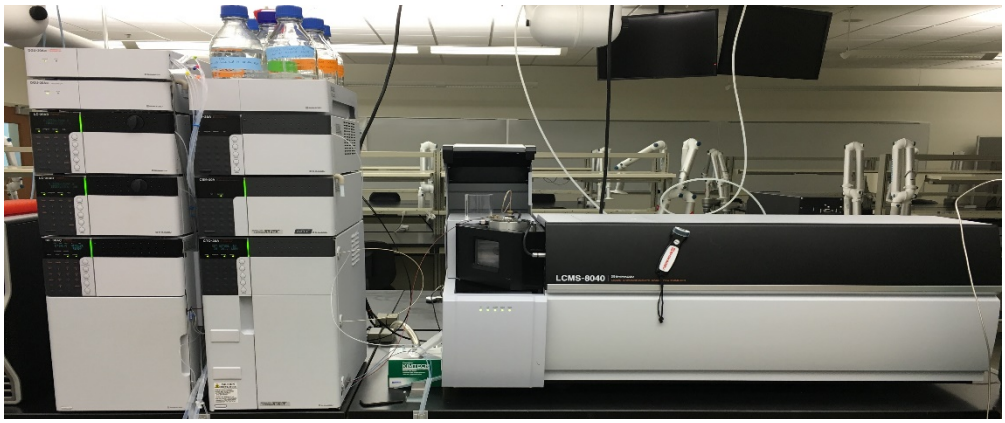
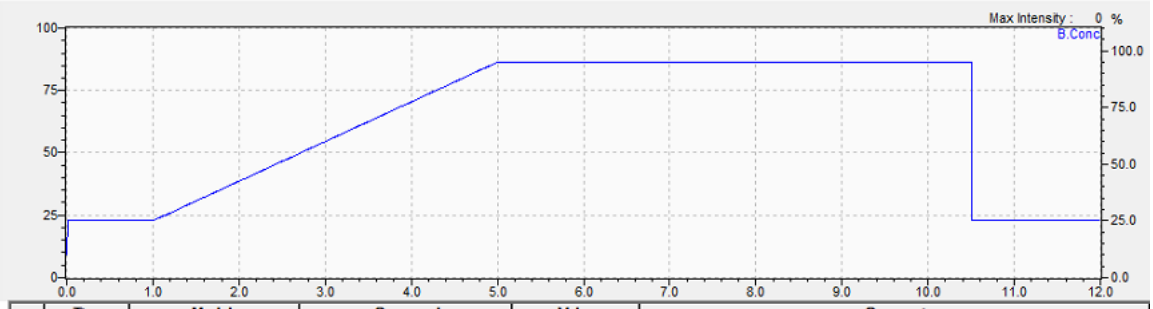


Figure 1. LC-MS/MS Shimadzu 8040 used in this study.

The chromatographic conditions were determined with the use of standard and internal standard mixes prepared in water to determine which conditions provide the best peak shape and separation. The conditions used by Restek Corporation[33] for the testing of the Raptor™ Biphenyl column were be used as a starting point. The final chromatography method utilized mobile phase A (MPA) consisting of 2mM ammonium formate and 0.1% formic acid in water. Mobile phase B (MPB) consisted of 0.1% formic acid in 9:1 acetonitrile:water. The mobile phase gradient is detailed in Figure 2.



	Time	Module	Command	Value	Comment
1	0.01	Pumps	Pump B Conc.	25	
2	1.00	Pumps	Pump B Conc.	25	
3	2.50	Column Oven	CTO.RVL	1	
4	5.00	Pumps	Pump B Conc.	95	
5	5.51	Column Oven	CTO.RVL	0	
6	10.50	Pumps	Pump B Conc.	95	
7	10.51	Pumps	Pump B Conc.	25	
8	12.00	Pumps	Pump B Conc.	25	
9	12.00	Controller	Stop		

Figure 2. Mobile phase B concentration gradient for the LC method

The mass spectrometer (MS) was run in positive mode and with electrospray ionization (ESI). Each drug and internal standard was optimized for detection using Lab Solutions' auto-optimization feature. The optimizations were manually adjusted as needed. Lab Solutions will also be used to collect and analyze the generated data.

Solid Phase Extraction Methods

The SPE extractions were carried out on the SPEware ALD device shown in Figure 3. For this study, two previously developed SPE methods were used. The first

method was an SPE method for synthetic cannabinoids in urine provided by SPEware. The method, described below, will be referred to as the SPEware method in this paper. The second method was a SPE method developed for the extraction of pain management, illicit, and other prescription drugs from urine provided by the Forensic Toxicology and Trace Laboratory at OSU-CHS. The method, described below, will be referred to as the FTTL method in this paper. Variables from each method were adjusted individually to determine the change in extraction efficiency, if any, for each variable. The variables analyzed were column bed depth, elution solvent composition, column ion affinity, wash solvent composition, and reconstitution solvent composition.

SPEware Solid Phase Extraction

For the SPEware sample preparation procedure, hydrolysis solution was prepared daily by combining 200 µL of 100 mM sodium acetate buffer (pH 5.0), 15 µL of β-glucuronidase solution, and 10 µL of internal standard stock solution per sample. Then 225 µL of hydrolysis solution and 200 µL of spiked urine were added to a CEREX® HP SAX 5 mg NBE column. The sample was mixed by repeated aspiration/dispense of a micropipette. The columns were then covered with aluminum foil and placed in a 68°C incubator for 30 minutes. After the incubation, the samples were cooled to room temperature before proceeding with the SPE method.

Samples were applied to the columns at a flow rate of 0.5 – 1 mL/minute. The columns were then washed with 0.5 mL of a 85:15:1 mixture of deionized water, acetonitrile, and ammonium hydroxide (H_2O:ACN:NH_4OH) at a flow rate of 0.5 – 1 mL/minute. The H_2O:ACN:NH_4OH wash was prepared each day before extraction. Next,

the columns were washed with 0.5 mL of a 70% methanol (MeOH) solution at a flow rate of 0.5 – 1 mL/minute. The columns were dried under 70 psi nitrogen for 10 minutes. The analytes were eluted with 200 µL of 98:2 ethyl acetate and formic acid (EtOAc:FA) and collected in a 1 mL 96-well plate at 0.5 – 1 mL/minute. The samples were evaporated to dryness under nitrogen for 20 minutes and reconstituted with 100 µl of MPA:MPB (9:1).

FTTL Solid Phase Extraction

Hydrolysis solution was prepared daily with 350 µL of 0.3 M sodium acetate buffer (pH 4.8), 10 µL of β-glucuronidase solution, and 10 µL of internal standard stock solution per sample. Then 370 µL of hydrolysis solution and 200 µL of spiked urine were added to a CEREX® HP SCX 5 mg NBE column. The samples were mixed by performing repeated aspiration/dispense steps. The columns were then placed in a 68°C incubator for 30 minutes. After incubation the samples were cooled to room temperature.

The samples were applied to the column at a flow rate of 0.5 – 1 mL/minute. The columns were then washed with 600 µL of H_2O, then 200 µL of 100 mM HCl, and finally 300 µL of H_2O. All three washes were applied at a flow rate of 0.5 – 1 mL/ minute. The columns were dried under nitrogen at 70 psi for 15 minutes. The analytes were eluted with 400 µL of 80:18:2 dichloromethane, isopropanol, and ammonium hydroxide (DCM:IPA:NH_4OH). Samples were collected in a 1 mL 96-well plate and evaporated to dryness under nitrogen. The samples were then reconstituted with 100 µL of 20% MeOH.

Column Bed and Elution Solvents

To test the effect of column depth and variation of elution on extraction

efficiency, four urine curves were extracted simultaneously with two different columns and two different elution solvents. Curve A was extracted according to SPEware method with the CEREX® HP SAX 5 mg NBE column and a EtOAc:FA (98:2) elution. Curve B was extracted according to the SPEware method with CEREX® HP SAX 5 mg NBE column, however, the elution solvent was replaced with 200 µL of an 80:18:2 dichloromethane, isopropanol, and formic acid (DCM:IPA:FA). Curve C was extracted using the CEREX® HP SAX 2.5 mg NBE column and EtOAc:FA (98:2) elution. Curve D was extracted using the CEREX® HP SAX 2.5 mg NBE column and DCM:IPA:FA (80:18:2) elution.

The curves for this portion of the study were prepared by a serial dilution of a 5 µg/mL stock solution, shown in Table 1. Each curve included ten samples: 1 ng/mL, 2 ng/mL, 5 ng/mL, 10 ng/mL, 20 ng/mL, 50 ng/mL, 100 ng/mL, 250 ng/mL, 500 ng/mL, and blank. The internal standard solution consisted of 7 deuterated internal standard. Deuterated internal standards were not available for all analytes, so the appropriate internal standard was chosen based on structural similarities. The internal standard groups are shown in Table 1.

The peak areas for each analyte were recorded and compared between the four curves to determine which column bed depth and elution solvent combination was most efficient for the extraction of the analytes. The peak shape and area for each analyte were used to determine the likely limits of detection (LODs) and limits of quantitation (LOQs) for each analyte in the method.

Water to Methanol Ratio in Wash

The next step in the study was to analyze the effect of altering the methanol (MeOH) content of the second SPE wash step. Six 500 ng/mL urine samples were prepared from the 5 µg/mL stock solution listed in Table 1. Three samples were extracted with the CEREX® HP SAX 5 mg NBE column and each was washed with 0.5 mL of 70% MeOH, 30% MeOH, or 100% H_2O during the second wash step. The other three samples were extracted with the CEREX® HP SAX 2.5 mg NBE extraction column and were washed with 0.5 mL of 70% MeOH, 30% MeOH, or 100% H_2O during the second wash step. Excluding the column and MeOH wash changes, the extraction was performed according the SPEware SPE procedure. The peak areas for each sample were compared to determine which wash resulted in the best recovery of all the analytes.

Table 1. List of analytes and their concentration in the first standard stock mix and internal standard stock mix

Analyte	Concentration (ug/mL)	Internal Standard Group
Standards		
5-Fluoro PB-22	5	4
AB-FUBINACA	5	3
AM2201 4-Hydroxypentyl metabolite	5	1
HU-210	5	6
JWH-018 Metabolite	5	2
JWH-019 5-Hydroxyheyxyl metabolite	5	2
JWH-073 Metabolite	5	2
JWH-122 5-Hydroxypentyl metabolite	5	3
JWH-250 5-Hydroxypentyl	5	4
PB-22	5	4
THC	5	5
THCA	5	6
UR-144	5	7
UR-144 5-Hydroxypentyl metabolite	5	7
XLR-11	5	7
XLR-11 4-Hydroxypentyl metabolite	5	7
Internal Standards		
IS AM2201 4-Hydroxypentyl metabolite D5	1	1
IS JWH-018 Metabolite-D5	1	2
IS JWH-122 4-Hydroxypentyl metabolite D5	1	3
IS JWH-250 4-Hydroxypentyl metabolite D5	1	4
IS THC-D3	10	5
IS THCA-D3	10	6
IS UR-144 5-Hydroxypentyl metabolite-D5	1	7

Column Ion Affinity and Eluent pH

The 5 µg/mL curve stock listed in Table 1 was used to spike eight, 500 ng/mL urine samples. Four of the samples were prepared according to the SPEware sample preparation method, and four of the samples were prepared according to the FTTL sample preparation method. Two samples from each sample preparation were added to a CEREX® HP SAX 5 mg NBE column, and two from each were added a CEREX® HP SCX 5 mg NBE column. The FTTL extraction procedure was followed until the elution

step, at which point one sample from each column type was eluted with 400 µL of DCM:IPA:NH4OH (80:18:2), and the other sample from each column type was eluted with 400 µL of DCM:IPA:FA (80:18:2). This process is diagramed in Figure 4. All samples were the evaporated to dryness and reconstituted in 100 µL MPA:MPB (9:1).

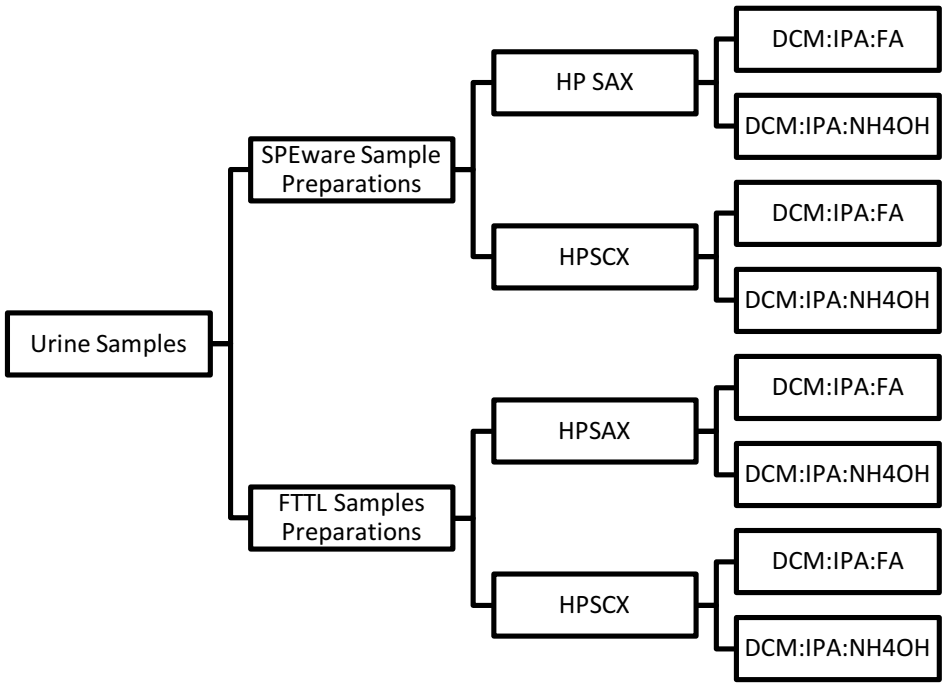


Figure 3. Diagram of the altered variables for the analysis of column ion affinity and eluent pH effect on synthetic cannabinoid extractions.

Reconstitution Solvent

Six 500 ng/mL urine samples were prepared from the 5 µg/mL standard stock solution listed in Table 1. The six urine samples were extracted according to the FTTL sample preparation and SPE methods using the CEREX® HP SAX 5 mg NBE column. Three of the samples were reconstituted with 100 µL of MPA:MPB (9:1), and three samples were reconstituted with 100 µL of 20% MeOH solution. Additionally, in effort to improve the detection of THCA, two urine samples spike at 500 ng/mL THCA were

extracted following the same procedure. The analyte peak areas for each reconstitution were averaged and the standard deviation was determined.

Final Solid Phase Extraction Method

The final SPE method was determined after reviewing the data from each variable study to determine the overall best procedure for extracting the synthetic cannabinoid and THC analytes from urine. The LOD and LOQ for each analyte were set at this time. For the purposes of this study, the LOD and LOQ were the same for each respective analyte, and any peak below the LOQ was considered a negative result. The LOQ for each analyte is shown in Table 2.

Sample preparation for the final method followed the FTTL sample preparation procedure. Hydrolysis solution containing 350 µL of 0.3 M sodium acetate buffer (pH 4.8), 10 µL of β-glucuronidase solution, and 10 µL of IS standard stock solution (shown in Table 3) per sample was prepared for each extraction. For each sample, 370 µL of hydrolysis solution was combined with 200 µL of urine in a CEREX® HP SAX 5 mg NBE column. The solutions were mixed by repeated aspiration/dispense steps. The columns were incubated at 68°C for 30 minutes. After incubation, the samples were cooled to room temperature.

The samples were applied to the column at a flow rate of 0.5 – 1 mL/minute. The columns were then washed with 600 µL of H_2O, followed by 200 µL of 100 mM HCl, and finally 300 µL of H_2O. Each wash was applied at a flow rate of 0.5 – 1 mL/minute. The columns were dried under nitrogen at 70 psi for 15 minutes. The analytes were eluted off the column with 400 µL of DCM:IPA:NH_4OH (80:18:2) and collected in a 1 mL 96-

well plate and evaporated to dryness under nitrogen. Finally, the samples were reconstituted with 100 μL of 20% MeOH.

Table 2. Limit of quantitation for analytes included in the final SPE and LC-MS/MS methods.

Analyte	LOQ (ng/mL)
5-Fluoro PB-22	1
AB-FUBINACA	1
AM2201 4-Hydroxypentyl metabolite	1
HU-210	400
JWH-018 Metabolite	1
JWH-019 5-Hydroxyheyxyl metabolite	1
JWH-073 Metabolite	1
JWH-122 5-Hydroxypentyl metabolite	1
JWH-250 5-Hydroxypentyl	2
PB-22	1
THC	200
UR-144	2
UR-144 5-Hydroxypentyl metabolite	2
XLR-11	2
XLR-11 4-Hydroxypentyl metabolite	2

Table 3. List of analytes and their concentration in the standard and internal standard stock mixes for the final SPE and LC-MS/MS method.

Analyte	Concentration (ug/mL)	Internal Standard Group
Standards		
5-Fluoro PB-22	0.25	4
AB-FUBINACA	0.25	3
AM2201 4-Hydroxypentyl metabolite	0.25	1
HU-210	50	5
JWH-018 Metabolite	0.25	2
JWH-019 5-Hydroxyheyxyl metabolite	0.25	2
JWH-073 Metabolite	0.25	2
JWH-122 5-Hydroxypentyl metabolite	0.25	3
JWH-250 5-Hydroxypentyl	0.5	4
PB-22	0.25	4
THC	25	5
UR-144	0.5	6
UR-144 5-Hydroxypentyl metabolite	0.5	6
XLR-11	0.5	6
XLR-11 4-Hydroxypentyl metabolite	0.5	6
Internal Standards		
IS AM2201 4-Hydroxypentyl metabolite D5	1	1
IS JWH-018 Metabolite-D5	1	2
IS JWH-122 4-Hydroxypentyl metabolite D5	1	3
IS JWH-250 4-Hydroxypentyl metabolite D5	1	4
IS THC-D3	10	5
IS UR-144 5-Hydroxypentyl metabolite-D5	1	6

Linearity and Carryover

Linearity and carryover were accessed by extracting six, six-point calibration curves consisting of a 1c, 2c, 5c, 10c, 15c, 25c, and Blank sample. The 1c for each analyte was the LOQ concentration listed in Table 3, except for THC with an LOQ corresponding to the 2c calibrator. The calibration curves were prepared by serial dilution of a blank urine sample spiked with the 250c stock solution shown in Table 3. The calibrators and blank samples were then extracted according to the fine SPE method procedure. Calibration curves were analyzed from 1c to 25c with the blank sample

following the 25c in order to access carryover from the highest concentration included in the method. Lab Solutions software was used to determine the best fitting line and weighting for each analyte.

Matrix Effects

Matrix effects were evaluated by analyzing two neat urine samples, five post-extraction spiked urine samples, and five pre-extraction spike urine samples. The neat urine samples were prepared by combining 2 µL of 250c standard stock solution, 10 µL of internal standard solution, and 88 µL of 20% MeOH in the well of a 1 mL 96-well plate. The first neat urine sample was injected three times, and the second neat urine sample was injected two time to make a total of five neat urine sample analyses.

The post-extraction spiked samples were prepared by adding 200 µL of blank urine to a CEREX® HP SAX 5 mg NBE column. All five blank samples were from a different individual. Next 370 µL of modified hydrolysis solution was added to each sample. The modified hydrolysis solution consisted of 350 µL of 0.3 M sodium acetate buffer (pH 4.8), 10 µL of MeOH, and 10 µL of H_2O per sample. The samples were then extracted according to the final SPE method through the sample evaporation to dryness step. Finally, the samples were reconstituted with 2 µL of 250c standard stock solution, 10 µL of internal standard solution, and 88 µL of 20% MeOH. Each sample was injected once.

The pre-extraction spiked samples were prepared by adding 2 µL of 250c standard stock solution and 200 µL of blank urine samples from five different individuals

to CEREX® HP SAX 5 mg NBE columns. The samples were then extracted according to the final SPE method and inject once.

The Matrix Effect (ME) was calculated by dividing the average peak area of the post-extraction urine samples by the peak area of the average peak area of the neat solution samples and multiplying the result by 100. This value is expressed as a percentage. The Recovery Efficiency (RE) was determined by dividing the average of the pre-extraction spike samples by the average of the post-extraction spike samples and multiplying by 100. The RE is also expressed as a percentage. Process Efficiency (PE) was determined by dividing the average of the pre-extraction spike samples by the average of the neat samples and multiplying by 100. The PE is also expressed as a percentage.

Statistical Analysis

The comparison studies of column bed depth, column ion affinity, elution solvent, elution pH, and wash solvent included only a single replicate for each variable. For this reason statistical analyses of these data could not be performed. In order to determine which modification was better suited for the analysis of the target molecules, the peak area data for each analyte were imported into an Excel file and compared visually. The modification from each comparison study that produced the highest peak areas consistently was determined to be most effective.

Samples were extracted in triplicate and reconstituted with one of two different solvents for the reconstitution comparison study. Peak area data for both reconstitution solvents were imported to Excel. A log10 transformation was performed on the raw data

in order to alleviate departures from normality. T-tests for each analyte were performed using GraphPad Prism software (San Diego, CA). The p-value from each test was used to determine the statistical difference, if any, between the average peak areas produced by the two reconstitution solvents.

Conclusion

Spiked urine samples were extracted by SPE and analyzed via LC-MS/MS. The final SPE method was determined to be the most effective procedure for extracting the synthetic cannabinoid analytes included in this method. The final method was determined by individually altering variables such as column depth and type; and wash, elution, and reconstitution solvents. The peak areas produced by the altered methods were then compared visually to determine which produced the best analysis for the target analytes. A statistical analysis was performed on the data gathered from the comparison of reconstitution solvents. The findings from all comparison studies were combined to determine the final method. Finally linearity, carryover, matrix effects, recovery efficiency, and process efficiency were determined for the final method.

CHAPTER IV

RESULTS

Column Bed and Elution Solvents

For most analytes there was little difference between the peak areas of the four extraction methods. As shown in Table 4, the DCM:IPA:FA (80:18:2) elutions had larger peak areas than the EtOAc:FA (98:2) elutions for the 500 ng/mL samples. Based on a visual comparison of the data, the performances of the 5 mg and 2.5 mg column beds were similar for the 500 ng/mL samples. When comparing the peak area data for all calibrator levels, no method consistently has larger peak areas for all analytes. However, the peak areas of the DCM:IPA:FA (80:18:2) elutions were larger more frequently than the EtOAc:FA (98:2) elution peak areas.

THCA, THCA-D3, Δ9-THC, Δ9-THC-D3, and HU-210 were not successfully extracted for any of the four methods. JWH-250 5-Hydroxypentyl was not detected below 5 ng/mL, UR-144 was not detected below 50 ng/mL, UR-144 5-Hydroxypentyl metabolite was not detected below 10 ng/mL, and XLR-11 was not detected below 10 ng/mL for any of the extraction methods.

Table 4. Peak areas for of 500 ng/mL analytes for the comparison of 5 mm and 2.5 mm column bed depths, and ethyl acetate and dichloromethane elution solvents. The highest peak area ratio for each analyte is indicate with **bold** font, and "-----" indicates that no peak was detected.

Analyte	5 mm Column, EtOAc Elution	2.5 mm Column, EtOAc Elution	5 mm Column, DCM Elution	2.5 mm Column, DCM Elution
5-Fluoro PB-22	3.32E+07	4.08E+07	4.39E+07	**4.57E+07**
AB-FUBINACA	1.29E+06	**2.35E+06**	2.18E+06	2.17E+06
AM2201 4-Hydroxypentyl metabolite	8.40E+06	9.03E+06	9.52E+06	**9.73E+06**
HU-210	-----	-----	-----	-----
IS AM2201 4-Hydroxypentyl metabolite D5	6.36E+05	8.07E+05	**9.46E+05**	9.06E+05
IS JWH-018 Metabolite-D5	1.90E+05	2.38E+05	2.82E+05	**2.88E+05**
IS JWH-122 4-Hydroxypentyl metabolite D5	1.11E+06	1.35E+06	1.59E+06	**1.61E+06**
IS JWH-250 4-Hydroxypentyl metabolite D5	4.58E+05	6.61E+05	**7.91E+05**	6.56E+05
IS THC-D3	-----	-----	-----	-----
IS THCA-D3	-----	-----	-----	-----
IS UR-144 5-Hydroxypentyl metabolite-D5	6.07E+05	7.81E+05	**1.02E+06**	8.73E+05
JWH-018 Metabolite	1.04E+07	1.61E+07	**1.63E+07**	1.62E+07
JWH-019 5-Hydroxyheyxyl metabolite	9.75E+06	1.09E+07	**1.26E+07**	1.25E+07
JWH-073 Metabolite	1.41E+07	1.77E+07	**1.94E+07**	1.88E+07
JWH-122 5-Hydroxypentyl metabolite	7.03E+06	1.16E+07	1.19E+07	**1.25E+07**
JWH-250 5-Hydroxypentyl	6.24E+06	6.45E+06	**7.63E+06**	6.72E+06
PB-22	2.97E+07	4.01E+07	3.93E+07	**4.06E+07**
THC	-----	-----	-----	-----
THCA	-----	-----	-----	-----
UR-144	1.67E+06	**1.95E+06**	1.55E+06	1.60E+06
UR-144 5-Hydroxypentyl metabolite	5.05E+06	7.27E+06	**8.25E+06**	7.73E+06
XLR-11	6.26E+06	7.05E+06	7.29E+06	**7.39E+06**
XLR-11 4-Hydroxypentyl metabolite	4.56E+06	4.34E+06	**5.71E+06**	4.61E+06

Water to Methanol Ratio in Wash

The unsuccessful extraction of THCA, THCA-D3, Δ9-THC, Δ9-THC-D3, and HU-210 in the previous study was thought to be a result of the high methanol content of the second wash in the SPEware SPE method. In attempt to recover the lost analytes, one set of 500 ng/mL urine samples was extracted and washed with the recommended 70% MeOH wash, one set was washed with less methanol (30% MeOH), and a third set was

washed without methanol. The analyte peak areas from the 5 mg column extractions are shown in Table 5, and the peak areas from the 2.5 mg column extractions are shown in Table 6.

Table 5. Peak areas for the effect of methanol content of the second wash on analyte extraction for 5 mg column bed. "-----" indicates that no peak was detected.

Analyte	70% MeOH	30% MeOH	100% H_2O
5-Fluoro PB-22	2.98E+07	7.55E+07	6.06E+07
AB-FUBINACA	1.72E+06	1.13E+07	1.94E+07
AM2201 4-Hydroxypentyl metabolite	6.27E+06	5.36E+07	3.68E+07
HU-210	-----	-----	-----
IS AM2201 4-Hydroxypentyl metabolite D5	3.59E+05	2.71E+06	2.51E+06
IS JWH-018 Metabolite-D5	1.28E+05	1.46E+06	7.17E+05
IS JWH-122 4-Hydroxypentyl metabolite D5	4.98E+05	4.61E+06	2.44E+06
IS JWH-250 4-Hydroxypentyl metabolite D5	2.44E+05	2.88E+06	4.91E+06
IS THC-D3	-----	-----	-----
IS THCA-D3	-----	-----	-----
IS UR-144 5-Hydroxypentyl metabolite-D5	5.21E+05	3.96E+06	4.00E+06
JWH-018 Metabolite	1.06E+07	5.67E+07	4.96E+07
JWH-019 5-Hydroxyheyxyl metabolite	8.21E+06	5.28E+07	3.86E+07
JWH-073 Metabolite	9.43E+06	6.36E+07	5.26E+07
JWH-122 5-Hydroxypentyl metabolite	8.03E+06	4.81E+07	2.96E+07
JWH-250 5-Hydroxypentyl	3.95E+06	5.28E+07	5.41E+07
PB-22	2.89E+07	5.00E+07	3.86E+07
THC	-----	-----	-----
THCA	-----	-----	-----
UR-144	1.10E+06	1.25E+06	1.18E+06
UR-144 5-Hydroxypentyl metabolite	4.77E+06	3.13E+07	4.51E+07
XLR-11	5.51E+06	1.10E+07	9.60E+06
XLR-11 4-Hydroxypentyl metabolite	2.70E+06	2.24E+07	2.71E+07

Table 6. Peak areas for effects of methanol content of the second wash on analyte extraction for 2.5 mg column bed. "-----" indicates that no peak was detected.

Analyte	70% MeOH	30% MeOH	100% H_2O
5-Fluoro PB-22	3.70E+07	7.52E+07	6.77E+07
AB-FUBINACA	2.29E+06	7.03E+06	1.32E+07
AM2201 4-Hydroxypentyl metabolite	9.10E+06	5.03E+07	3.93E+07
HU-210	-----	-----	-----
IS AM2201 4-Hydroxypentyl metabolite D5	3.71E+05	2.35E+06	2.62E+06
IS JWH-018 Metabolite-D5	1.91E+05	1.19E+06	7.75E+05
IS JWH-122 4-Hydroxypentyl metabolite D5	7.70E+05	4.25E+06	3.23E+06
IS JWH-250 4-Hydroxypentyl metabolite D5	3.06E+05	1.29E+06	3.11E+06
IS THC-D3	-----	-----	-----
IS THCA-D3	-----	-----	-----
IS UR-144 5-Hydroxypentyl metabolite-D5	7.25E+05	2.27E+06	2.84E+06
JWH-018 Metabolite	1.04E+07	5.41E+07	5.14E+07
JWH-019 5-Hydroxyheyxyl metabolite	1.25E+07	5.21E+07	5.01E+07
JWH-073 Metabolite	9.87E+06	5.75E+07	5.12E+07
JWH-122 5-Hydroxypentyl metabolite	8.06E+06	4.83E+07	4.01E+07
JWH-250 5-Hydroxypentyl	6.37E+06	3.37E+07	4.01E+07
PB-22	4.08E+07	5.30E+07	5.04E+07
THC	-----	-----	-----
THCA	-----	-----	-----
UR-144	1.51E+06	1.64E+06	1.12E+06
UR-144 5-Hydroxypentyl metabolite	4.52E+06	1.65E+07	3.49E+07
XLR-11	8.18E+06	1.22E+07	1.14E+07
XLR-11 4-Hydroxypentyl metabolite	3.99E+06	1.38E+07	1.82E+07

Decreasing the methanol content of the second wash step did not improve the extraction and detection of THCA, THCA-D3, Δ9-THC, Δ9-THC-D3, or HU-210. However, the peak areas for all other analytes were greatly increased when less methanol was used in the second wash step. These results indicated that the high methanol content (70% MeOH) of the recommended wash step resulted in the loss of synthetic cannabinoid analyte during the extraction process. Based on these results, the SPEware SPE method was determined to be inappropriate for the extraction of the analytes of interest in this study due to high methanol concentration in the second wash step. Further analyses were

completed by following the FTTL SPE method which had no methanol in any of the three wash solutions.

Column Ion Affinity and Eluent pH

The FTTL SPE method was previously shown to successfully extract THCA, JWH-018 metabolite, and JWH-073 metabolite with a cation exchange column, (CEREX® HP SCX 5 mg NBE column), and a basic eluent consisting of DCM:IPA:NH_4OH (80:18:2). Previous analyses during this study were competed with anion exchange columns, (CEREX® HP SAX 5 mg and 2.5 mm NBE columns), and acid eluents containing 2% formic acid.

In order to determine which column type and eluent pH combination was most effective for the extraction of the analytes in this study, four 500 ng/mL samples were extracted following the SPEware sample preparation procedure and the FTTL SPE procedure. The first sample was extracted with a HP SAX column paired with an acidic DCM:IPA:FA (80:18:2) eluent. A second sample was extracted with the HP SCX and the acidic DCM:IPA:FA (80:18:2) eluent. The HP SAX and HP SCX column were then used for the third and fourth samples respectively, and paired with a basic DCM:IPA:NH_4OH eluent. The same four extractions were then repeated using the FTTL sample preparation procedure.

The peak areas for the samples prepared by the SPEware procedure are shown in Table 7. The HP SCX with FA eluent and HP SAX with NH_4OH eluent produced the highest peak areas for most analytes. From these data it was determined that acid eluents are better for the recovery of synthetic cannabinoids from cation exchange columns, while basic eluents are better for the recovery of synthetic cannabinoids from anion exchange columns.

Table 7. Peak areas for analysis of the effect of the column ion affinity and acid or basic elution on the extraction of synthetic cannabinoids. Samples prepared via the SPEware SPE method sample preparation procedure. The largest peak area for each column/elution combination is indicated with **bold** font.

Analyte	HP SAX DCM:IPA: FA	HP SCX DCM:IPA: FA	HP SAX DCM:IPA: NH4OH	HP SCX DCM:IPA: NH4OH
5-Fluoro PB-22	3.36E+07	5.24E+07	4.94E+07	**6.54E+07**
AB-FUBINACA	1.85E+07	**4.83E+07**	4.76E+07	4.53E+07
AM2201 4-Hydroxypentyl metabolite	2.04E+07	4.54E+07	**6.52E+07**	4.57E+07
HU-210	1.23E+04	6.09E+04	**2.78E+05**	1.04E+04
IS AM2201 4-Hydroxypentyl metabolite D5	9.95E+05	**3.43E+06**	2.36E+06	2.43E+06
IS JWH-018 Metabolite-D5	5.06E+05	9.36E+05	**1.25E+06**	8.28E+05
IS JWH-122 4-Hydroxypentyl metabolite D5	1.69E+06	4.77E+06	**5.64E+06**	3.49E+06
IS JWH-250 4-Hydroxypentyl metabolite D5	2.31E+06	**8.93E+06**	2.20E+06	7.23E+06
IS THC-D3	4.10E+03	8.38E+04	**1.83E+05**	5.58E+03
IS THCA-D3	7.53E+03	**1.00E+04**	4.98E+03	5.86E+03
IS UR-144 5-Hydroxypentyl metabolite-D5	3.22E+06	6.70E+06	**7.65E+06**	6.32E+06
JWH-018 Metabolite	2.54E+07	5.69E+07	**5.95E+07**	5.56E+07
JWH-019 5-Hydroxyheyxyl metabolite	2.46E+07	**5.60E+07**	5.14E+07	5.01E+07
JWH-073 Metabolite	2.40E+07	5.41E+07	**5.73E+07**	5.27E+07
JWH-122 5-Hydroxypentyl metabolite	1.26E+07	4.11E+07	**4.27E+07**	2.77E+07
JWH-250 5-Hydroxypentyl	3.85E+07	**6.48E+07**	4.55E+07	5.83E+07
PB-22	2.40E+07	3.19E+07	**5.18E+07**	3.98E+07
THC	4.19E+03	6.65E+04	**1.27E+05**	5.57E+03
THCA	5.76E+03	**1.43E+04**	9.28E+03	7.47E+03
UR-144	9.23E+05	9.48E+06	**1.50E+07**	1.10E+06
UR-144 5-Hydroxypentyl metabolite	2.12E+07	5.33E+07	**5.54E+07**	5.31E+07
XLR-11	6.25E+06	1.74E+07	**2.27E+07**	7.97E+06
XLR-11 4-Hydroxypentyl metabolite	1.58E+07	4.01E+07	3.96E+07	**4.34E+07**

The peak areas for the samples prepared via the FTTL procedure are shown in Table 8. The HP SAX column paired with the basic NH_4OH eluent produced the highest peak area for most analytes. Overall for the two sample preparation methods, the FTTL preparation with the HP SAX column and DCM:IPA:NH_4OH elution performed best. The HP SCX column with either eluent produced higher peak areas for THCA, however the increase in peak area was minimal, and the HP SAX column showed better recovery of HU-210. A higher peak area for HU-210 and the other synthetic cannabinoids was given priority over the higher peak area of THCA for this

method. Therefore, the FTTL sample preparation with the HP SAX column and DCM:IPA:NH_4OH elution was determined to be the best method for the extraction of the synthetic cannabinoid analytes included in this study.

Table 8. Peak areas for analysis of the effect of the column ion affinity and acid or basic elution on the extraction of synthetic cannabinoids. Samples prepared via the FTTL SPE method sample preparation procedure. The largest peak area for each column/elution combination is indicated with **bold** font.

Analyte	HP SAX DCM:IPA: FA	HP SCX DCM:IPA: FA	HP SAX DCM:IPA: NH4OH	HP SCX DCM:IPA: NH4OH
5-Fluoro PB-22	3.70E+07	4.83E+04	**7.09E+07**	4.76E+07
AB-FUBINACA	4.23E+07	3.70E+07	**5.76E+07**	3.75E+07
AM2201 4-Hydroxypentyl metabolite	4.00E+07	3.13E+07	**5.37E+07**	3.16E+07
HU-210	2.14E+04	2.50E+04	**2.76E+05**	6.08E+03
IS AM2201 4-Hydroxypentyl metabolite D5	1.84E+06	1.55E+06	**3.33E+06**	1.30E+06
IS JWH-018 Metabolite-D5	6.77E+05	5.34E+05	**1.40E+06**	6.98E+05
IS JWH-122 4-Hydroxypentyl metabolite D5	2.63E+06	2.14E+06	**6.69E+06**	2.24E+06
IS JWH-250 4-Hydroxypentyl metabolite D5	2.52E+06	**4.42E+06**	3.94E+06	3.87E+06
IS THC-D3	9.87E+03	1.34E+04	**6.62E+04**	2.05E+03
IS THCA-D3	3.20E+03	**4.99E+03**	3.89E+03	3.26E+03
IS UR-144 5-Hydroxypentyl metabolite-D5	4.47E+06	3.36E+06	**9.20E+06**	4.79E+06
JWH-018 Metabolite	4.22E+07	3.39E+07	**6.76E+07**	3.76E+07
JWH-019 5-Hydroxyheyxyl metabolite	4.27E+07	3.36E+07	**5.96E+07**	3.95E+07
JWH-073 Metabolite	5.07E+07	4.28E+07	**6.26E+07**	3.84E+07
JWH-122 5-Hydroxypentyl metabolite	1.87E+07	1.36E+07	**5.41E+07**	1.85E+07
JWH-250 5-Hydroxypentyl	4.75E+07	4.74E+07	**5.72E+07**	5.12E+07
PB-22	2.24E+07	7.92E+04	**6.07E+07**	1.83E+07
THC	1.58E+04	2.18E+04	**8.81E+04**	2.57E+03
THCA	1.08E+04	**1.33E+04**	7.02E+03	6.15E+03
UR-144	1.72E+06	2.35E+06	**8.52E+06**	4.32E+05
UR-144 5-Hydroxypentyl metabolite	3.79E+07	2.83E+07	**6.48E+07**	3.74E+07
XLR-11	6.71E+06	6.82E+06	**1.70E+07**	3.85E+06
XLR-11 4-Hydroxypentyl metabolite	3.96E+07	3.47E+07	**4.83E+07**	2.82E+07

Reconstitution Solvent

The last adjustment to the SPE method was the content of the reconstitution solvent. All previous samples were reconstituted with 100 µL of 9:1 MPA:MPB, which is 10% acetonitrile in water. However, the FTTL SPE procedure was previously shown to successfully extract THCA,

JWH-018 metabolite, and JWH-073 metabolite in clinical urine samples by reconstituting with 100 µL of 20% MeOH. In addition, the data from the analysis of the MeOH content of SPE wash solutions indicated that synthetic cannabinoids are easily displaced off the SPE column by methanol. Therefore, a 20% MeOH reconstitution solvent may increase the recovery of dried analytes.

Six samples were extracted following the same procedure; three samples were reconstituted with 100 µL of MPA:MPB (9:1), and three were reconstituted with 100 µL of 20% MeOH. The three peak areas for each reconstitution step were averaged and the standard deviation was calculated. The first sample for the 20% MeOH reconstitution had much lower peak areas than the other two samples. This indicated that there may have been a bad injection on the instrument or a mistake during the extraction. The inaccurate data were substituted with a data from a 20% MeOH reconstitution sample run at a later date.

The average peak areas for the log10 transformed data, relative standard deviations, and p-values of the t-tests for all analytes are shown in Table 9. At an alpha value of 0.1 – 0.05, there is a significant difference in analyte recovery between the two reconstitution methods for AM2201 metabolite and AM2201 D-5, UR-144 metabolite, and XLR-11. At and alpha value of 0.05 – 0.01, the average peak area ratios were significantly higher with the 20% MeOH reconstitution for 5-fluoro PB-22, JWH-018 and JWH-018 D-5, UR-144 metabolite D-5, and JWH-019 metabolite,JWH-073 metabolite, JWH-122 metabolite, and JWH-250 metabolite. These results indicate that a methanol based reconstitution provides better recovery of synthetic cannabinoids than an acetonitrile based reconstitution.

However, there was still not good recovery of THCA or THCA-D3 with either reconstitution method. Two 500 ng/mL THCA samples were extracted and reconstituted with each reconstitution solution. The data for the two THCA samples are shown in Table 10. The

detection of THCA still not sufficiently improved when potential interference from other analytes were removed. Based on these data, the method was determined to be insufficiently sensitive for the detection of THCA. THCA and THCA-D3 were not included in the final method.

Table 9. Average peak area and standard deviation of samples reconstituted with 9:1 MPA:MPB and 20% MeOH. Standard deviations are reported as percent standard deviation relative to the mean. †Calculated with a fourth sample substituted for the first sample. *Denotes statistical difference at alpha 0.1 – 0.05. **Denotes statistical difference at alpha 0.05 – 0.01.

Analyte	Average MPA:MPB	RSD	†Average 20% MeOH	RSD	p-value
5-Fluoro PB-22	7.9	0.7%	8.0	0.3%	0.03**
AB-FUBINACA	7.7	0.6%	7.7	0.7%	0.25
AM2201 4-Hydroxypentyl metabolite	7.7	1.2%	7.8	0.3%	0.09*
HU-210	5.6	7.6%	5.8	1.4%	0.33
IS AM2201 4-Hydroxypentyl metabolite D5	6.5	1.2%	6.6	0.4%	0.06*
IS JWH-018 Metabolite-D5	6.1	1.5%	6.3	0.8%	0.04**
IS JWH-122 4-Hydroxypentyl metabolite D5	6.9	1.6%	7.0	0.9%	0.12
IS JWH-250 4-Hydroxypentyl metabolite D5	6.4	1.9%	6.7	0.5%	0.02**
IS THC-D3	5.0	4.4%	5.3	4.7%	0.26
IS THCA-D3	3.9	2.7%	4.1	3.0%	0.16
IS UR-144 5-Hydroxypentyl metabolite-D5	6.9	1.3%	7.1	0.3%	0.03**
JWH-018 Metabolite	7.8	0.2%	7.9	0.1%	0.01**
JWH-019 5-Hydroxyheyxyl metabolite	7.8	0.3%	7.9	0.1%	0.01**
JWH-073 Metabolite	7.8	0.3%	7.9	0.2%	0.03**
JWH-122 5-Hydroxypentyl metabolite	7.8	0.3%	7.9	0.2%	0.02**
JWH-250 5-Hydroxypentyl	7.7	0.5%	7.8	0.3%	0.02**
PB-22	7.8	1.1%	7.9	0.8%	0.21
THC	5.1	4.2%	5.4	4.6%	0.29
THCA	4.0	3.2%	4.1	5.4%	0.33
UR-144	7.1	3.2%	7.3	4.7%	0.36
UR-144 5-Hydroxypentyl metabolite	7.8	0.3%	7.8	0.1%	0.06*
XLR-11	7.5	1.4%	7.7	1.2%	0.05*
XLR-11 4-Hydroxypentyl metabolite	7.7	0.3%	7.7	0.2%	0.02**

Table 10. Peak areas of the 500 ng/mL THCA samples reconstituted with 9:1 MPA:MPB and 20% MeOH.

Analyte	9:1 MPA:MPB	20% MeOH
THCA	24,035	22,146
IS THC-D3	30,467	25,348

Linearity and Carryover

Linearity and carryover studies were performed on the final method. For the linearity study six calibration curves were extracted and analyzed. Each calibration curve consisted of a 1c at the concentration of the Lower limit of quantitation (LLOQ), 2c, 5c, 10c, 15c, and 25c at the Upper limit of quantitation (ULOQ). Except for the Δ9-THC calibration curve, which had consisted of a 2c at the concentration of the LLOQ, 5c, 10c, 15c, and 25c at the ULOQ. Table 11 provides the LLOQ, ULOQ, calibration fit, and weighting for each analyte.

Table 11. Lower limit of quantitation (LLOQ), Upper limit of quantitation (ULOQ), calibration curve fit type, and weighting used for each analyte.

Analyte	LLOQ	ULOQ	FIT TYPE	WEIGHT
5-Fluoro PB-22	1	25	Quadratic	1/C^2
AB-FUBINACA	1	25	Linear	1/C^2
AM2201 4-Hydroxypentyl metabolite	1	25	Linear	1/C
HU-210	400	5000	Quadratic	1/C
JWH-018 Metabolite	1	25	Quadratic	1/C^2
JWH-019 5-Hydroxyheyxyl metabolite	1	25	Linear	1/C^2
JWH-073 Metabolite	1	25	Linear	1/C^2
JWH-122 5-Hydroxypentyl metabolite	1	25	Quadratic	1/C^2
JWH-250 5-Hydroxypentyl	2	50	Linear	1/C^2
PB-22	1	25	Quadratic	1/C^2
THC	200	2500	Linear	None
UR-144	2	50	Quadratic	1/C^2
UR-144 5-Hydroxypentyl metabolite	2	50	Linear	1/C
XLR-11	2	50	Quadratic	1/C
XLR-11 4-Hydroxypentyl metabolite	2	50	Linear	1/C^2

Blank urine samples were extracted and analyzed after the highest concentration calibrator to access the carryover for all analytes in the method. No peaks were detected above the LLOQ for any analytes at the 25c level. The method was determined to have no carryover within the quantitation range.

Matrix Effects

Five samples each of neat, pre-extraction spike, and post-extraction spike samples were analyzed to determine the Matrix Effects (ME), Recovery Efficiency (RE), and Process Efficiency (PE) of each analyte. The ME values provides a measure of ion suppression and enhancement. Values should ideally be within 40% of the target concentration. RE measures the efficiency of analyte recovery of the extraction method. Ideally the RE will be greater than 50%. PE measures the measures the efficiency of analyte detection on the instrument. RE and PE values less than 50% may be acceptable if the method is sufficiently selective and sensitive enough to produce forensically or medically relevant results. The ME, RE, and PE values for all analytes are shown in Table 11.

There is not significant ion suppression for any analytes, but 5-Fluoro PB-22, PB-22, and HU-210 all have significant ion enhancement. The enhancement is most likely caused by the other analytes within the method. Therefore, chromatography must be adjust before these three analytes can pass validation. The REs of 5-Fluoro PB-22 and PB-22 are 25% and 17%, respectively, which is far below the lowest ideal value of 50%. HU-210 also has significantly high values for RE (158%) and PE (417%). The REs and PEs of UR-144 and XLR-11 are also below 50%.

Table 12. Matrix effects (ME), Recovery efficiency (RE), and Process efficiency (PE) for all analytes. ME values with **bold** font indicate ion suppression or enhancement greater than or less than 40%. RE and PE values with **bold** font indicate less than 50% efficiency.

Analyte	Matrix Effects (ME)	Recovery Efficiency (RE)	Process Efficiency (PE)
5-Fluoro PB-22	**242%**	**25%**	60%
AB-FUBINACA	75%	143%	107%
AM2201 4-Hydroxypentyl metabolite	110%	59%	65%
HU-210	**265%**	158%	417%
JWH-018 Metabolite	74%	91%	68%
JWH-019 5-Hydroxyheyxyl metabolite	87%	77%	67%
JWH-073 Metabolite	79%	83%	66%
JWH-122 5-Hydroxypentyl metabolite	121%	61%	74%
JWH-250 5-Hydroxypentyl	99%	67%	67%
PB-22	**270%**	**17%**	**46%**
THC	104%	79%	82%
UR-144	108%	**12%**	**12%**
UR-144 5-Hydroxypentyl metabolite	74%	78%	58%
XLR-11	64%	**29%**	**19%**
XLR-11 4-Hydroxypentyl metabolite	98%	82%	80%

CHAPTER V

CONCLUSION

The purpose of this study was to provide an improved extraction and detection method for synthetic cannabinoids in urine. An additionally goal was to successfully detect a natural cannabinoid, Δ9-THC, and its metabolite, THCA. The final method included 10 synthetic cannabinoid metabolites, 4 synthetic cannabinoid parent drugs, and Δ9-THC. All of the synthetic cannabinoids included in the method are currently controlled by the Drug Enforcement Agency as Schedule I controlled substances.[34]

The size of the column bed did not make a much impact on the extraction of synthetic cannabinoids. The data from the first comparison study show little difference in the peak areas of the 5 mg and 2.5 mg columns. However, the first study did show that eluent polarity impacts the elution of synthetic cannabinoids. The dichloromethane elution peak areas were consistently lager than the peak areas of the ethyl acetate elutions.

The comparison study of the methanol content in SPE washes showed that synthetic cannabinoids are partially eluted off the SPE column by high concentrations of methanol. The 70% MeOH washed samples showed notably lower peak areas than the samples washed with little or no methanol. These data indicate that methanol should not be included in any wash step for the SPE extraction of synthetic cannabinoids.

The synthetic cannabinoids in this study were best extracted with a anion exchanging column (HP SAX) paired with a basic eluent (DCM:IPANH_4OH 80:18:2). The best results for

HP SAX and DCM:IPA:NH_4OH combination were achieved when using 350 µL of 0.3 M sodium acetate buffer (pH 4.8) during the sample preparation according to the FTTL procedure. The THCA peak areas were largest with a cation exchanging column (HP SCX) paired with an acidic eluent (DCM:IPA:FA 80:18:2). If including THCA in the synthetic cannabinoid method was high priority, the HP SCX and DCM:IPA:FA elution would be the most favorable column/eluent combination.

The final comparison study showed that reconstituting the dried samples with 20% MeOH had significantly higher peak areas than the samples reconstructed with an acetonitrile solutions (MPA:MPA 9:1) for 4 analytes at an alpha of 0.1 – 0,05 and 10 analytes at an alpha of 0.05 – 0.01. The increase in peak area indicates that the synthetic cannabinoids are more soluble in methanol than acetonitrile.

Validation studies performed on the final method showed no analyte carryover at the 25c calibrator. The linearity study showed that while some analytes fit a linear calibration model, other were better fit with a quadratic calibration model. Matrix effects studies showed no major ion suppression, but significant ion enhancement was present for HU-210, 5-Fluoro PB-22, and PB-22. The ion enhancement of these drugs must be addressed and corrected before further validation can occur. PB-22, 5-Fluoro PB-22, UR-144, and XLR-11 had recovery efficiencies below the minimum desired value of 50%. PB-22, UR-144, and XLR-11 had process efficiency values below the minimum desired value of 50%. However, recovery and process efficiencies below 50% are acceptable as long as the method is sufficiently sensitive enough to provide medically or forensically relevant results.

The information gained during the comparison studies enabled the improvement of existing SPE methods for the extraction of synthetic cannabinoids from urine. With the original SPE extraction method, THC, HU-210, and THCA could not be detected at or below 500 ng/mL. With the improved method, THC and HU-210 were detectable down to 200 ng/mL and 400 ng/mL respectively, and although THCA was not included in the final method it was detectable at 500 ng/mL. The peak areas were greatly increased for all synthetic cannabinoids included in this study. The knowledge gained during this study can be applied to the continued improvement of synthetic cannabinoid detection and the inclusion of more synthetic cannabinoids in the existing method.

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APPENDICES

A1. Data for the three replicates of the 9:1 MPA:MPB reconstitution gathered during the reconstitution solvent comparison study			
Analyte	Sample 1	Sample 2	Sample 3
5-Fluoro PB-22	6.31E+07	7.66E+07	7.99E+07
AB-FUBINACA	4.74E+07	4.58E+07	5.55E+07
AM2201 4-Hydroxypentyl metabolite	3.83E+07	5.14E+07	5.76E+07
HU-210	1.19E+05	6.98E+05	5.94E+05
IS AM2201 4-Hydroxypentyl metabolite D5	3.40E+06	2.60E+06	3.70E+06
IS JWH-018 Metabolite-D5	1.28E+06	1.21E+06	1.79E+06
IS JWH-122 4-Hydroxypentyl metabolite D5	6.14E+06	6.53E+06	9.76E+06
IS JWH-250 4-Hydroxypentyl metabolite D5	3.06E+06	1.98E+06	3.33E+06
IS THC-D3	6.12E+04	1.47E+05	1.50E+05
IS THCA-D3	6.33E+03	9.21E+03	1.00E+04
IS UR-144 5-Hydroxypentyl metabolite-D5	7.21E+06	6.96E+06	1.02E+07
JWH-018 Metabolite	6.61E+07	6.60E+07	7.07E+07
JWH-019 5-Hydroxyheyxyl metabolite	6.21E+07	5.79E+07	6.50E+07
JWH-073 Metabolite	6.22E+07	6.08E+07	6.80E+07
JWH-122 5-Hydroxypentyl metabolite	5.97E+07	5.93E+07	6.55E+07
JWH-250 5-Hydroxypentyl	5.18E+07	4.71E+07	5.58E+07
PB-22	5.39E+07	7.50E+07	7.79E+07
THC	7.68E+04	1.76E+05	1.88E+05
THCA	7.08E+03	8.90E+03	1.27E+04
UR-144	6.78E+06	1.71E+07	1.61E+07
UR-144 5-Hydroxypentyl metabolite	5.90E+07	5.85E+07	6.51E+07
XLR-11	2.14E+07	3.14E+07	3.36E+07
XLR-11 4-Hydroxypentyl metabolite	4.44E+07	4.23E+07	4.78E+07

A2. Data for the three replicates of the 20% methanol reconstitution gathered during the reconstitution solvent comparison study			
Analyte	Sample 1	Sample 2	Sample 3
5-Fluoro PB-22	9.04E+07	9.21E+07	1.01E+08
AB-FUBINACA	5.87E+07	6.14E+07	4.86E+07
AM2201 4-Hydroxypentyl metabolite	6.05E+07	6.56E+07	6.61E+07
HU-210	6.78E+05	5.79E+05	8.46E+05
IS AM2201 4-Hydroxypentyl metabolite D5	4.08E+06	4.63E+06	4.19E+06
IS JWH-018 Metabolite-D5	2.02E+06	1.92E+06	2.38E+06
IS JWH-122 4-Hydroxypentyl metabolite D5	1.08E+07	8.60E+06	1.14E+07
IS JWH-250 4-Hydroxypentyl metabolite D5	4.53E+06	5.08E+06	5.16E+06
IS THC-D3	1.37E+05	1.47E+05	3.80E+05
IS THCA-D3	8.86E+03	1.35E+04	1.50E+04
IS UR-144 5-Hydroxypentyl metabolite-D5	1.16E+07	1.15E+07	1.27E+07
JWH-018 Metabolite	7.50E+07	7.54E+07	7.86E+07
JWH-019 5-Hydroxyheyxyl metabolite	7.21E+07	7.27E+07	7.42E+07
JWH-073 Metabolite	7.10E+07	7.66E+07	7.25E+07
JWH-122 5-Hydroxypentyl metabolite	7.35E+07	6.88E+07	7.02E+07
JWH-250 5-Hydroxypentyl	6.11E+07	6.61E+07	6.72E+07
PB-22	7.86E+07	7.64E+07	9.90E+07
THC	1.75E+05	1.61E+05	4.46E+05
THCA	1.39E+04	2.28E+04	8.08E+03
UR-144	1.52E+07	1.26E+07	5.35E+07
UR-144 5-Hydroxypentyl metabolite	6.84E+07	6.52E+07	6.72E+07
XLR-11	4.30E+07	4.06E+07	5.92E+07
XLR-11 4-Hydroxypentyl metabolite	4.98E+07	5.31E+07	5.24E+07

VITA

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