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Detection and Characterization of Delta-9-Tetrahydrocannabinol and Novel Synthetic Cannabinoid Metabolites using Human Liver Microsomes

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DETECTION AND CHARACTERIZATION OF Δ-9-TETRAHYDROCANNIBINOL AND NOVEL SYNTHETIC CANNABINOID METABOLITES USING HUMAN LIVER MICROSOMES

by
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A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

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ABSTRACT

SEEMA MURUGAN: DETECTION AND CHARACTERIZATION OF Δ-9-TETRAHYDROCANNIBINOL AND NOVEL SYNTHETIC CANNABINOID METABOLITES USING HUMAN LIVER MICROSOMES
(Under the direction of Dr. Murrell Godfrey)

Marijuana and synthetic cannabinoids are the most commonly used and trafficked illicit drug. Δ^9-tetrahydrocannabinol (THC) is the primary active compound in marijuana. Synthetic cannabinoids were created as a method to study the effects of cannabinoids on the endocannabinoid system, which consists of endogenous cannabinoids, cannabinoid receptors, and the synthetic and degrading enzymes responsible for the synthesis and degradation of endocannabinoids. Cannabinoids activate the same receptors as endocannabinoids. Synthetic cannabinoids gained popularity as recreational drugs due to their ability to avoid detection. Synthetic cannabinoids have been found to have a greater binding affinity to the cannabinoid CB_1 receptor than THC. They are also shown to have a greater affinity at the CB_1 receptor than the CB_2 receptor. With the current legalization status of marijuana for both recreational and medicinal use, it is important to know the effects that these compounds can have on the body. The purpose of this experiment was to characterize the metabolites of THC and three other synthetic cannabinoids metabolized by human liver microsomes. The three synthetic cannabinoids chosed for this project are JWH-302, JWH-237, and mepirapim. These cannabinoids are all related to very popular synthetic cannabinoids. An AB SCIEX 3200 mass spectrometer (LC/MS/MS) in multiple reaction monitoring (MRM) mode with electrospray ionization (ESI) was used to characterize the metabolites. This is part of a bigger project that aims to use rat models to find the effects of THC and synthetic cannabinoids on the cardiovascular and renal systems.

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LIST OF ABBREVIATIONS

CBD	cannabidiol
CB1	cannabinoid receptor type 1
CB2	cannabinoid receptor type 2
CYP	cytochrome P450
EH	epoxide hydrolase
ESI	electrospray ionization
FMO	Flavin-containing monooxygenase
G6P	glucose-6-phosphate
G6PD	glucose-6-phosphate dehydrogenase
GC-MS	gas chromatography-mass spectrometry
HPLC	high pressure liquid chromatography
K_i	dissociation constant
LC/MS/MS	liquid chromatography with tandem mass spectrometry
MPCC	Mississippi Poison Control Center
MRM	multiple reaction monitoring
m/z	mass-to-charge
$NADP^+$	nicotinamide adenine dinucleotide phosphate
RT	retention time
THC	Δ^9-tetrahydrocannabinol
THC-COOH	11-nor-9-carboxy-Δ^9-tetrahydrocannabinol
UDP	uridine diphosphate
UGT	UDP-glucuronosyltransferase

11-OH-THC	11-hydroxy-Δ^9-tetrahydrocannabinol

INTRODUCTION

Four patients were evaluated at the University of Mississippi Medical Center on April 2, 2015 for delirium after the use of synthetic cannabinoids. Over the next couple of days, 24 more patients were assessed with similar symptoms related to synthetic cannabinoid use. There was a statewide alert requesting that health care providers report suspected cases of synthetic cannabinoid use and intoxication to the Mississippi Poison Control Center (MPCC) after the death of a patient. A patient showing at least two of the symptoms—sweating, psychosis, and severe agitation—was considered a suspected case. Another statewide alert was issued after eleven days, instructing all Mississippi emergency departments to submit lists of suspected patients to MPCC every day. There were approximately 400 reported cases, which included eight deaths all linked to synthetic cannabinoid use (Kasper et al., 2015). The outbreak of hospitalization related to synthetic cannabinoid use in Mississippi was part of a larger multistate outbreak (Law et al. 2015). This was the largest outbreak of the adverse effects related to synthetic cannabinoid use recorded (Drenzek et al. 2013, Ghosh et al. 2013, & Murphy et al., 2013).

The debate on the legalization of marijuana in the United States received significant media attention in 2012 (Wang et al., 2013). Marijuana prohibition began 80 years ago when the federal government banned the sale, cultivation, and use of the cannabis plant. At a federal level, it remains illegal (Berke, 2019). Public opinion has shifted dramatically towards support for the legalization of marijuana (Galston & Dionne, 2013). Two-thirds of Americans say that the use of marijuana should be legal, and the share of American adults who opposed legalization has fallen 20% since 2010 (Daniller, 2019). Support is especially strong among the younger generations, and those 65 and older staunchly oppose the legalization of marijuana. The proportion of Americans who see marijuana as a gateway drug is steadily decreasing (Galston & Dionne, 2013). Marijuana has been

legalized for recreational and medical use in 11 states for adults over the age of 21 and legalized for medical use in 33 states (Berke, 2019).

The Colorado state constitution was modified in November 2000 in order to allow for the use of medical marijuana for patients with chronic incapacitating medical conditions. Few patients were using medical marijuana until the US Attorney General created guidelines for the federal prosecution of the possession and use of marijuana. Jurisdiction of marijuana law enforcement was given to the state government. This meant that medical marijuana licenses could be issued without an expiration date to anyone with one of the conditions outlined in the Colorado law. This drastically increased the number of licenses from 2008 to 2014 (Monte et al., 2015). The increased availability of marijuana has led to increased health care problems related to marijuana exposure (Wang et al., 2013). The scope of the problem with marijuana use is often difficult to fully quantify because there are often other factors involved (Monte et al., 2015).

The rising prevalence of marijuana and other synthetic cannabinoids has become an issue because the potential health effects associated with their use are still unclear. The legalization of marijuana has increased opportunities for scientists to study the positive and negative effects of marijuana, but the federal designation of marijuana as a schedule 1 drug limits the quality and scope of trials that researchers can conduct (Monte et al., 2015). With policies moving towards the legalization of cannabis, the adverse health effects are a source of controversy. Most policies have been framed by the media as being either wholly harmless or harmful, without a complete understanding of the effects of marijuana (Hall, 2009). Marijuana has been advocated for its medicinal value because of the activity of Δ^9-tetrahydrocannabinol (THC). The rapid absorption of THC occurs with cannabis smoking, as THC is detectable in the plasma seconds after the first smoke of a marijuana cigarette (ElSohly et al., 2018).

Δ^9-tetrahydrocannabinol is the primary psychoactive compound in the cannabis plant. THC is associated with psychosis, anxiety, and depression symptoms, which leads to the inevitable exacerbation of underlying psychiatric disorders (Monte et al., 2015 & ElSohly et al., 2018). There is a lack of laboratory-based data that directly evaluates the psychological effects of cannabinoid compounds (D'Souza et al., 2004). The endocannabinoid system consists of endogenous cannabinoids, cannabinoid receptors, and the synthetic and degrading enzymes responsible for the synthesis and degradation of endocannabinoids (Mackie, 2008). The endocannabinoid system is an important pathway for the regulation of appetite and emesis (Martin, 2002, Di Marzo et al., 2001, Darmani et al., 2003, Darmani, 2001, Sharkey et al., 2014, Schlicker & Kathmann, 2001, & Howlett et al., 2002). Cannabinoids activate the same receptors as endocannabinoids (ElSohly et al., 2000). The identification of cannabinoid receptors grew out of a desire to understand the psychoactive effects of THC. A binding assay for this receptor showed high levels of receptors present in certain brain regions (Mackie, 2008).

Ingestion of marijuana results in THC metabolites in the urine of the person using the substance. The methods of drug testing for marijuana work based on the detection of THC carboxy metabolite in the urine. This has been a relatively effective and reliable method (Elsohly et al., 2011). Drug users have been trying to evade the detection of marijuana use by contamination (Elsohly et al., 1997 & Tsai et al., 1998). Drug testing programs have now implemented specimen validity testing as part of their drug testing protocol (Elsohly et al., 2011).

Synthetic cannabinoids are a heterogeneous group of compounds developed to investigate the endogenous cannabinoid system or as potential medicines. Covert laboratories used published data to develop variations in synthetic cannabinoids marketed as designer drugs that could be abused. Synthetic cannabinoids because popular in the early 2000's because of their ability to

escape detection by standard cannabinoid screening tests. The majority of synthetic cannabinoids detected in herbal products have a greater binding affinity to the cannabinoid CB_1 receptor than does THC. They also show a greater affinity at the CB_1 receptor than the CB_2 receptor. Synthetic cannabinoids are more potent than THC, therefore, creating more intense psychoactive effects. There is a need for further study pertaining to these compounds (Castaneto et al., 2014, Castaneto et al., 2015, & Gandhi et al., 2015). The three synthetic cannabinoids being studied in this experiment are JWH-302, JWH-237, and mepirapim.

JWH-302 (1-pentyl-3-(3-methoxyphenylacetyl) indole) is an analgesic chemical from the phenlacetylindole family. This acts as a cannabinoid agonist with a moderate affinity at both the CB_1 and CB_2 receptors. This synthetic cannabinoid is a possible isomer of the more common drug JWH-250. JWH-302 is less potent with a K_i of 17 nM at the CB_1 receptor compared to a K_i of 11 nM for JWH-250 (Manera et al. 2008). These two compounds have identical molecular weight and similar fragmentation patterns. It is quite difficult to distinguish between these two using GC-MS testing (Huffman et al. 2005). This substance is a Schedule I controlled substance. The structures of both JWH-302 and JWH-250 are shown in **Figure 1** in order to compare the two. The only difference between JWH-302 and JWH-250 is the orientation of the methoxy group: meta for JWH-302 and ortho for JWH-250.

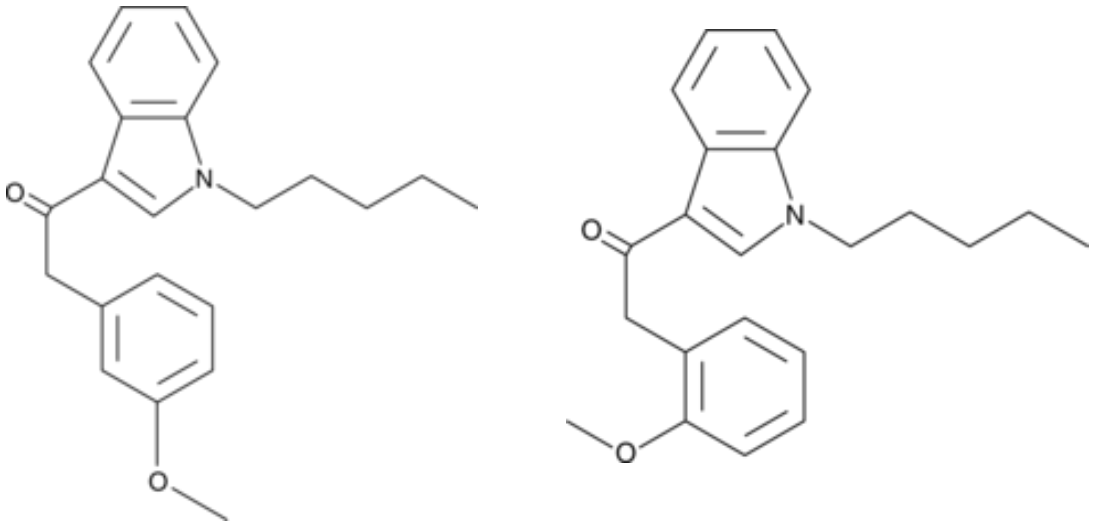


Figure 1. Structure of JWH-302 on the left and JWH-250 on the right.[1]

JWH-237 (2-(3-chlorophenyl)-1-(1-pentyl-1*H*-indol-3-yl)ethan-1-one) is also known as JWH-203 3-chlorophenyl isomer. JWH-237 is an analog of the phenylacetylindole cannabinoid agonist JWH-203. JWH-203 acts as a cannabinoid receptor at both the CB_1 and CB_2 receptors. It has a phenylacetyl group in place of the naphthoyl ring used in most aminoalkylindole cannabinoid compounds and has the strongest *in vitro* binding affinity for the cannabinoid receptors of any of the synthetic compounds in the phenylacetyl group. While JWH-203 has been studied, the activity of JWH-237 has not yet been determined (Huffman et al., 2005). The structures for JWH-237 and JWH-203 are shown in **Figure 2**. They are presented side by side for comparison. The only difference is in the position of the chlorine atom. In JWH-237 the chloride atom is meta, and in JWH-203 the chloride atom is ortho.

[1] Figures of all three synthetic cannabinoids and synthetic cannabinoids used for comparison are placed in the supplementary information section for easy access

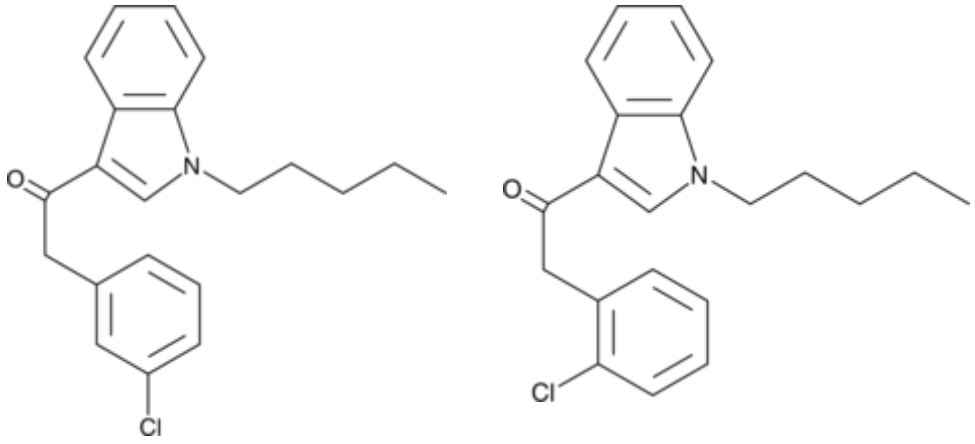


Figure 2. Structures of JWH-237 on the left and JWH-203 on the right.

Mepirapim (4-methylpirperazin-1-yl)-(1-pentylindol-3-yl)methanone) is an indole-based cannabinoid. It differs from JWH-018 by having a 4-methylpiperazine group in place of the naphthyl group. More is known about JWH-018 because it is a more researched compound because of its large prominence in spice and K2 (ElSohly et al., 2011). JWH-018 has an affinity for the cannabinoid receptor that is five times greater than that of THC. It is from the naphthoylindole family and acts as a full agonist at the CB_1 and CB_2 receptors. It has some selectivity for the CB_2 receptor. This compound works by mimicking the endocannabinoid hormones that the body naturally produces. This can cause either inhibition or activation of nerve signaling (Brents et al., 2013). Mepirapim has been used as an active ingredient in synthetic cannabinoid products. It was first identified in Japan in 2013 alongside FUBIMINA (Uchiyama et al., 2014). The structures of mepirapim, JWH-018, and FUBIMINA are shown in **Figure 3-4.** The difference between the two compounds comes from the mepirapim having 4-methylpiperazine group instead of the naphthyl group.

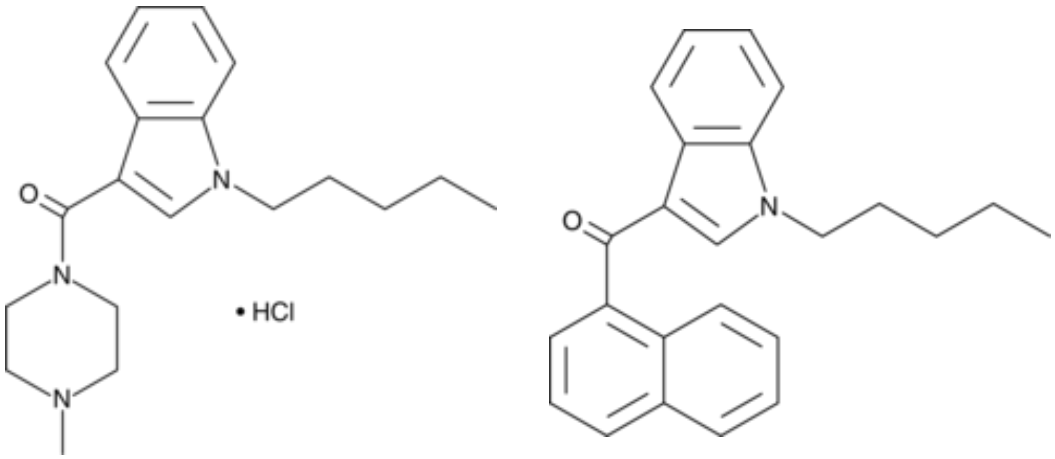


Figure 3. Structure of mepirapim on the left and JWH-018 on the right.

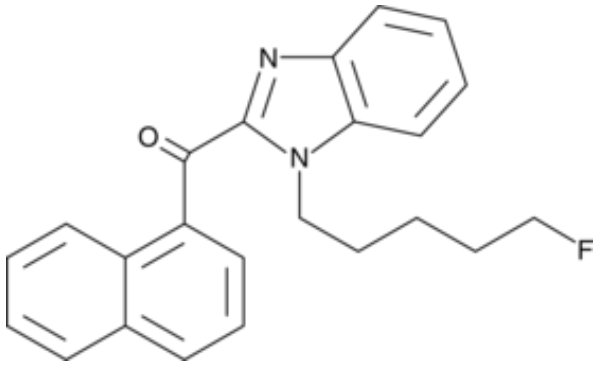


Figure 4. Structure of FUBIMINA.

This is a part of a larger product that aims to study the effects of synthetic cannabinoids compared to THC on the renal and cardiovascular systems using animal studies. After this project is complete, another group of undergraduates will take this study further to study the effects of synthetic cannabinoids and THC on the renal and cardiovascular systems in rat models. Instead of using rat models, this study will use human liver microsomes. The use of the human liver microsomes can help characterize these compounds and show how the liver metabolizes these compounds (Human and Animal Liver Microsomes Thawing and Incubation Protocol). The liver

is the largest internal organ in the human body, located between the digestive tract and the other parts of the body. The liver is rich is heme-containing enzymes, CYP450. This enzyme plays a big role in phase I oxidation reactions. CYPs can transform nontoxic chemicals into reactive intermediates that are toxic (Asha et al., 2009). The liver metabolizes endogenous substrates as well as exogenous drugs (Human and Animal Liver Microsomes Thawing and Incubation Protocol).

Human liver microsomes contain a wide variety of drug metabolizing enzymes and are commonly used in support *in vitro* absorption, distribution, metabolism, and excretion studies (Human Liver Micrsomes). There are several *in vitro* tools that are available for researches to study the metabolic fate of drugs. These include liver slices, cryopreserved hepatocytes, and subcellular fractions such as liver microsomes and S9 fractions (Human and Animal Liver Microsomes Thawing and Incubation Protocol). Human liver microsomes are used for the evaluation of phase I oxidation with the addition of a cofactor like $NADP^+$. Liver micrsomes can be prepared using liver slices, cell lines, and primary hepatocytes. Human liver microsomes can be used to identify critical CYP involved in the metabolism of the drug. It is advantageous to use human liver micrsomes because of the low cost, simplicity of use, easy storage, best characterized *in vitro* model for drug metabolism research, and enzyme activity can be kept frozen for many years (Asha et al., 2009).

The liver microsomes are prepared from the liver through a series of homogenization and ultracentrifugation. Lower speed centrifugation of liver homogenate at 10,000g produces the supernatant, which is known as the S9 fraction. The S9 fraction contains all phase I and phase II enzymes. Further centrifugation at 100,000g yields the endoplasmic reticulum derived microsomes. Microsomes are an enriched source of cytochrome P450 (CYP) and flavin

monooxygenase (FMO) enzymes. Some phase II enzymes, certain uridine glucuronide transferases (UGT) isoforms and epoxide hydrolase (EH) enzymes are present, as well in the microsomes. Individual or pooled donor microsomes can be used for metabolism-related studies. Pooled donors can represent the average human population or particular factors of research interest (Human and Animal Liver Microsomes Thawing and Incubation Protocol). Human liver micrsomes are prepared by differential centrifugation and stored at -80 °C until use. The microsomes are incubated with a mixture consisting of NADPH generating compounds, $NADP^+$ and glucose-6-phosphate dehydrogenase. The addition of compounds to create the NADPH system initiates the reaction. There is also the addition of buffers like a potassium phosphate buffer (Asha et al., 2009).

MATERIALS AND METHODS

Preparation of Stock Solutions

A series of stock solutions had to be prepared before the start of the work with the human liver microsomes. These solutions included a potassium phosphate buffer, a $NADP^+$ solution, a glucose-6-phosphate solution, a magnesium chloride solution, and a glucose-6-phosphate dehydrogenase solution. In order to create the 1 M potassium phosphate buffer with a pH of 7.4, two solutions were created using dibasic and monobasic potassium phosphate. Both potassium phosphate monobasic and potassium phosphate dibasic were obtained from Sigma-Aldrich. The first solution of 1 M of potassium phosphate dibasic (K_2HPO_4) was created by mixing 17.42 grams of K_2HPO_4 with 100 mL of deionized water. The second solution of 1 M of potassium phosphate monobasic (KH_2PO_4) was created by mixing 13.60 grams of KH_2PO_4 with 100 mL of deionized water. The pH of 7.4 was created by the addition of 80.2 mL of K_2HPO_4 and 19.8 mL of KH_2PO_4. The next solution that was created was the 50 mM $NADP^+$ solution. A total of 0.0783 grams was added to 2 mL of water to create the solution. The sample was separated into aliquots: 5 vials of 100 μL and 8 vials of 300 μL. The next solution prepared was the 100 mM G6P solution. A total of 0.1855 grams of G6P was added to 6 mL of water. The sample was separated into aliquots: 3 vials of 350 μL and 3 vials of 1200 μL. The G6PD was used directly from Sigma-Aldrich. It was prepared as 200 units/mg of protein. Aliquots were created: 5 vials of 50 μL and 5 vials 150 μL. The last solution prepared was the 1 M magnesium chloride solution. A total of 0.2845 grams of $MgCl_2$ was dissolved in 3 mL of water. The dissolved solution was separated into aliquots: 10 vials of 50 μL and 5 vials of 400 μL.

Preparation of Positive Controls

A positive control is a group in an experiment that receives treatment with a known result, and therefore should show a particular change during the experiment. It is used to control for unknown

variables in the experiment and to give something to compare with the test group. The purpose of the positive control was to see if the test compounds would be metabolized by the human liver microsomes with the method used. Two different positive controls were used for the experiment: THC and testosterone. The total volume of the reaction mixture was 1.5 mL. This reaction mixture (1.5 mL) was prepared by mixing 30 μL of $NADP^+$ (1 mM), 150 μL of G6P (10 mM), 15 μL of G6PD (2 U/mL), 15 μL of $MgCl_2$ (10 mM), 150 μL of KPO_4 buffer (100 mM), and 75 μL of human liver microsomes. This was done twice for the two positive controls. The amount of THC and testosterone added to the mixture was calculated for the 1.5 mL mixture. This calculation led to the addition of 4.7 μL of THC (10 μM) and 1.5 μL of testosterone (10 μM). The solution was diluted with water to bring it to the 1.5 mL volume. The solution was agitated in the shaking water bath at 37 ˚C. A total of nine samples of 100 μL of each solution was collected at intervals of 15 minutes starting at 0 up until 2 hours. A total of 200 μL of ice-cold acetonitrile was added to the solutions at the time the sample was collected to stop the reaction. The samples were vortexed for 2 minutes. After the vortex, the samples were incubated at -60 ˚C for 1 hour. The samples were then centrifuged at 16128 RCF for 15 minutes. The clear supernatant was collected and analyzed using LC/MS/MS.

Preparation of Negative Control

The preparation of the negative control had all the components present in the positive controls and reaction except for the liver microsomes. Since the negative control does not contain liver microsomes, there should be no response in terms of the metabolism of the test compound. It controls the unknown variable during the experiment and provides something to compare with the test group. The negative control was prepared by adding a total of 30 μL of $NADP^+$ (1 mM), 150 μL of G6P (10 mM), 15 μL of G6PD (2 U/mL), 15 μL of $MgCl_2$ (10 mM), and 150 μL of KPO_4

buffer (100 mM). A total of 4.7 μL of THC was added to the mixture. Water was added to the compound to make up for the loss of volume from human liver microsomes and to bring the volume to 1.5 mL. The same procedure was performed for the negative control. The solution was agitated in the shaking water bath at 37 ˚C. A total of nine samples of 100 μL of each solution was collected at intervals of 15 minutes starting at 0 up until 2 hours. A total of 200 μL of ice-cold acetonitrile was added to the solutions at the time the sample was collected to stop the reaction. The samples were vortexed for 2 minutes. After the vortex, the samples were incubated at -60 ˚C for 1 hour. The samples were then centrifuged at 16128 RCF for 15 minutes. The clear supernatant was collected and analyzed using LC/MS/MS.

Preparation of Vehicle Control

The vehicle control is used in studies in which a substance (e.g., saline, mineral oil, organic solvents) is used as a vehicle to dissolve the experimental compound. For the vehicle control, the substance is administered in the same manner in which it will be used with the experimental control. For the positive control of THC in this experiment, methanol was used as a vehicle. For the other compounds in the experiment, DMSO was used as the vehicle. The vehicle control was a reaction mixture of 1.5 mL. The reaction mixture was prepared by adding a total of 30 μL of $NADP^+$ (1 mM), 150 μL of G6P (10 mM), 15 μL of G6PD (2 U/mL), 15 μL of $MgCl_2$ (10 mM), 150 μL of KPO_4 buffer (100 mM), and 75 μL of human liver microsomes. Depending on the compound used, either 4.7 μL of methanol or 1.5 μL of DMSO was added to the reaction mixture. The same procedure was further followed as mentioned above to collect and analyze samples for LC/MS/MS. The solution was agitated in the shaking water bath at 37 ˚C. A total of nine samples of 100 μL of each solution was collected at intervals of 15 minutes starting at 0 up until 2 hours. A total of 200 μL of ice-cold acetonitrile was added to the solutions at the time the sample was

collected to stop the reaction. The samples were vortexed for 2 minutes. After the vortex, the samples were incubated at -60 ˚C for 1 hour. The samples were then centrifuged at 16128 RCF for 15 minutes. The clear supernatant was collected and analyzed using LC/MS/MS.

Preparation of Synthetic Cannabinoids

The three synthetic cannabinoids – JWH-302, JWH-237, and mipirapim – were obtained from Cayman Chemicals. JWH-302 was prepared as a 5 mg solution in methyl acetate. The solution was diluted to 5 mM with the addition of 33.5 μL of JWH-302 from Cayman Chemicals with 966.5 μL of DMSO. JWH-237 was prepared as a 5 mg solution in methanol. The solution was diluted to 5 mM with the addition of 33.99 μL of JWH-237 with 966.0 μL of DMSO. Mepirapim was prepared as a 5 mg crystalline solid. The 5 mg solid was mixed with 2.86 mL of DMSO.

Reaction with Synthetic Cannabinoids

The test compounds for the reaction were prepared in the same method as the positive controls. A total of 30 μL of $NADP^+$ (1 mM), 150 μL of G6P (10 mM), 15 μL of G6PD (2 U/mL), 15 μL of $MgCl_2$ (10 mM), 150 μL of KPO_4 buffer (100 mM), and 75 μL of human liver microsomes were mixed together. This was done in three different vessels for the three compounds: JWH-302, JWH-237, and mipirapim. A total of 3 μL of the test compound was added. One of the three test compounds was added to each of the vessels. The solution was diluted with water to bring it to the 1.5 mL volume. The solution was agitated in the shaking water bath at 37 ˚C. A total of nine samples of 100 μL of each solution was collected at intervals of 15 minutes starting at 0 up until 2 hours. A total of 200 μL of ice-cold acetonitrile was added to the solutions at the time the sample was collected to stop the reaction. The samples were vortexed for 2 minutes. After the vortex, the samples were incubated at -60 ˚C for 1 hour. The samples were then centrifuged at 16128 RCF for 15 minutes. The clear supernatant was collected and analyzed using LC/MS/MS.

RESULTS AND DISCUSSION

Instrumentation Theory

In this experiment, THC was subjected to *in vitro* human liver microsomal metabolism in which the compound was incubated with the microsomes for 30 minutes at 37 ºC in the presence of $NADP^+$ and G6PD. The THC (positive control) and negative control were analyzed using LC/MS/MS after the extraction of the reaction mixture. The instrument used in this lab was the AB SCIEX 3200 mass spectrometer using MRM with ESI, attached with a Shimadzu HPLC using a Phenomenex C-18 reverse phase column. Water and acetonitrile, both with 0.1% formic acid, were used as solvents. LC/MS/MS is the most common bioanalytical method in use today. It is a very versatile, robust, and sensitive method. The AB SCIEX 3200 is best suited for quantitative and qualitative analysis of small molecules and qualitative analysis of proteins and peptides. This experiment largely focuses on the quantitative analysis of small molecules with the molecular weight of the synthetic cannabinoids in addition to the resulting metabolites.

HPLC uses high pressure to push samples through a chromatography column to separate the compounds that make up the sample. A reverse phase column was used in this experiment. This is the opposite of normal phase, but it also separates molecules based on polarity. It does this through the use of a non-polar stationary phase and a polar mobile phase. The molecules were eluted from the chromatography column under high pressure. The continuous flow cannot be directly detected by the mass spectrometer because they operate in a vacuum and require liquid to be passed through an interface. The interface removes the mobile phase used in the chromatography step and transfers the analyte to the mass spectrometry unit. The interface used in this experiment is an electrospray ionization (ESI) system. The liquid is nebulized into a fine spray, ionized, and transferred to the mass spectrometer detector.

A triple quadrupole mass spectrometer was used in this experiment. The mass spectrometry detector measures the mass-to-charge (m/z) ratio of the compounds by exposing the ions to a magnetic or electric field, which alters the movement of the compounds. This movement allows the analysis based on mass. The use of two mass spectrometer detectors allows for molecules with certain m/z ratios to be sorted through and further analyzed by fragmenting. High-sensitivity multiple reaction monitoring is used for this experiment. MRM involves a MS/MS scan where an ion is passed through the Q1 quadrupole and fragmented in the Q2 collision cell; the Q3 quadrupole is then used to specify which fragment ion can reach the detector. The mass spectrum of the sample will provide information about the structural and chemical properties of the synthetic cannabinoids used in this experiment. A schematic for LC/MS/MS obtained from ResearchGate is shown in **Figure 5.**

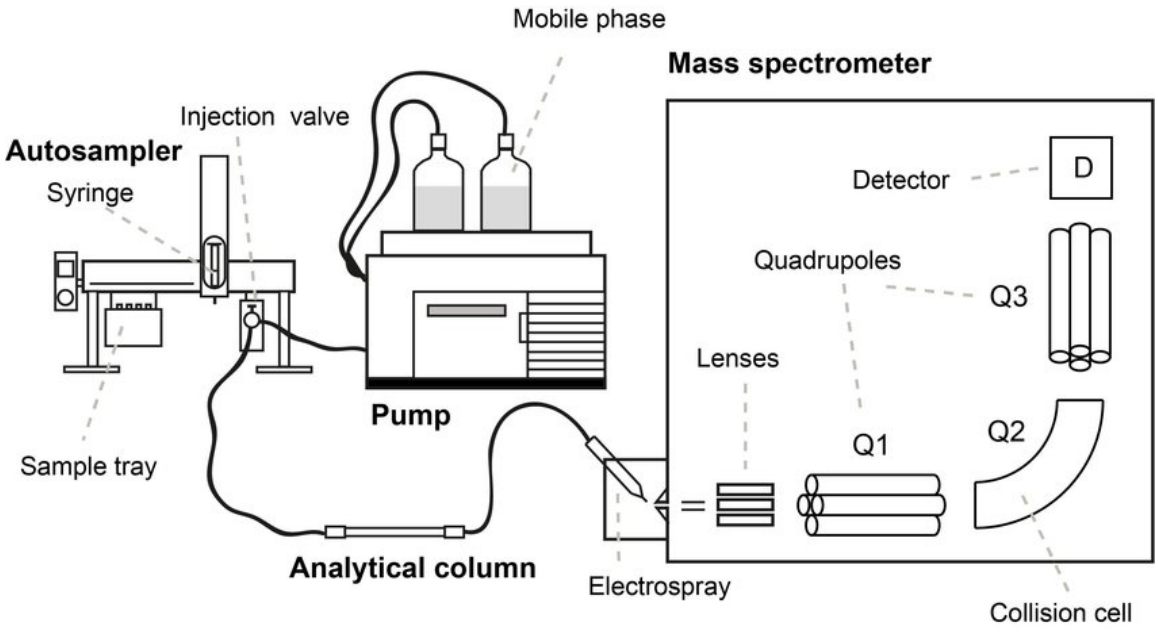


Figure 5. Schematic for LC/MS/MS

Standards

An internal standard for THC was run before the controls in order to confirm the metabolites obtained in our sample. The compounds for each of the retention times for the THC standard were identified in **Figure 6.** The THC was metabolized into three major metabolites: 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH), 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC), and cannabidiol (CBD). These compounds are labeled. The structure of THC is shown in **Figure 7** and the structures of its metabolites are shown in **Figures 8-10.** The progression of the metabolism of THC went from THC to 11-OH-THC to THC-COOH. The following transition ions were monitored by multiple reaction monitoring (MRM): m/z 314.960 > 193.050 Da and 314.960 > 122.880 Da for THC/CBD/CBC, m/z 331.300 > 201.200 Da and 331.300 > 193.200 Da for 11-OH-THC, m/z 345.200 > 299.100 Da and 345.200 > 193.100 Da for THC-COOH.

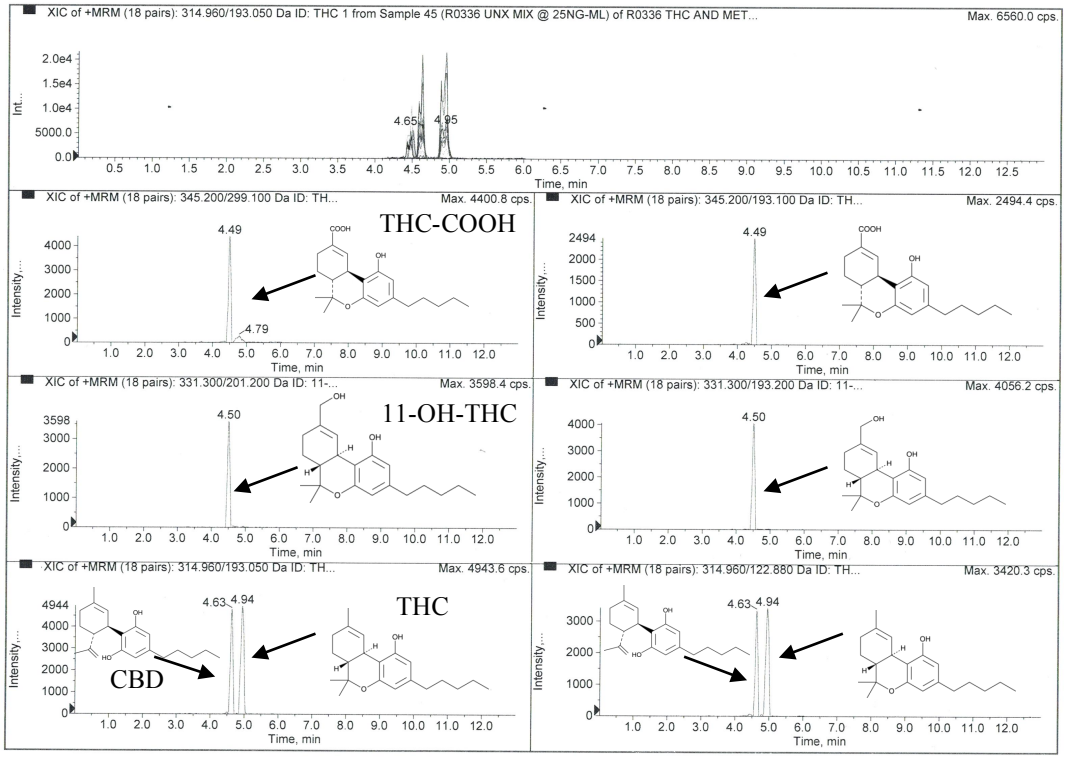


Figure 6. LC/MS/MS chromatogram of the THC standard. THC and its metabolites are labeled in the chromatogram. The right side of the chromatogram has the deuterated samples.

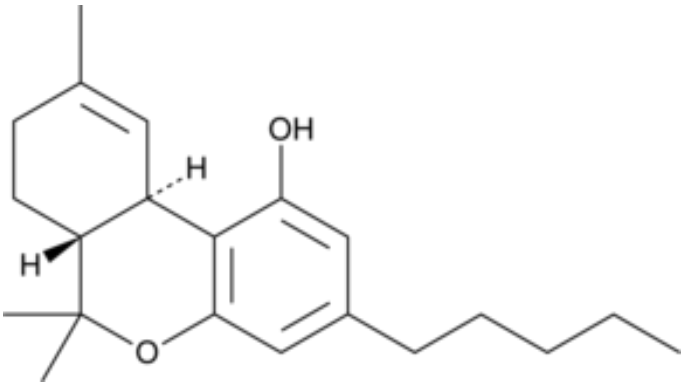


Figure 7. Structure of THC

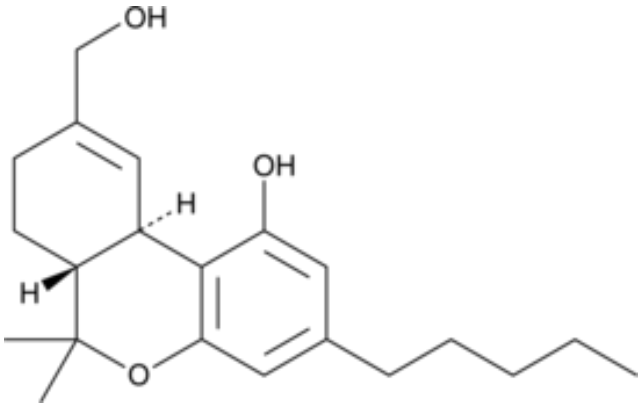


Figure 8. Structure of 11-OH-THC

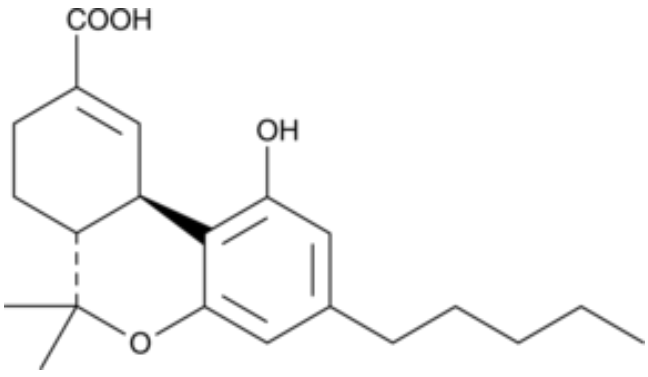


Figure 9. Structure of THC-COOH

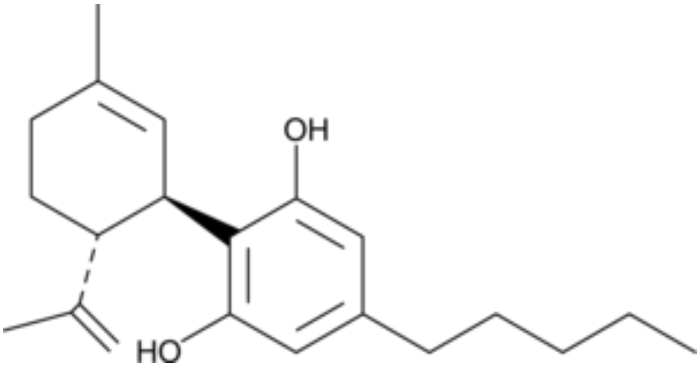


Figure 10. Structure of cannabidiol

THC Positive Control

The retention times (RT) present in the internal standards were compared with the LC/MS/MS results obtained from the THC positive control. The LC/MS/MS retention times shown in the standard matched with the retention times from the THC positive control. The THC positive control was measured in 9 aliquots that varied with the time that the reaction was run. The samples of THC started at 0 minutes and went to 2 hours in 15-minute increments. **Figures 11-19** show the results for the LC/MS/MS. **Figure 11** is the THC at 0 minutes, **Figure 12** is the THC at 15 minutes, **Figure 13** is the THC at 30 minutes, and continues in 15-minute increments until **Figure 19** with the THC at 2 hours. Through the attenuated changes in the intensity of the THC peak located at a RT of 4.96 present in the data with time, it is shown that THC becomes fully metabolized around one hour.

There is a high-intensity peak present at 0 minutes with a RT of 4.95. The intensity of the peak was 24000 cps with a mass of m/z 314.96 Da. This indicates the strong presence of THC. There is also a very weak intensity peak (approximately 1500 cps) present with a retention time of 4.51 and a mass of m/z 331.300 Da. The m/z indicates the presence of 11-OH-THC. This is shown in **Figure 11.** As the time changes to 15 minutes, the high-intensity peak now becomes less intense

(4.95 RT and intensity of 600 cps). The less intense peak becomes more intense (4.51 RT and intensity of 2868 cps). This indicates that most of the THC is getting converted into 11-OH-THC. This is shown in **Figure 12.** The THC and 11-OH-THC peaks are still visible in the data at 30 minutes, but the intensity has decreased to around 278 and 351, respectively. The low level of intensity shows that the THC and 11-OH-THC are converted into other metabolites. The additional peak at 4.49 RT indicates the presence of THC-COOH. This is shown in **Figure 13.** At 45 minutes, there is no longer a THC peak present at 4.95 RT. However, the other metabolites previously mentioned were found at low intensity. This is shown in **Figure 14.** It is shown in **Figure 15** that there are no prominent THC peaks. The intensity of the peaks changes from being 24000 cps to 278 cps. The peak present for the THC at 0 minutes at a RT of 4.95 is no longer visible in the data. The peaks from the LC/MS/MS do not change after the 1-hour mark; the peaks remain similar for the next four collections. This is shown in **Figures 16-19.** The positive control data show that the method being used is appropriate for the experiment and that the human liver microsomes were successful in metabolizing THC.

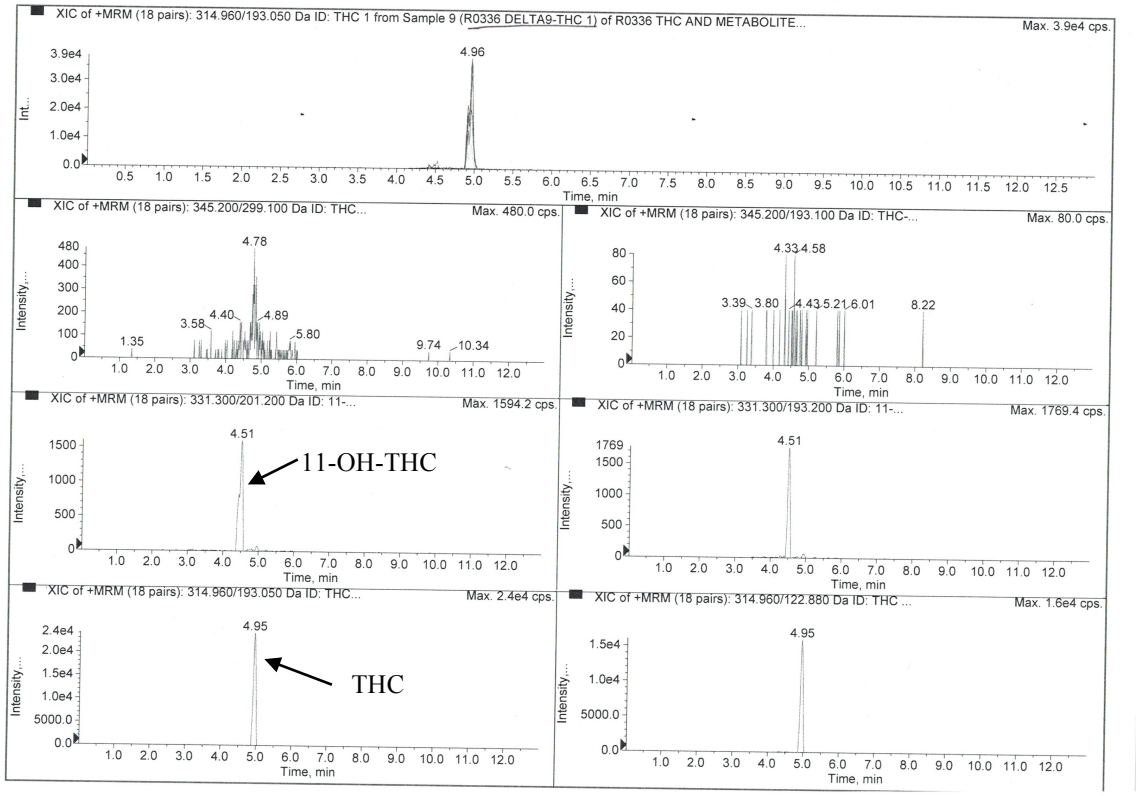


Figure 11. LC/MS/MS chromatogram of the THC positive control at 0 minutes. There is a strong peak present for THC at an intensity at 24,000 cps. There is weak peak already present at 1500 cps for 11-OH-THC. The THC has is spontaneously metabolizing into 11-OH-THC.

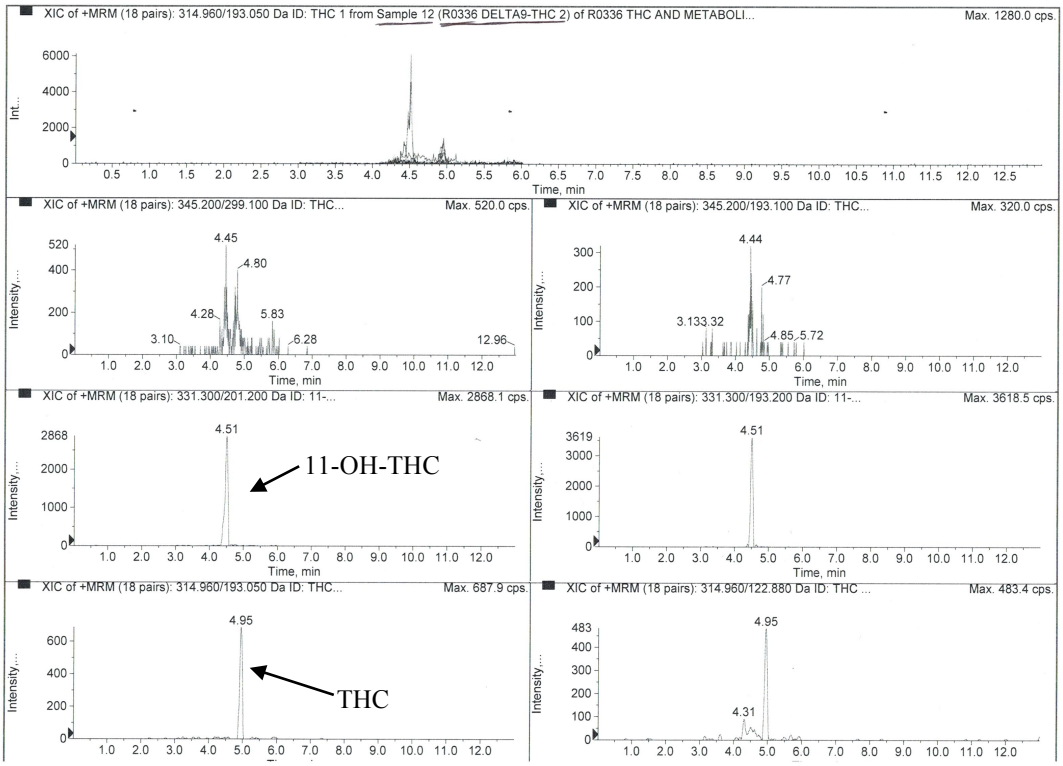


Figure 12. LC/MS/MS chromatogram of the THC positive control at 15 minutes. The THC peak has decreased in intensity to 600 cps. The 11-OH-THC peak has increased in intensity to 2868 cps. This means that the THC is being metabolized into 11-OH-THC.

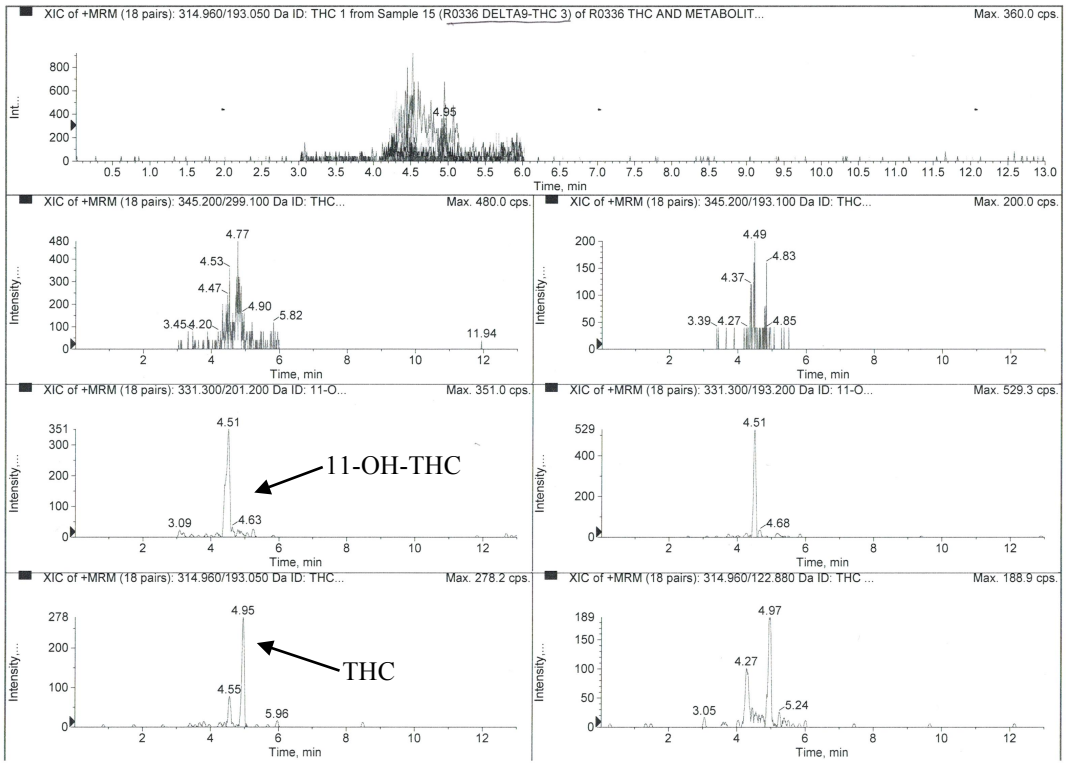


Figure 13. LC/MS/MS chromatogram of the THC positive control at 30 minutes. Both the THC and the 11-OH-THC peak have decreased in intensity. THC was being metabolized into 11-OH-THC, and the 11-OH-THC was being metabolized further.

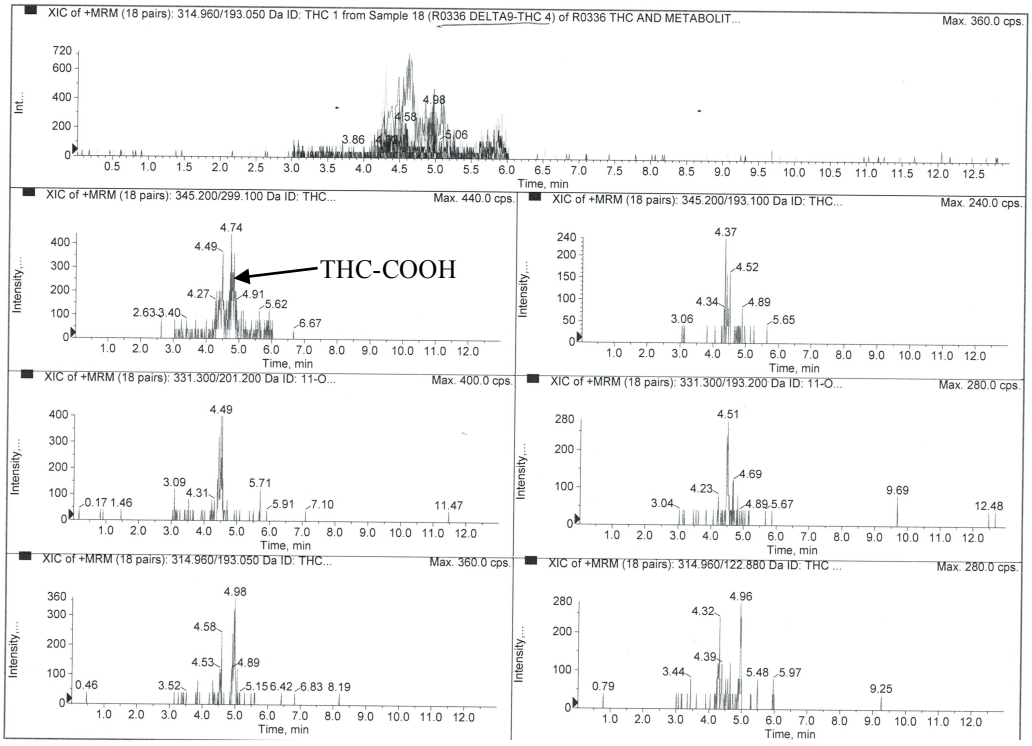


Figure 14. LC/MS/MS chromatogram of the THC positive control at 45 minutes. There are no longer peaks present for THC or 11-OH-THC. This means that the THC and the 11-OH-THC have completely been metabolized. There is also a peak present for THC-COOH from the metabolism of 11-OH-THC.

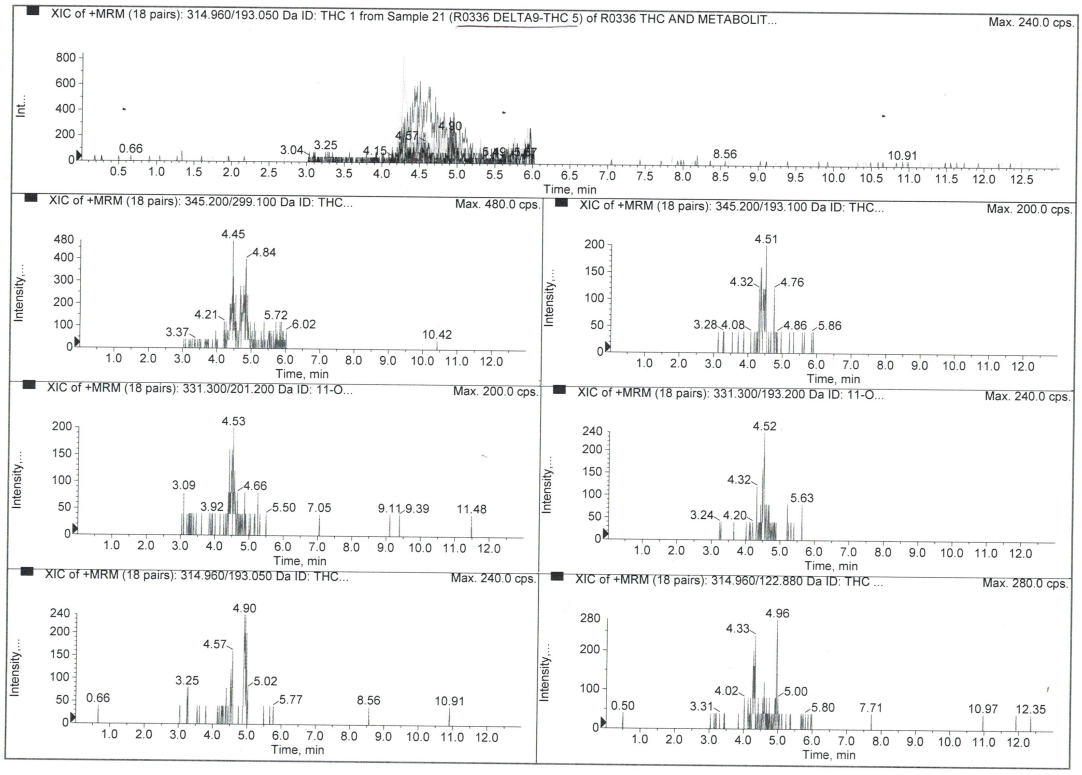


Figure 15. LC/MS/MS chromatogram of the THC positive control at 1 hour. There are no longer any peaks present for THC or its metabolites. THC and its metabolites have been completed metabolized. After the 1 hour mark, the chromatograms look around similar for the next hour.

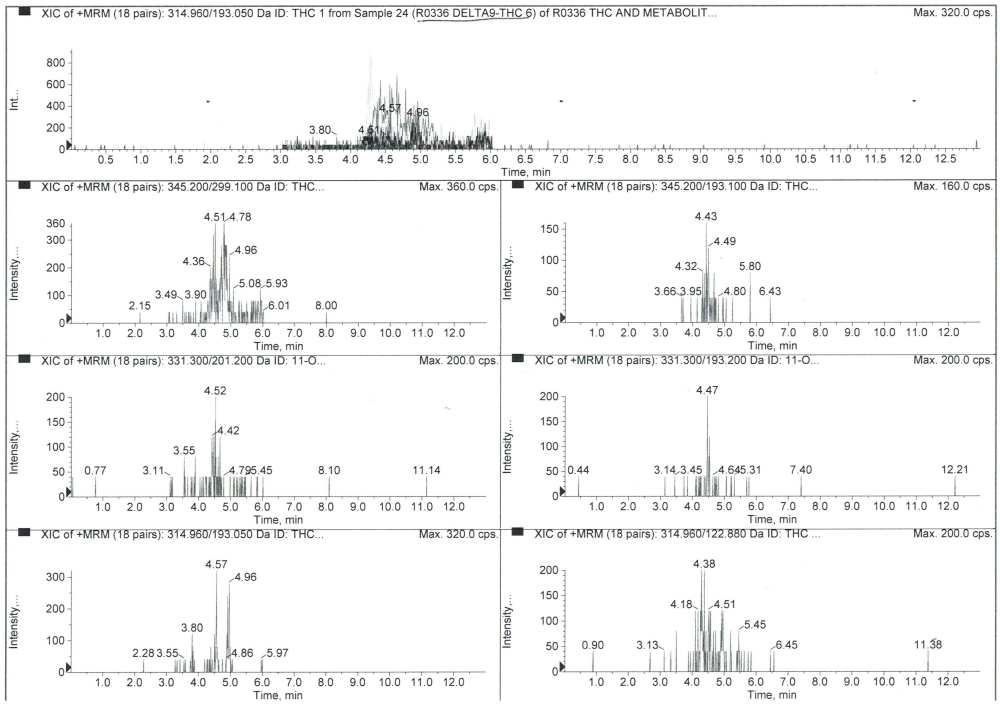


Figure 16. LC/MS/MS chromatogram of the THC positive control at 1 hour 15 mintues

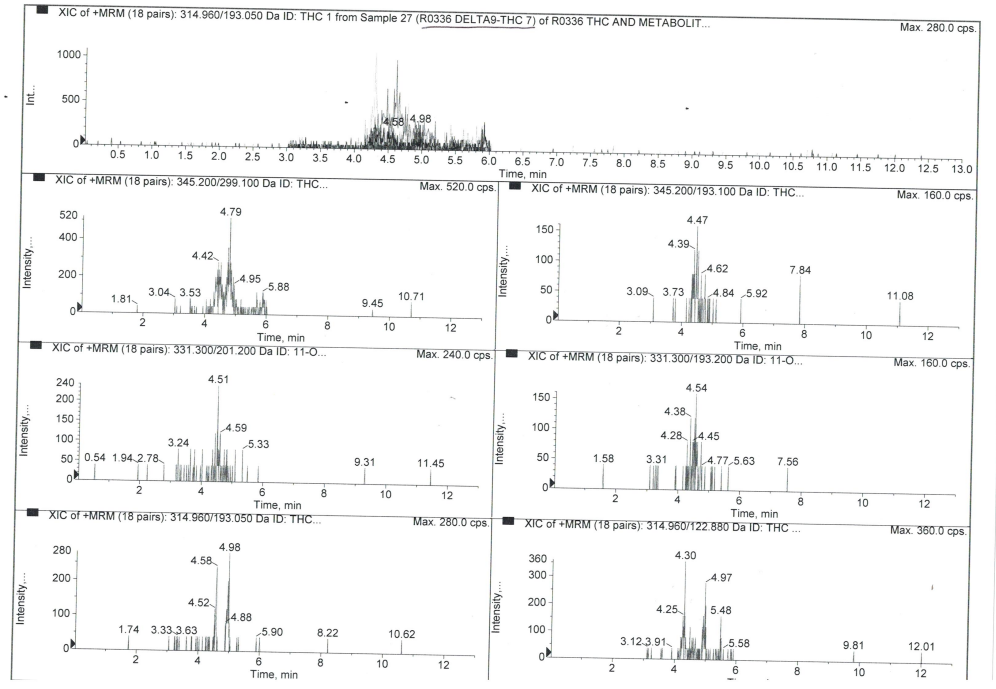


Figure 17. LC/MS/MS chromatogram of the THC positive control at 1 hour 30 minutes

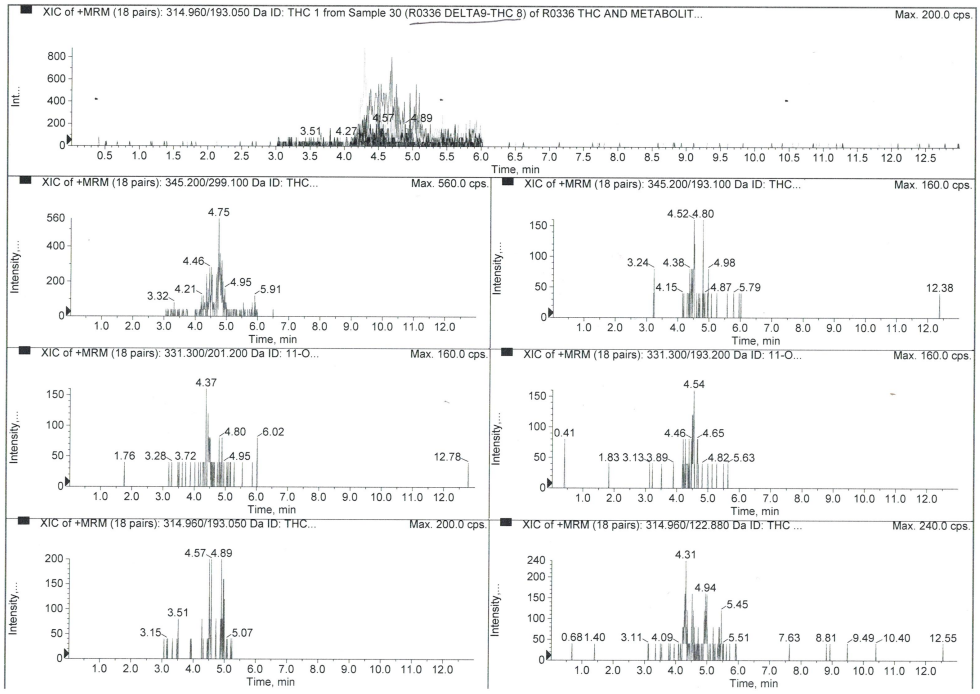


Figure 18. LC/MS/MS chromatogram of the THC positive control at 1 hour 45 minutes

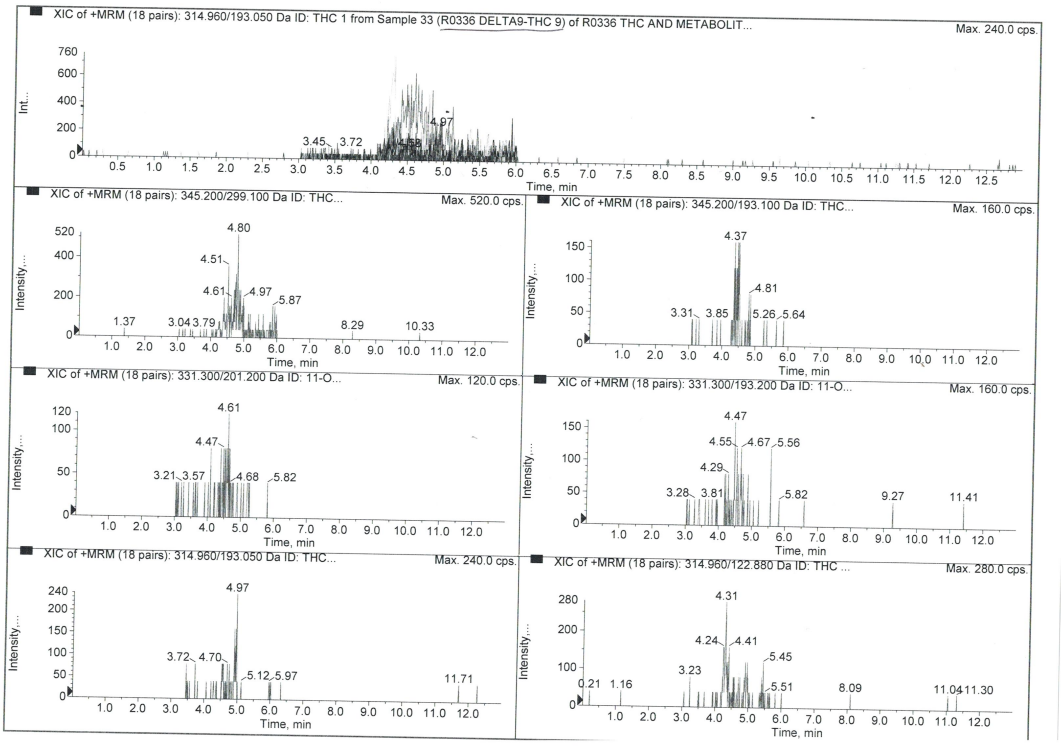


Figure 19. LC/MS/MS chromatogram of the THC positive control at 2 hours

Negative Control

The negative control was performed with all the components used in the experiment except for the pooled human liver microsomes. The negative control was measured at 0 minutes, 1 hour, and 2 hours. The LC/MS/MS data for these are shown in **Figures 20-22.** The negative control was analyzed in order to confirm that the method did not have confounding variables. It was important that the metabolism that occurred was a result of the human liver microsomes and not caused by the other compounds that were used in the experiment. It was confirmed by the LC/MS/MS that the human liver microsomes were responsible for metabolizing the test compound. The analysis of the THC did not change throughout the time interval. All three graphs showed similar activity. The THC peak at the retention time of 4.95 remained prominent throughout the 2 hours.

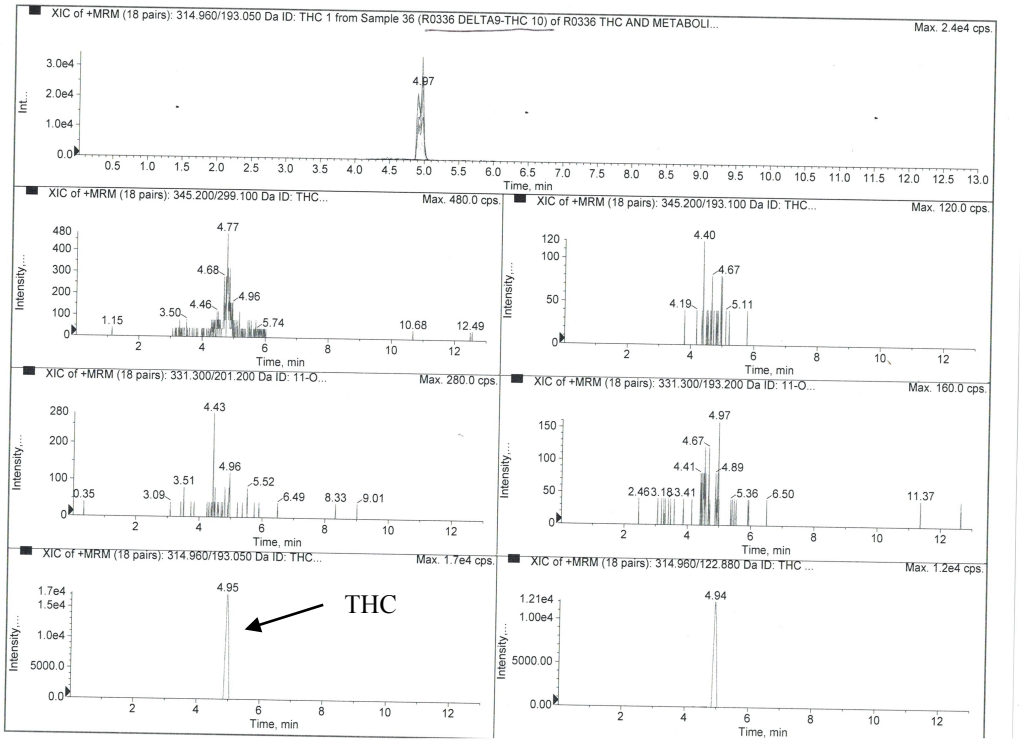


Figure 20. LC/MS/MS chromatogram of the THC negative control at 0 minutes. There is a peak present at an intensity of 17,400 cps for THC.

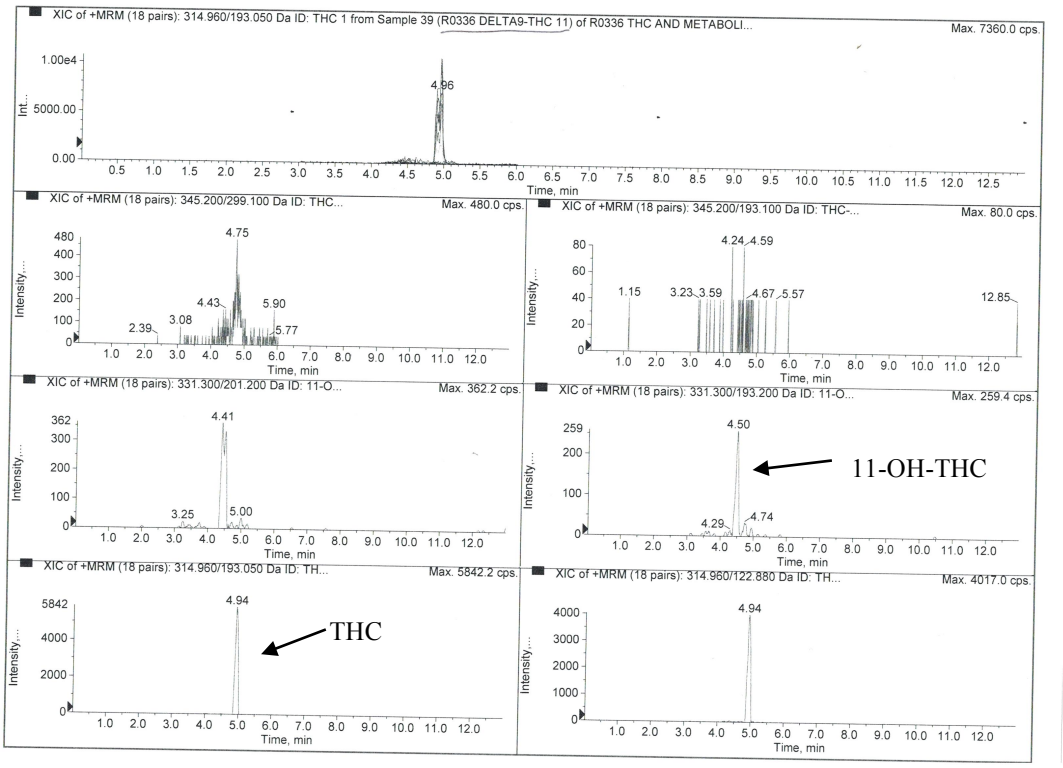


Figure 21. LC/MS/MS chromatogram of the THC negative control at 1 hour. The THC peak has decreased in intensity to 5842 cps. There is also a peak present at 259 cps for 11-OH-THC. The THC is being metabolized into its metabolite 11-OH-THC.

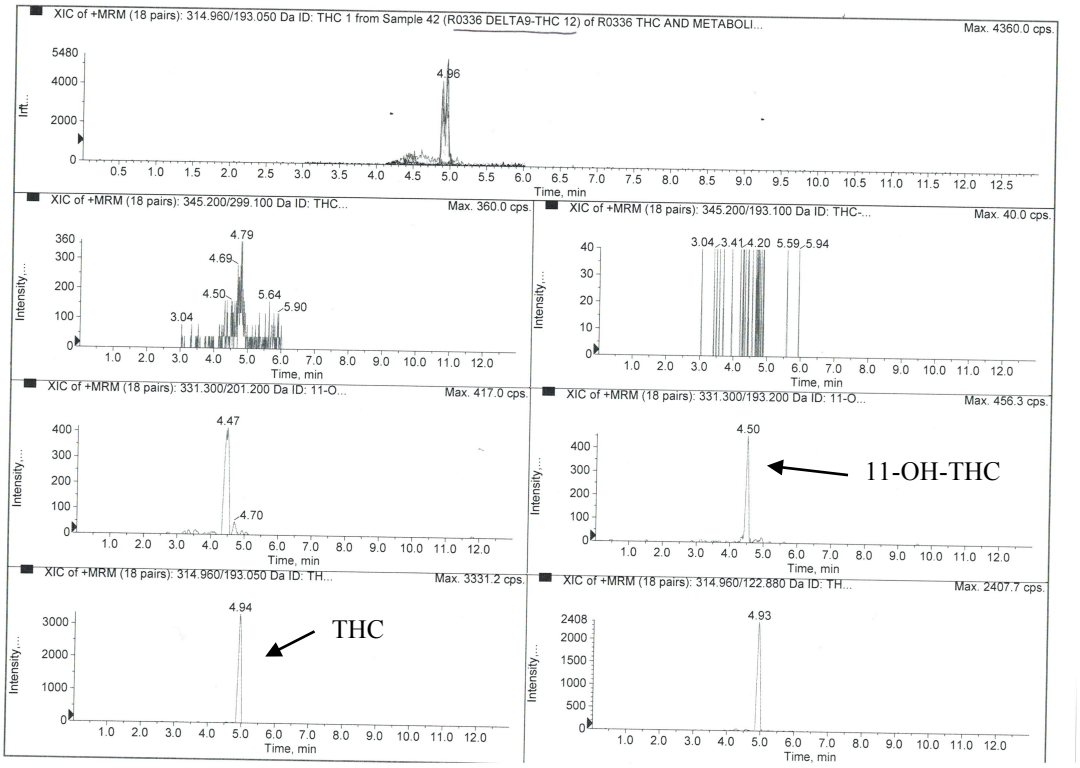


Figure 22. LC/MS/MS chromatogram of the THC negative control at 2 hours. The THC peak has further decreased in intensity to 3000 cps. The peak for 11-OH-THC increased in intensity to 400 cps. The THC is being metabolized further into its metabolite 11-OH-THC.

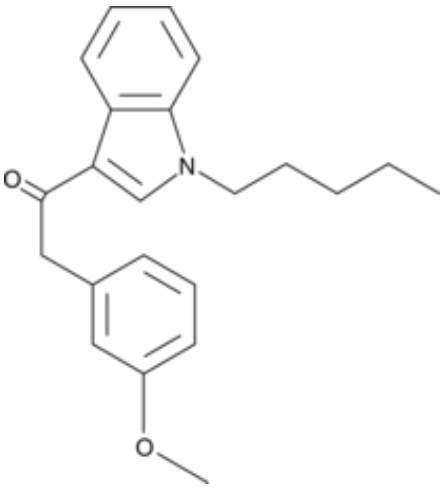
Further Experiment Halted by COVID-19

The experimental procedure was conducted up to the point of the preparation of the synthetic cannabinoid samples. The reaction mixtures with the three novel synthetic cannabinoids were never able to be prepared and analyzed because of the closure of the chemistry department for safety reasons concerning COVID-19. This portion was still included in the procedure for the completeness purposes. The purpose for completing this research was to test the metabolism of the three novel synthetic cannabinoids (JWH-302, JWH-237, and mepirapim) in human liver microsomes. If the LC/MS/MS chromatogram for the three synthetic cannabinoids could have been obtained, an analysis similar to the one performed for the THC positive control would have been included in this thesis. It is quite disappointing that all the literary reviews and work done to find novel compounds never led to a completed final product. While it saddens me to turn in a thesis that does not reach my initial expectations, I am proud that I was able to have some research to present.

CONCLUSION

The purpose of this experiment was to characterize the metabolites of THC and three other synthetic cannabinoids metabolized by human liver microsomes. The three novel synthetic cannabinoids JWH-302, JWH-237, and mepirapim were not analyzed using LC/MS/MS because of the constraints placed on the chemistry department and The University of Mississippi due to COVID-19. The reaction mixtures that were prepared before the pandemic included the THC positive control and negative control. These two reaction mixtures were analyzed using liquid chromatography with tandem mass spectrometry (LC/MS/MS). A LC/MS/MS chromatogram for a THC standard was obtained before analyzing the controls. The chromatogram for the standard showed the THC and its subsequent metabolites. The LC/MS/MS chromatogram for the THC positive control showed a high intensity peak at 4.95 RT at time zero. After 15 minutes had elapsed, the THC peak at 4.95 RT attenuated to approximately 700 cps while the THC metabolite, 11-OH-THC at RT 4.51, peak increased from 1500 cps to 2868 cps. At the 30-minute mark, there are two really weak peaks present that represent THC (approximately 280 cps) and 11-OH-THC (approximately 350 cps). The low intensity of the peaks indicated that most of the THC and 11-OH-THC had been converted into other metabolites. 11-COOH-THC is present with a peak with a retention time of 4.49. There is no longer a THC peak present at 45 minutes, incidating that all of the THC had been matabolized. The positive control showed that the method being used is appropriate for the experiment and that the human liver microsomes were successful in metabolizing THC. No changes were observed in the LC/MS/MS chromatograms for the negative control which suggests that no metabolism of the test compound occurred in the absence of human liver microsomes. This is evident through the presence of the 4.95 RT of THC peak throughout the 2 hours.

SUPPLEMENTARY MATERIAL



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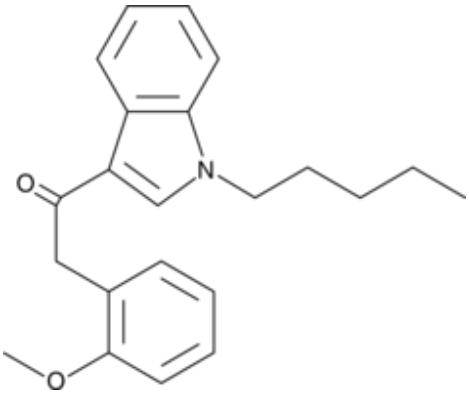
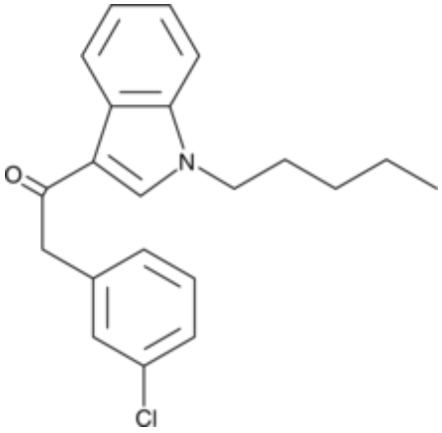
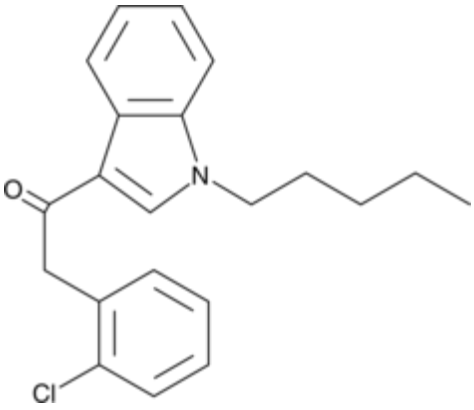


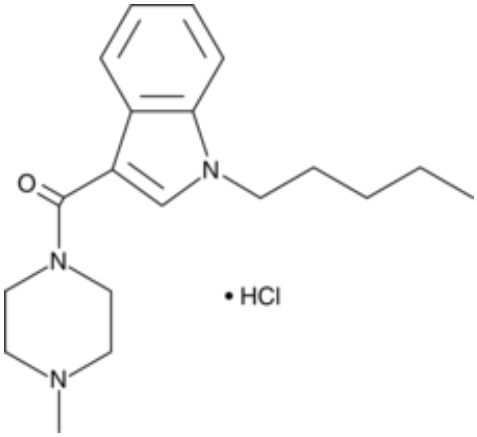
JWH-250



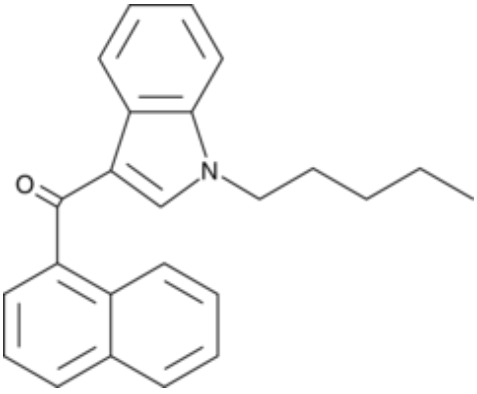
JWH-237



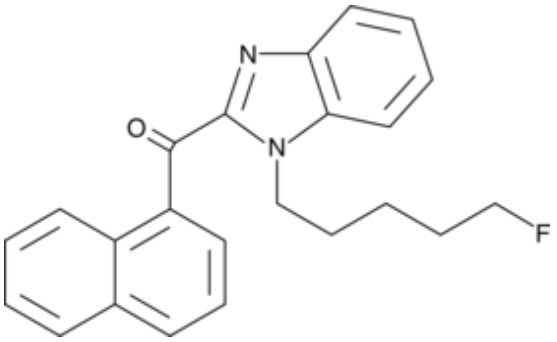
JWH-203



mepirapim



JWH-018



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