GC-MS Studies on a Series of 1-pentylacylindoles: Regioisomeric Compounds Related to the Synthetic Cannabinoids

by

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Abstract

This thesis is primarily focused on analytical approaches to differentiate twenty isomeric compounds related to designer drugs of the1-pentyl-acylindoles class. The compounds investigated represent a series of synthetic cannabinoids substances associated mainly with naphthoylindole and benzoylindole structures. These compounds represent potential designer modification of banned drugs. Electron-ionization gas chromatography-mass spectrometry (EI-GC-MS) was used as the initial method to differentiate and separate the target compounds.

Chapter one is a literature review of synthetic cannabinoids history, general pharmacology, metabolism and elimination, in addition to a brief review on the general analytical methods used on synthetic cannabinoids. The project rationale and statement of research objectives are also included. Chapter two compares the analytical properties of the first banned naphthoylindole compound in clandestine samples 1-n-pentyl-3-(1-naphthoyl)indole, JWH-018, with three possible regioisomers, 1-n-pentyl-3-(2-naphthoyl) indole, 1-(1-naphthoyl)-3-n- pentylindole and 1-(2-naphthoyl)-3-n-pentylindole. Chapter three compares the six regioisomers of 1-n-pentyl-3-(dimethoxybenzoyl)-indoles. Chapter four compares JWH-018 with eleven possible regioisomeric 1-n-pentyl-naphthoylindoles.

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List of Abbreviations

µl	Micro liter
µm	Micrometer
°C	Degree centigrade
EI	Electron impact
eV	Electron volt
GC-IRD	Gas chromatography coupled to infrared detection
GC-MS	Gas chromatography- mass spectrometry
GC-TOF-MS	Gas chromatography with time of flight mass spectrometry
HPLC	High performance liquid chromatography
i.d.	Internal diameter
IR	Infrared
m	Meter
min	Minute
ml	Milliliter
mm	Millimeter
MS	Mass spectrometry

Chapter 1

Literature review

1.1. Introduction:

Over the last decade, a number of synthetic cannabinoid substances have emerged in the illicit drug market as alternatives to cannabis and represent a major class of "designer drugs". These designer cannabinoids act as full agonists at two cannabinoid receptors, CB1 and CB2, and provide a psychoactive effect similar to delta (9)-tetrahydrocannabinol (THC), the main psychoactive component of the natural cannabis plant (Figure 2) [33]. The synthetic cannabinoids chemical structures are completely different from the cannabis plant component THC and represent a new category of drugs of abuse. Synthetic cannabinoid compounds are mainly available on the internet or in some open markets and marketed as "herbal incense" products as dried plant material [15]. These compounds are sold under many different brand names such as "Spice," "K2" and "synthetic marijuana" and labeled "not for human consumption," in an attempt to avoid drug laws and regulation [5, 53]. The synthetic cannabinoids can be classified based upon their chemical structure [18, 44] into the five main novel classes [1] classical cannabinoids (I) non-classical cannabinoids (II) hybrid cannabinoids (III) aminoalkylindoles (IV) and eicosanoids (V). A representative compound for each class of the synthetic cannabinoids is shown in the Figure 1.

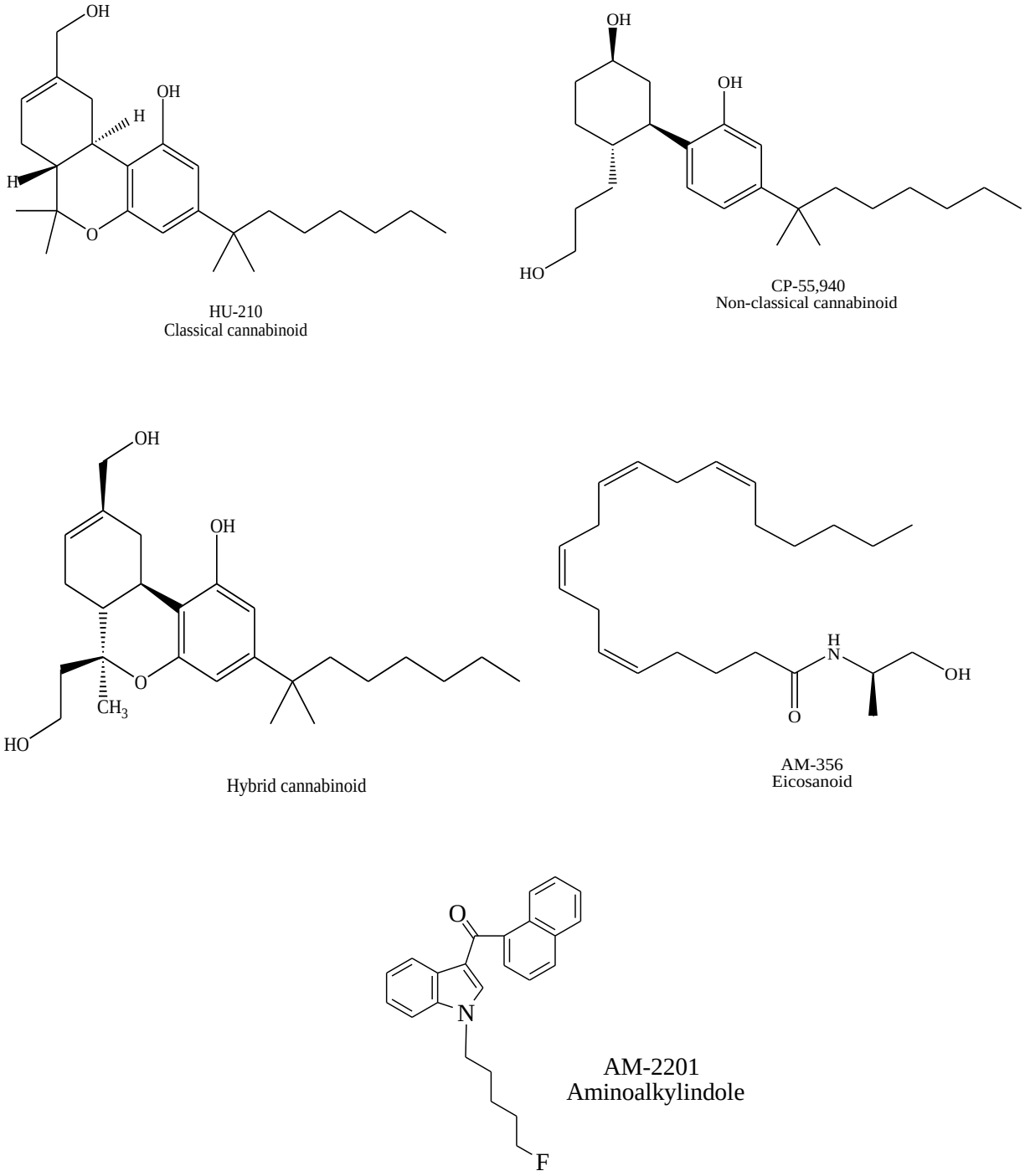
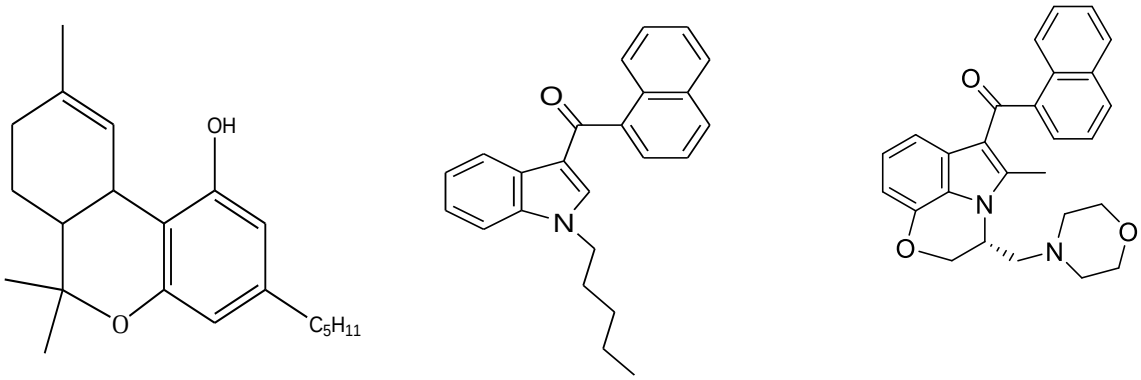


Figure 1: Representative compound for each class of the synthetic cannabinoids.

Currently, the most prevalent class of synthetic cannabinoids in clandestine samples is the aminoalkylindole and this is perhaps due to the relative ease of synthesis compared to other classes of cannabinoid agonists. These compounds can be synthesized without advanced laboratory equipment by using commercially available reagents and chemicals [1]. The prototype compound in the aminoalkylindole series is WIN55, 212-2 (Figure 2) and it inspired the idea for synthesis of alkyacylindole substances. The aminoalkylindoles series can be further divided into many different groups including naphthoylindoles, benzoyl indoles, phenylacetylindoles and others, as well as many derivatives and analogs. The most popular group, in terms of abuse, in this class is the JWH series. This series contains one of the most prevalent and well-known cannabimimetic agent called 1-n-penthyl-3-(1-napthoyl) indole, JWH-018 (Figure 2). JWH-018 was one of the first compounds synthesized in this series and reported as a potent cannabinoid receptor agonist by Huffman et al. in 1998 (Figure 1) [5].

Clandestine or illegal manufacturing laboratories are often operated to circumvent legal restrictions with the goal of creating drugs for distribution on the black market. When synthetic drug substances such as the cannabinoids are controlled by governments, clandestine chemists are able to create or reformulate analogs or regioisomeric forms to replace the existing one. The Controlled Substances Analogue Act (CSA) provides enforcement laws with the authority to investigate and upgrade the penalties associated with clandestine chemists who had been manufacturing and distributing new analogs of a structural series. However, the most challenging aspect in forensic science laboratories these days is to identify the new synthetic cannabinoid compounds and other designer drugs as they appear in new street drug samples.



Δ9-THC JWH-018 WIN55, 212-2

Figure 2: Chemical structures of delta (9)-tetrahydrocannabinol (THC), 1-n-penthyl-3-(1-napthoyl) indole (JWH-018) and WIN55, 212-2

1.2 History of synthetic cannabinoids:

The use of marijuana and other constituents of the cannabis plant (e.g., hashish) has been reported for thousands of years for medicinal and recreational purposes and marijuana remains one of the most widely used psychoactive drugs throughout the world [30]. The cannabis plant contains more than 460 known chemical structures of which more than 60 structures belong to a class known as the cannabinoids [8, 27]. However, the production and administration of cannabis have been illegal in most countries since the early 20th century. A group of synthetic cannabinoids was synthesized in the research laboratory of Dr. John W. Huffman at Clemson University [22] in an effort to explore drug-receptor interactions in the cannabinoid system and to create new cannabimimetic compounds for medical research purposes [22-23]. These studies occurred over many years before these compounds appeared as abused drugs.

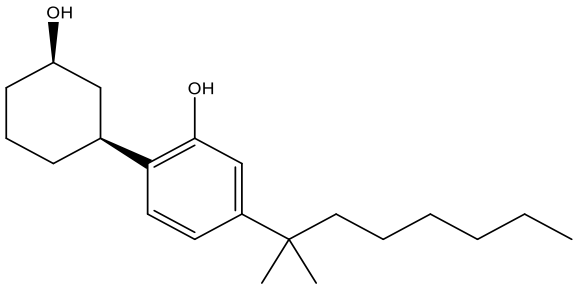
Most of synthetic cannabinoids in forensic samples are manufactured in foreign clandestine laboratories in Asia, especially in China, without advanced laboratory equipment and without quality control standards. The first use of the synthetic cannabinoid substances appeared in several European countries around 2004 as legal recreation and relaxation product alternatives to smoking marijuana [6, 38]. Two years later, these drugs were being sold predominantly as smoking tobacco substitute on the internet and in some open markets such as the local smoke shops and gas stations under the street name “Spice” and “K2”; these two names have persisted as the generic term for all synthetic cannabis products [38]. After two years, in November 2008, the first appearance of “spice” in the United States was officially reported and analyzed in government forensic laboratories by U.S. Customs and Border Protection [32].

The cannabicyclohexanol, C8 homolog of the non-classical cannabinoid CP-47,497(CP-47,497-C8), and a cannabimimetic aminoalkylindole, 1-penthyl-3-(1-napthoyl) indole (JWH-018) were identified as the main active ingredient of spice by Uchiyama et al. 2009 (Figure 2) [49]. Since then, synthetic cannabinoids have gained popularity in the United States and Europe marketed as "legal alternatives to cannabis" or "legal highs". Numerous public health authorities and poison control centers in some countries have reported serious and dangerous side effects associated with the use of these compounds such as hypertension, agitation, hallucinations, recurring seizures and overdose deaths. Because these substances pose a threat to the public health and safety, many governments have focused on preventing the abuse of these compounds. At the beginning of 2009, legislation in several European countries subjected the synthetic cannabinoid compounds found in the spice products to the Narcotics Law and banned the selling of these compounds on the internet and open stores [1].

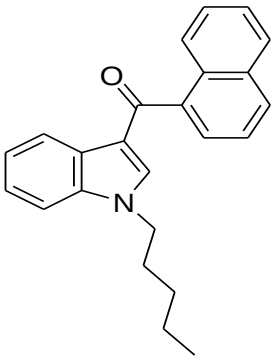
In the United States, the US Drug Enforcement Agency (DEA) used its temporary scheduling authority to place five of the most popular synthetic cannabinoids as the primary psychoactive components of spice on its Schedule I of CSA in March 1, 2011 and 10 states banned these five drugs: Alabama, Georgia, Illinois, Kansas, Kentucky, Louisiana, Michigan, Mississippi, Missouri and Tennessee. These five banned compounds are 1-pentyl-3-(1-naphthoyl)indole (JWH-018), 1-butyl-3-(1-naphthoyl) indole (JWH-073), 1-[2-(4-morpholinyl)ethyl]-3-(1-naphthoyl)indole (JWH-200), 5-(1,1-dimethylheptyl)-2-[(1R,3S)-3-hydroxycyclohexyl]-phenol (CP-47,497) and 5-(1,1-dimethyloctyl)-2-[(1R,3S)-3-hydroxycyclohexyl]-phenol(cannabicyclohexanol), CP- 47,497 /C8 homologue. Figure 3 shows the chemical structures of these compounds.

One year later, these five drugs as the first generation of synthetic cannabinoids was permanently placed in Schedule I as controlled substance by the United States Congress in June 2012. That means consumption and selling of these compounds or any products containing these substances are considered illegal in the United States [43,35]. Once the permanent ban was in place, first-generation synthetic cannabinoids were replaced by new compounds in the same class with similar structure. Thus, clandestine chemists were able to synthesize different analogs and derivatives over a very short period of time. Furthermore, the brand name of these new compounds was changed which makes these products difficult to identify and study. Therefore, the Controlled Substance Analog Act decided to place all cannabimimetic agents as Schedule I drugs in May 2012.

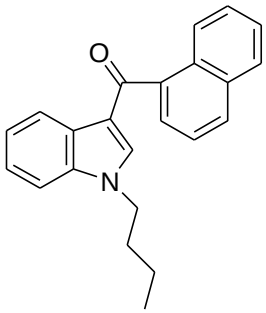
These new generations of restricted drugs often take a long time to be detected and regulated. Therefore, detection of new designer drugs remains an analytical challenge of many governments because the manufacturing of different analogs or isomers of banned compounds has been increasing with time. [9]. Recently, 51 synthetic cannabinoid compounds were identified and named in the Controlled Substances Analogue Act (CSA) compared to only five identified in 2009. However, there are over 200 synthetic cannabinoid compounds sold on the street under different brand names and ingredients while only 51 of them are currently listed in the U.S. as federal Class I drugs [32].



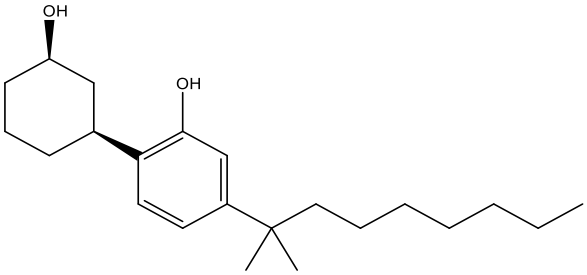
CP- 47,497



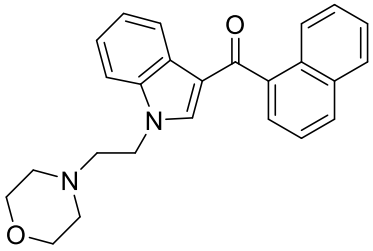
JWH-073



JWH-018



CP- 47,497 C8



JWH-200

Figure 3: The chemical structure of five synthetic cannabinoid drugs permanently placed in Schedule I of CSA by the Congress in 2012.

1.3. General pharmacology:

1.3.1 Pharmacodynamics:

The psychoactive component of natural marijuana and synthetic cannabinoid compounds activate two known subtypes of G-protein coupled receptors (GPCRs), CB1 and CB2. Most of the synthetic cannabinoid substances act as full agonists at the CB1 and CB2 with varying receptor potency, affinity and selectivity whereas the main psychoactive component of natural marijuana (THC) acts as a partial agonist at both CB receptors (CB1 and CB2) [7]. Many of the synthetic cannabinoids are more potent as agonists than THC at the CB1 receptor. For example, the indole JWH-018 has affinity for the cannabinoid brain (CB1) receptor five times greater than that of THC. However, there are very little data about the pharmacological action of these synthetic cannabinoids substances and few human studies have been published concerning their action in the human body.

The CB1 receptors are central receptors located mainly in the brain and are responsible for the psychoactive effect similar to THC and play a significant role in the modulation of GABA and glutamate neurotransmission [17]. The psychoactivity of CB1 is characterized by acute euphoria, relaxation, sensory and perceptual distortions and disruption of motor coordination, as well as appetite stimulation and analgesia [28]. The CB2 receptors are peripheral receptors located mainly on immune cells and act to modulate immunosuppression by inducing apoptosis inhibition of proliferation, and suppression of cytokine and chemokine production [37].

Biologically, the activation of CB1and CB2 cannabinoid receptors results in the modulation of multiple intracellular signal transduction pathways resulting in neuronal hyperpolarization and a decrease in neurotransmitter release. This activation includes inhibition of cyclic adenosine monophosphate (c AMP) production, modulation of ion channels, and promotion of mitogen-activated protein kinase (MAPK) activation [19, 48]. Therefore any ligand, such as the synthetic cannabinoid compounds, able to bind to CB1 and act as an agonist is considered an alternative to marijuana.

1.3.2 Route of administration:

Inhalation was reported as the most common route to administer the synthetic cannabinoids either by smoking like conventional cannabis or using a vaporizer. These compounds are synthesized in oil or a crystalline powder dosage form which is usually mixed directly with various herbal plants such as bay bean, blue lotus or red clover which act as a carrier for these substances. Synthetic cannabinoids powder dissolved in a chemical solvent and then sprayed onto dried plant material is another way to prepare the spice products. These compounds usually are smoked using a pipe or rolling in cigarette papers, or dissolved in a chemical solvent such as acetone to be vaporized and inhaled using e-cigarettes or hookah pens [31, 42]. However, there are few reports on the oral consumption, such as taken with food or prepared as a tea. Other types of administration, such as intravenous injection or snorting have not been reported [47].

1.3.3 Toxicology and acute adverse effects:

Synthetic cannabinoid compounds provide a similar psychoactive effect to THC such as elevated mood, relaxation and altered perception. Synthetic cannabinoids can produce fatalities and life threating adverse health effects. According to the Drug Enforcement Agency (DEA) and the American Association of Poison Control Centers, fatalities have been reported associated with overdose intoxication of "spice" mixture. The overdose death associated with these street chemicals reportedly is related to heart attack, suicides, acute psychosis and panic attack [39].

In addition, the frequency and severity of synthetic cannabinoids adverse effects are much greater than natural marijuana, perhaps because the synthetic cannabinoids act as full agonists at the CB receptors [12, 13]. The DEA has also reported patients having severe kidney injury often requiring hospitalization and dialysis. Another case of severe toxicity associated with emesis, agitation, mydriasis, mild tachycardia and seizure was reported one hour after smoking Spice [39]. Furthermore, there are other serious and dangerous adverse effects reported including high blood pressure, increase of heart rate, tremors, palpitations, clenched muscles, dizziness, coma, unconsciousness, terrible headaches with vomiting and inability to speak [2, 3].

1.3.4 Chronic and long Term Effects

Unfortunately, there is no information about the chronic and long term toxicity of the spice while the acute adverse effects and toxicity of these compounds are reported. Nevertheless, one study reported that some of the aminoalkylindole metabolites, naphthylindole compounds in particular, may have carcinogenic potential [25]. The chronic effects associated with the use of spice products have been reported to be similar to cannabis use [9, 54]. The cardiovascular problems and psychological disorders associated with prolonged use of marijuana, and typically with spice, are dependent on the age of users and frequency of use [29]. A number of behavioral and psychological studies indicate that prolonged use of the synthetic cannabinoid compounds may increase the risk of psychosis symptoms. These symptoms are similar to those reported in prolonged use of marijuana, for example, increased risk of developing schizophrenia with adolescent use, impairments in memory and cognition, auditory and visual hallucinations, paranoid delusions, anxiety, violent behavior and stupor and suicidal ideation.

1.3.5 Addictive potential and withdrawal syndrome:

Some studies have reported dependence and withdrawal syndrome associated with chronic use of spice because patients appear to develop tolerance very quickly. In comparing with marijuana, a number of synthetic cannabinoid substances have a higher dependence and withdrawal syndrome such as irritability, insomnia, decreased appetite, anxiety and drug craving [21]. In a case report published by Zimmermann et al. in 2009 from Germany [56], tolerance and dependence occurred after chronic and frequent use of spice with rapidly increasing dose to 3 g per day. Withdrawal symptoms include inner unrest, drug craving, nocturnal nightmares, profuse

sweating, nausea, tremor, and headache. Synthetic cannabinoid addiction is a serious medical condition that requires treatment by chemical dependency specialists.

1.4 Metabolism and elimination:

There are few metabolic studies of synthetic cannabinoid compounds in the published literature to date. All of the published research articles on cannabinoid metabolism was performed based on human liver microsomes or biological samples of drug users, in vitro and in vivo studies [11, 41, and 55]. Altogether, in vivo and in vitro studies have generally showed consistent results [11, 55]. The aminoalkylindoles compounds are metabolized smoothly by various hepatic enzymes of CYP450 family, mainly by CYP2C9 and CYP1A2.

One of the first in vivo studies of human urinary metabolites of mixtures containing JWH-018, CP-47, 497C-8 homologs was reported by Sobolevsky et al. (2010) [41]. The investigators found that there are three metabolic target sites in parent drug: aryl or napthyl substituents (I) the indole ring (II) the alkyl side chains (III) and no unchanged drug was detected. These compounds undergo multiple metabolic pathways which included mainly N-dealkylation, multiple hydroxylation, (mono-, di- and tri- hydroxylation) a combination of hydroxylation and dealkylation and oxidation of the terminally hydroxylated N-alkyl moiety to a carboxylic acid. Monohydroxylated compounds are the main metabolites for the aminoalkylindoles. In addition, all of the metabolites are mainly excreted in the urine by conjugation with glucuronic acid via uridine diphosphate-glucuronosyltransferase (UDP-UGT). Other elimination pathways, such as feces, sweat, oral fluid, and hair, have also been reported [10].

A later study was reported that omega-minus one (ω-1) monohydroxylation at the alkyl side chain is the main metabolite of JWH-018 and JWH-073 which was easily detected in human urine. This analysis was performed using LC-MS/MS and LC-TOF [26]. The structure of these primary metabolites are shown in Figure 4.

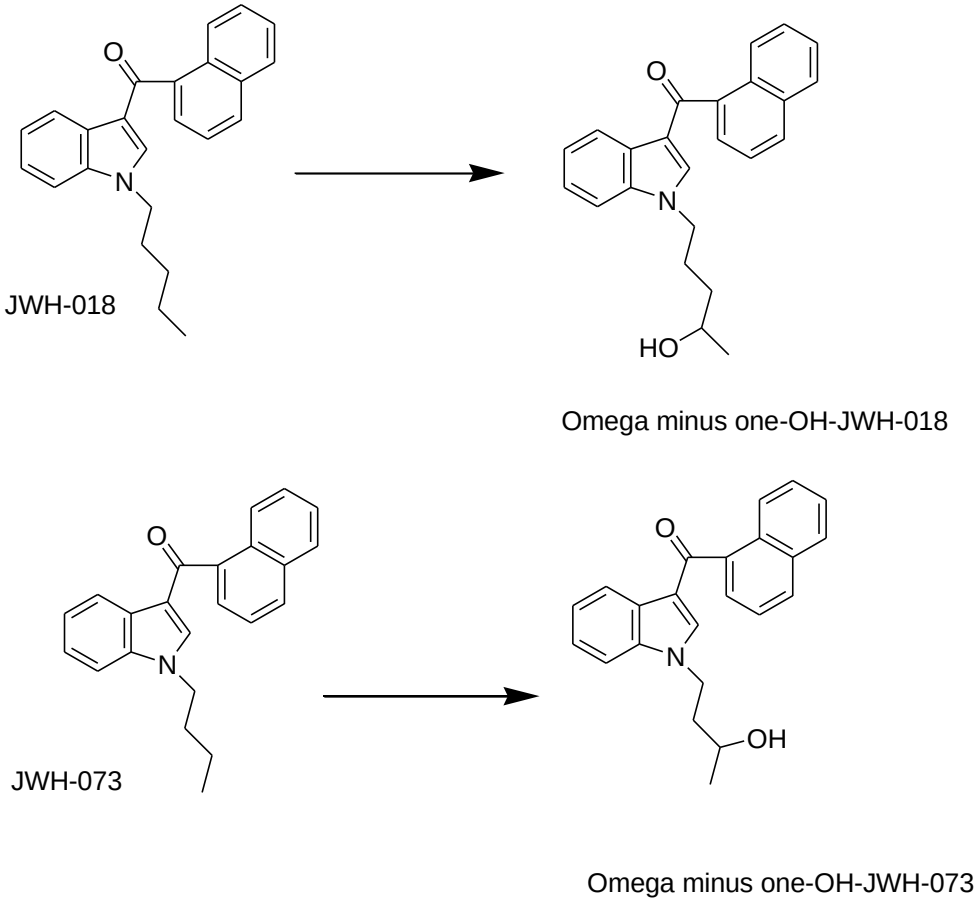


Figure 4: Structures of JWH-018 and JWH-073 and their major metabolites [8].

1.5 General analytical methods used on synthetic cannabinoid substances:

Since the emergence of synthetic cannabinoids, various analytical studies have been published to detect and identify these compounds in forensic products and their metabolites in biological samples. Different analytical methods have been reported in the chemistry and toxicology literature and gave successful results for identification and differentiation of synthetic cannabinoids from each other. The main analytical methods discussed in review articles of synthetic cannabinoids were performed by GC-MS and LC-MS/MS. Hudson et al. in 2011 [20] reported that EI-GC-MS was the preferred analytical technique because it provides extensive detail in fragmentation patterns that helps for initial identification of unknown compounds and structure elucidation. However, consecutive confirmation can be provided by using LC-MS/MS. In addition to the separation and fragmentation technique, the LC/GC-TOF provides the accurate mass determination for parent molecules as well as fragments that play an important role in facilitating structure elucidation and molecular formula determination. In addition, several articles described other methods used only to provide additional confirmation and structural elucidation by using GC-IR, NMR, and CI-MS [26, 49, 50].

The mass spectrum (MS) technique often provides a high-quality analysis and specific "fingerprint" for an individual compound. However, most of the cannabinoid compounds are isomers and analogs and yield similar MS information. Therefore, there is a limitation of the MS technique to differentiate between the cannabinoid analogs and isomers. The alternative analytical method to distinguish these analogs and isomers is to separate them chromatographically or use other analytical techniques for those isomers overlapping or co-eluting in the chromatography system such as the GC-IRD and IR.

1.5.1. Gas chromatography-mass spectrometry (GC-MS):

In forensic science, gas chromatography (GC-MS) is often called the "gold standard" analytical methods for detection and identification of unknown compounds due to its simplicity, sensitivity and effectiveness. Electron ionization (EI) is the preferred ionization method because it provides distinguishing detail in fragmentation patterns. The EI-GC was one of the different analysis techniques that Uchiyama et al. (2009) used to identify the JWH-018 and CP-47,497-C8 as the main components of spice in Japan [49]. In a late study of the same group, Uchiyama et al. (2010) identified new synthetic cannabinoid compounds, JWH-073 and CP-47,497, found in a new generation of spice products using EI-GC-MS [50]. The structures of these compounds are shown in Figure 2 and the major GC-MS fragment ions of these synthetic cannabinoids are shown in Table 1.

Table 1: The major GC-MS fragment ions for the synthetic cannabinoid identified by Uchiyama [49, 50].

Compound	Major GC-MS ions (*m/z*)
JWH-018	$341M^+$, 324, 284, 214.
JWH-073	$327M^+$, 310, 284, 200.
CP-47,497	$318M^+$, 300, 233, 215.
CP-47,497-C8	$332M^+$, 314, 233, 215.

In some published studies, the EI-GC has successfully differentiated a series of aminoalkylindole isomers. These isomers usually have identical molecular formulae, nominal mass and yield almost the same fragment ions in their EI mass spectra. The sensitivity of EI-GC mass spectra to differentiate between aminoalkylindole isomers was studied in five possible regioisomers of JWH-018 by Clark et al, 2015 [45]. The naphthoyl group of these sets of compounds is attached at each of the 6 possible ring substituent positions of the indole ring 2-, 3-, 4-, 5-, 6- and 7-naphthoyl-1-n-pentylindole. These compounds have almost an identical mass spectral with a difference between them in their relative abundance and some major fragment ions. In this study, the EI- mass spectra of JWH-018 showed a higher relative abundance of both the *m/z* 144 ion and the *m/z* 324 ion compared with the other five regioisomers mass spectra [45]. The difference among these compounds and their other 6 analogs will be discussed in detail in chapter 4. In a separate article, Clark et al. (2015) reported that EI mass spectra perfectly differentiates between JWH-018 and its specific inverse-isomer, 1 naphthoyl-3-n-pentylindole. The mass spectrum of the inverse isomer shows only two fragment ions at *m/z* 155 and m/a 127 [46]. The difference among these two compounds and their analogs will be discussed in detail in chapter 2. In addition, analysis studies of identifying JWH-018 and JWH-073 metabolites in human blood and urine using GC-MS were reported by Stoykova S. et al in 2014 [40].

1.5.2 Liquid chromatography–tandem mass spectrometry (LC–MS/MS) and high-resolution liquid chromatography time-of-flight mass spectrometry (LC-TOF-MS):

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) is a powerful technique that has a significant role for both qualitative and quantitative analysis of synthetic cannabinoids. Many forensic researchers have reported the use of LC-MS/MS for detection of herbal mixtures or pure powder. Liquid chromatography coupled to quadrupole time-of-flight (LC-QTOF) was used for accurate mass determination of these compounds and aided for molecular formula calculations, which is useful information for identifying novel or unknown compounds [20, 24]. These two instruments provided detailed information for identification and elucidation of the structure of compounds (JWH-018 and other compounds) found in pure bulk powder as reported in 2012 by Jankovics et al. 2012 [24]. In addition, LC-MS/MS is considered a basic method for detecting these compounds directly in a biological sample. One of the first in vivo studies of human urinary metabolites of mixtures containing JWH-018 and a CP-47 and 497C-8 homologs was reported by Sobolevsky et al. (2010) [41]. In addition, detection of some synthetic cannabinoids and their metabolites in human urine and blood sample using LC-MS/MS has been reported [5, 26]. However, these two techniques are not able to differentiate between isomers since LC-TOF identifies compounds based on their accurate molecular mass and LC-MS/MS provides less fragmentation patterns.

1.5.3. Nuclear magnetic resonance (NMR) spectroscopy.

Several researchers reported the use of NMR spectroscopy in their studies of cannabinoids. In forensic laboratories, this technique is utilized for further confirmation of structural elucidation of new compounds and synthesis confirmation purposes. Compared with other techniques, NMR has an advantage which allows for differentiation of isomers and analogues and the capability to analyze nonvolatile compounds. Ernst et al. (2011) reported using NMR to determine the structure of a new naphthoylindole compound (JWH-122) that had become popular in Germany and sold on the internet under the brand named "Lava Red" after the banning of "Spice" brand products [14]. However, NMR is not common in most forensic laboratories facilities due to the high cost of this instrumentation, the technical expertise required for analysis and the poor sensitivity comparing with other instruments.

1.6 Rationale and statement for the Research:

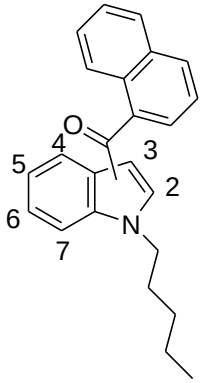
Designing new drugs of the synthetic cannabinoid class will continue and it is likely these will appear in clandestine drug samples. There are several factors which determine the development and growth of a new generation of banned compounds [35] including manufacturers' attempts to avoid drug laws and regulation, the demand for producing a high potent psychoactive drug and attempts to avoid the detection of these new drugs in traditional urine drug testing.

The overall goal of this study is to provide a complete analytical profile as well as methods of differentiation of isomeric and designer substances of well-known banned compounds in the indole class of synthetic cannabinoids that have not yet been found in clandestine samples. This complete data is important for rapid identification of these substances in order to include it in a database containing the indole class and to prevent misidentification in the future. Perhaps the US Controlled Substance Analog Act would include all these isomers in controlled substances.

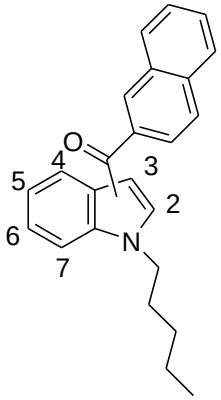
This project will focus on the evaluation and differentiation of fourteen possible regioisomers and analogs of JWH-018 and six regioisomers of dimethoxy-benzoyl substituted-1-n-pentylindole. The previous study has shown the MS differentiation and separation of JWH-018 and its specific inverse-isomer, 1 naphthoyl-3-n-pentylindole [46]. In this study, these two compounds and their analogs with the 2-naphthoyl group instead of the 1-naphthoyl group, will be evaluated and differentiated. Another previous study has shown the MS differentiation and separation of JWH-018 with a number of compounds having the naphthoyl group attached at each of the 5 possible positions in the indole ring, 2-, 4-, 5-, 6- and 7-naphthoyl-1-n-pentylindole [45]. In this study, these 6 compounds will be compared and differentiated with their possible 6 analogs where the 1-naphthoyl is replaced with 2-naphthoyl group.

In addition, the research will provide the complete sets of six possible regioisomeric benzoyl-ring substituted, 1-n-pentyl-3-dimethoxybenzoyl-indoles. These isomers and analogs compounds were synthesized in our lab. The general structure of 20 compounds involved in this study are shown in the Figure 5 which represents potential designer modifications of the synthetic cannabinoid drugs. This study will:

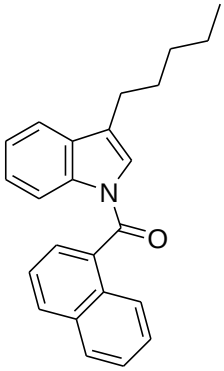
1. Provide a complete mass spectral analytical profile for each compound.
2. Provide a complete chromatographic study to separate/resolve the mixture of the interesting regioisomers in different types of stationary phases
3. Provide complete structural confirmation methods of these compounds by chemical CI and LC-TOF.



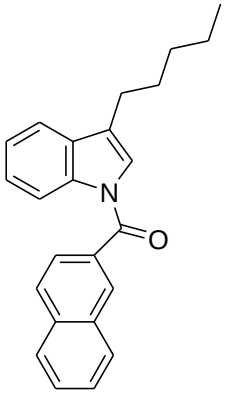
2-, 3-, 4-, 5-, 6- and 7-(1-naphthoyl)-1-n-pentylindole.



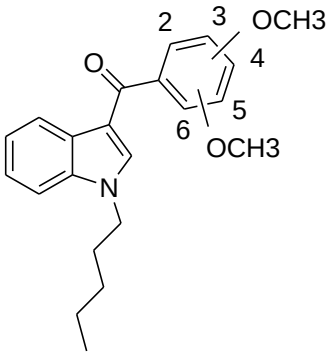
2-, 3-, 4-, 5-, 6- and 7-(2-naphthoyl)-1-n-pentylindole.



1-(1-naphthoyl)-3-n-pentylindole



1-(2-naphthoyl)-3-n-pentylindole



Six possible regioisomers of 1-n-pentyl-3-(dimethoxybenzoyl)-indoles

Figure 5: The general structures of 20 compounds involved in this study.

Chapter 2

Analytical Studies of 1-n-pentyl-3-(1-naphthoyl) indole (JWH-018), 1-n-pentyl-3-(2-naphthoyl) indole and the inverse isomer 1-(1-naphthoyl)-3-n-pentylindole and 1-(2-naphthoyl)-3-n-pentylindole

2.1 Introduction:

Identification of new synthetic isomers that have the structural features similar to controlled drugs is a continuous challenge for forensic scientists. One of the more common drugs of abuse is JWH-018, 1-n-pentyl-3-(1-naphthoyl) indole. As we have already mentioned, this drug has been listed as Schedule I in the Controlled Substances Act (CSA). However, clandestine chemists are able to create new regioisomers by small structural designer modifications often resulting in a new drug with similar pharmacological action. In this chapter, we will compare JWH-018 (compound **1**) with three possible regioisomers. One of these regioisomers (compound **3**) differs with the JWH-018 by reverse positions of the alkyl and acyl substituents attached to the indole ring, 1-(1-naphthoyl)-3-n-pentylindole. The other two regioisomers have the 2-naphthoyl group instead of 1-naphthoyl as in JWH-018 and compound **2**. Compound **3**, 1-(1-naphthoyl)-3-n-pentylindole, is the inverse regioisomer of JWH-018. Compound **4**, 1-(2-naphthoyl)-3-n-pentylindole, is the inverse regioisomer of compound **2**. Because of this structural similarity, it is easy to misidentify of JWH-018 with its other potential isomers. The structures of four compounds in this section are shown in Figure 6.

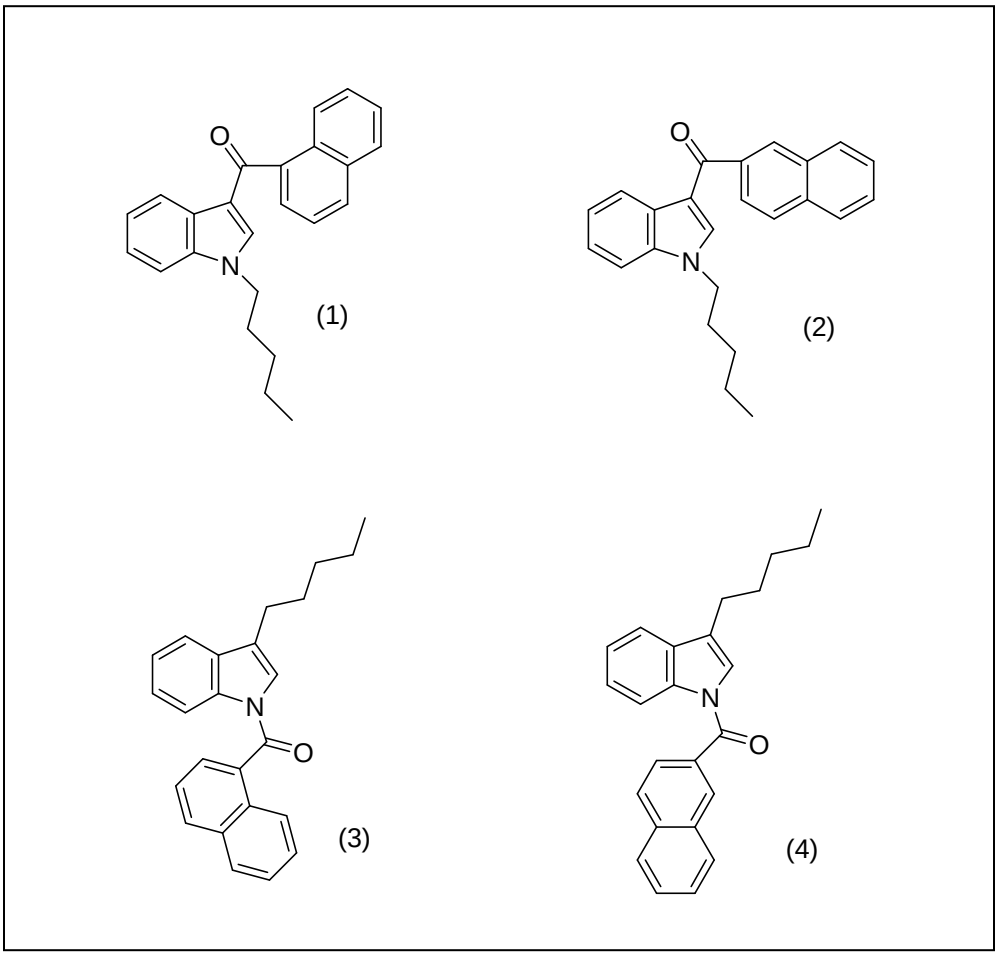


Figure 6: The structures of four compounds in this study, **1**= 1-n-pentyl-3-(1-naphthoyl) indole (JWH-018), **2**= 2-n-pentyl-3-(1-naphthoyl) indole, **3**= 1-(1-naphthoyl)-3-n-pentylindole, **4**= 1-(2-naphthoyl)-3-n-pentylindole.

The mass spectra of 1-n-pentyl-3-(1-naphthoyl) indole (JWH-018) and its specific inverse isomer 1 naphthoyl-3-n-pentylindole (compound **3**) were reported by Clark et al. in 2015 [46]. The authors suggested structures for a number of the major fragment ions of these compounds as shown in Table 4 in section 2.2.2.1. Another previous study reported the EI-MS of the six regioisomers 2, 3, 4, 5, 6 and 7-(1-naphthoyl)-1-n-pentylindole as shown in Figure 7 [45].

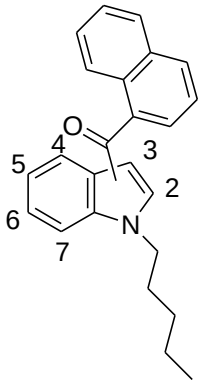


Figure 7: Structure of 2, 3, 4, 5, 6 and 7-(1-naphthoyl)-1-n-pentylindole.

2.2 Differentiation of JWH-018, 1-n-pentyl-3-(2-naphthoyl) indole and their inverse isomers:

In this chapter, we will discuss different kinds of analytical methods for the structural differentiation between these four regioisomers. Additionally, we will make a comparison between 1-n-pentyl-3-(1-and 2-naphthoyl) indole and their inverse isomers 1-(1-and 2-naphthoyl)-3-n-pentylindole spectra, as well as the separation of the mixture of these four compounds by different chromatographic methods.

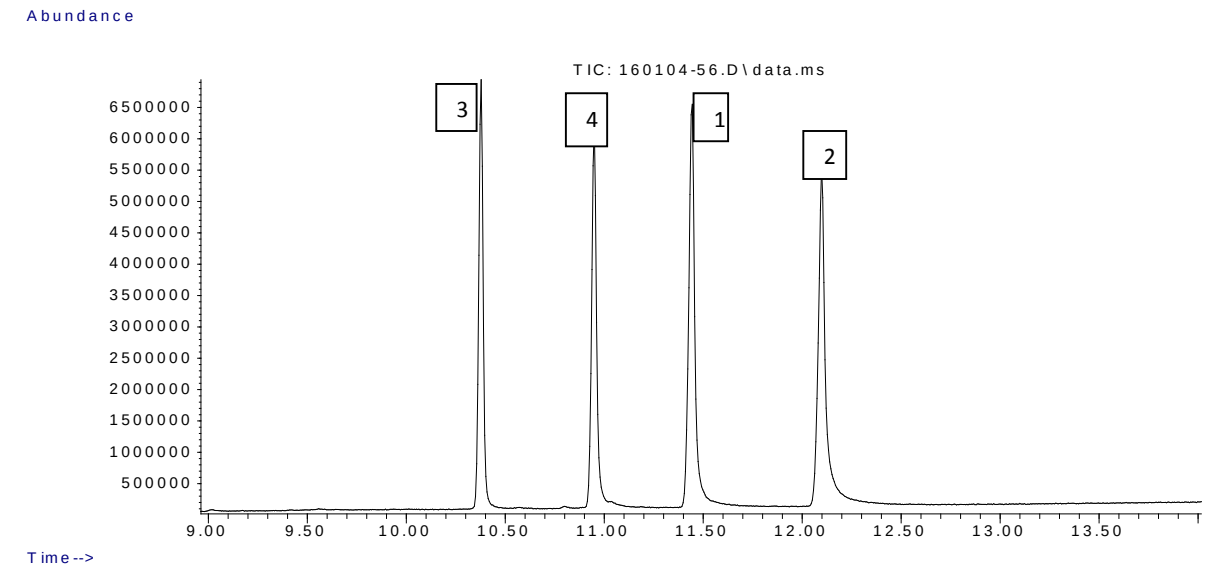
2.2.1 Chromatography separation:

The separation of JWH-018 and its regioisomeric equivalents is challenging due to similarities in their structures. Chromatography is an important tool in forensic drug laboratories for separation and identification of forensic drugs. Gas chromatography (GC) coupled with mass spectrometry (MS) detector and high-performance liquid chromatography (HPLC) with UV/VIS were employed in this study to evaluate procedures for separation of the synthetic cannabinoid JWH-018 and its regioisomers (compounds **1- 4**).

2.2.1.1 GC separation:

Several stationary phases and different temperature programs were evaluated in an effort to improve the separation of these four regioisomeric compounds. Four types of stationary phases were used in this study (Rtx®-5, Rxi®-35Sil, Rxi®-17 and Rtx®-200) and the retention properties of these four compounds were compared on these columns. The GC separation of these regioisomers is shown in Figure 8. The four regioisomers were chromatographed individually by a temperature program named TP1 first to record their retention times. Table 2 shows the observed retention times of these four isomers

A)


Abundance
TIC: 160104-56.D\data.ms
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2
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4500000
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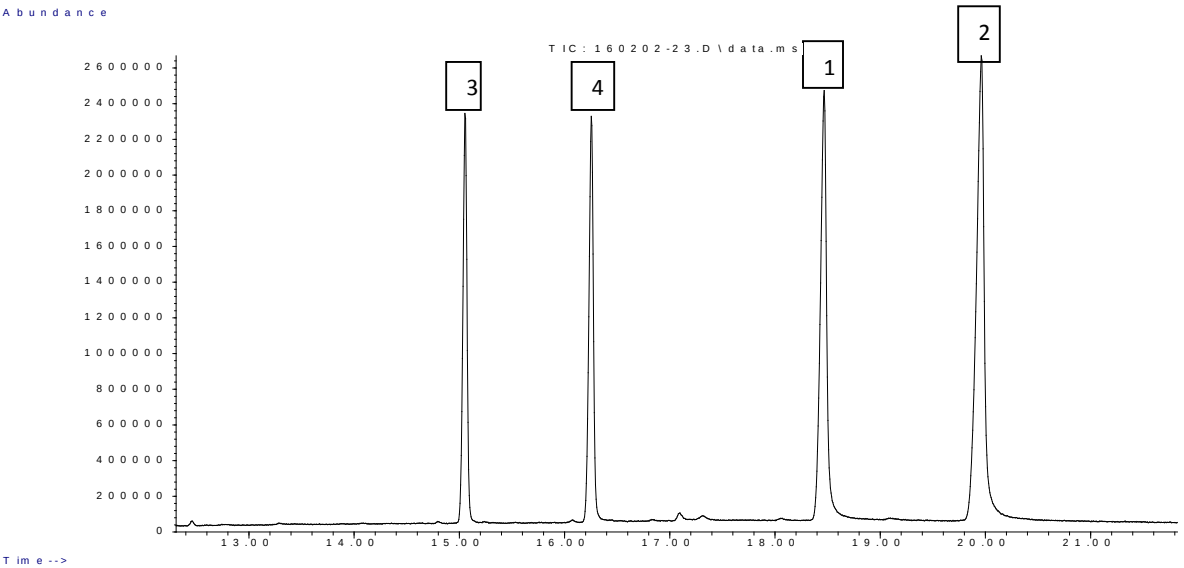
11.00
11.50
12.00
12.50
13.00
13.50
Time-->

B)


Abundance
TIC: 160202-23.D\data.ms
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4
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2
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24000000
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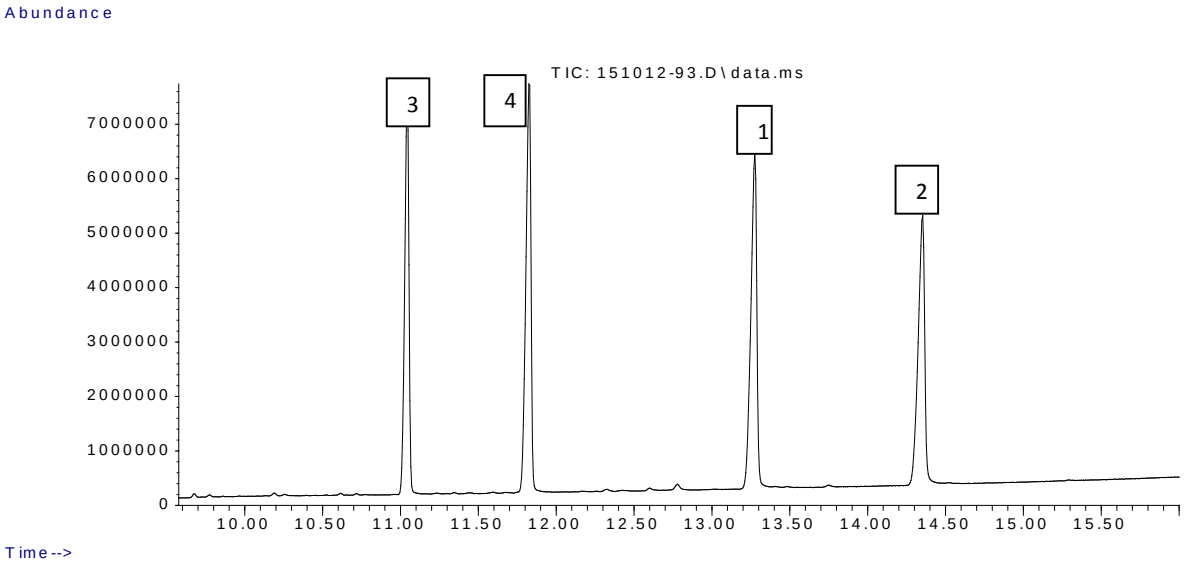
20000000
18000000
16000000
14000000
12000000
10000000
8000000
6000000
4000000
2000000
0
13.00
14.00
15.00
16.00
17.00
18.00
19.00
20.00
21.00
Time-->

C)



D)

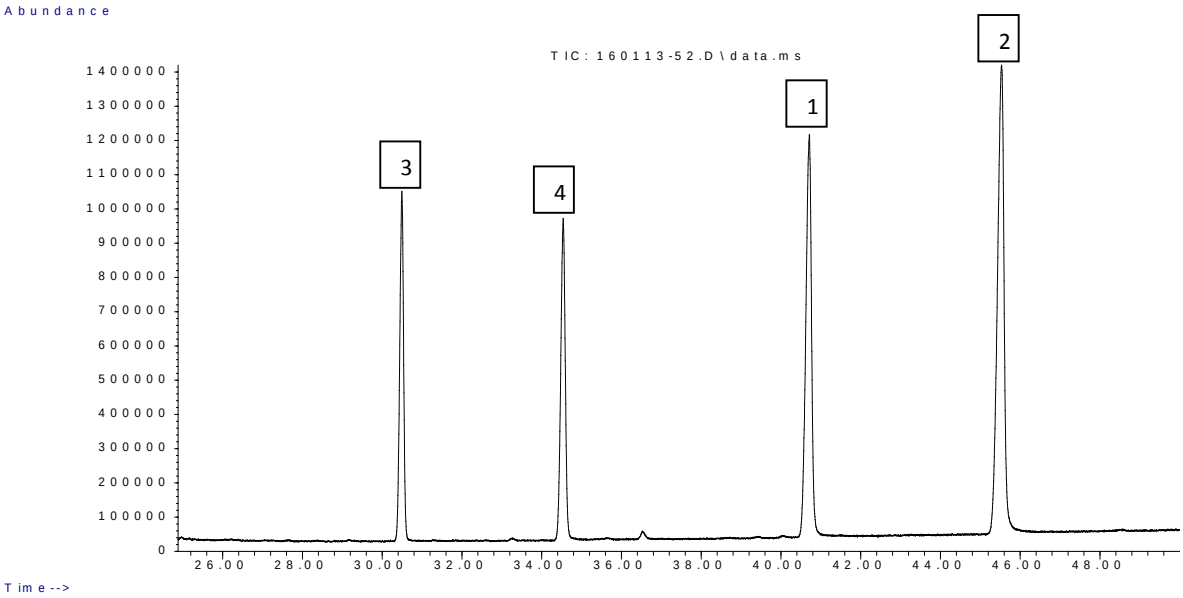


Figure 8: GC separation of the four regioisomers using column Rtx®-5 (panel A), Rxi®-17 (panel B), Rtx®-200 (panel C) and Rxi®-35Sil (panel D).

Table 2: Retention time (min.) of compounds **1-4**, using temperature program TP1 and TP2.

Column Used	Temperature programs*	Compound Number **3**	**4**	**1**	**2**	
Rtx®-5	TP1	10.379	10.950	11.439	12.104	
Rxi®-17Sil	TP1	15.053	16.254	18.468	19.955	
Rtx®-200	TP1	11.043	11.824	13.275	14.354	Rt (min).
Rxi®-35Sil	TP2	30.493	34.538	40.711	45.531	

*details of the programs are in the experimental section

All these stationary phases showed the same elution order with differences among them in the retention time. The elution order appears related to the position of two substituted groups (1- and 2-naphthoyl- and pentyl-group). The amide isomers (the inverse isomer of compound JWH-018 and compound **2**) are the least retained and elute first on all the capillary columns used in this study, however, the compound with the 1-naphthoyl group eluted before the compound with the 2-naphthoyl substituted groups. According to results in Figure 8, compound **3** with the 1-naphthoyl group elutes first then compound **4** with the 2-naphthoyl group. These two early eluting compounds have the common substituted position, 1-naphthoyl-3-n-pentylindole. Compound **1** with (1-naphthoyl) then compound **2** with (2-naphthoyl) are the next eluted compounds, and each of these compounds has a naphthoyl group in position 3 in the indole ring.

Column one was a 30m x 0.25mm-i.d coated with 0.5 μm 5% diphenyl and 95% dimethyl polysiloxane (Rtx®-5). The separation was performed using a temperature program consisting of an initial temperature hold at 80 °C for 1.0 min, ramped up to 300 °C at a rate of 30 °C/min, held at 300 °C for 0.5 min then ramped to 340 °C at a rate of 5.0 °C/min and held at 340 °C for 5.0 min with a total runtime of 21 min. (TP1). The four regioisomers are completely resolved with full baseline resolution on this stationary phase. However, the last two peaks showed slightly tailing but this tailing does not affect the quality of the separation as shown in Figure 8 panel A.

Column two was a 30m x 0.25mm-i.d, capillary coated with 0.5 μm film of 50% phenyl and 50% dimethyl polysiloxane (Rxi®-17Sil MS). Column three was a 30m x 0.25mm-i.d, capillary coated with 0.5 μm film of 100% trifluoropropyl methyl polysiloxane (Rtx®-200). The separation on these two columns was performed using the same temperature program as column one (TP1). Although the last two peaks showed some degree of tailing on the second column, the individual components of this mixture were well separated from each other. This tailing did not significantly influence the quality of separation as shown in Figure 8 panel B. However, the third column shows that the four regioisomers are completely resolved with symmetric peaks in this column with an observed slight upward shift of the baseline as shown in Figure 8 panel C.

Column four was a 30m x 0.25mm-i.d, capillary coating with 0.5 μm film of of 35% phenyl, 65% dimethyl polysiloxane (Rxi®-35Sil MS). The separation was performed using a temperature program consisting of an initial temperature hold at 80 °C for 1.0 min, ramped up to 300 °C at a rate of 15 °C/min, held at 300 °C for 0.5 min then ramped to 340 °C at a rate of 1.0 °C/min and held at 340 °C for 5.0 min with a total runtime of 61.0 min. (TP2). On this column, the run time was increased to allow for the complete elution of sample components. It takes more than forty minutes to get a complete separation with slow upward baseline shift as show in Figure 8 panel D. Otherwise, the separation was excellent with symmetric peak shape and complete baseline separation.

According to the data in Table 2, the temperature program was the same in the first three columns. The retention time of these compounds significantly differs according to differences in column polarity. The regioisomeric compounds are found to be less retained in nonpolar columns Rtx®-5 compared to the more polar column Rtx®-200 and Rxi®-17sil. On the other hand, a different temperature program was used in column Rxi®-35sil to prolong the runtime in order to get a complete separation.

2.2.1.2 LC separation:

High performance liquid chromatography coupled with UV/VIS is a less selective technique used to provide additional confirmation of identify and separate the target compounds. The HPLC-UV system consisted of an Agilent 1200 series (Agilent Technologies, Santa Clara, CA, USA). The instrument is composed of a binary pump, vacuum degasser and UV/VIS multiple wavelength detector and connected to a computer loaded with the Agilent ChemStation Software. The HPLC separation of JWH-018 and its regioisomers are shown in Fig 9.

The excellent separation was obtained by using the reversed phase column allure PFP Propyl (4.6x150 mm, 5µ particle size) purchased from Restek Corporation (Bellefonte PA, USA). HPLC separation was performed using an isocratic elution [65% acetonitrile: 35% water] over twenty minutes. The best flow rate was at 1 mL/min. All studied compounds exhibited considerable UV absorption over the range 200-340, where 254 nm was found suitable for recording their chromatogram. In addition, the eluents were monitored by UV detector from 190 to 400 nm. This chromatographic conditions showed symmetric peaks. Unlike the GC separation, 1-alkyl-3-acylindoles isomers eluted before the inverse regioisomers 1-naphthoyl-3-n-pentylindole. The elution order is based on the relative lipophilicity of these compounds, thus retention time increases with compounds have the naphthoyl group on the nitrogen. JWH-018 eluted first, followed by 1-n-pentyl-3-(2-naphthoyl)-indole then 3-n-pentyl-1-(1-naphthoyl)indole eluted third, followed 3-n-pentyl-1-(2-naphthoyl)indole as shown in Table 3.

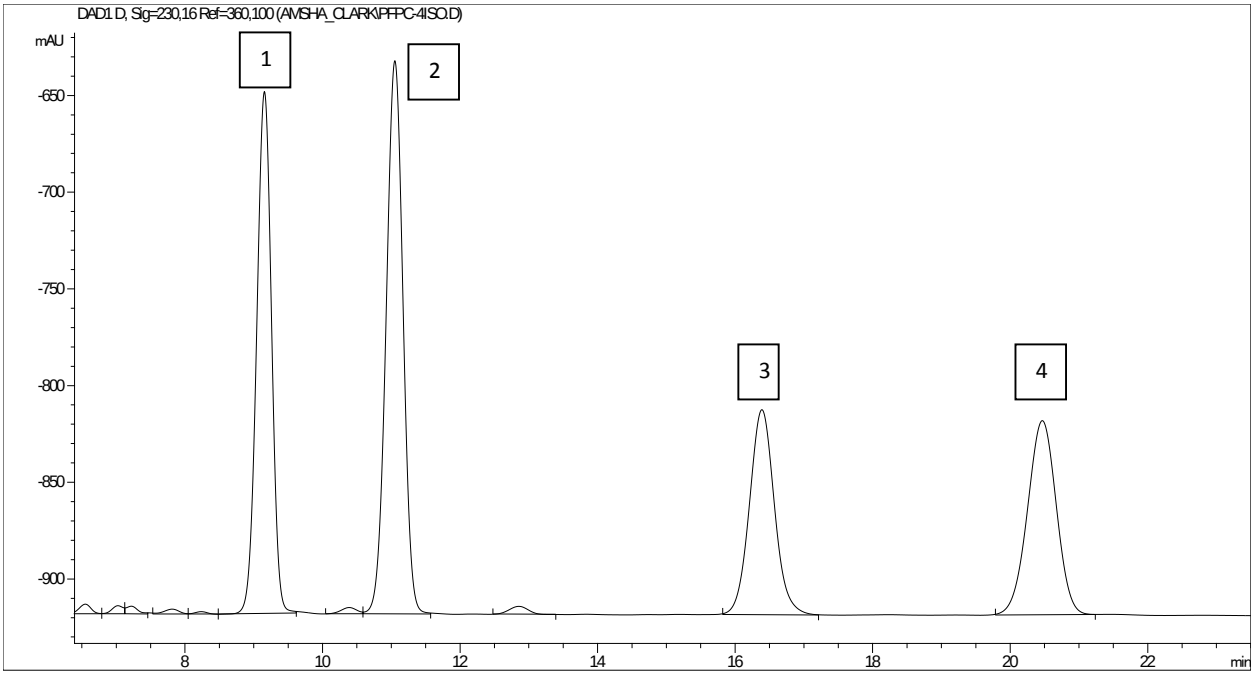


Figure 9: HPLC-UV separation of four isomers mixture by using allure PFP Propyl column, where peak 1 = JWH-018, peak 2 =1-n-pentyl-3-(1-naphthoyl) indole, peak 3 = 3-n-pentyl-1-(1-naphthoyl) indole and peak 4= 3-n-pentyl-1-(2-naphthoyl) indole

Table 3: Reversed phased LC separation of JWH-018 and the three possible regioisomers.

Compound	Rt (min).
JWH-018	9.153
1-n-pentyl-3-(1-naphthoyl) indole	11.050
3-n-pentyl-1-(1-naphthoyl) indole	16.387
3-n-pentyl-1-(2-naphthoyl) indole	20.465

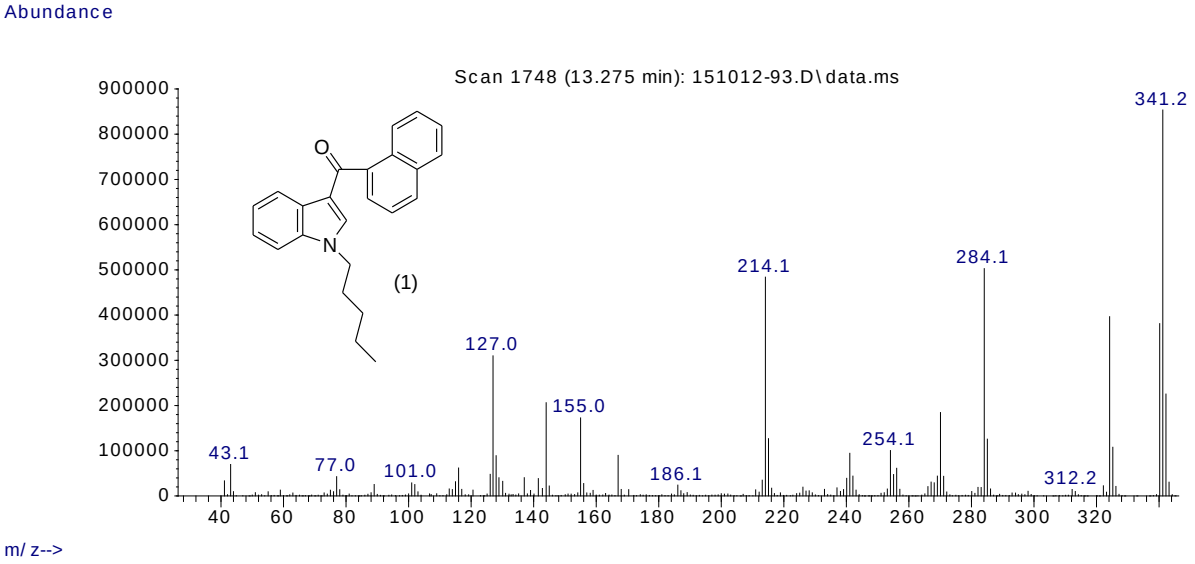
2.2.2 Mass Spectral Studies:

2.2.2.1 Electron impact ionization-mass spectrometry (EI-MS):

Mass spectrometry is the primary method for confirming the identity of drugs in forensic samples. EI-MS yields significant fragmentation patterns that provide structural information to help identify and differentiate these compounds. Figure 10 shows the EI mass spectra of the four regioisomeric compounds (panels A, B, C and D). The EI mass spectral results clearly show a number of unique ions which allow for differentiation of 1-pentyl-3-(1-and 2- naphthoyl) indoles from the inverse isomers 1-(1-and 2- naphthoyl) - 3-pentylindoles. The structures of the major fragment ions in the spectrum for four compounds are shown in Table 4.

The EI mass spectra of 1-n-pentyl-3-(1-naphthoyl) indole (JWH-018) and its specific inverse isomer 1 naphthoyl-3-n-pentylindole was reported by Clark et al. in 2015 [45]. There are unique features in the mass spectra that can be used to easily differentiate1-alkyl-3-acylindoles from 1-acyl-3-alkylindoles based on the fragment ions. However, the two inverse amide regioisomers, 1-(1-and 2-naphthoyl)-3-n-pentylindoles, yield similar fragment ions in their EI mass spectra with similarity in their relative abundance. Thus, unfortunately, the mass spectra alone do not provide specific confirmation of identity or differentiate these two isomers. On the other hand, the two regioisomers of 3-(naphthoyl)-1-n-pentylindole yield very similar fragment ions in their mass spectra with only the 3-(1-naphthoyl)-1-n-pentylindole, JWH-018, showing one unique major fragment ion at *m/z* 324 $[M-17]^+$.

A)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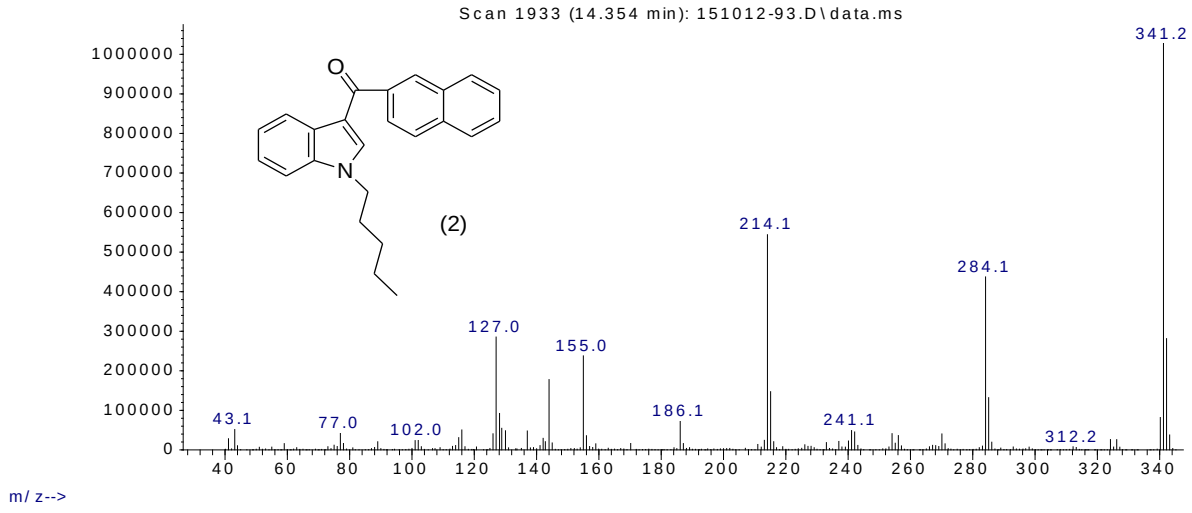
700000
600000
500000
400000
300000
200000
100000
0
341.2
214.1
284.1
127.0
155.0
254.1
43.1
77.0
101.0
186.1
312.2
O
N
(1)
40 60 80 100 120 140 160 180 200 220 240 260 280 300 320
m/z-->

B)


Abundance
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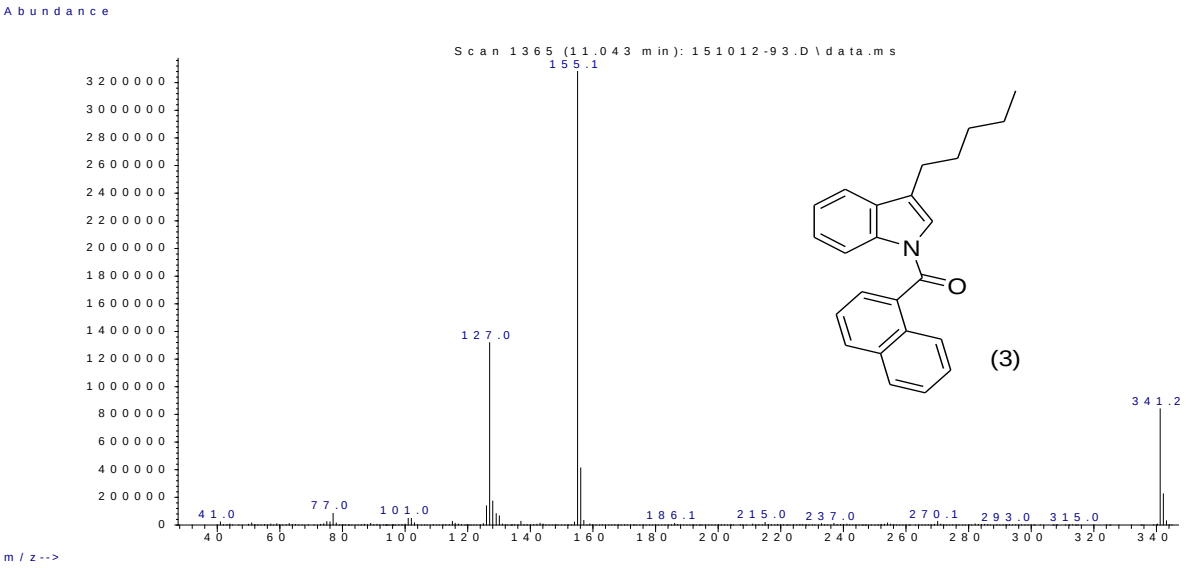
800000
700000
600000
500000
400000
300000
200000
100000
0
341.2
214.1
284.1
127.0
155.0
43.1
77.0
102.0
186.1
241.1
312.2
O
N
(2)
40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340
m/z-->

C)



D)

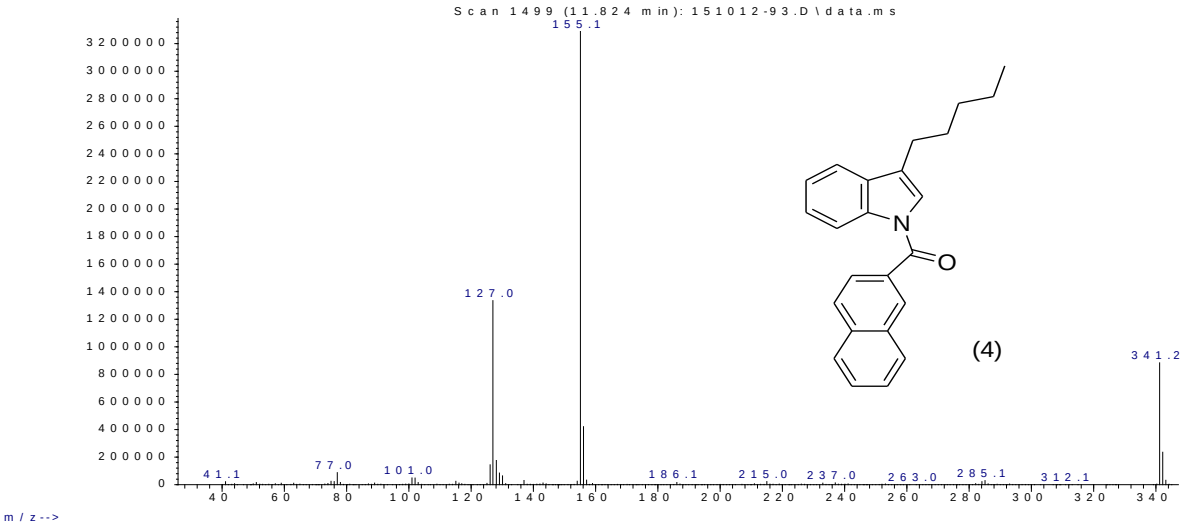
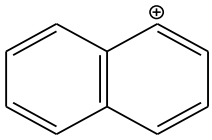
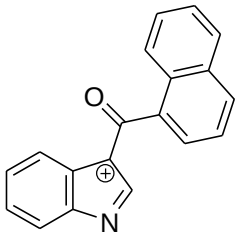
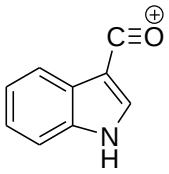
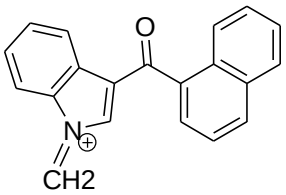
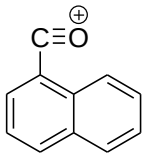
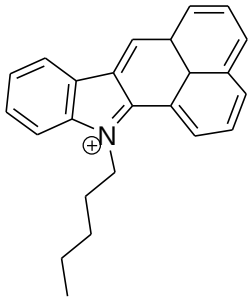
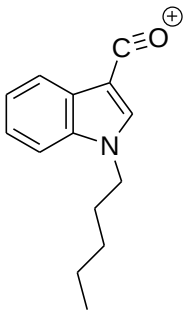
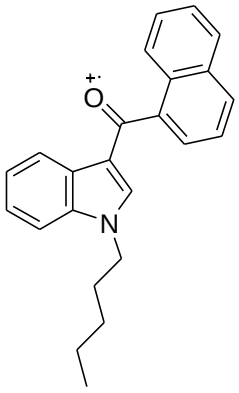


Figure 10: EI- Mass spectra of the model compound of 1-n-pentyl-3-(1-and 2-naphthoyl) indole panel (A and B), 1-(1-and 2-naphthoyl) 3-n-pentylindole panel (C and D) respectively.

Table 4: structures for the major fragment ions in the EI-MS in Figure 10 by Clark et al. [3]

Fragment ion	Mass-to-charge *m/z*	Fragment ion	Mass-to-charge *m/z*
	127		270
	144		284
	155		324
	214		341

A number of studies have reported the EI mass spectra for 1-alkyl-3-acylindoles, JWH-018, and some reports have suggested structures for a number of the major fragment ions as shown in the Table 4 [45, 49, 50]. A direct comparison between 3-(1-naphthoyl)-1-n-pentylindole and 3-(2-naphthoyl)-1-n-pentylindole isomers spectra (panel A and B in Figure 10) revealed a unique fragment ion in the JWH-018 compound at *m/z* 324 $[M-17]^+$.

Previous stable isotope studies using the regionally deuterated analog 3-(1-(D7) naphthoyl)-1-pentylindole [46] have reported that this ion primarily formed as a result of loss of a hydroxyl radical ($^{\bullet}OH$), the carbonyl oxygen and one hydrogen atom comes from the 8-position of naphthalene ring of the molecular radical ion $M^{+\bullet}$. The OH loss represents a hydrogen migration to the carbonyl oxygen followed by elimination of the OH radical to yield the *m/z* 324 fragment cation. However, this $[M–17]^+$ ion is characteristic not only for JWH-018 but in many indole derivatives substituted at the 3-position with the 1-naphthoyl group such as JWH-122, JWH-210, and AM-2201.

In 2-naphthoyl compound, because the thru-space proximity of the hydrogen at naphthalene in the 1-naphthoyl substituted indoles allows for migration but this distance appears to be too great for efficient hydrogen transfer in the 2-naphthoyl indoles. The fragment ion at *m/z* 324 for JWH-018 indicated that it can be easily differentiated by EI-MS from the 2-naphthoyl isomers and other acyl substituted in the 3-position such as the benzoylindole series.

However, both isomers showed the base peak occurring at *m/z* 341, molecular ion peak. The major high-mass fragments in these two isomers were observed at *m/z* 284 $[M-57]^+$ and 270 $[M-71]^+$ with the loss of a butyl radical $[C_4H_9^{\bullet}]$ and a pentyl radical $[C_5H_{11}^{\bullet}]$ from the molecular radical ion $M^{+\bullet}$. These ions occur by alpha-cleavage initiated on the indole nitrogen radical site.

Other major fragment ions occurring at *m/z* 214, 186 and 144 and have been observed in a number of 1-pentyl-3-acylindoles and these fragment ions do not contain the substituted naphthoyl portion. The ions at *m/z* 214 and *m/z* 186 represent cleavage of either of the bonds to the carbonyl carbon with the charge remaining on the indole portion of the molecule. The *m/z* 214 ion represents the loss of the naphthyl group and the *m/z* 186 represents the loss of the naphthoyl radical.

However, the *m/z* 144 ion is the product of a hydrogen rearrangement from the *m/z* 214 cation with the elimination of the entire nitrogen substituted alkyl side chain [C_5H_{10}]. Thus, the *m/z* 144 is formed by the loss of 70 Da from the ion at *m/z* 214 and does not occur directly from the molecular ion. Labeling studies have confirmed these proposed structures and suggested a mechanism of these fragment ions by using deuterium isotope labeling of the naphthalene ring, indole ring and n-pentylindole groups of JWH-018 [46]. In addition, the ion trap mass spectrometry was used for the *m/z* 214 fragment in the JWH-018 isomer (compound **1**) to confirm that the *m/z* 144 ion is a decomposition product of *m/z* 214 ion and does not occur directly from the molecular ion. The MS/MS spectrum of the *m/z* 214 fragment gave *m/z* 144 ion as the major peak and the only ion of significance in this MS/MS experiment as shown in Figure 11.

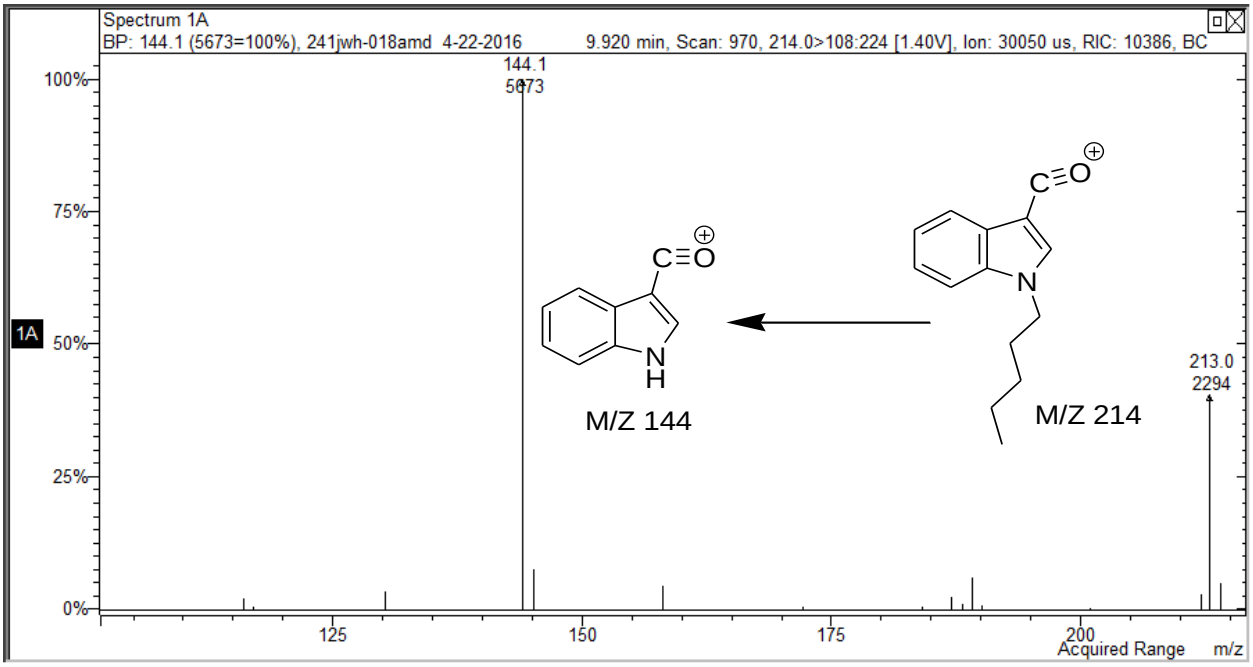


Figure 11: MS/MS spectrum of the *m/z* 214 fragment ion for JWH-018 (operated in MS/MS mode).

All these four regioisomeric compounds have in common two fragment ions at *m/z* 155 and *m/z* 127 which confirms the presence of an acyl group attached to the indole ring. However, the mass spectra of amide isomers (compound **3** and **4**) show only these two fragments (*m/z* 155 and *m/z* 127) with the same relative abundance. Both ions result from cleavage of the amide bond attached to the indole ring with the base peak occurring at *m/z* 155. The base peak at *m/z* 155 is the naphthoyl cation $[C_{10}H_7NO]^+$, and the *m/z* 127 is the naphthalene cation $[C_{10}H_7]^+$. Therefore, the 1-(1 and 2)-naphthoyl-3-n-pentylindole inverse regioisomers can be easily differentiated from the model compound, JWH-018, and other traditional synthetic cannabinoid compounds. However, the mass spectra cannot easily differentiate amide isomers from each other.

2.2.2.2 Chemical ionization (CI)-mass spectrometry:

Chemical ionization (CI) is a soft ionization technique in GC-MS. The main benefit of this technique is to provide a simpler mass spectrum that easily confirms the molecular weight of these compounds. Methanol was used as the CI gas reagent in this study. This reagent gas will interact with ionizing electrons and produce reagent ions, then the ionized methanol collides directly with the compound molecule and proton transfer occurs to generate ionized molecular $[M+H]^+$ and produce one protonated molecular fragment ion. The protonated molecular ion of all these compounds show the expected ion in mass spectrum at *m/z* 342. The CI –MS spectrum of JWH-018, as an example of chemical ionization of this set of compounds is shown in Figure 12. This technique does not provide any additional data for discrimination among these four regioisomeric compounds which have the same elemental composition and only confirms the molecular weight of the compound.

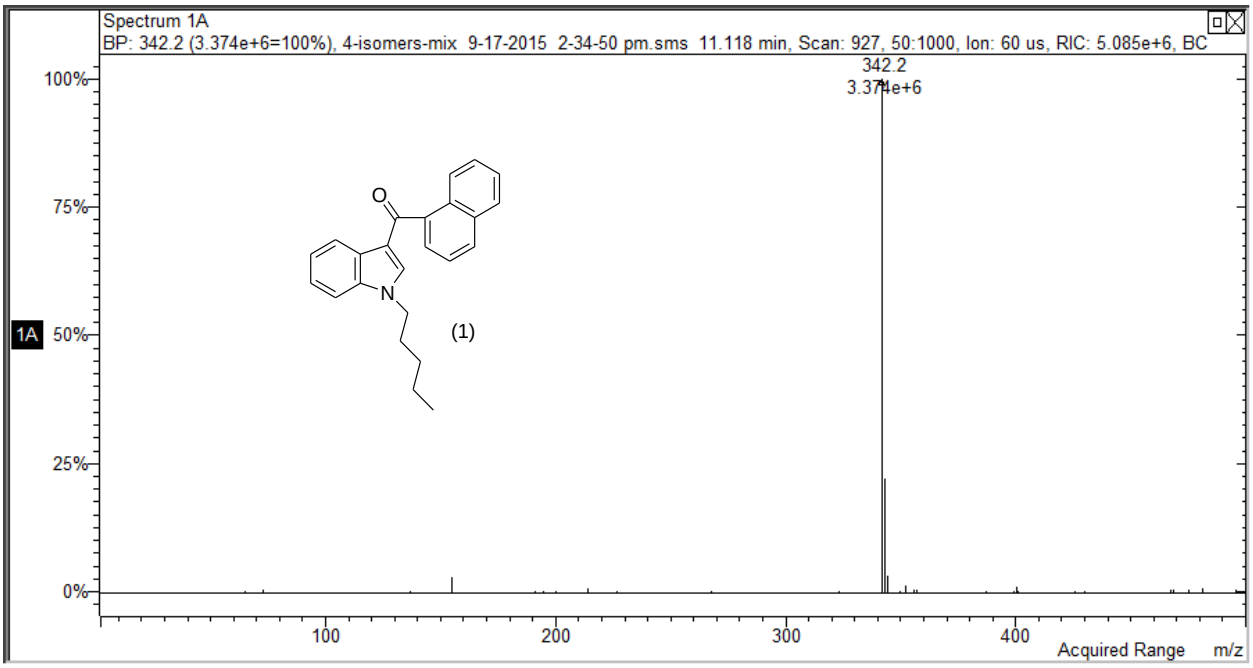


Figure 12: CI Mass spectrum of the model compound JWH-018.

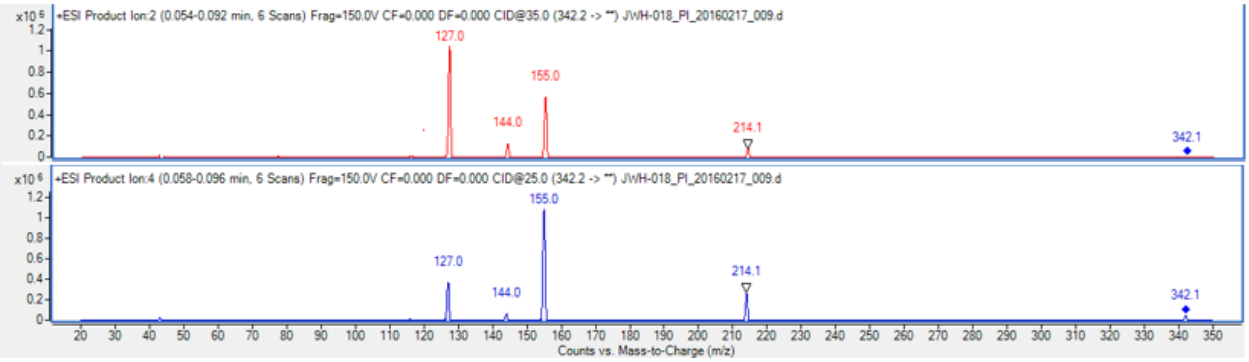
2.2.2.3 LC-MS/MS mass spectral studies:

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) is a sensitive and specific technique and used in this study to give additional information to confirm the identity and discriminate JWH-018 from its regioisomers. The MS/MS investigates the ions of interest by collision-induced dissociation (CID) mass spectra of molecular ion colliding with a collision nitrogen gas by a different energy. The experiment was employed in positive ion mode by electrospray ionization (ESI^+) and detection mode by multiple reaction monitoring (MRM). The LC-MS/MS analysis in this study was performed using an Agilent 1290 HPLC and reversed phase Zorbax Eclipse Plus C18 column (2.5 x 50 mm, 1.8 μm) with isocratic elution. Isocratic elution was performed using 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) (35%:65%) at a flow rate of 0.5 mL/min. In general, the product fragment ions at *m/z* 155 and 127 are expected for acylalkylindole derivatives.

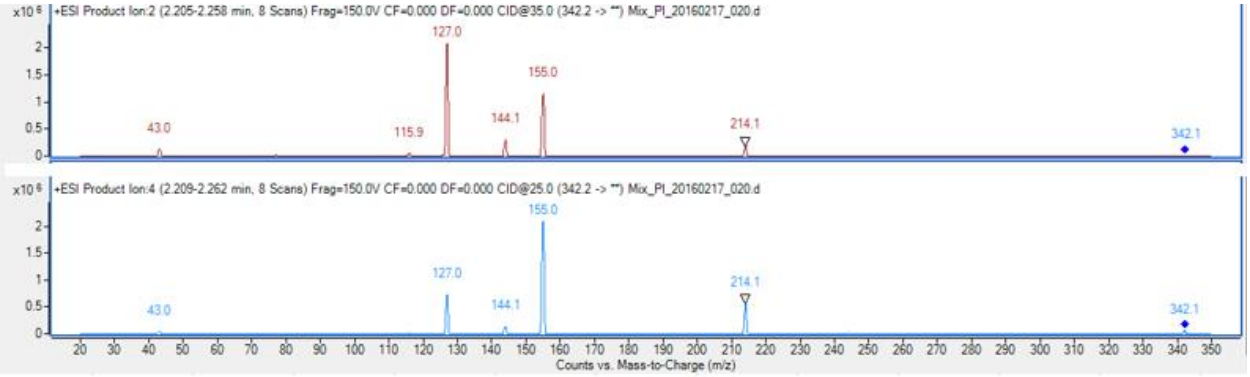
All these four compounds gave $[M+H]^+$ at 342 as a precursor ion and show major fragment ions at *m/z* 155 and *m/z* 127 called product ions. All these regioisomers show the major product ion at *m/z* 155 in 25 eV and at *m/z* 127 in 35 eV. These product ions reflect the chemical structures of the naphthoyl group resulting from cleavage of the acyl group attached to the indole ring. On the other hand, the LC–MS/MS mass spectra of 1-n-pentyl-3-naphthoylindole isomers have shown minor fragment ions at *m/z* 43, 116, 214 and 144. The *m/z* 214 ion represents the loss of the naphthyl group and the *m/z* 144 ion is a hydrogen rearrangement product of the *m/z* 214 ion resulting from the loss of pentene (C_5H_{10}) and does not come directly from the molecular radical ion. The structure and mechanism of these ions are described in the previous section. However, the isomer containing 1-naphthoyl substituted group attached to the indole ring could not be differentiated from the isomer containing the 2-naphthoyl group by LC-MS/MS mass spectra. In

conclusion, this technique did not provide any discrimination among these compounds since the main fragment ions are identical in these regioisomers.

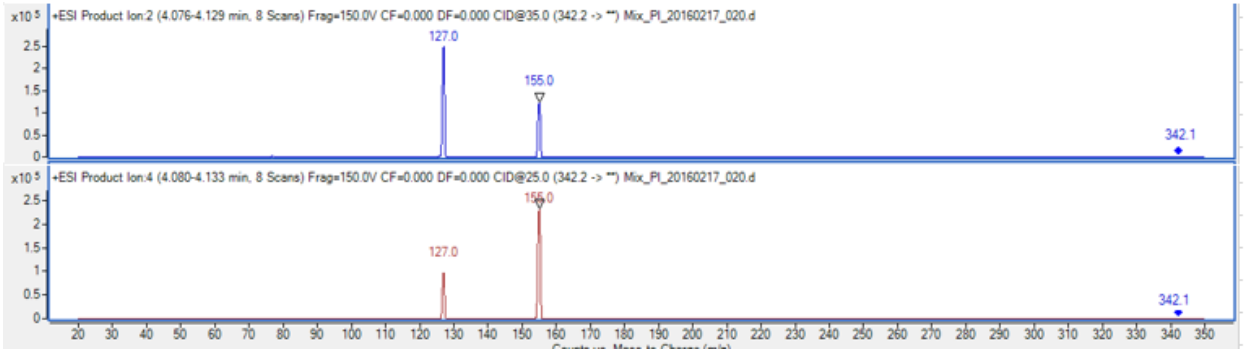
A)



B)



C)



D)

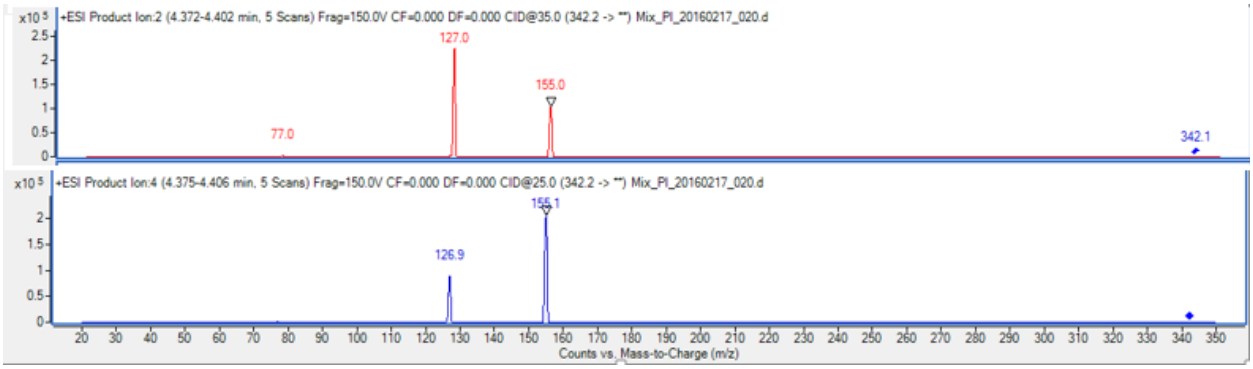


Figure 13: Electrospray ionization (ESI^+) MS/MS spectra (positive mode) of 1-n-pentyl-3-(1- and 2-naphthoyl) indole panel (A and B), 1-(1-and 2-naphthoyl)3-n-pentylindole panel (C and D), respectively.

2.2.3 LC-TOF studies for exact mass determination:

LC-TOF provides accurate mass information (four decimal places) for these compounds to enable calculation of molecular formula. The accurate-mass information is essential for the structural elucidation which provides excellent information for identifying unknown compounds and for confirming the presence of a specific compound. The accurate-mass measurements of these compounds was achieved using an Agilent 6520 with TOF mass analyzer, and the protonated molecular ion information [M+H] from ESI^+. This instrument allows determination of the *m/z* of ionic formula based on the mass sufficiency of the constituent atoms within the acceptable error limits ±5 ppm and the isotope abundance scores were above 95% for all ion compounds. The LC-TOF accurate mass results are shown in Table 5. These results are compatible with molecular formula $C_{24}H_{23}NO$ and supported the results of LC–MS/MS and GC–MS results. The four regioisomeric compounds have the same molecular formula of $C_{24}H_{23}NO$, nominal mass of 341, and the calculated measured mass of 341.1780. In conclusion, LC-TOF-MS confirms the elemental composition of these four regioisomers within acceptable experimental error.

Table 5: Results of the accurate mass measurement by LC–TOF–MS of compounds **1-4.**

Compound No.	Generated formula	Absolute difference (ppm)	abundance score	Exact mass	Accurate mass
1	$C_{24}H_{23}NO$	0	100	341.1780	341.1780
2	$C_{24}H_{23}NO$	-0.23	99.87	341.1780	341.1779
3	$C_{24}H_{23}NO$	-0.23	99.87	341.1780	341.1779
4	$C_{24}H_{23}NO$	-0.23	99.87	341.1780	341.1779

2.3 Conclusion:

JWH-018 and its possible regioisomeric involved in this study have the same molecular formula and nominal mass but are different in their structures. 1-n-pentyl-3-naphthoylindoles can be easily differentiated by EI mass spectra from 1-naphthoyl-3-n-pentylindoles. 1-n-pentyl-3-(1- and 2-naphthoyl)indoles yield very similar fragment ions in their EI-GC-MS and ESI-LC-MS/MS mass spectra with only the JWH-018 showing one unique major fragment ion $[M-17]^+$ at *m/z* 324 by EI-GC-MS and this ion is essentially absent in the spectrum of the 2-naphthoyl isomer. On the other hand, 1-(1-and 2-naphthoyl)-3-n-pentyl indoles yield only two fragment ions at *m/z* 155 and 127 with the same relative abundance in their EI-MS and ESI-LC-MS/MS. Thus, the mass spectral studies do not provide any additional information for discrimination between these two regioisomers. LC-TOF-MS and CI-MS provide an additional means of molecular formula confirmation. The four regioisomers were successfully resolved on the different GC and LC stationary phase involved in this study and temperature programming conditions.

Chapter 3

Analytical Studies of six regioisomeric 3-dimethoxybenzoyl-1-pentylindoles

3.1 Introduction:

The six regioisomeric 1-n-pentyl-3-(dimethoxybenzoyl)-indoles represent potential designer modifications in the synthetic cannabinoid drug category. These novel synthetic cannabinoid compounds contain two rings, a benzoyl ring and a substituted indole ring. The benzoyl ring possesses dimethoxy groups substituted in all possible positions on the aromatic ring. The six compounds have the same elemental composition $C_{22}H_{25}NO_3$and the same substituents attached to the indole ring. This chapter compares the analytical properties of the six regioisomeric 1-n-pentyl-3-dimethoxybenzoylindoles and describes different methods for separation and differentiation among these compounds. The structures of six compounds evaluated in this section are shown in Figure 14.

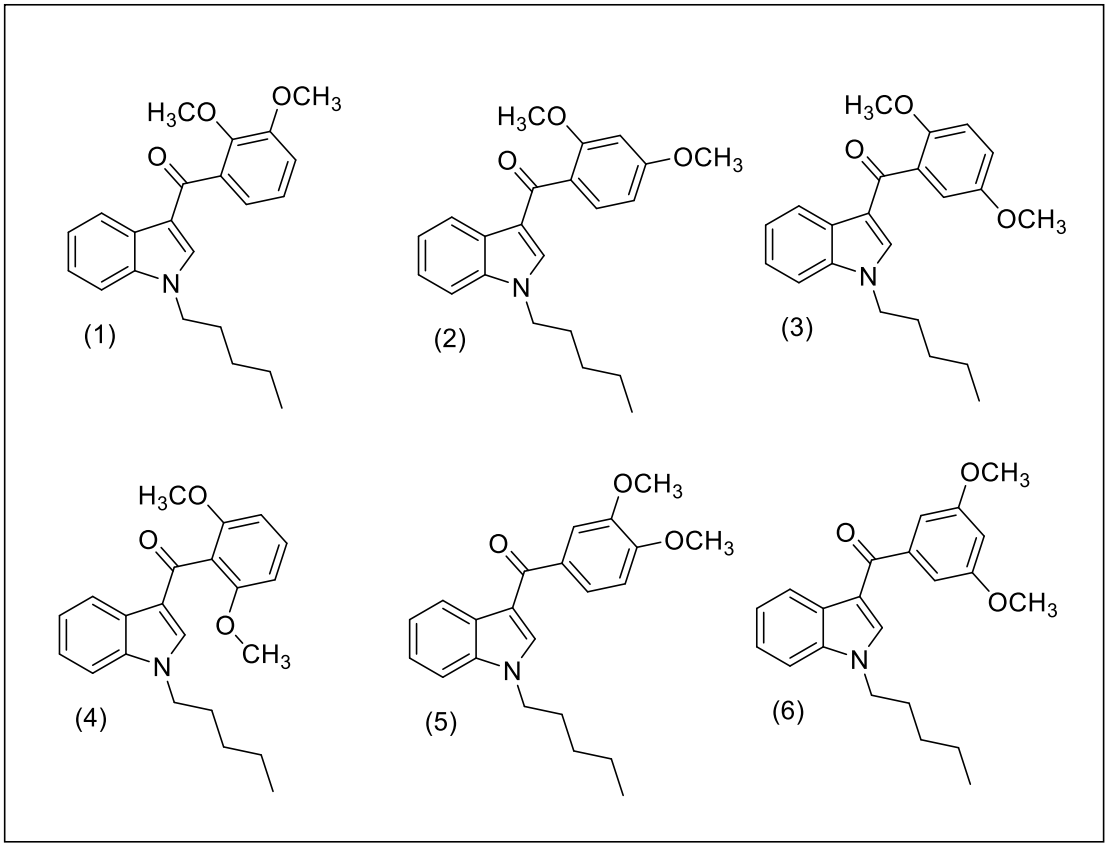


Figure 14: Structures of the six regioisomeric 3-(dimethoxybenzoyl)-1-n-pentylindoles in this study, **1**= (2,3 dimethoxybenzoyl), **2**= (2,4 dimethoxybenzoyl), **3**= (2,5 dimethoxybenzoyl), **4**= (2,6 dimethoxybenzoyl), **5**= (3,4 dimethoxybenzoyl) and **6**= (3,5 dimethoxybenzoyl).

3.2 Differentiation of six 1-n-pentyl-3- (dimethoxybenzoyl)-indole isomers:

3.2.1 Gas Chromatography Separation:

The separation of the 1-n-pentyl-3-(dimethoxybenzoyl)-indole isomers is challenging due to the similarities in their structures. Chromatography is considered the method of choice in forensic drug laboratories for separation and identification of forensic drugs. Gas chromatography (GC) coupled with mass spectrometry (MS) was used in this study to provide a procedure for separation of these six regioisomers. GC separation of the six 1-n-pentyl-3-(dimethoxybenzoyl)-indole isomers using EI-MS detection was carried out using three stationary phases (Rtx®-200, Rxi®-35Sil and Rxi®-17sil).

Column one was a 30m x 0.25mm-i.d coated with 0.5 μm midpolarity phase consisting of 50% phenyl and 50% dimethyl polysiloxane (Rxi®-17sil). The separation was performed using a temperature program consisting of an initial temperature hold at 80 °C for 1.0 min, ramped up to 300 °C at a rate of 30 °C/min, held at 300 °C for 0.5 min then ramped to 340 °C at a rate of 5.0 °C/min and held at 340 °C for 5.0 min with a total runtime of 21 min. (TP1). Although the peaks shapes showed slight tailing on this column, the individual components of the mixture were well separated from each other. This tailing did not significantly influence the quality of separation as shown in Figure 15 panel A.

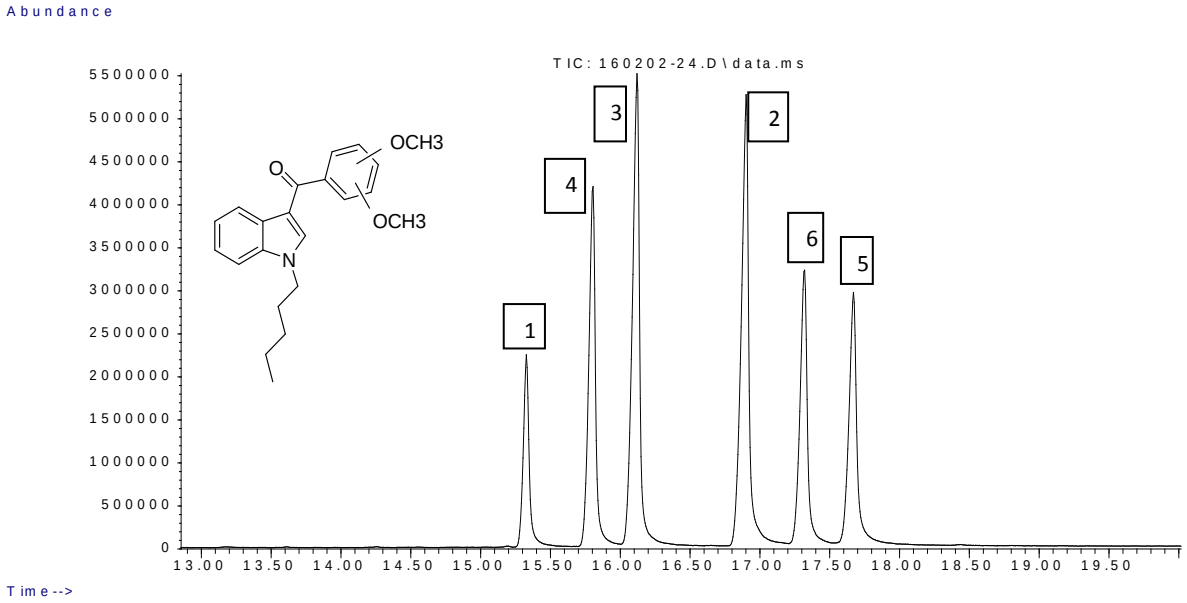
Column two was a 30m x 0.25mm-i.d, capillary coated with 0.5 μm film of midpolarity phase consisting of 35% phenyl and 65% dimethyl polysiloxane (Rxi®-35Sil). The temperature program for this column consisted of an initial temperature at 80 °C for 1.0 min, ramped up to 300 °C at a rate of 30.0 °C/min, held at 300 °C for 0.5 min then ramped to 340 °C at a rate of 2.5 °C/min and held at 340 °C for 5.0 min with a total runtime of 29.0 min. (TP3). The six regioisomers are completely resolved with symmetric peaks and full baseline resolution by this stationary phase (Rxi®-35sil) as shown in Figure 15 panel B.

Column three was a 30m x 0.25mm-i.d, capillary coating with 0.5 μm film of 100% trifluoropropyl methyl polysiloxane (Rtx®-200). The separation was performed using a temperature program consisted of an initial temperature at 80 °C for 1.0 min, ramped up to 300 °C at a rate of 2.0 °C/min, held at 300 °C for 30.0 min with a total runtime of 141.0 min. (TP4). The complete mixture separation on this column required an extremely long runtime (over 2 hours). It takes more than two hours to get a complete separation with slow upward baseline shift as show in Figure 15 panel C.

The six isomers were chromatographed individually by temperature program named TP1 first to record their retention times. All these stationary phases showed the same elution order with differences among them in their relative ersolution. The elution order appears to be related to the degree of substituent crowding in the dimethoxybenzoyl portion of the molecules. Table 6 shows the observed retention times of all six isomers on the three columns.

The most sterically crowded substitution pattern in the 2,3- and 2,6-dimethoxybenzoyl isomers (compounds **1** and **4**) are the least retained and elute first on all the capillary columns used in this study. Compound **1** elutes first then compound **4** and these two early eluting compounds have in common all three groups substituted in a 1,2,3 manner on the benzene ring. Compound **3** then compound **2** are the next eluted compounds, and each of these compounds has one methoxy group located ortho to the carbonyl group and one methoxy group as an isolated substituent, more distant from the carbonyl group. The compounds showing the highest retention and eluting last (compounds **6** and **5**) do not have a methoxy group adjacent to the carbonyl. The GC separation of six isomers compounds is shown in Figure 15, the compound eluted first is 2,3-Dimethoxybenzoyl-1-pentylindole, second is 2,6-Dimethoxybenzoyl-1-pentylindole, third is 2,5-Dimethoxybenzoyl-1-pentylindole, fourth is 2,4-Dimethoxybenzoyl-1-pentylindole, fifth is 3,5-Dimethoxybenzoyl-1-pentylindole and last is 3,4-Dimethoxybenzoyl-1-pentylindole.

A)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3500000
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2500000
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1500000
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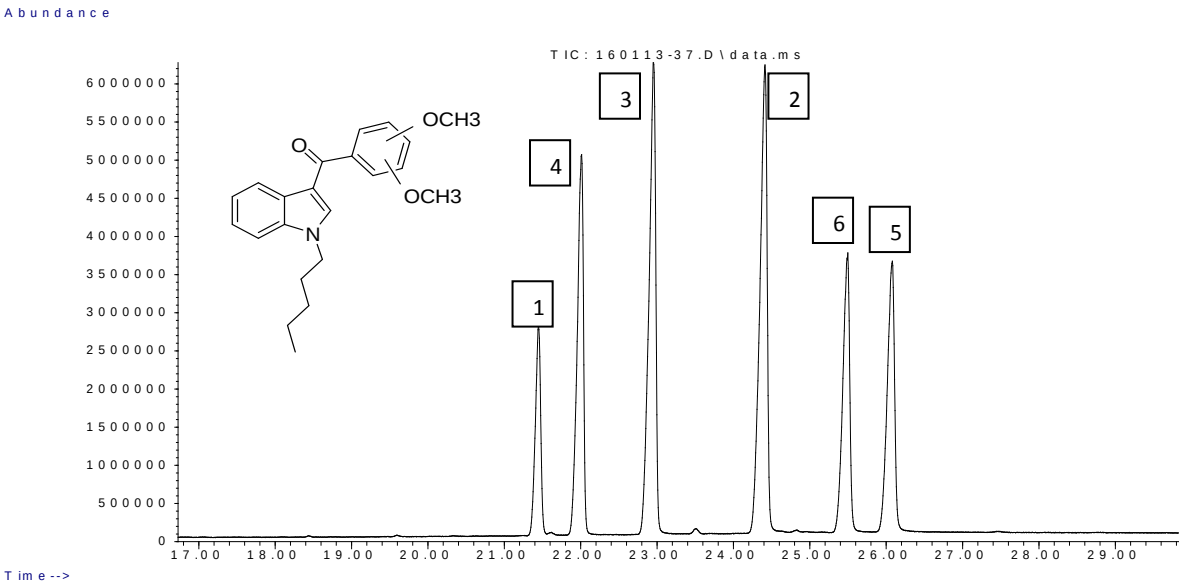
500000
0
13.00 13.50 14.00 14.50 15.00 15.50 16.00 16.50 17.00 17.50 18.00 18.50 19.00 19.50
Time-->
OCH3
OCH3
O
N
1
2
3
4
5
6

B)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O
N
1
2
3
4
5
6

C)

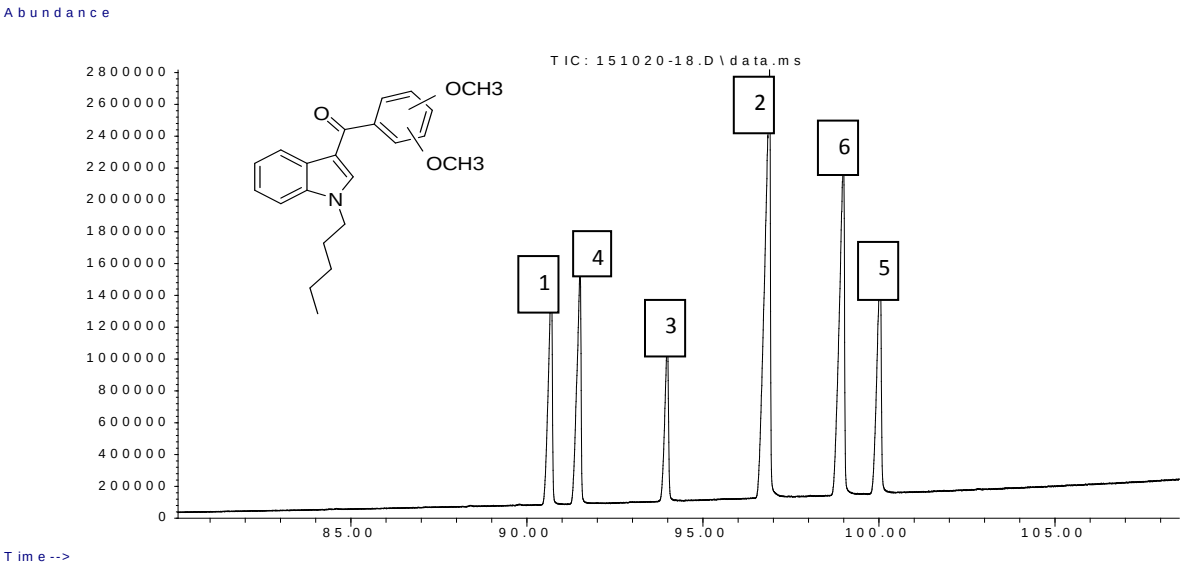


Figure 15: The GC-MS separation of six isomers using, Rxi®-17sil, Rxi®-35sil and Rtx®-200 in panel (A, B and C) respectively. The number over the peak corresponds to the compound number according to the elution order. Peak 1= (2,3-dimethoxybenzoyl), peak 2= (2,6-dimethoxybenzoyl), peak 3= (2,5-dimethoxybenzoyl), peak 4= (2,4-dimethoxybenzoyl), peak 5= (3,5-dimethoxybenzoyl) and peak 6= (3,4-dimethoxybenzoyl).

Table 6: Retention time (min.) of six dimethoxybenzoyl isomers (1-6).

Column Used	Temperature programs*	Compound Number **1**	**4**	**3**	**2**	**6**	**5**	
Rxi®-17sil	TP1	15.445	15.3974	16.292	17.016	17.427	17.75	Rt (min)
Rxi®-35sil	TP3	21.447	22.013	22.951	24.414	25.498	26.081	
Rtx®-200	TP4	90.686	91.507	93.990	96.887	98.991	100.029	

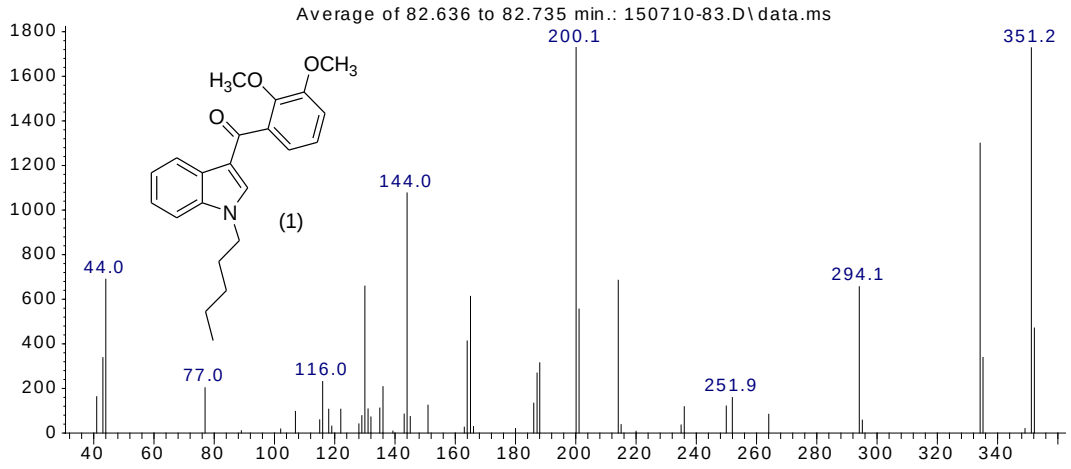
*details of the programs are in the experimental section

3.2.2 Mass Spectral Studies:

3.2.2.1 Electron impact ionization-mass spectrometry (EI-MS):

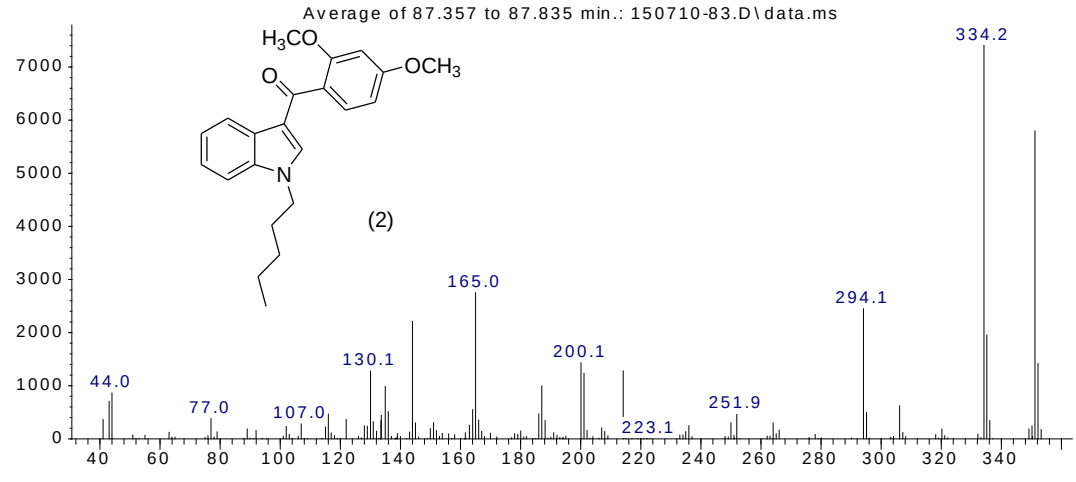
Mass spectrometry is the primary method for confirming the identity of drugs in forensic science and can provide a specific "fingerprint" for an individual forensic compound. During the course of this research project on these six regioisomeric dimethoxybenzoylindoles, the mass spectra provides excellent structural information that can be used to differentiate these compounds from each other. The EI mass spectra of the six regioisomeric 1-n-pentyl-3-(dimethoxybenzoyl)-indoles using GC-MS and the Rxi®-35sil stationary phase are shown in Figure 16. The structures for the major fragments ions in the spectra for these six regioisomeric compounds are shown in Figure 17

Abundance

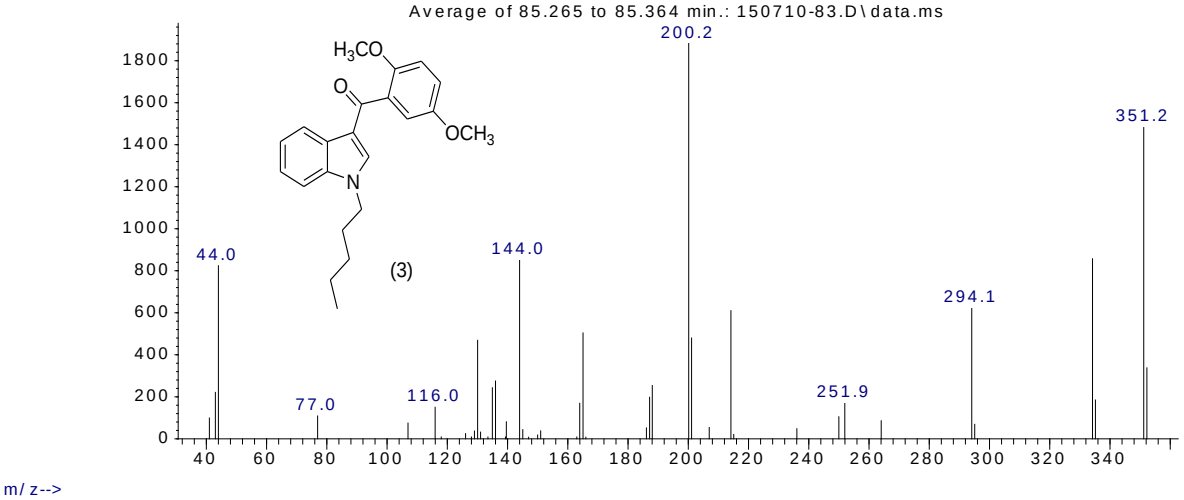


m/z-->

Abundance



m/z-->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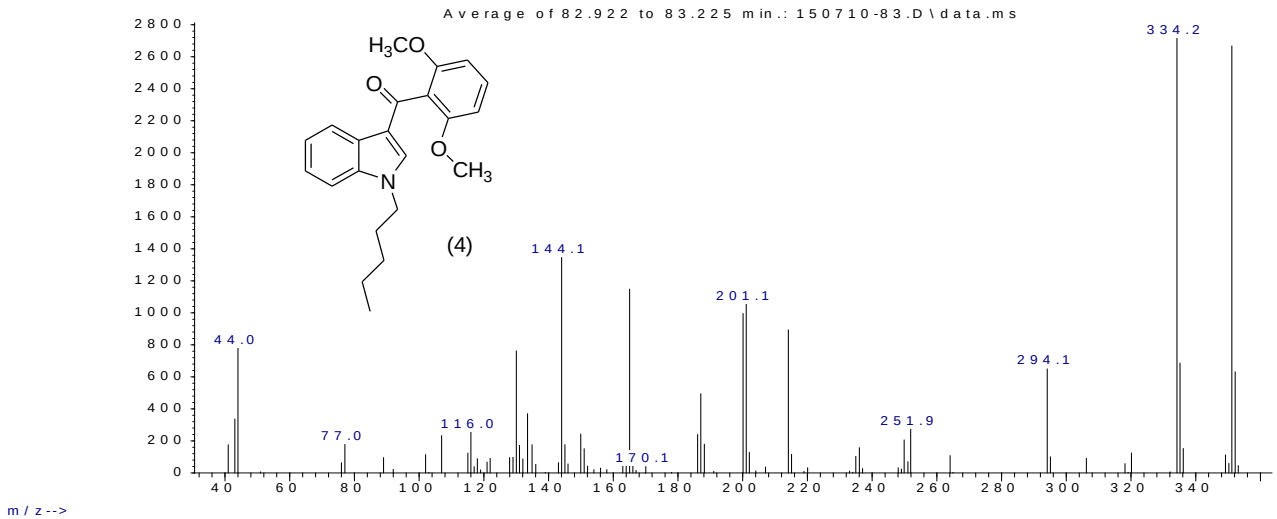
(3)
m/z-->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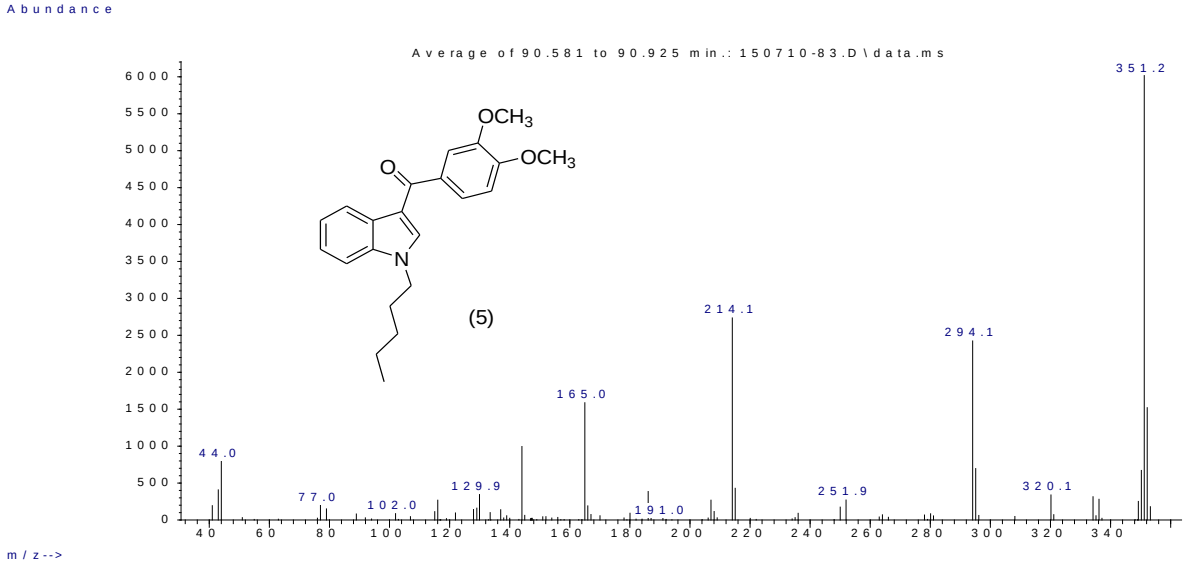
m/z-->



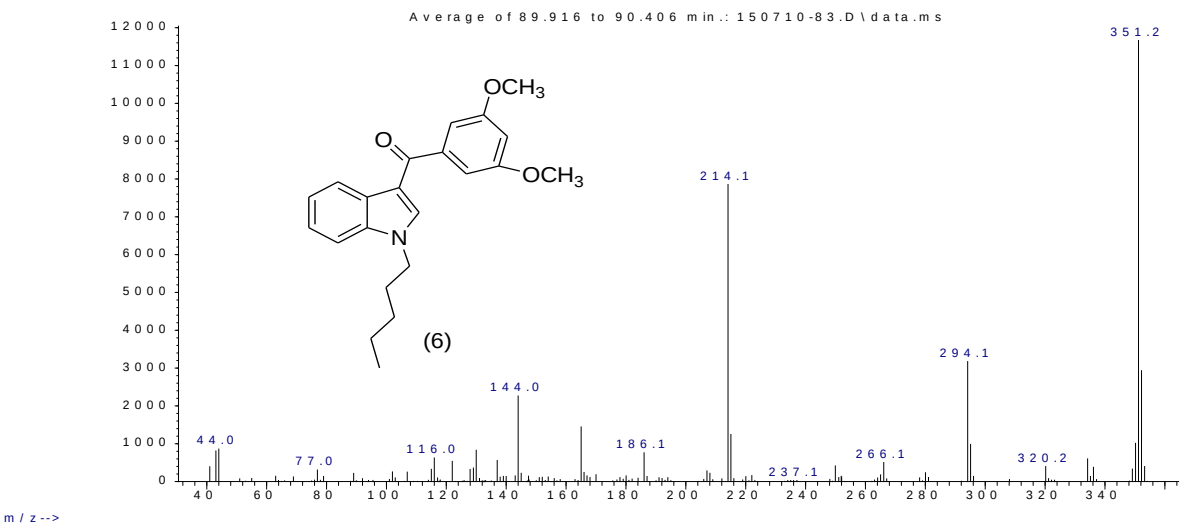


Figure 16: Mass spectra for the individual 3-(dimethoxybenzoyl)-1-n-pentylindoles regioisomers.

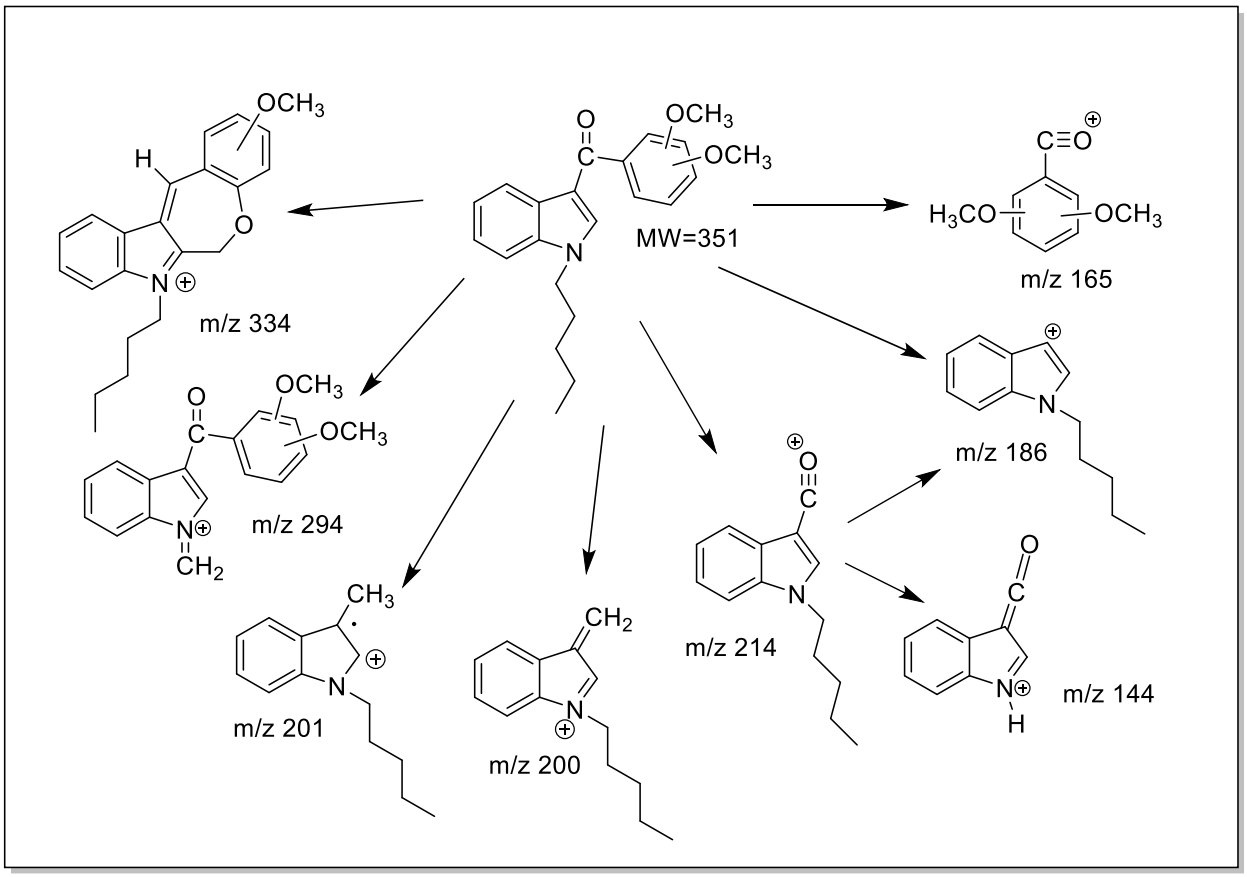


Figure 17: General fragmentation scheme for the 3-(dimethoxybenzoyl)-1-n-pentylindoles.

There are a number of unique features in the mass spectra of these compounds that can be used to differentiate these regioisomers from each other. These unique features appear to be based on the relative position of the two regioisomeric methoxy groups in the benzoyl ring. The four isomers having at least one methoxy group at the 2-position (ortho to the carbonyl group) show unique fragments of significant relative abundance at *m/z* 334 $[M-17]^+$ and at *m/z* 200 $[M-151]^+$. The two isomers having the second methoxy group in direct conjugation with the ortho-methoxy substituent, compounds **1** and **3**, show the *m/z* 200 fragment as the base peak. The other two isomers having the second methoxy group not in direct conjugation with the ortho-methoxy group (compounds **2** and **4**) yield the *m/z* 334 ion as the base peak. The remaining two isomers (compound **5** and **6**) that do not have a methoxy group ortho to the carbonyl group show the molecular ion as the base peak. Thus, we can subdivide these compounds into three groups based on their base peaks in the EI- mass spectra, each group contining two compounds:

1. The *m/z* 200 ion is the base peak for the 2,3- and 2,5-dimethoxybenzoyl isomers (compounds **1** and **3**).
2. The *m/z* 334 ion is the base peak for the 2,4- and 2,6-dimethoxybenzoyl isomers (compounds **2** and **4**).
3. The *m/z* 351 ion is the base peak for the 3,4- and 3,5-dimethoxybenzoyl isomers (compounds **5** and **6**).

The first group includes compounds **1** and **3** having a fragment ion at *m/z* 200 as the base peak. As we have mentioned above, the fragment at *m/z* 200 represents the loss of 151 Da from the molecular ion. This ion is only significant in the four ortho-methoxy containing regioisomers and is completely absent for both compounds that do not contain an ortho methoxy group (compounds **5** and **6**). We expected that methyl group migration from the ortho methoxy substituent to the indole ring with elimination of a hydroxyl-methoxy benzoyl group is the only obvious process for formation of *m/z* 200. However, the *m/z* 200 ion is accompanied by a significant *m/z* 201 radical cation in the EI spectra for these compounds. This ion likely arises from elimination of the substituted benzoyl group without hydrogen migration. Both deuterium (D) labeling and exact mass analysis of the *m/z* 200 ion was used to confirm the elemental composition of this fragment and will be discussed later in section 3.2.2 and 3.2.3, respectively.

The second group has a base peak at *m/z* 334 $[M-17]^+$, this group includes compound **2** and **4**. As shown in the mass spectra in Figure 16, the fragment ion at *m/z* 334 is a the major high mass fragment in these four compounds having at least one methoxy group ortho to the carbonyl on the benzene ring (compounds **1**, **2**, **3**, and **4**). However, previous studies on indole derived cannabinoids, that have structures similar to these compounds, have shown that the $[M-17]^+$ ion result from the loss of a hydroxyl radical ($^{\bullet}OH$) from the molecular radical ion $M^{+\bullet}$. As a further proof to this mechanism, deuterium labels of 2-D3-methoxy-5-methoxybenzoyl)-indole was carried out during this research and is described in section 3.2.2.

There is a common set of significant major and minor fragment ions observed in all the spectra of these six regioisomeic compounds with varying levels of relative intensity. The major fragment ions occur at *m/z* 294, *m/z* 214, *m/z* 186, *m/z* 165, *m/z* 144 and *m/z* 77, and the minor fragment ion occurs at *m/z* 320.The minor fragment at *m/z* 320 observed in many of the spectra is likely the result of the loss of a methoxy group from the molecular ion in this series of compounds. The major high mass fragment occurs at *m/z* 294 by the loss of a butyl radical [C4H9•] from the molecule by alpha-cleavage initiated at the indole nitrogen radical site. This mechanism was confirmed by one earlier study using deuterium isotope labeling of the pentyl side chain of JWH-018 [2]. Another major fragment ion occurs at *m/z* 165, representing a substituted benzoyl group ion and provides structural information about the nature of the acyl group substituent but not the position of the dimethoxy ring substituents.

The fragment ions at *m/z* 214, 186 and 144 have been observed in a number of 1-pentyl-3-acylindoles. These fragment ions do not contain the substituted dimethoxybenzoyl ring portion of these compounds. The ions at *m/z* 214 and *m/z* 186 represent cleavage of either of the bonds to the carbonyl carbon with the charge remaining on the indole portion of the molecule. The *m/z* 214 ion represents the loss of the substituted dimethoxybenzene radical, and the *m/z* 186 represents the loss of the substituted dimethoxybenzoyl radical. The *m/z* 144 ion is the product of a hydrogen rearrangement from the *m/z* 214 cation with the elimination of the entire nitrogen substituted alkyl side chain as the alkene [C_5H_{10}]. The proposed structures and suggested mechanism of these fragment ions are previously confirmed by using deuterium isotope labeling studies of the deuterated naphthalene ring of JWH-018 [2].

The ion trap mass spectrometry was used for the *m/z* 214 fragment in the 3,5-dimethoxybenzoyl isomer (compound **6**) to confirm that this fragment ion is a decomposition product of *m/z* 214 ion and did not occur directly from the molecular ion. The MS/MS spectrum of the *m/z* 214 fragment gave an *m/z* 144 ion as the major peak and was the only ion of significance in this MS/MS experiment as shown in Figure 18.

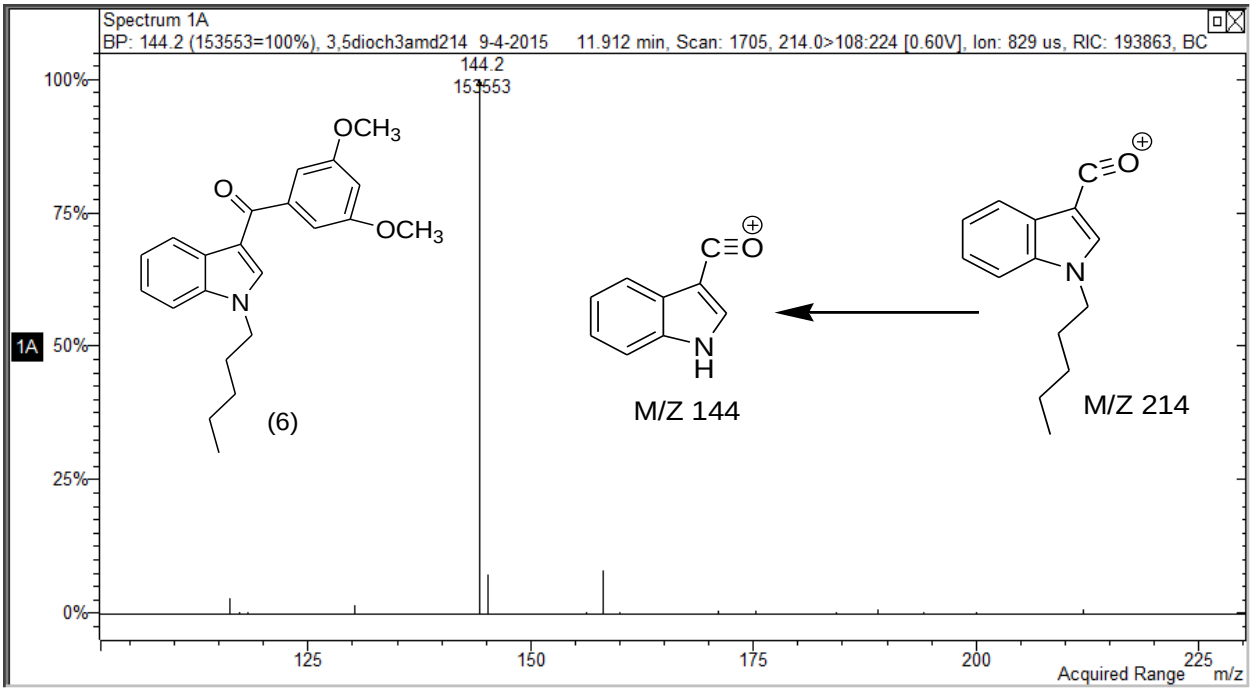


Figure 18: MS/MS spectrum of the *m/z* 214 fragment for 3-(3,5 dimethoxybenzoyl)-1-pentylindole (operated in MS/MS mode).

3.2.2.2 Deuterium labeling studies to confirm the fragment ions at 200 and *m/z* 334:

Deuterium labeled samples were used in this study to confirm the mass spectrometry fragmentation mechanisms and the elemental composition of fragment ions at *m/z* 200 and *m/z* 334 in compounds **1**, **2**, **3** and **4**. The proposed structure for the *m/z* 200 $[C_{14}H_{18}N]^+$ and 334 $[C_{21}H_{24}NO_2]^+$ ions are shown in the fragmentation pattern in Figure 17. These ions of interest have been supported by the mass spectrum of trideutero labeled form of the 1-pentyl-3-(2-D3-methoxy-5-methoxybenzoyl)-indole.

The suggested mechanism for fragment ion at *m/z* 200 is illustrated in Figure 20. It starts with a migration of the methyl group from the ortho methoxy group of the substituted benzoyl moiety to the 3-position of the indole ring followed by hydrogen radical transfer to form the eliminated hydroxy-methoxybenzoyl radical (151 Da).This mechanism can also be confirmed by the mass spectrum of the 1-pentyl-3-(2-D3-methoxy-5-methoxybenzoyl)-indole as shown in Figure 19 as the corresponding fragment is shifted 2 Da higher to become *m/z* 202 and represents the loss of 152 Da from the molecular ion of this labeled compound. Thus, the *m/z* 202 ion confirms that only two deuterium atoms from the 2-D3 methoxy group remain in the fragment while the molecular ion increased by 3 Da as expected.

In addition, the EI mass spectrum of 1-pentyl-3-(2-D3-methoxy-5-methoxybenzoyl)-indole further provided confirmation that the loss of a hydroxyl radical to yield *m/z* 334 is from the molecular radical ion $M^{+\bullet}$. The fragment of interest in Figure 19 occurs at *m/z* 336 and represents $[M-18]^+$ for this D3-analogue with a molecular ion at *m/z* 354. Thus, the elimination of 18 Da in this deuterium labeled species identifies the ortho methoxy group as the source of the eliminated hydrogen in these compounds. The proposed mechanism for the formation of this $[M-17]^+$ species is shown in Figure 21.

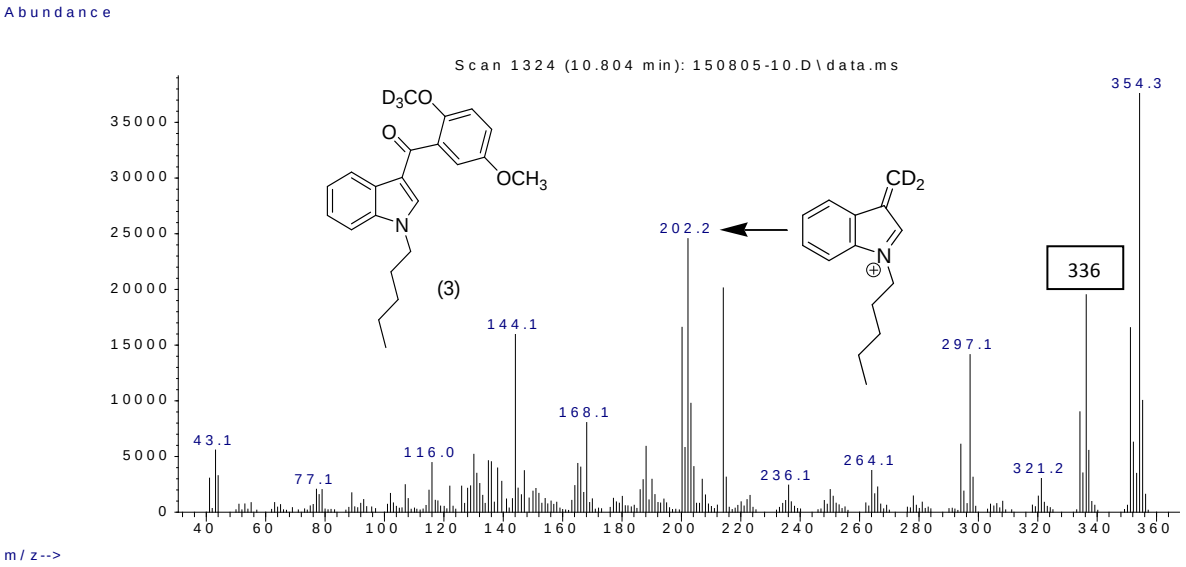


Figure 19: EI-MS of 1-pentyl-3-(2-D3-methoxy-5-methoxybenzoyl)-indole.

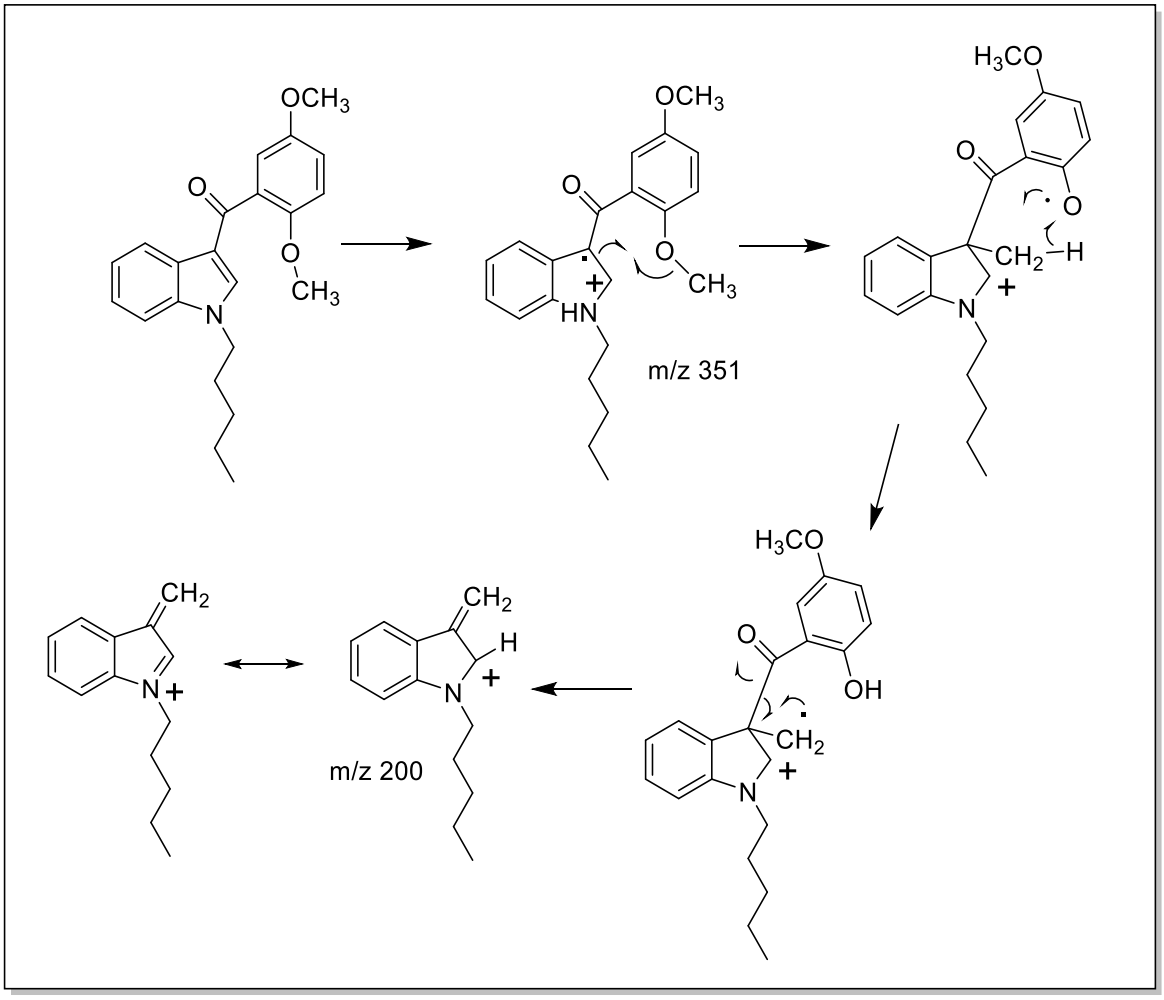


Figure 20: Mechanism for the formation of the *m/z* 200 ion for 2,5-dimethoxybenzoylindole.

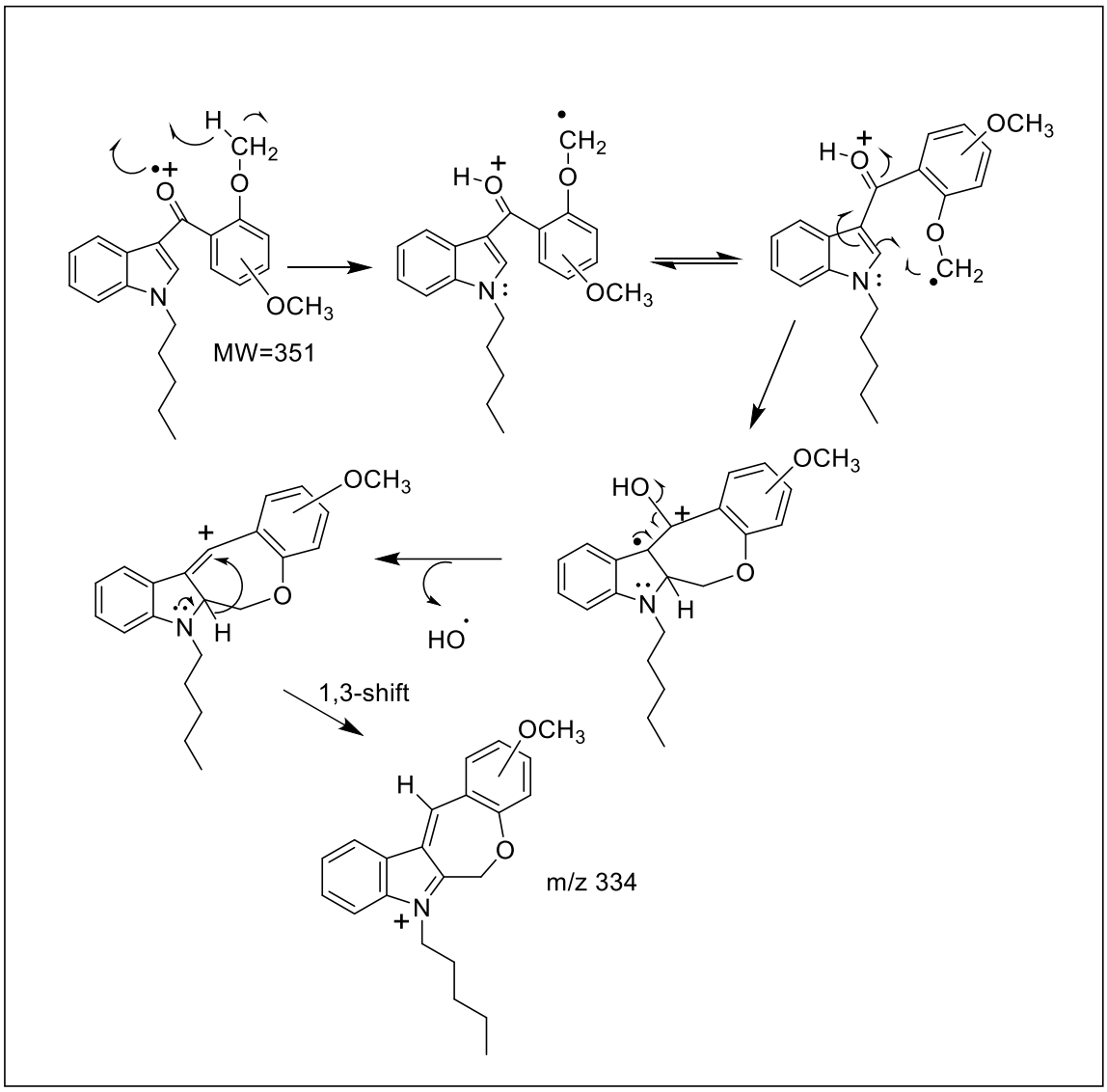
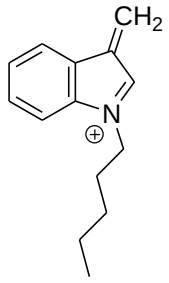


Figure 21: Mechanism for the formation of the *m/z* 334 ion for 2,5-dimethoxybenzoylindole.

3.2.2.3 GC-TOF analysis on 2,3-dimethoxybenzoyl-1-pentylindoles *m/z* 200 fragment:

GC-TOF is an analytical method that can provide high resolution exact mass molecular formula information for fragments in the mass spectrum. As further proof of this ion mechanism and structure, it is an important approach for confirming the elemental composition of *m/z* 200. As mentioned above the ion at *m/z* 200 is only significant in the four ortho-methoxy containing regioisomers (compounds **1**, **2**, **3** and **4**) and the base peak for (compounds **1** and **3**). TOF data on *m/z* 200 fragment of compound **3** is shown in Table 7.

Table 7: GC-TOF data on *m/z* 200 fragment of 3-(2,5 dimethoixybenzoyl)-1-pentylindole

Measured Mass	200.1431
Calculated Mass	200.1439
Deviation (in PPM)	- 4.00
Fragment Formula	$C_{14}H_{18}N$ (DBE=6.5)
Best Fit Ion Structure	

Exact mass analysis using GC-TOF-MS confirmed the *m/z* 200 ion has the elemental composition $C_{14}H_{18}N$ with the mass deviation of - 4.00 ppm (-0.8 mDa). The experimental mass deviation (- 4.00 ppm) is within the acceptable error limits ±5 ppm for high resolution mass spectral data. This fragment ions represents the loss of 151 Da [$C_8H_7O_3$] from the molecular ion and it has a suggested formula of $C_{14}H_{18}N$. Thus, the methyl group migration from the ortho-methoxy substituent to the indole ring with elimination of a hydroxyl-methoxy benzoyl group is a possible process for this fragment. The proposed structure and mechanism for the formation of the *m/z* 200 $C_{14}H_{18}N$ ion is shown in Figure 20. However, the exact mass measurement of *m/z* 200 does not differentiate between the four ortho-methoxy containing regioisomeric compounds.

3.2.2.4 Chemical ionization (CI)-mass spectrometry

The chemical ionization (CI) is a soft ionization technique in GC-MS. The main benefit of this technique is to provide a simpler mass spectrum that easily identifies and confirms the structure of these compounds. Methanol was used as the reagent gas in this study. The ion trap (GC-CI-MS) utilizes chemical ionization to identify the molecular ion of these regioisomers. The reagent gas will interact with ionizing electrons and produce reagent ions and the ionized methanol collides directly with the dimethoxybenzoylindole molecule where the proton transfer generates the protonated molecular fragment ion $[M+H]^+$ at *m/z* 352. The CI-MS spectra of all six compounds were the same. The CI-MS spectrum of 2,3-dimethoxybenzoyl-1-pentylindoles, as an example of chemical ionization of these compounds, is shown in Figure 22. This technique does not provide any additional data for discrimination among these six regioisomeric compounds which have the same elemental composition.

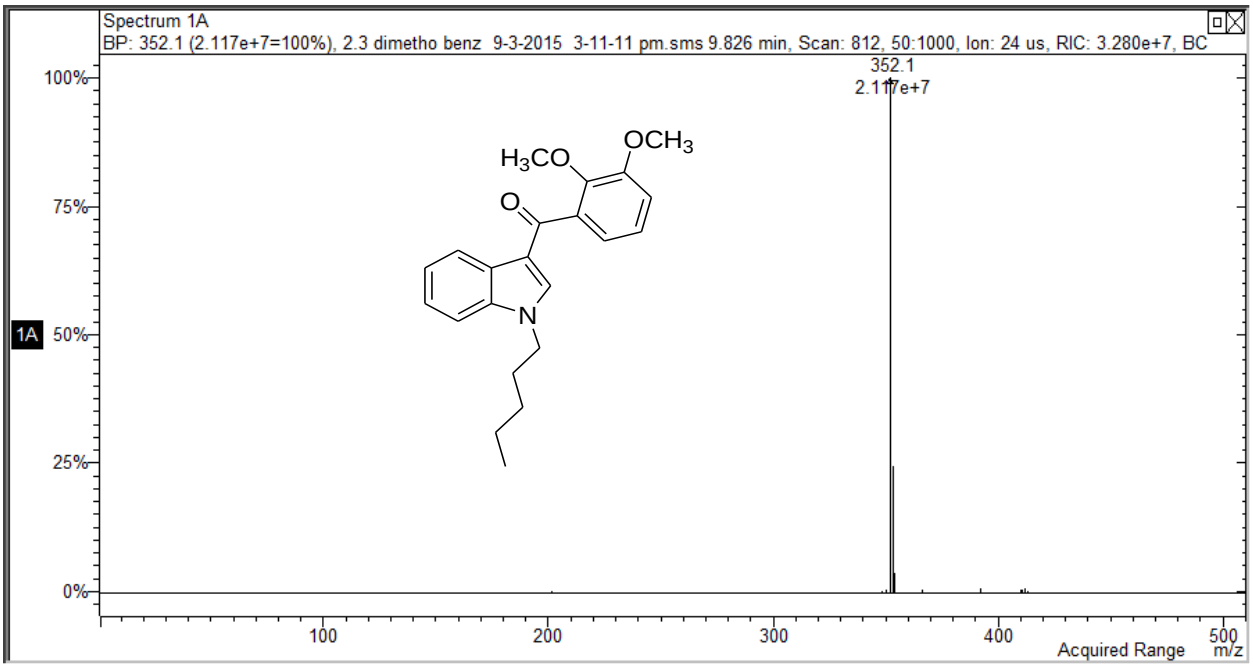


Figure 22: CI –MS spectra of 3-(2,3-dimethoxybenzoyl)-1-n-pentylindoles.

3.2.3 LC-TOF studies for exact mass determination:

LC-TOF provides accurate mass information (four decimal places) for these compounds to enable calculation of molecular formula. The accurate-mass information is essential for the structural elucidation which provides excellent information for identifying unknown compounds and for confirming the presence of a specific compound. The accurate-mass measurements of these compounds was achieved using an Agilent 6520 with TOF mass analyzer, and the protonated molecular ion information $[M+H]^+$ from ESI. This instrument allows the determination of the *m/z* of ionic formula based on the mass sufficiency of the constituent atoms within the acceptable error limits ±5 ppm and the isotope abundance scores were above 95% for all ion compounds. The LC-TOF accurate mass results are shown in Table 8. These results are

compatible with molecular formula $C_{22}H_{25}NO_3$ and supported the results of LC–MS/MS and GC–MS results. The four regioisomeric compounds have the same molecular formula of $C_{22}H_{25}NO_3$, nominal mass of 351, doubled bond equivalent of 11, and the calculated measured mass of 351.1834. In conclusion, LC-TOF-MS confirms the elemental composition of these four regioisomers within acceptable experimental error.

Table 8: Results of the accurate mass measurement by LC–TOF–MS of six dimethoxybenzoyl isomers.

Compound No.	Generated formula	Absolute difference (PPM)	Abundance score	Exact mass	Accurate mass
1	$C_{22}H_{25}NO_3$	-0.2848	77.58	351.1834	351.1833
2	$C_{22}H_{25}NO_3$	3.1323	86.68	351.1834	351.1845
3	$C_{22}H_{25}NO_3$	-4.2713	81.73	351.1834	351.1819
4	$C_{22}H_{25}NO_3$	-3.4170	83.61	351.1834	351.1822
5	$C_{22}H_{25}NO_3$	4.5560	76.04	351.1834	351.1850
6	$C_{22}H_{25}NO_3$	1.4238	98.02	351.1834	351.1839

3.3 Conclusion:

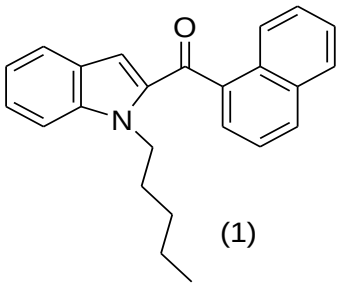
The six regioisomeric 3-(dimethoxy benzoyl)-1-pentyl-indoles involved in this study have the same molecular formula and nominal mass but are different in their chemical structures. The six isomers in this series display fragment ions at equivalent masses with some differences in relative abundance of these ions. The ortho-substituted isomers (compounds **1**- **4**) show unique fragment ions occurring at *m/z* 334 $[M-17]^+$ and *m/z* 200 $[M-151]^+$ which are completely absent in compounds **5** and **6**. The fragment ion at *m/z* 334 $[M-17]^+$ is considered as the base peak in compounds **2** and **4** while fragment ion at *m/z* 200 is the base peak in compound **1** and **3**. Stable isotope deuterium labeling studies of the ortho-methoxy group allow confirmation of the structures of these two fragments. In addition, the exact mass determination analysis of the *m/z* 200 ion allow confirmation of the elemental composition of *m/z* 200 ion. LC-TOF-MS and CI-GC-MS provide an additional confirmation of the molecular formula determination. These six regioisomers were well resolved on three different GC stationary phases involved in this study, Rxi®-35Sil, Rtx®-200 and Rxi®-17, and under different temperature programming conditions.

Chapter 4

Analytical Studies on six regioisomeric 1-naphthoyl and six 2-naphthoyl substituted-1-n-pentylindoles: Regioisomeric related to the synthetic cannabinoid JWH-018

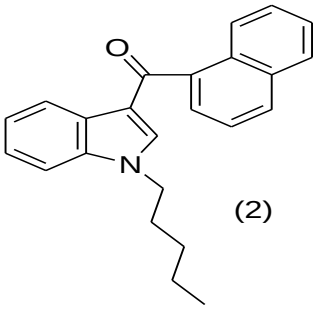
4.1 Introduction:

Many of the synthetic cannabinoid drugs of abuse are 1-alkyl-3-acyl indole derivatives. One of the most commonly encountered drugs in this series is JWH-018, 1-n-pentyl-3-(1-naphthoyl)-indole. As we have already mentioned before, this drug has been listed as Schedule I in the Controlled Substances Act (CSA). In this chapter, we will compare JWH-018 (compound 2) with its eleven possible regioisomeric 1-n-pentyl-naphthoylindoles. These twelve regioisomeric compounds contain both naphthyl and indole rings joined by a carbonyl group in the center of the compound with the pentyl side chain substitution attached directly to the nitrogen atom of the indole ring. The carbonyl group in these compounds attached to the two possible positions in the naphthalene ring: (I) 1-naphthyl also called "alpha-naphthol" (II) 2-naphthyl also called "beta-naphthol" to give compounds having the 1-and 2-naphthoyl-group. These groups are attached at each of the 6 possible ring substituent positions of the indole ring 2-, 3-, 4-, 5-, 6- and 7-naphthoyl-1-n-pentylindole. The structures of these regioisomeric compounds of the 1-naphthoyl group are shown in Figure 23 (compound **1- 6**) while the 2-naphthoyl group compounds are shown in Figure 24 (compounds **7- 12**).



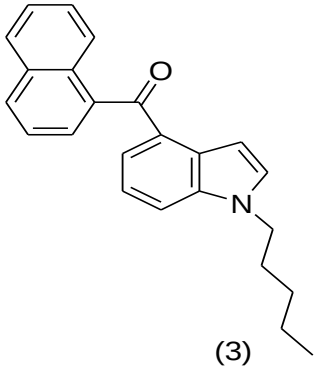
(1)

2-(1-naphthoyl)-1-n-pentylindole



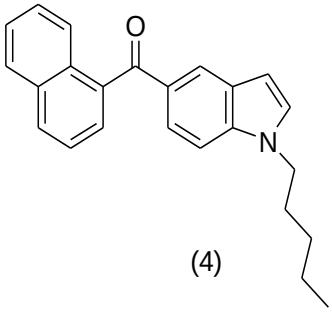
(2)

3-(1-naphthoyl)-1-n-pentylindole (JWH-018)



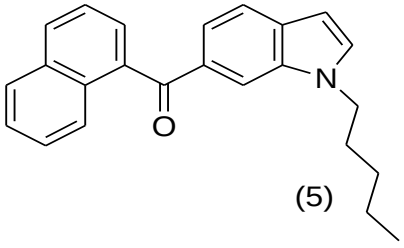
(3)

4-(1-naphthoyl)-1-n-pentylindole



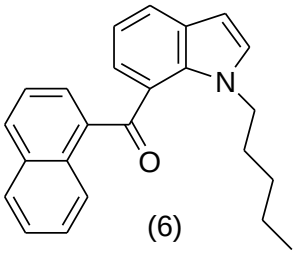
(4)

5-(1-naphthoyl)-1-n-pentylindole



(5)

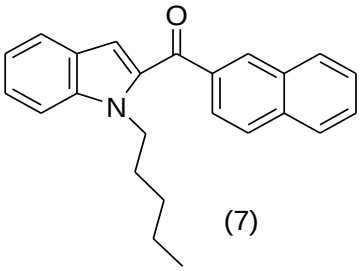
6-(1-naphthoyl)-1-n-pentylindole



(6)

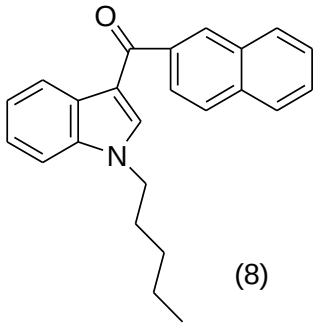
7-(1-naphthoyl)-1-n-pentylindole

Figure 23: Structures of the regioisomeric 2-, 3-, 4-, 5-, 6-, and 7-(1-naphthoyl)-1-pentylindoles.



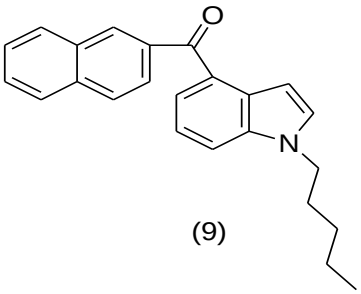
(7)

2-(2-naphthoyl)-1-n-pentylindole



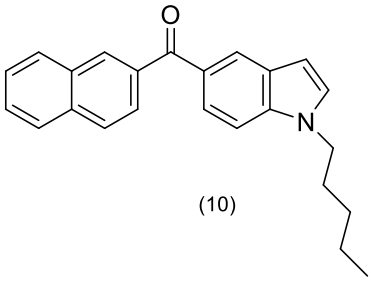
(8)

3-(2-naphthoyl)-1-n-pentylindole



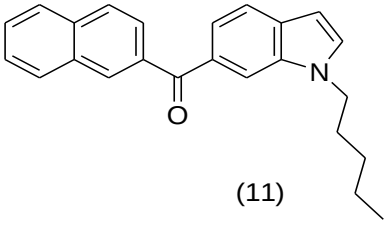
(9)

4-(2-naphthoyl)-1-n-pentylindole



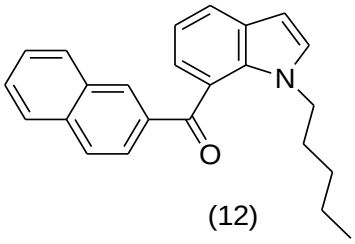
(10)

5-(2-naphthoyl)-1-n-pentylindole



(11)

6-(2-naphthoyl)-1-n-pentylindole



(12)

7-(2-naphthoyl)-1-n-pentylindole

Figure 24: Structures of the regioisomeric 2-, 3-, 4-, 5-, 6-, and 7-(2-naphthoyl)-1-pentylindoles.

4.2 Differentiation of six regioisomeric 1-naphthoyl and six regioisomeric 2-naphthoyl substituted-1-n-pentylindoles: Regioisomeric related to the synthetic cannabinoid JWH-018.

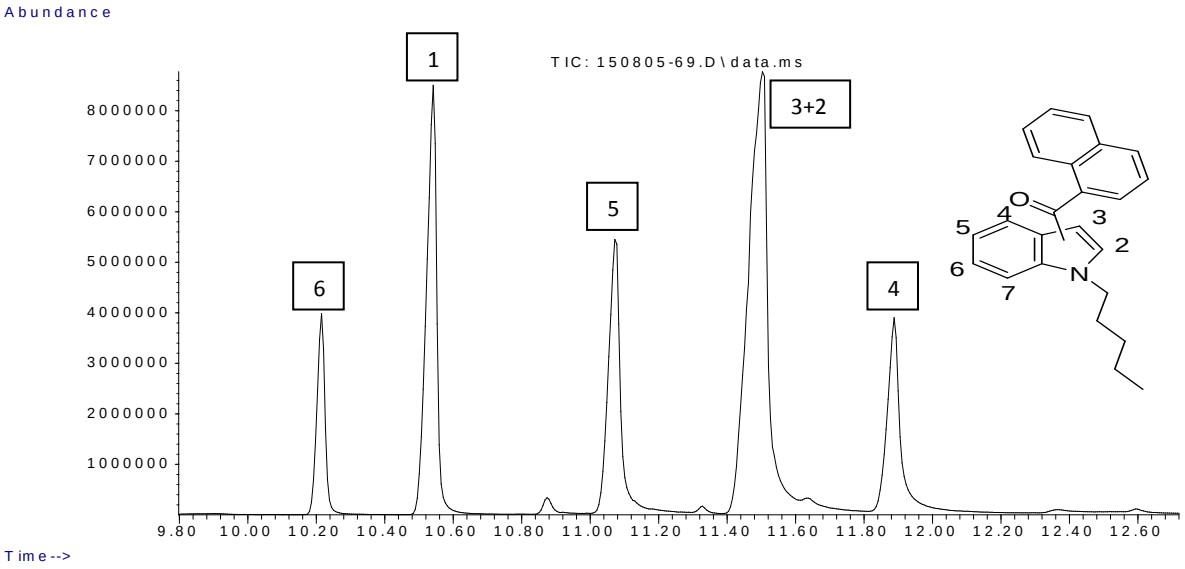
The six regioisomers of 1-naphthoyl substituted 1-n-pentyl-indoles (compounds **1-6**), were reported previously by Clark et al in 2015. This study showed that these compounds could be separated and differentiated by GC-MS [45]. This chapter compares the GC separation, EI / CI-MS spectral, LC-MS/MS spectral studies and exact mass determination of all 6 regioisomeric 1-naphthoyl and all 6 regioisomeric 2-naphthoyl substituted-1-n-pentylindoles compounds (**1-12**).

4.2.1 Gas Chromatography Separation:

GC-MS was used in this study to separate these twelve regioisomers. GC separation of the six regioisomeric 1-naphthoyl mixture and all six regioisomeric 2-naphthoyl substituted-1-n-pentylindoles mixture using EI-MS detection was carried out using four stationary phases (Rtx®-5, Rtx®-200, Rxi®-35Sil and Rxi®-17sil). The separation of JWH-018 and its eleven regioisomeric equivalents has been challenging due to similarities in their structures. The twelve regioisomers were chromatographed individually by temperature program named TP1 first to record their retention times. Table 9 shows the observed retention times of the 1-naphthoyl isomers and Table 10 shows the observed retention times of the 2-naphthoyl isomers.

Both mixtures showed the same elution order on all four stationary phases with differences among them in the retention time and resolution. The elution order appears related to the degree of substituent crowding on the indole ring (pentyl-group and naphthoyl-group). The compounds with the minimum distance between the two groups attached to the indole ring are the least retained and elute first on all the capillary columns used in this study. Starting with 1-naphthoyl mixture separation, compound **6** elutes first then compound **1** and these two early eluting isomers have in common the most sterically crowded substitution patterns on the indole ring (the naphthoyl group at position 7 and position 2, respectively). The pentyl and 1-naphthoyl groups substituted on the same side of the indole ring and the interaction between these two substituent groups is expected in these two isomers which elute first. The other four isomers show minimum intramolecular crowding between pentyl and 1-naphthoyl groups and the retention time obviously increases in these isomers with 6-(1-naphthoyl)-1-n-pentylindole (compound **5**) followed by 4-(1-naphthoyl)-1-n-pentylindole (compound **3**) being the next eluting compounds. The compounds showing the highest retention and eluting last are 3-(1-naphthoyl)-1-n-pentylindole (compound **2**) and 5-(1-naphthoyl)-1-n-pentylindole (compound **4**). The six 2-naphthoyl regioisomers showed the same elution order as that reported for the 1-naphthoyl series.

A)



B)

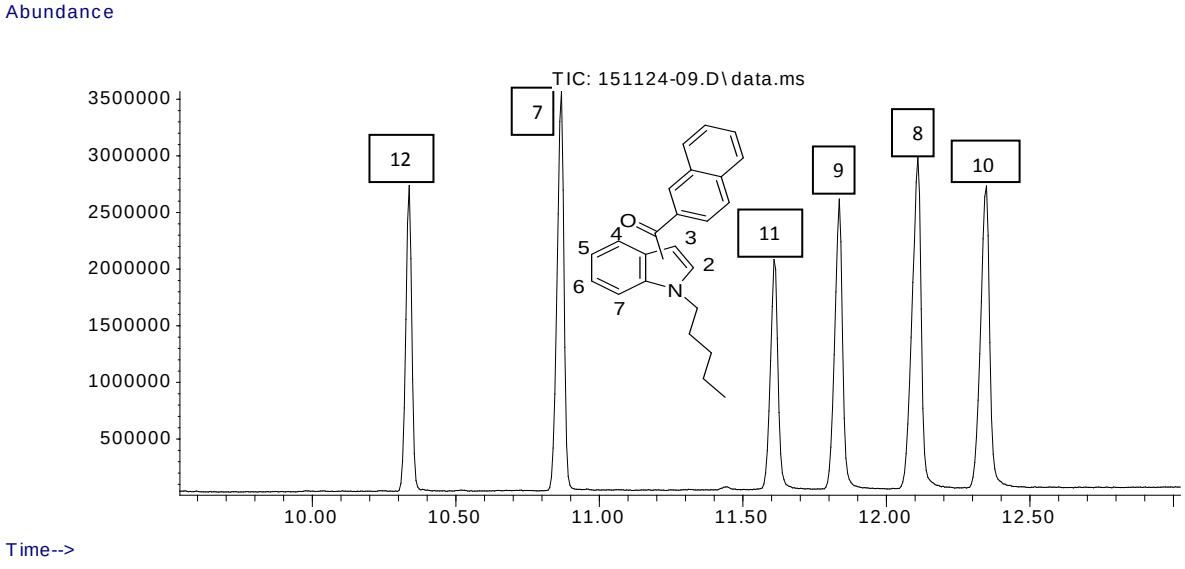
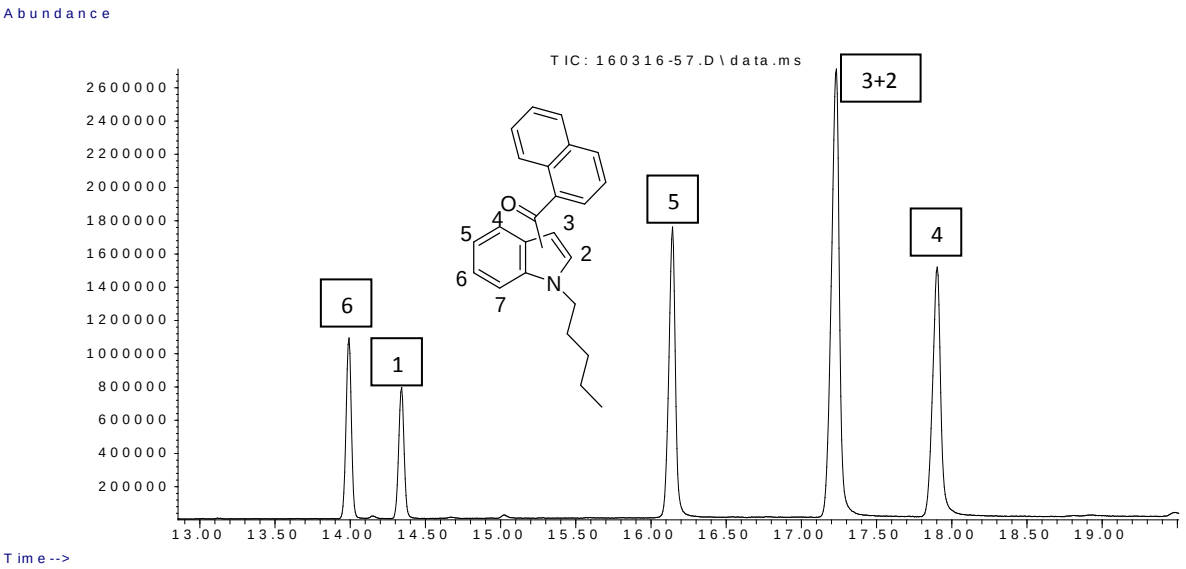


Figure 25: GC separation of 1-n-pentyl-3-(1-naphthoyl) indole mixture (panel A) and separation of 1-n-pentyl-3-(2-naphthoyl) indole mixture (panel B) on Rtx®-5 stationary phase.

A)



B)

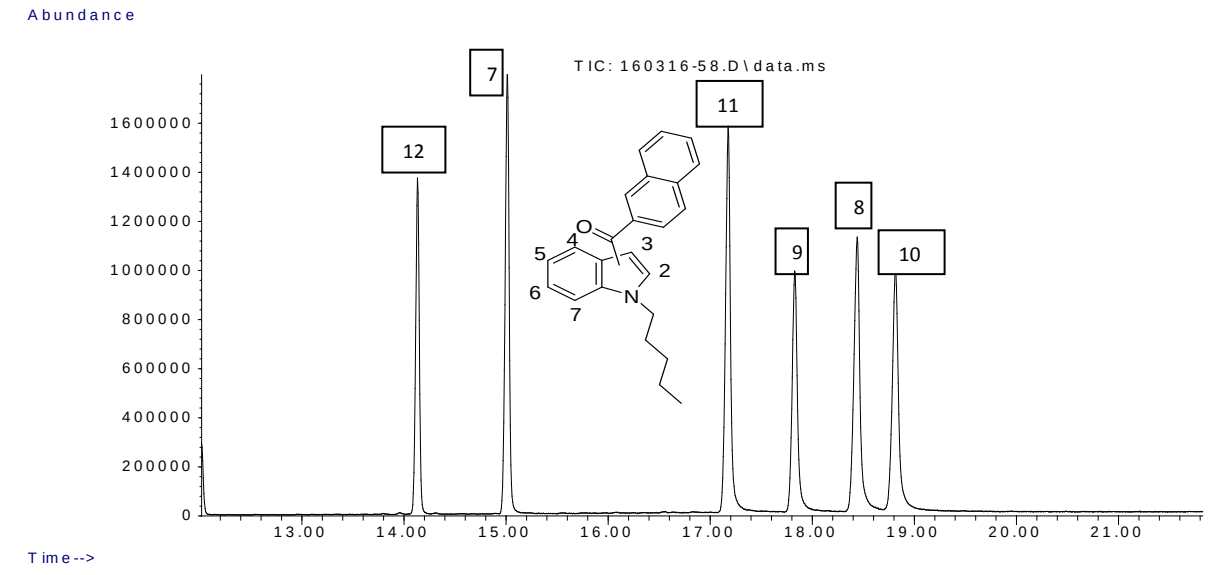
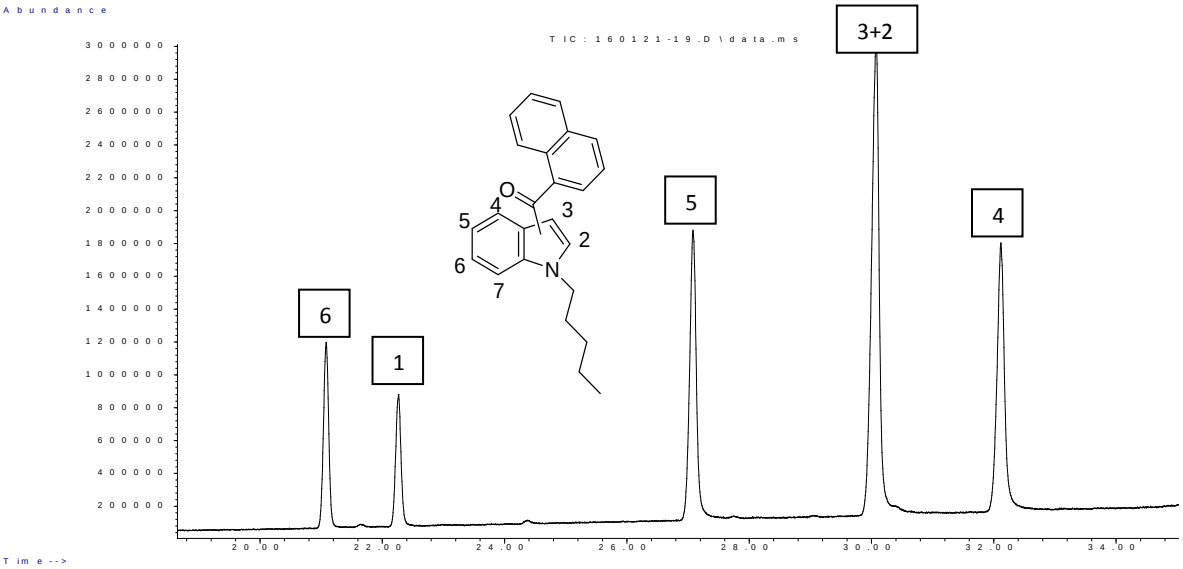


Figure 26: GC separation of 1-n-pentyl-3-(1-naphthoyl) indole mixture (panel A) and separation of 1-n-pentyl-3-(2-naphthoyl) indole mixture (panel B) on Rxi®-17Sil stationary phase.

A)



B)

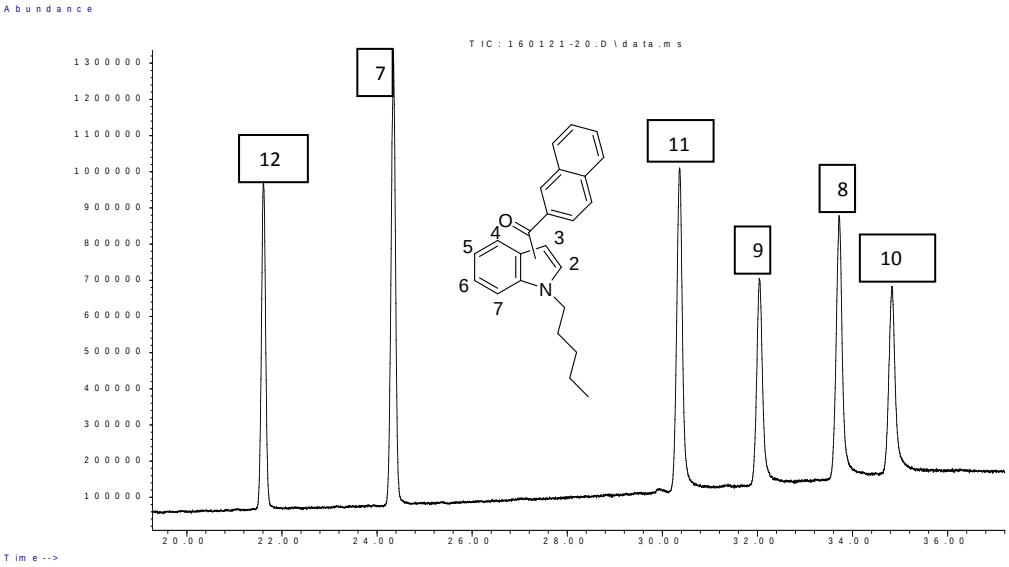
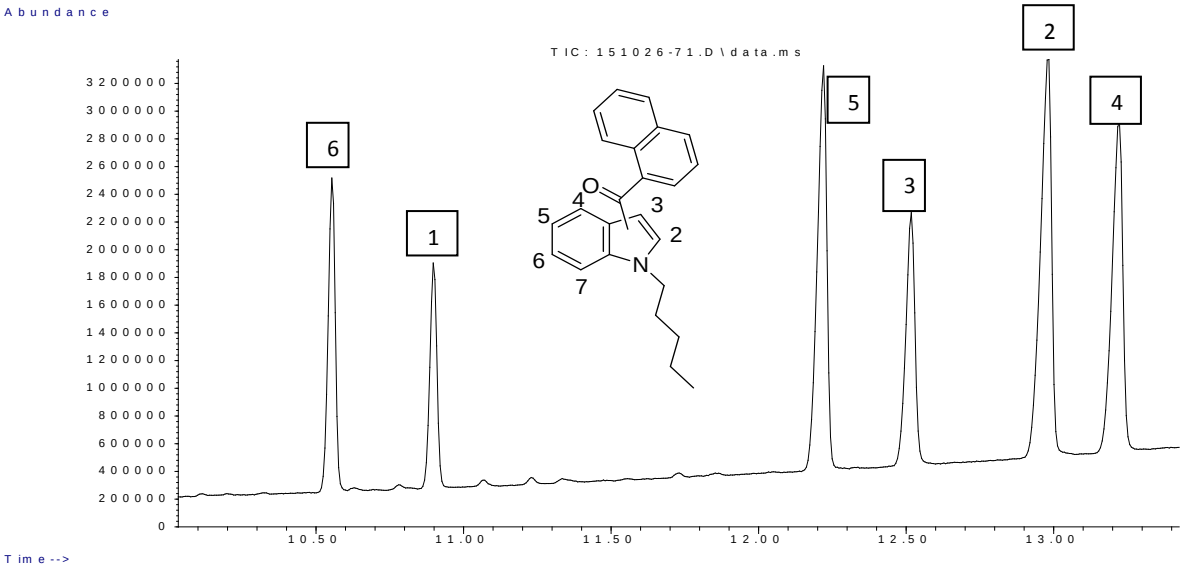


Figure 27: GC separation of 1-n-pentyl-3-(1-naphthoyl) indole mixture (panel A) and separation of 1-n-pentyl-3-(2-naphthoyl) indole mixture (panel B) on Rxi®-35Sil stationary phase.

A)



B)

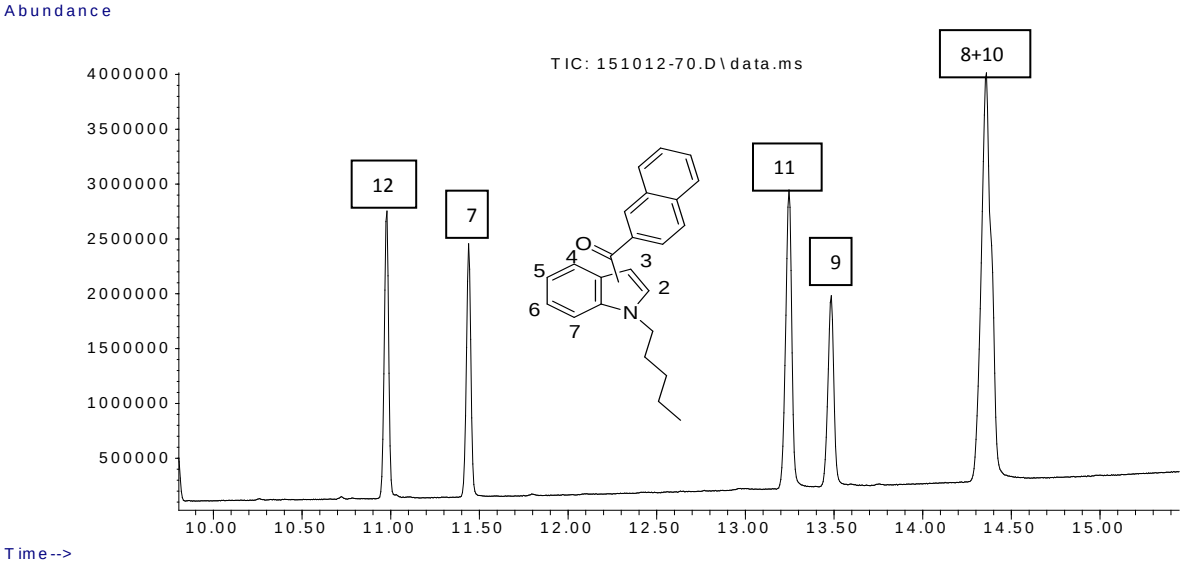


Figure 28: GC separation of 1-n-pentyl-3-(1-naphthoyl) indole mixture (panel A) and separation of 1-n-pentyl-3-(2-naphthoyl) indole mixture (panel B) on Rtx®-200 stationary phase.

Table 9: Retention time (min.) of compounds **1-6**, using temperature program TP1 on Rtx®-5, Rtx®-200 and Rxi®-17sil columns and TP2* on Rxi®-35Sil column.

Column Name	Compounds No. **6**	**1**	**5**	**3**	**2**	**4**	
Rtx®-5	10.215	10.542	11.071	11.503	11.503	11.888	
Rxi®-17Sil	13.986	14.342	16.143	17.233	17.233	17.903	Rt (min.)
Rxi®-35Sil	21.080	22.263	27.083	30.073	30.073	32.125	
Rtx®-200	10.553	10.897	12.220	12.517	12.978	13.223	

*details of the programs are in the experimental section

Table 10: Retention time (min.) of compounds **7-12**, using temperature program TP1 on Rtx®-5, Rtx®-200 and Rxi®-17sil columns and TP2* on Rxi®-35Sil column.

Column Name	Compounds No. **12**	**7**	**11**	**9**	**8**	**10**	
Rtx®-5	10.332	10.862	11.602	11.830	12.337	12.517	
Rxi®-17Sil	14.132	15.013	17.175	17.827	18.439	18.818	Rt (min.)
Rxi®-35Sil	21.610	24.344	30.371	32.055	33.722	34.836	
Rtx®-200	10.979	11.439	13.246	13.485	14.459	14.459	

*details of the programs are in the experimental section.

Column one was a 30m x 0.25mm-i.d coated with 0.5 μm 5% diphenyl and 95% dimethyl polysiloxane (Rtx®-5). Column two was a 30m x 0.25mm-i.d coated with 0.5 μm midpolarity phase consisting of 50% phenyl and 50% dimethyl polysiloxane (Rxi®-17sil). Column three was a 30m x 0.25mm-i.d, capillary coating with 0.5 μm film of 100% trifluoropropyl methyl polysiloxane (Rtx®-200). The separation was performed using the same temperature program on all these stationary phases. The temperature program used for separation consisted of an initial temperature hold at 80 °C for 1.0 min, ramped up to 300 °C at a rate of 30 °C/min, held at 300 °C for 0.5 min then ramped to 340 °C at a rate of 5.0 °C/min and held at 340 °C for 5.0 min with a total run 21 min. (TP1). Column four was a 30m x 0.25mm-i.d, capillary coated with 0.5 μm film of midpolarity phase consisting of 35% phenyl and 65% dimethyl polysiloxane (Rxi®-35Sil). The temperature program for this column consisted of an initial temperature at 80 °C for 1.0 min, ramped up to 300 °C at a rate of 15 °C/min, held at 300 °C for 0.5 min then ramped to 340 °C at a rate of 1.0 °C/min and held at 340 °C for 5.0 min with a total run 61.0 min (TP2).

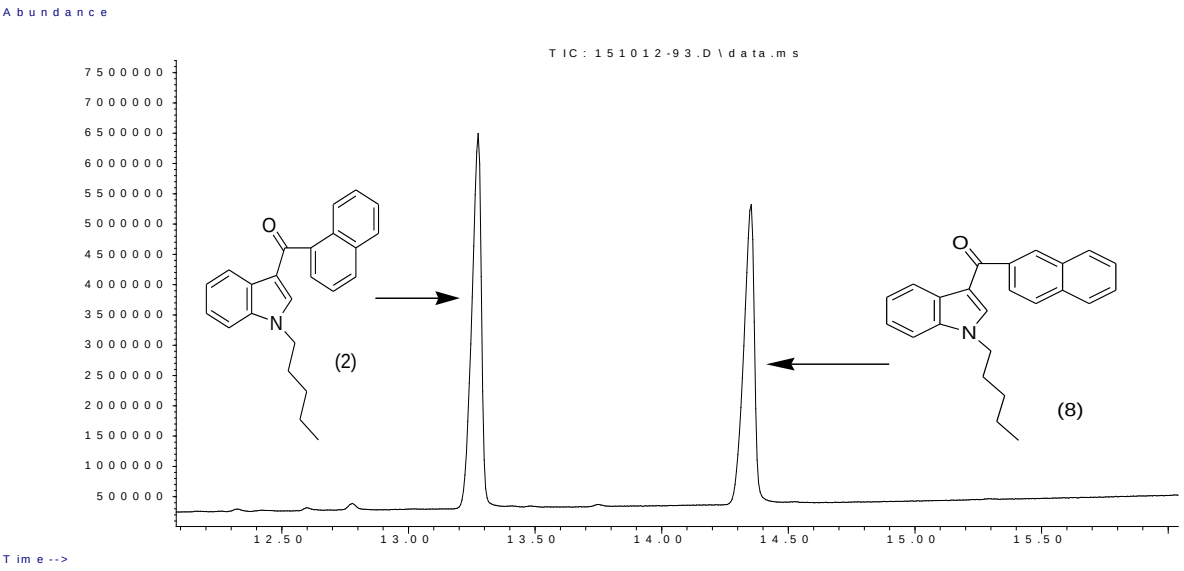
Figure 25, 26, 27 and 28 show the separation of the 1-naphthoyl mixture in panel A and 2-naphthoyl mixture in panel B on Rtx®-5, Rxi®-17sil , Rxi®-35Sil and Rtx®-200, respectively. The 1-naphthoyl group mixture was completely separated on only the Rtx®-200 column with an observed slight upward shift of the baseline. Unfortunately, the separation of this mixture of compounds was not successful on any of the other three columns due to the extensive overlap and co-elution of the JWH-018 and 4-(1-naphthoyl)-1-pentylindole (compound **2** and **3**). To make sure of this observation, mixtures of these co-eluted isomers were prepared and run in these three columns and as expected only one peak appeared and give the mass spectra like JWH-018.

Unlike 1-naphthoyl, the 2-naphthoyl regioisomers mixture were well resolved on all stationary phase columns except Rtx®-200. There is an extensive overlap and co-elution of the 3-(1-naphthoyl)-1-pentylindole and 5-(1-naphthoyl)-1-pentylindole (compound **8** and **10**) on Rtx®-200. The mixture is completely resolved with full baseline resolution on Rtx®-5 and Rtx®-17Sil. However, the last four peaks showed slight tailing but this tailing does not affect the quality of the separation. On the other hand, the run time was increased on the Rxi®-35Sil column to allow for the complete elution of sample components. It takes more than thirty minute to get a complete separation with slow upward baseline shift. Otherwise, the separation was excellent with slightly tailing in the last four compounds, but this tailing does not affect the quality of the separation.

According to the data in Table 9 and 10, the retention times of the studied compounds differ due to differences in column polarity. Alkylacylindoles are found to be less retained in non-polar columns such as Rtx®-5 compared to the polar column Rtx®-200 and Rxi®-17Sil. However, we used a different temperature program to prolong the runtime in column Rxi®-35silM in order to get a complete separation.

The 1-naphthoyl compounds usually eluted before the 2-naphthoyl compounds in regioisomeric comparison between the 1-naphthoyl and 2-naphthoyl compounds substituted at the equivalent indole position. Figure 29 shows the separation of 1-naphthoyl and 2-naphthoyl mixtures of the compounds at the 3-position (panel A) and 7-position of the indole ring (panel B). In the both example 1-naphthoyl isomer elutes before the 2-naphthoyl isomer.

A)



B)

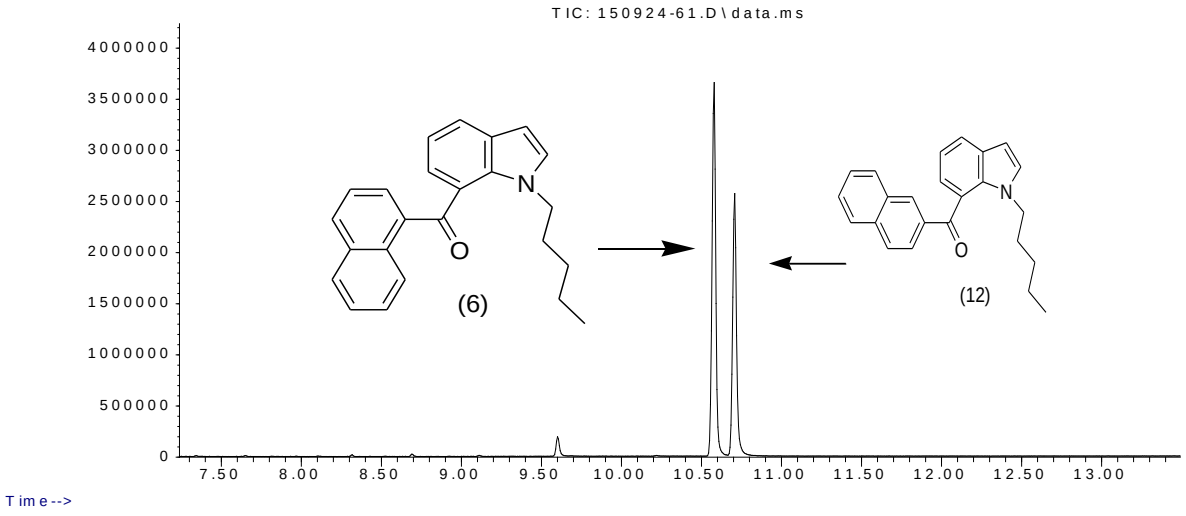
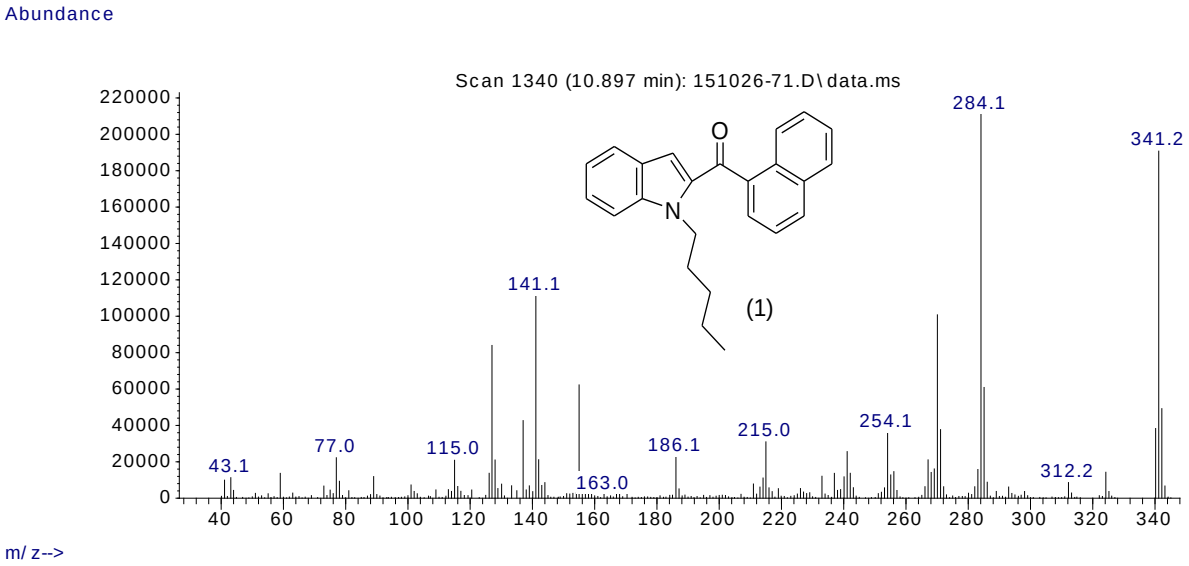


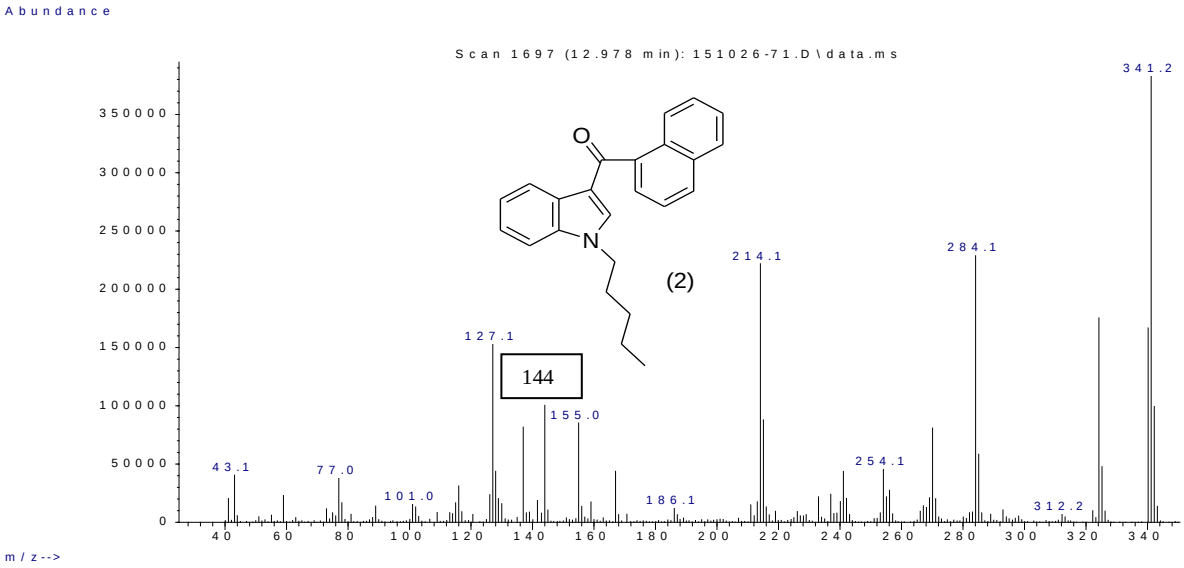
Figure 29: GC separation of 1-n-pentyl and 2-naphthoyl mixture of the compounds at the 3-position (panel A) and 7-position (panel B) on Rxi®-17Sil stationary phase.

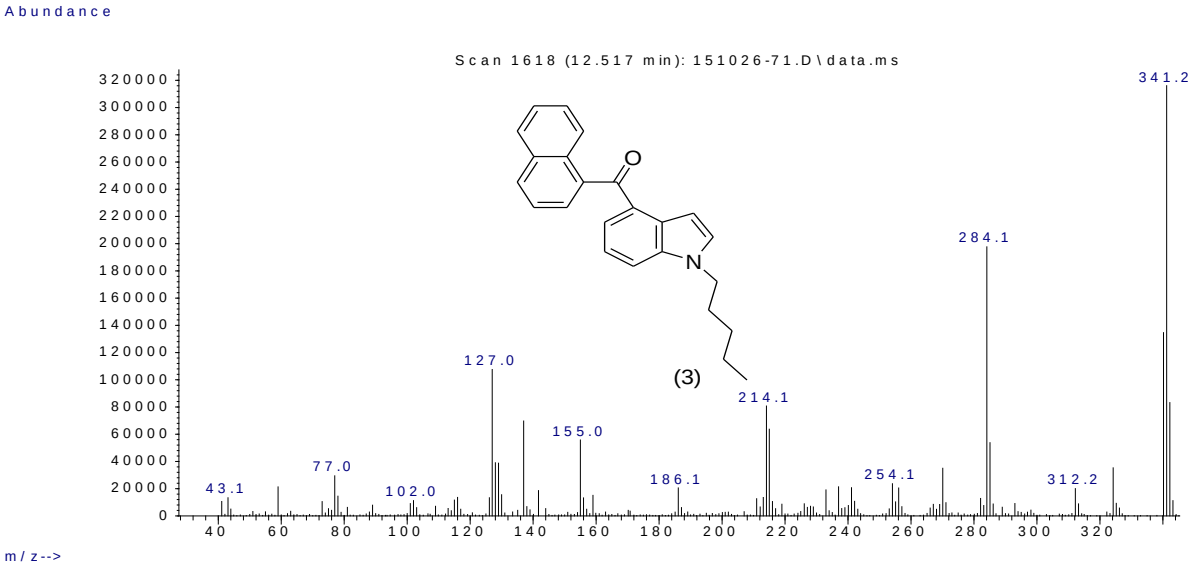
4.2.2 Mass Spectral Studies:

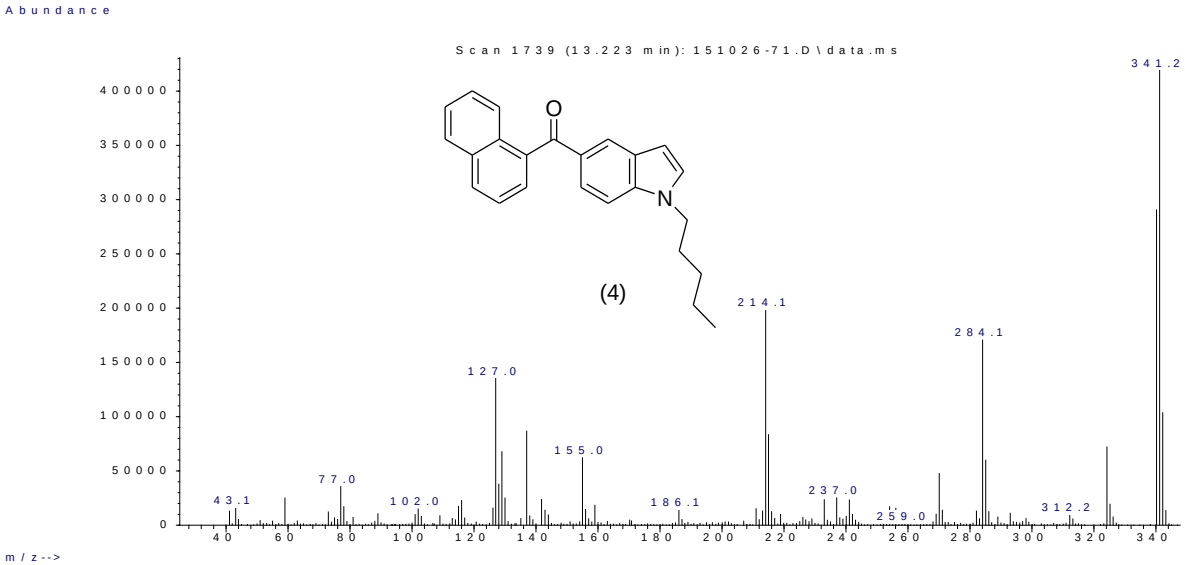
4.2.2.1 Electron impact ionization-mass spectrometry (EI-MS):

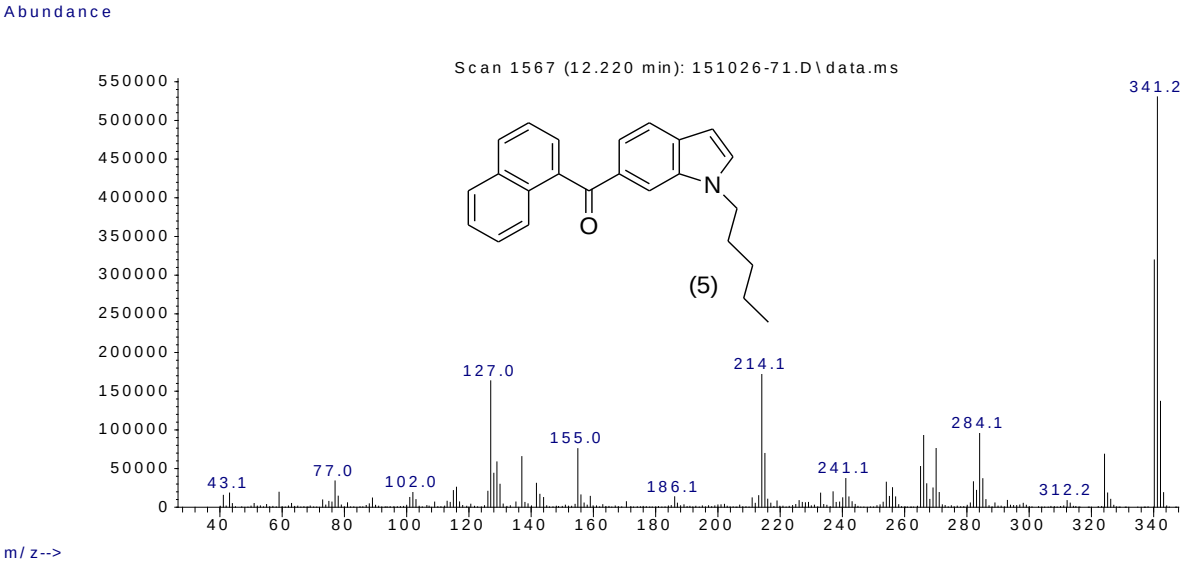
The electron ionization mass spectra of these compounds show equivalent regioisomeric major fragments resulting from cleavage of the groups attached to the central indole nucleus. Figure 30 shows the EI mass spectra of all twelve 1-n-pentyl-3-naphthoylindole regioisomers, compounds **1-12**. The mass spectra of all these compounds are almost identical to each other, especially for those compounds having a naphthyl group at the same position. A previous study reported the deuterium labeling studies on the d11-n-pentyl-, d5-indole- and d7-naphthoyl-analogues of JWH-018 in order to confirm the EI-MS fragmentation processes for these compounds [3].

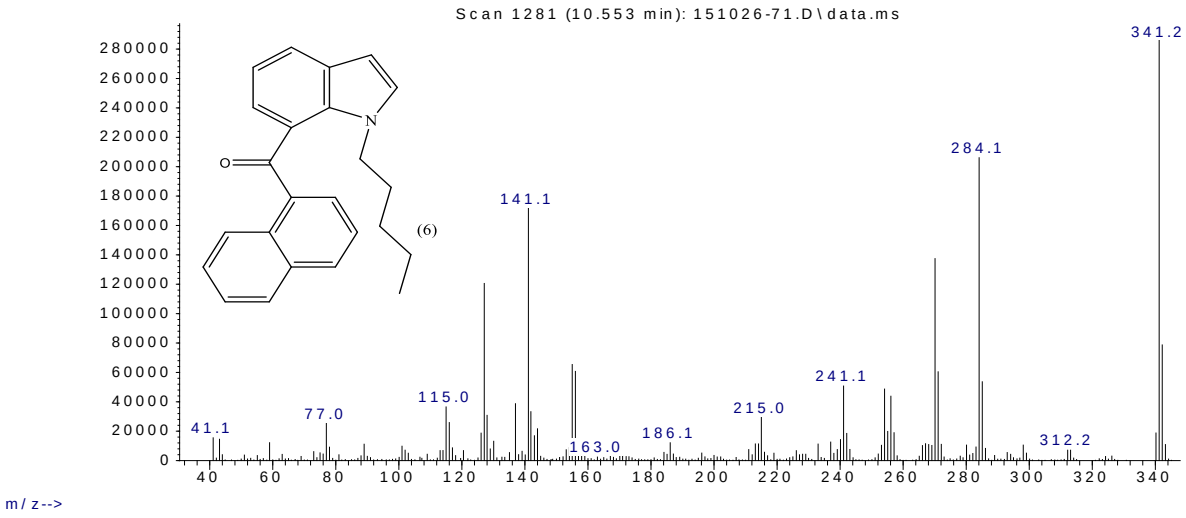

Abundance
Scan 1340 (10.897 min): 151026-71.D\data.ms
O
N
(1)
284.1
341.2
141.1
77.0
115.0
43.1
163.0
186.1
215.0
254.1
312.2
m/z-->

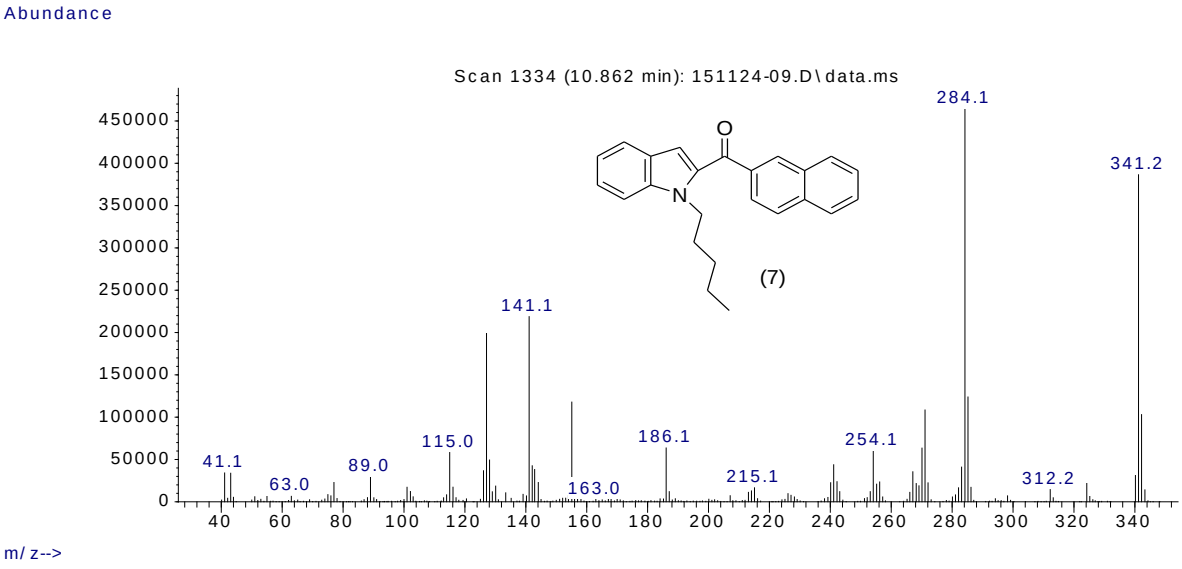

Abundance
Scan 1697 (12.978 min): 151026-71.D\data.ms
O
N
(2)
341.2
214.1
284.1
127.1
144
155.0
43.1
77.0
101.0
186.1
254.1
312.2
m/z-->

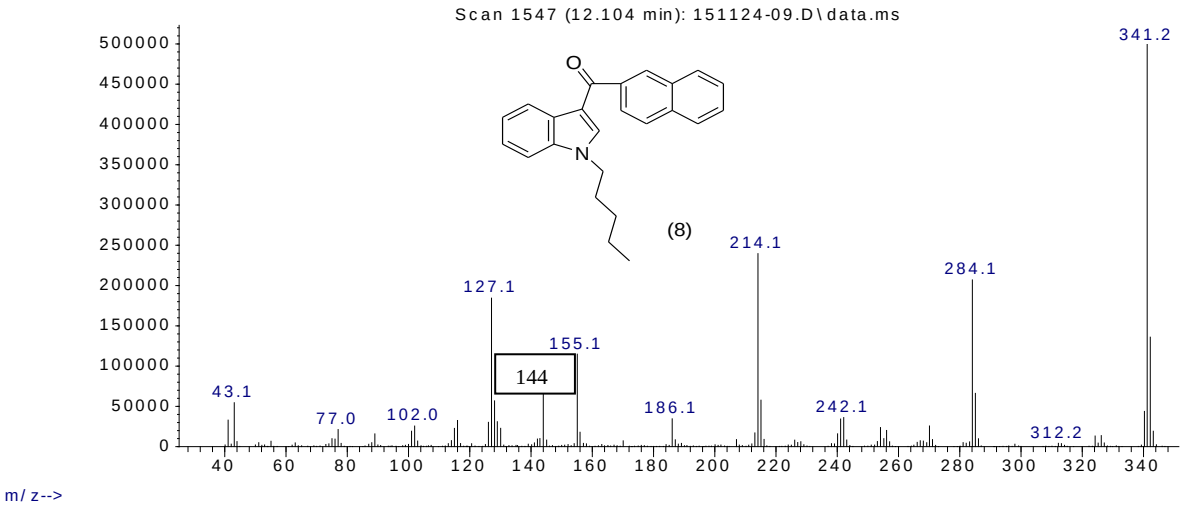

Abundance
Scan 1618 (12.517 min): 151026-71.D\data.ms
341.2
284.1
127.0
214.1
155.0
77.0
43.1
102.0
186.1
254.1
312.2
(3)
m/z-->

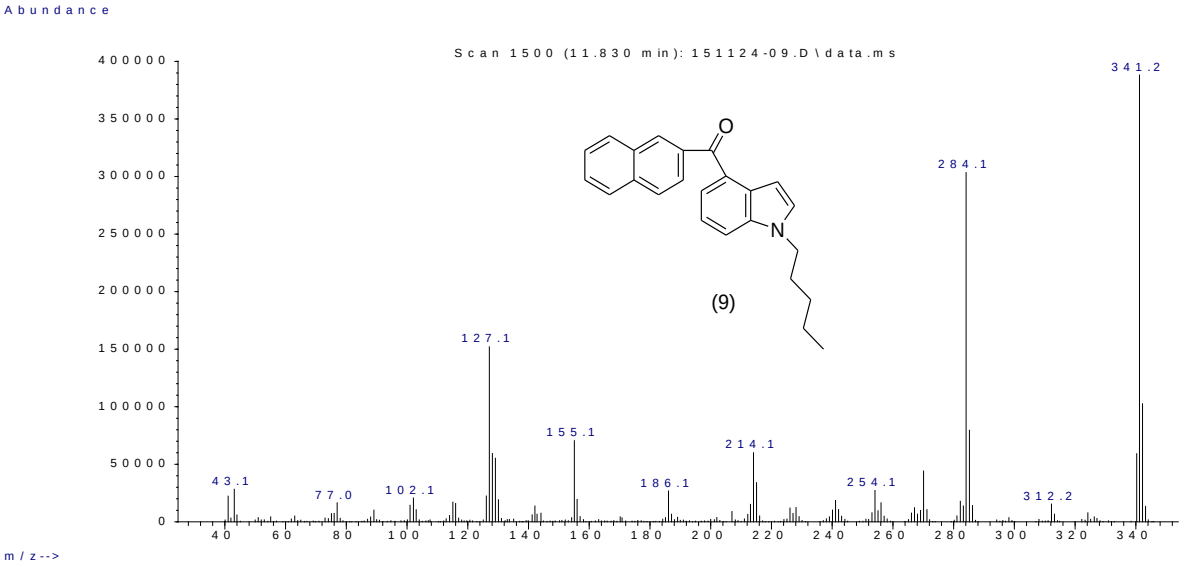

Abundance
Scan 1739 (13.223 min): 151026-71.D\data.ms
341.2
284.1
214.1
127.0
155.0
77.0
43.1
102.0
186.1
237.0
259.0
312.2
(4)
m/z-->

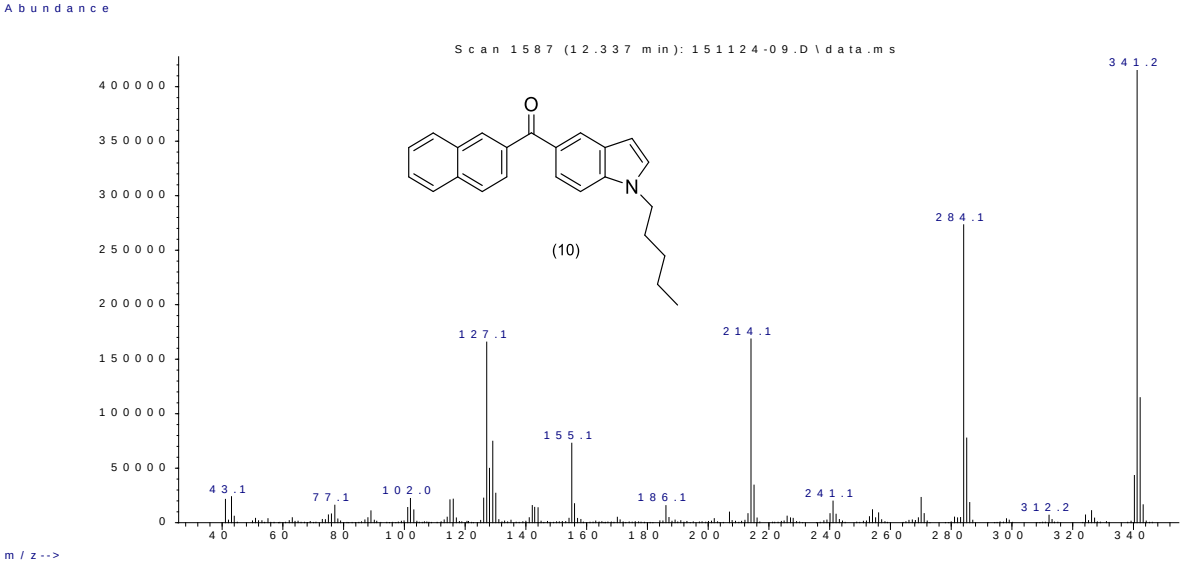

Abundance
Scan 1567 (12.220 min): 151026-71.D\data.ms
550000
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
341.2
127.0
214.1
155.0
284.1
43.1
77.0
102.0
186.1
241.1
312.2
O
N
(5)
40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340
m/z-->


Abundance
Scan 1281 (10.553 min): 151026-71.D\data.ms
280000
260000
240000
220000
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
341.2
284.1
141.1
115.0
241.1
215.0
77.0
41.1
186.1
163.0
312.2
O
N
(6)
40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340
m/z-->


Abundance
Scan 1334 (10.862 min): 151124-09.D\data.ms
284.1
341.2
141.1
115.0
186.1
254.1
41.1
63.0
89.0
163.0
215.1
312.2
(7)
m/z-->


Abundance
Scan 1547 (12.104 min): 151124-09.D\data.ms
341.2
214.1
284.1
127.1
155.1
144
43.1
77.0
102.0
186.1
242.1
312.2
(8)
m/z-->


Abundance
Scan 1500 (11.830 min): 151124-09.D\data.ms
400000
350000
300000
250000
200000
150000
100000
50000
0
341.2
284.1
127.1
155.1
214.1
43.1
77.0
102.1
186.1
254.1
312.2
O
N
(9)
40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340
m/z-->


Abundance
Scan 1587 (12.337 min): 151124-09.D\data.ms
400000
350000
300000
250000
200000
150000
100000
50000
0
341.2
284.1
127.1
214.1
155.1
43.1
77.1
102.0
186.1
241.1
312.2
O
N
(10)
40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340
m/z-->

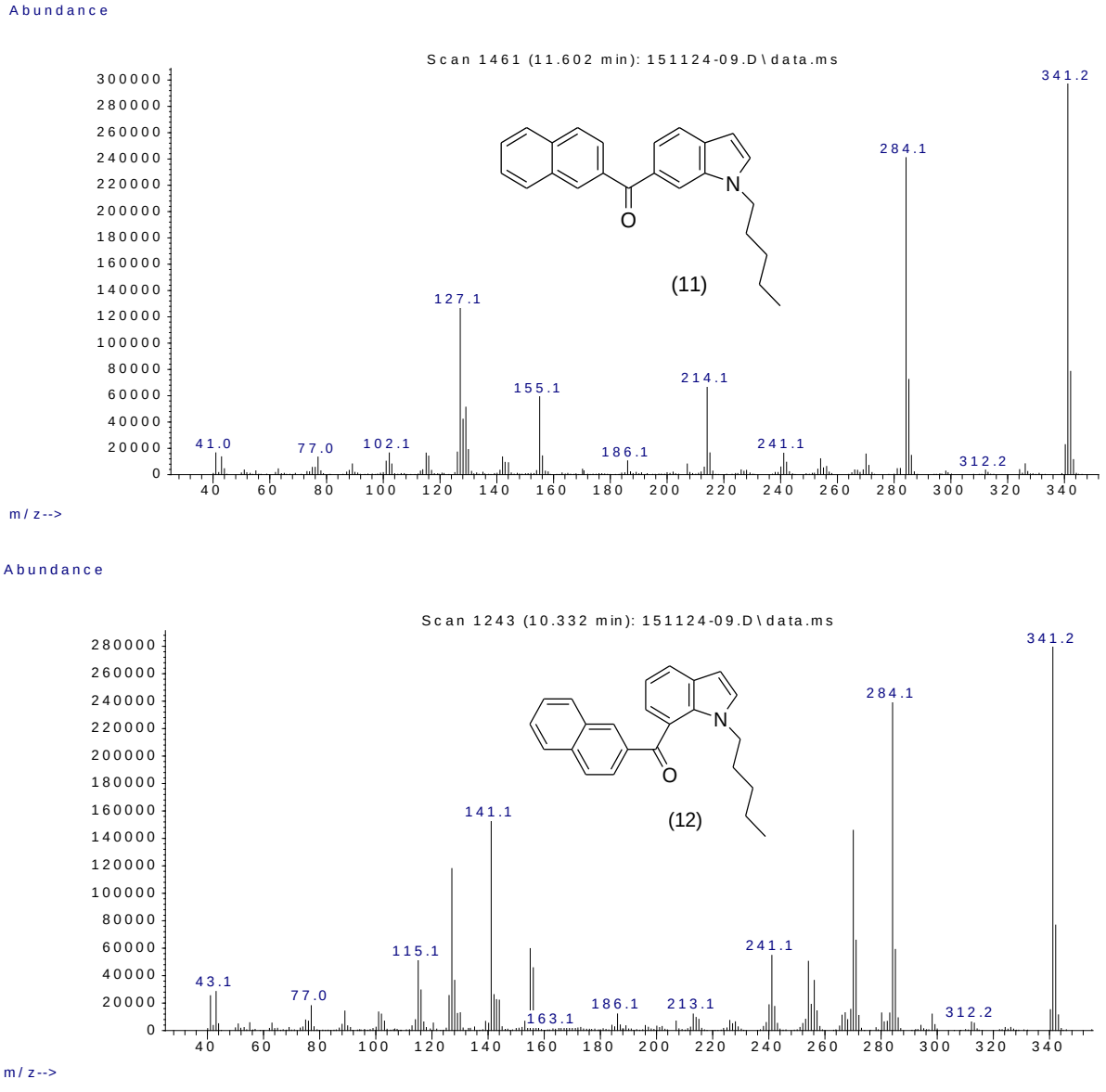


Figure 30: mass spectra for the twelve (1-and 2-naphthoyl) 1-n-pentylindoles, isomeric compounds **1-12.**

All twelve compounds show a major molecular ion peak at *m/z* 341 with a high relative abundance and this ion is the base peak for all the regioisomers involved in this study except the compounds that have a naphthoyl group at the 2-position of the indole ring (compound **1** and **7**). The base peak of the two compounds that have the naphthoyl group attached to the indole ring at the 2-position is observed at *m/z* 284. The fragment ion at *m/z* 284 $[M-57]^+$ occur with the loss of a butyl radical [$C4H9^{\bullet}$] from the molecular radical ion $M^{+\bullet}$ and is observed as a major fragment ion in all compounds. However, all 1-pentylindole compounds of varying acyl groups attached to the indole ring show fragmentation of the acyl and/or pentyl groups to yield the cations at *m/z* 270, 214 and 186. The proposed structure and the fragmentation mechanism of these ions was described in detail in Chapter 2. However, the relative abundances for the ions in the spectra for the twelve isomers are slightly different and these results indicate that very little specific structural information is available for differentiation among these isomers.

The EI mass spectral results show a number of ions which allow for differentiation of some of these compounds. The regioisomers having the naphthoyl group substituted at the 3-position, 3-(1- and 2-naphthoyl)-1-n-pentylindole, show a significant fragment ion at *m/z* 144 fragment. This ion is the product of a hydrogen rearrangement from the *m/z* 214 cation with the elimination of the entire nitrogen substituted alkyl side chain [C_5H_{10}]. Labeling studies have confirmed these proposed structures and a possible mechanism of formation of these fragment ions was reported in a previous study [46].

JWH-018 and compounds **1**, **3**, **4**, and **5** show a significant fragment ion at *m/z* 324. As mentioned in chapter 2, this ion is primarily formed as a result of loss of a hydroxyl radical ($^{\bullet}OH$), the carbonyl oxygen and one hydrogen atom which comes from the 8-position in the naphthalene ring of the molecular radical ion $M^{+\bullet}$. The OH loss represents a hydrogen migration

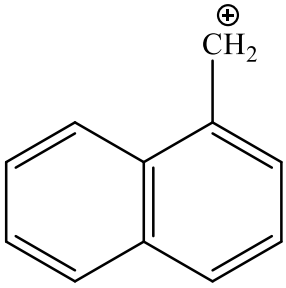
to the carbonyl oxygen followed by elimination of the OH radical to yield the *m/z* 324 fragment cation. However, this ion is significant only in 1-naphthoyl series because the close proximity of the hydrogen at the 8-position in the naphthalene ring in the 1-naphthoyl substituted indoles allows for migration, but this distance appears to be too great for efficient hydrogen transfer in the 2-naphthoyl indoles. On the other hand, compound **2** (JWH-018) has the greatest relative abundance for this ion, perhaps, due to the efficiency of indole-nitrogen resonance stabilization of the carbocation species at the 3-position of indole. Thus, the major point of differentiation of the cannabinoid isomer JWH-018 (compound **2**) from the other elven isomers is the high relative abundance of both the *m/z* 144 ion and the *m/z* 324 ion as shown in the JWH-018 (compound **2**) spectrum.

The four crowded compounds having the naphthoyl group on the same side of the indole ring at position 7 and 2 (compound **1**,**6** ,**7** and **12**) also showed a unique fragment ion at *m/z* 141 in their mass spectra as a major fragment with high relative abundance. This ion may occur by intramolecular interaction of the two substituted groups since it is not seen in the other less crowded indole isomers. The ions at *m/z* 141 are of very high relative abundance in compounds **1**, **6**, **7** and **12** as seen in the EI-MS for these four compounds. Previous deuterium (D) labeling studies [3] of the 7-(1-naphthoyl)-1-(D11)-pentylindole reported that the fragment ion at *m/z* 141 shifted to *m/z* 143 and is the product of migration of the methylene moiety from the N-1 position in *m/z* 284 to the naphthalene aromatic ring followed by fragmentation of the entire indole ring with carbonyl moiety to yield the expected unique fragment ion. Exact mass analysis of the *m/z* 141 ion was carried out in this study to confirm the elemental composition of this fragment and will be discussed later in section 4.2.2.

4.2.2.2 GC-TOF analysis on 7-(1-naphthoyl)-1-n-pentylindole *m/z* 141 fragment:

GC-TOF is an analytical method that can provide high resolution accurate and elemental composition information for any fragments in the mass spectrum. As a further proof to this ion mechanism and structure, it is an important approach for confirming the elemental composition of *m/z* 141. As mentioned above the ion at *m/z* 141 is only significant in the compounds that have the naphthoyl group at the 7 and 2 positions (compounds **1**, **6**, **7** and **12**). TOF data on the *m/z* 141 fragment of compound **6** is shown in Table 11.

Table 11: GC-TOF data on *m/z* 141 fragment of 7-(1-naphthoyl)-1-n-pentylindole

Measured Mass	141.0700
Calculated Mass	141.0704
Deviation (in PPM)	-2.8
Fragment Formula	$C_{11}H_9$ (DBE=7.5)
Best Fit Ion Structure	

Exact mass analysis using GC-TOF-MS confirmed the *m/z* 141 ion as the elemental composition $[C_{11}H_9]^+$ with the mass deviation of [-2.8] ppm (-0.4 mDa). The experimental mass deviation (-2.8 ppm) is within the acceptable error limits ±5 ppm for high resolution mass spectral data. This fragment ion represents the loss of 143 Da $[C_9H_7NO]$ from the fragment ion at *m/z* 284 and it has a suggested formula of $[C_{11}H_9]^+$. Thus, the migration of the methylene moiety at the N-1 position in the *m/z* 284 cation to the naphthalene aromatic ring followed by fragmentation of the entire indole ring with carbonyl moiety is the expected process for this fragment. The proposed structure and mechanism for the formation of the *m/z* 141 ion is shown in Figure 31. However, the exact mass measurement of *m/z* 141 does not differentiate between the 1- and 2-naphthoyl groups containing regioisomeric compounds.

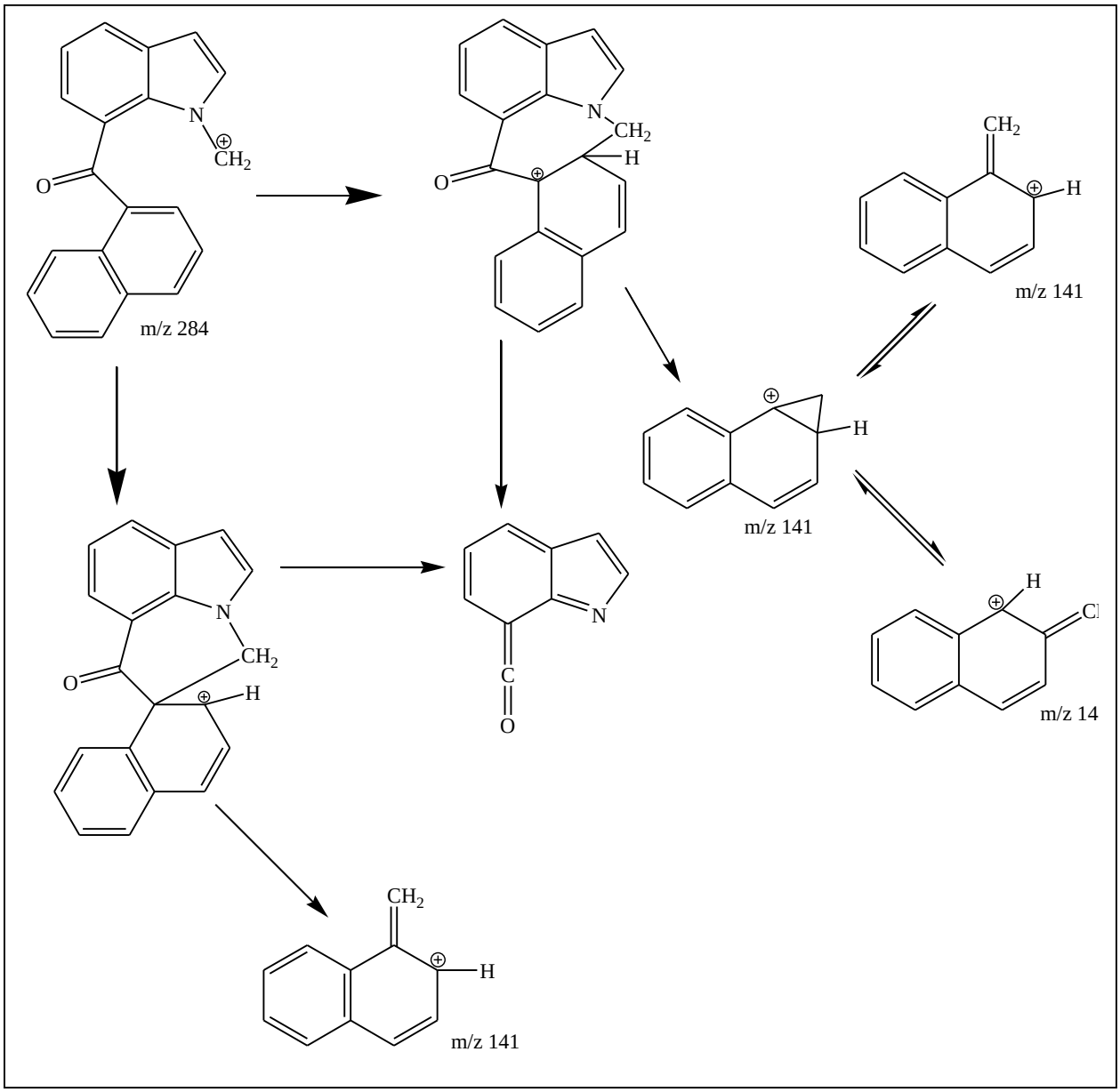
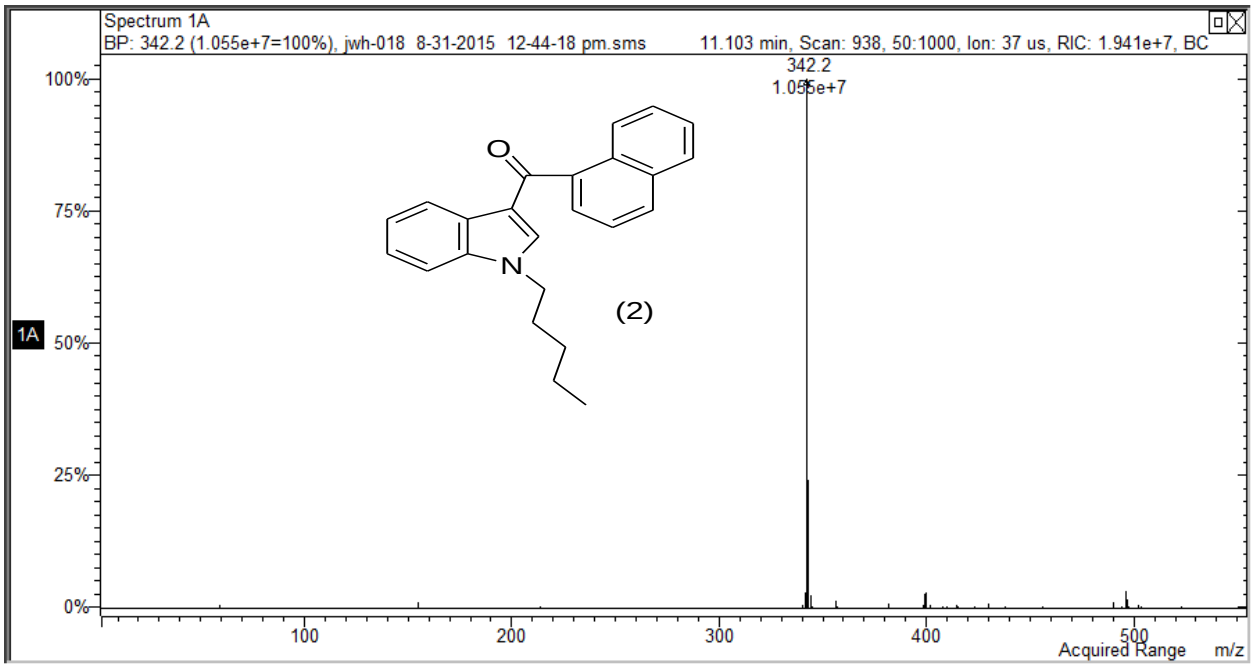


Figure 31: Mechanism for the formation of the *m/z* 141 ion for 7-(1-naphthoyl)-1-n-pentylindole (compound **6**).

4.2.2.3 Chemical ionization (CI)-mass spectrometry:

The chemical ionization (CI) is a soft ionization technique in GC-MS. The main benefit of this technique is to provide a simpler mass spectrum that easily identifies and confirms the molecular weight of these compounds. The ion trap (GC-CI-MS) utilizes chemical ionization to identify the molecular ion of these regioisomers. Methanol was used as the CI gas reagent in this study. This reagent gas will interact with ionizing electrons and produce reagent ions and then the ionized methanol collides directly with the molecule of interest for the proton transfer to generate the ionized molecule $[M+H]^+$ and produce the protonated molecular fragment ion. The protonated molecular ion of these compounds shows the expected *m/z* 342. All these twelve compounds have identical CI mass spectra. The CI-MS spectrum of JWH-018, as an example of chemical ionization of 1-naphthoyl compounds and the 3-(2-naphthoyl)-1-n-pentylindole mass spectrum as an example of 2-naphthoyl compounds, are shown in Figure 32 panels A and B, respectively. This technique does not provide any additional data for discrimination among these twelve regioisomeric compounds which have the same elemental composition and only confirms the molecular weight of the compound.

A)



B)

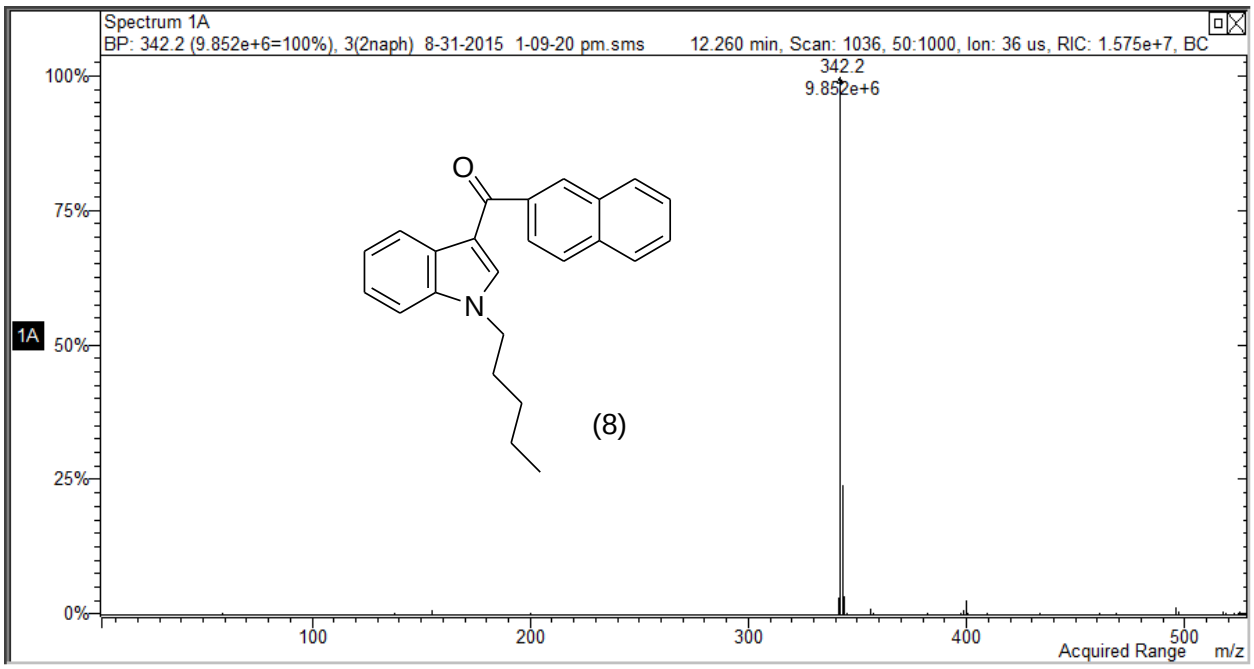


Figure 32: CI Mass spectrum of the model compound JWH-018 panel (A) 3-(2-naphthoyl)-1-n-pentylindole panel (B).

4.2.2.4 LC-MS/MS mass spectral studies

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) was used in this study to give additional information to confirm the identity and potentially discriminate JWH-018 and its regioisomers. LC-MS/MS mass spectral studies used the data concerning the precursor mass $[M+H]^+$ ion and product fragment ions. In general, the product fragment ions at *m/z* 155 and 127 are expected for alkylaminoindoles derivatives. The MS/MS investigates the ions of interest by collision-induced dissociation (CID) mass spectra of molecular ion colliding with a collision nitrogen gas by a different energy. In addition, it was employed in positive ion mode by electrospray ionization (ESI^+) and detection mode by multiple reaction monitoring (MRM). The LC-MS/MS analysis in this study was performed using an Agilent 1290 HPLC and reversed phase Zorbax Eclipse Plus C18 column (2.5 x 50 mm, 1.8 μm) with isocratic elution. Isocratic elution was performed with of 0.1% formic acid with water (solvent A) and acetonitrile (solvent B), (35%:65%) at a flow rate of 0.5 mL/min.

The twelve compounds involved in this study gave $[M+H]^+$ at 342 as a precursor ion and show major fragment ions at *m/z* 155 and *m/z* 127 called product ions. These regioisomers show the major product ion at *m/z* 155 at 25 eV and *m/z* 127 at 35 eV. These product ions reflect the chemical structures of the naphthoyl group resulting from cleavage of the acyl group attached to the indole ring. LC–MS/MS mass spectra of these compounds have shown minor fragment ions at *m/z* 186, *m/z* 116, *m/z* 214 and *m/z* 144. However, the isomer containing the 1-naphthoyl substituted group attached to the indole ring could not be differentiated from the isomer containing the 2-naphthoyl group by LC-MS/MS mass spectra. In conclusion, this technique did not provide any discrimination among these compounds since the main fragment ions are identical in all these regioisomers. The LC-MS/MS mass spectrum of JWH-018, as an example

of the 1-naphthoyl compounds and the 3-(2-naphthoyl)-1-n-pentylindole mass spectrum as an example of the 2-naphthoyl compounds, is shown in chapter 2 Figure 13 panles A and B, respectively.

4.2.3 LC-TOF studies for exact mass determination:

LC-TOF provides accurate mass information (four decimal places) for these compounds to enable calculation of molecular formula. The accurate-mass information is essential for the structural elucidation which provides excellent information for identifying unknown compound and for confirming the presence of a specific compound. The accurate-mass measurements of these compounds was achieved using an Agilent 6520 with TOF mass analyzer, and the protonated molecular ion information [M+H] from ESI^{+}. This instrument allow for the determination of the *m/z* of ionic formula based on the mass sufficiency of the constituent atoms within the acceptable error limits ±5 ppm and the isotope abundance scores were above 95% for all ion compounds. The LC-TOF accurate mass results are shown in Table 12. These results are compatible with molecular formula $C_{24}H_{23}NO$ and supported the results of LC–MS/MS and GC–MS results. The four regioisomeric compounds have the same molecular formula of $C_{24}H_{23}NO$, nominal mass of 341, and the calculated measured mass of 341.1780. In conclusion, LC-TOF-MS confirms the elemental composition of these four regioisomers within acceptable experimental error.

Table 12: Results of the accurate mass measurement by LC–TOF–MS of JWH-018 and eleven isomers.

Compound No.	Generated formula	Absolute difference (PPM)	Abundance score	Exact mass	Accurate mass
1	$C_{24}H_{23}NO$	0.23	98.53	341.1780.	341.1781
2	$C_{24}H_{23}NO$	0	100	341.1780.	341.1780
3	$C_{24}H_{23}NO$	-0.46	98.43	341.1780.	341.1778
4	$C_{24}H_{23}NO$	0.93	97.5	341.1780.	341.1784
5	$C_{24}H_{23}NO$	-0.46	98.43	341.1780.	341.1778
6	$C_{24}H_{23}NO$	0.93	97.5	341.1780.	341.1784
7	$C_{24}H_{23}NO$	0.64	99.48	341.1780.	341.1782
8	$C_{24}H_{23}NO$	-0.23	97.87	341.1780.	341.1779
9	$C_{24}H_{23}NO$	0.46	97.03	341.1780.	341.1782
10	$C_{24}H_{23}NO$	0	100	341.1780.	341.1780
11	$C_{24}H_{23}NO$	0.93	95.12	341.1780.	341.1784
12	$C_{24}H_{23}NO$	-0.23	99.87	341.1780.	341.1779

4.3 Conclusion:

The twelve compounds involved in this chapter have the same elemental composition $C_{24}H_{23}NO$ yielding identical nominal, exact masses and the same substituents attached to the indole ring. The EI-GC mass spectra of all these regioisomers share equivalent major fragment ions resulting from cleavage of the groups attached to the central indole nucleus with slight difference in the relative abundances. However, some mass spectra show a number of unique ions which allow for differentiation of these compounds from each other. The regioisomers having the naphthoyl group substituted at the 3-position, 3-(1- and 2-naphthoyl)-1-n-pentylindole, show a significant fragment ion at *m/z* 144 fragment. The compounds that have the 1-naphthoyl group at the 2-,3-,4-,5-, and 6-position show significant fragment ions at *m/z* 324. The major point of differentiation of the model cannabinoid isomer JWH-018 (compound **2**) from the other eleven isomers in this study is the high relative abundance of both the *m/z* 144 ion and the *m/z* 324 ion as shown in the JWH-018 mass spectrum. The four crowded ring compounds, those with the naphthoyl group and the pentyl group on the same side of the indole ring at position 7 and 2 (compound **1**,**6** ,**7** and **12**) also showed a unique fragment ion at *m/z* 141 in their mass spectra with high relative abundance. The elemental composition and suggested structure for the *m/z* 141 fragment ion formation are supported by accurate mass analysis. CI-MS, LC-MS/MS and LC-TOF were used in this study in order to provide confirmation of structure for these compounds. The 6 regioisomers of the 1-naphthoyl group mixture were successfully resolved on the Rtx®-200 GC column while The 6 regioisomers of the 2-naphthoyl group mixture were successfully resolved on three GC columns (Rtx®-5, Rxi®-17Sil and Rxi®-35Sil).

Chapter 5

Materials, Instruments, GC-Columns, Temperature Programs

5.1. Materials:

The twenty synthetic cannabinoid compounds involved in this research were prepared in our laboratory as a part of a project supported by Award No. 2012-DN-BX-K026, awarded by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice. All solvents used in the study (acetonitrile, isopropyl alcohol and water) were HPLC-grade (Fisher Scientific, Fairlawn, NJ). Individual solutions and mixture of the studied compounds were prepared in acetonitrile (HPLC grade). All samples were dissolved and diluted in high performance liquid chromatography-grade acetonitrile. In GC instruments, the sample was introduced via the auto injector using an injection volume of 1 μL.

5.2. Instruments:

GC−MS System 1 consisted of an Agilent Technologies (Santa Clara, CA) 7890A gas chromatograph and an Agilent 7683B auto injector coupled with a 240 Agilent Ion Trap mass spectrometer. The mass spectral scan rate was 2.86 scans/s. The GC was operated in splitless mode with a helium (grade 5) flow rate of 0.7 mL/min and the column head pressure was 10 psi. The MS is composed of a combination ion source which allows electron impact (EI) /chemical ionization (CI) mode of analysis. Methanol was used as the reagent gas in CI mode. In addition, tandem mass spectrometry (MS/MS) were performed in the ion trap. Both modes were operated

using an ionization voltage of 70 eV and a source temperature of 230 °C. The GC injector was maintained at 250 °C and the transfer line at 280 °C.

GC−MS System 2 consisted of an Agilent Technologies (Santa Clara, CA) 7890A gas chromatograph and an Agilent 7683B auto injector coupled with a 5975C VL Agilent mass selective detector. The mass spectral scan rate was 2.86 scans/s. The GC was operated in splitless mode with a helium (grade 5) flow rate of 0.7 mL/min, and the column head pressure was 10 psi. The MS was operated in the electron impact (EI) mode using an ionization voltage of 70 eV and a source temperature of 230 °C. The GC injector was maintained at 300 °C and the transfer line at 325 °C.

Liquid chromatography- ultraviolet detection (HPLC-UV) system consisted of Agilent 1200 series (Agilent Technologies, Santa Clara, CA, USA). The instrument is composed of binary pump, vacuum degasser and UV/VIS multiple wavelength detector and connected to a computer loaded with Agilent ChemStation Software. A Rheodyne manual injector with 20 µL loop was used in HPLC-UV. The eluents were monitored by the UV detector from 190 to 400 nm.

A rapid resolution LC 1200 series coupled with Agilent 6520 accurate mass Q-TOF spectrometer (Agilent Technologies, Little Falls, DE, USA) equipped with dual electrospray ionization (ESI) source was used for the analysis. Data acquisitions were collected under positive (+) mode. For the mass spectrometer conditions, the nebulizing and drying gases were nitrogen supplied at a flow rate of 25 Psig and 10 ml/min, respectively. Drying gas temperature was 350 °C. Ionization source was dual ESI (electrospray ionization) in the positive mode with a capillary voltage of 3200 V. for tandem mass spectrometry MS/MS condition, the ions of interest detected by collision-induced dissociation (CID). The nebulizing and drying gases were nitrogen supplied at a flow rate of 45 Psig and 10 ml/min, respectively. The drying gas temperature was 400 °C.

Ionization source was ESI (electrospray ionization) in the positive mode with a capillary voltage of 4000V and detection mode by multiple reaction monitoring (MRM).

5.3. Columns:

Different capillary GC columns were evaluated throughout the course of this work, however only columns showed best results between resolution and analysis time are illustrated in Table 13. All columns used were purchased from Restek Corporation (Bellefonte PA, USA) and have the same dimensions, 30m x 0.25mm-i.d. Inlet pressure was converted according to the constant flow mode and the total flow was 60 ml/min. The injection was in the splitless mode with an injector temperature at 250 °C.

Table 13: List of columns in GC used and their composition.

Column Name	Column Composition
Rtx®-5	Coated with 0.10 μm film of 5% diphenyl, 95% dimethyl polysiloxane.
Rxi®-35Sil MS	Midpolarity phase with 0.10 μm of 35% phenyl, 65% dimethyl polysiloxane.
Rxi®-17Sil MS	Midpolarity phase with 0.10 μm of 50% phenyl, 50% dimethyl polysiloxane
Rtx®-200	Coated with 0.5 μm film of 100% trifluoropropyl methyl polysiloxane.

In addition, different LC stationary phase columns were evaluated throughout the course of this work. In LC-UV/VIS, only 3 stationary phases (allure PFP Propyl column, Ultra Cyano Columns, and Kromasil C18) showed best results between resolution and analysis time. All columns used were purchased from Restek Corporation (Bellefonte PA, USA) and have the same dimensions, 150x 4.6mm-i.d, 5 μm particle size. In the LC-MS/MS, analysis was performed using reversed phase Zorbax Eclipse Plus C18 column (2.5 x 50 mm, 1.8 μm) and was purchased

from Agilent. All separations were carried out at room temperature, and the best flow rate was from 0.5 mL/min to 1 mL/min. A Rheodyne manual injector with 20 μL loop was used.

5.4. Temperature Programs:

In GC/MS, different temperature programs were evaluated throughout the course of this work, however only programs showing the best results between resolution and analysis time are illustrated in Table 14.

Table 14: List of temperature programs used in this project.

Temperature program name	Program set up
TP1	hold column temperature at 80 °C for 1.0 min, ramped up to 300 °C at a rate of 30 °C/min, held at 300 °C for 0.5 min then ramped to 340 °C at a rate of 5.0 °C/min and held at 340 °C for 5.0 min with a total run 21 min.
TP2	hold column temperature at 80 °C for 1.0 min, ramped up to 300 °C at a rate of 15 °C/min, held at 300 °C for 0.5 min then ramped to 340 °C at a rate of 1.0 °C/min and held at 340 °C for 5.0 min with a total run 61.0 min.
TP3	hold column temperature at 80 °C for 1.0 min, ramped up to 300 °C at a rate of 30.0 °C/min, held at 300 °C for 0.5 min then ramped to 340 °C at a rate of 2.5 °C/min and held at 340 °C for 5.0 min with a total run 29.0 min.
TP4	Hold column temperature at 80 °C for 1.0 min, ramped up to 300 °C at a rate of 2.0 °C/min, held at 300 °C for 30.0 min with a total run 141.0 min.

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