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Stability of Synthetic Cannabinoids in Biological Specimens: Analysis through Liquid Chromatography Tandem Mass Spectrometry

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Stability of Synthetic Cannabinoids in Biological Specimens:

Analysis through Liquid Chromatography Tandem Mass Spectrometry

By

Chelsea Elyse Fort

A THESIS

APPROVED FOR THE W. ROGER WEBB FORENSIC SCIENCE INSTITUTE

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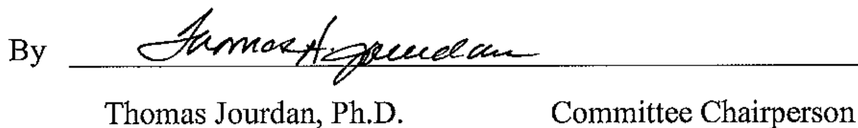
By 

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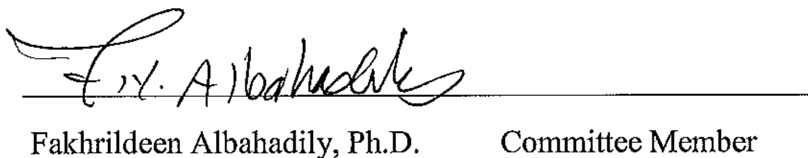

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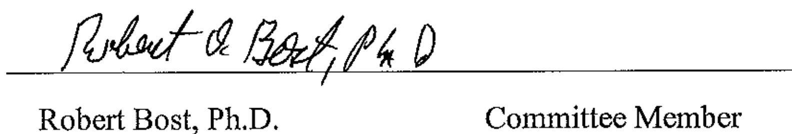

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Abstract

Synthetic cannabinoids, known as “spice” or “K2” among others, create an ever-changing challenge for the forensic chemist or toxicologist. The spectrum of compounds is constantly growing and evolving. Understanding the stability and degradation timelines for these under-characterized substances will be valuable for forensic chemists across the globe as they endeavor to manage their case loads.

The focus of this study was to determine the stability of four specific synthetic cannabinoids, XLR-11, UR-144, AB-Pinaca, and AB-Fubinaca. The study used human blood spiked with the compounds of interest to mimic real forensic laboratory samples submitted for synthetic cannabinoid analysis. These whole blood samples were stored under three different temperature conditions, room or ambient temperature (22°C), refrigerator temperature (4°C), and freezer conditions (-20°C). Scheduled testing on days 0, 3, 7, 14, 21, 28, 35, 42, 56, 70, and 84 were spanned the study’s nearly three month duration, monitoring stability and degradation of the analytes spiked into whole blood samples. Whole blood samples were then extracted using a forward alkaline extraction at pH 10.2 and analyzed using a liquid chromatograph tandem mass spectrometer (LC-MS/MS). Results showed that AB-Fubinaca, AB-Pinaca, and UR-144 were relatively stable, while XLR-11 significantly degraded at ambient and refrigerated conditions. Frozen storage conditions were the only tested parameter able to preserve and stabilize all four compounds over the three month period. Therefore, it should be suggested that forensic blood evidence suspected of containing synthetic cannabinoid compounds should be stored in frozen conditions.

Introduction

Manufactured to mimic and/or exceed the pharmacological effects of tetrahydrocannabinol (THC) found in marijuana, synthetic cannabinoid compounds have hundreds of variants and isomers, and are in a constantly shifting market of compounds (Logan, 2012). Twenty-one substances known as synthetic cannabinoids have been controlled by the Drug Enforcement Agency (DEA) under its Schedule I of the Controlled Substances Act as of 2014. However, many more synthetic cannabinoids exist and are being sold under product names like "K2" or "Spice" (Logan, Reinhold, Xu, Diamond, 2012). Easy accessed, these drugs in seemingly harmless packages are sold under euphemistic trade names such as "K2", "Kush", "Sexy Monkey", "Black Diamond", and "Dead Man Walking". Most users are unconvinced or unconcerned about the potentially harmful side effects that can accompany smoking these deceptively-marketed compounds.

Synthetic cannabinoids have been designed to mimic the effects of marijuana by activating the body's cannabinoid CB_1 and CB_2 receptors. However, these drugs have been discovered to have an even stronger binding potential to these receptors than does delta 9-tetrahydrocannabinol, and thus a greater probability of toxic effects. The appeal of a quasi "legal" high and easy accessibility makes these drug compounds extremely popular with devotees (Harris and Brown, 2012). Such smoking mixtures can often be purchased at smoke or head shops, and also on the internet.

Since these drug compounds are becoming more popular, and controlled by the DEA as they are identified, the demand for their analysis in the forensic laboratory is increasing. In a survey conducted by The Center for Forensic Science Research and Education (2013), results

showed that practicing toxicologists believe that the full range of synthetic cannabinoids should be routinely pursued in applicable cases, demonstrating the general concern amongst forensic chemists and toxicologists that synthetic cannabinoids are of analytical concern. While extraction methods have been established for specific synthetic cannabinoids, little has been done toward studying their stability, especially in whole blood. Ante-mortem forensic chemistry and toxicology laboratories often analyze biological fluids, such as serum and urine, but whole blood proves the most useful for true drug quantitation at the time of a crime or a death. However, in the forensic laboratory setting, the case may not be processed immediately after obtaining custody, especially in cases involving "driving under the influence" thus victimless crashes versus those involving a death. Therefore, sample holding-time in response to case prioritization backlog in the laboratory could negatively impact the concentration of synthetic cannabinoids in the sample or samples in question.

Assessment of the stability of these drug compounds in biological matrices is necessary for the forensic laboratory testing evidence specimens and for those collecting such evidence. Establishing more specific data about the most recently-detected synthetic cannabinoids can directly impact casework prioritization in forensic laboratories. Assessing a wide range of temperature conditions is also important spanning the range of conditions to which biological evidence may be subjected.

Statement of the Problem

Establishing stability data for synthetic cannabinoids has numerous benefits for law enforcement and death investigation. In this study, not only are stability data being established for these commonly-encountered drugs of abuse, but a validated method for their quantitation in

whole blood is as well undertaken. Both of these pieces of information are vital to the forensic chemist or toxicologist who is currently working these types of cases in the laboratory. Stability of drugs in biological matrices is an analytical issue that has impacted forensic chemistry researchers. Simply put, drugs degrade. Whether related to shelf-life or in biological specimens, most if not all drugs or analytes of interest suffer from some type of degradation related to temperature conditions, storage container and preservatives, and specific biological matrices.

As a hypothetical illustration, take the example of a state trooper patrolling an interstate who notices an oncoming driver driving erratically. The driver is swerving in and out of lanes, thus endangering the other drivers. The highway patrolman stops the suspicious driver, suspecting that they are under the influence of alcohol and/or drugs. After failing a field sobriety test followed by a negative Breathalyzer assessment for alcohol, the suspect is taken in for an examination by a drug recognition expert, after which a blood draw is undertaken to assess illicit substances that may be present. It is then the obligation of that trooper, or officer, to turn the blood sample over to the proper authorities or forensic laboratory for testing. The interval between the blood draw and lodging, as well as the conditions experienced by the blood sample during this time, may be variable. The next hurdle to be overcome by the blood specimen is within the forensic examiner's case load prioritization scheme. On a related front, what if the situation involves a discovered body, of which the time of death is uncertain? How might the time since death and ambient temperature have affected the blood and its contents?

The questions above are commonly asked in court by opposing counsel of law enforcement personnel having undertaken of these types of sample collections. However, similar questions can be asked of all forensic laboratories about specimen storage and testing conditions. It is customary for forensic laboratories to store biological specimens under refrigerated and

frozen conditions for preservation; but what about a specimen that is accidentally left out or in the mail at ambient temperature for a time? These are all key quality assurance questions to be asked related to specimen degradation. Specimen and analyte degradation are a real concern in these scenarios.

Stability of analytes is a key factor in the processing of samples in the forensic laboratory. Drug compounds can be subject to degradation and instability from storage conditions, matrix effects, extraction methods, or instrumental conditions. Due to drug acidity or basicity, polarity, volatility, and chemical affinity, detection of drugs and other analytes of interest can be suppressed within a biological specimen, if not lost completely. This further demonstrates the importance of establishing a rigorous analysis protocol that produces reliable, reproducible results, and addresses specimen collection, transportation, and storage for the extraction, and instrument processing. Since synthetic cannabinoids are somewhat new on the drug market and their molecular structures are constantly evolving, little is known about their stability in biological specimens. Extraction methods have been used and validated by previous authors towards the specific cannabinoids of interest for this project (Shanks, Dahn, and Terrell, 2012), but their specific stabilities have not been established.

It has been shown that some related cannabinoids of the JWH group, named for John W. Huffman, are relatively stable for 30 days under all conditions in human whole blood (Kacinko et al., 2011). But cases are not always assessed and analyzed in 30 days or less. If a substantial backlog exists for a forensic laboratory, it may take weeks if not possibly months for the case of interest to be analyzed and reported. Establishing a longer stability timeline provides more data as to how these synthetic cannabinoids may or may not possibly degrade based on their specific storage conditions. If substantial data and evidence of stability or degradation are established, a

predictive model may be projected to other cannabinoids of similar chemical structure or moieties.

Due to the lack of stability data for synthetic cannabinoids in blood in particular, there is a fundamental need to establish some sort of structural integrity timeline for these compounds. Their prevalence in the drug market is growing despite DEA Scheduling efforts, and if more information is not gathered about these compounds in a timely fashion, forensic laboratories will soon face additional difficulties.

Background

Although research is limited as to degradation of the specific synthetic cannabinoids targeted in this study, some literature is available to assist in the analytical process, extraction techniques, and possible degradation timelines from previous structurally non-related stability studies. A short review of this relevant literature is provided as background to the present undertaking.

Synthetic cannabinoids are typically sold at head shops, smoke shops, and online across the United States and around the globe. They are often marketed under trade names such as "incense", "Spice", or "K2" and all usually come with the warning, "Not for Human Consumption." One of the first synthetic cannabinoids synthesized was JWH-018, also known as

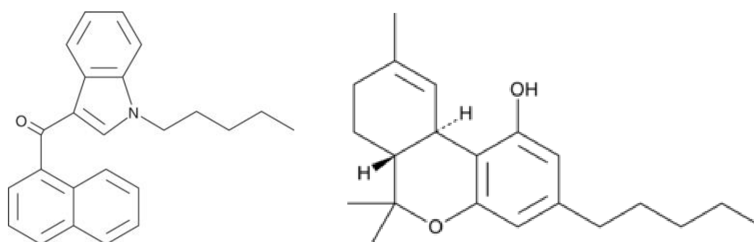


Figure 1. Molecular structures of JWH-018 and THC from Cayman Chemical (2014)

1-pentyl-3-(1-napthoyl) indole. It was synthesized by the Huffman research group as a cannabinoid agonist, especially for the CB_1 receptor in a fashion similar to tetrahydrocannabinol (THC), the pharmacologically active ingredient of marijuana. The CB_1 receptor is mainly expressed in the brain, and more importantly facilitates the central nervous system response to cannabinoid-like compounds (Ernst et al. 2012). Synthetic cannabinoid compounds often exhibit a greater binding affinity and activation efficacy for the cannabinoid receptors compared to THC, which leads to significant side effects such as seizures, agitation, anxiety, tachycardia, hallucinations and psychotic episodes, or death.

After JWH-018 became a DEA Schedule I compound, other derivatives and moieties became available and were sold over-the counter (OTC), including: JWH-073, JWH-122, CP-47, and CP-497. The structures of these compounds are provided below.

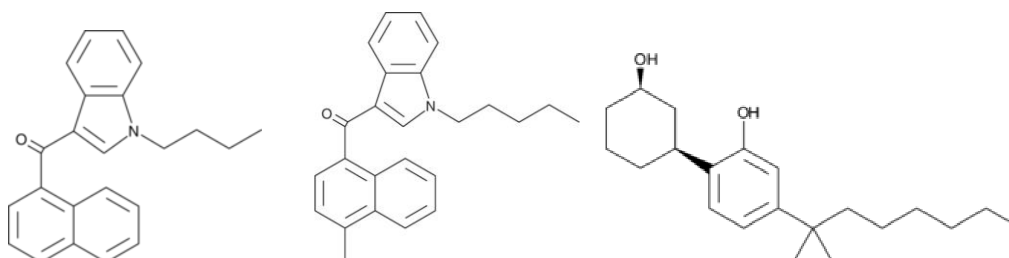


*Figure2.*Molecular structures of JWH-073, JWH-122 and CP-47, 497 (left to right, respectively)

Cayman Chemicals (2014)

Ultimately, these derivatives would become controlled Schedule I substances, in the DEA's efforts to control the growing synthetic cannabinoid market (Dunham et al., 2012). Current, casework observed at the Office of the Chief Medical Examiner of Oklahoma and trending literature suggests that AB-Pinaca, AB-Fubinaca, ADB-Pinaca, PB-22, UR-144 and XLR-11 are the predominant species encountered in drug evidence and in blood work.

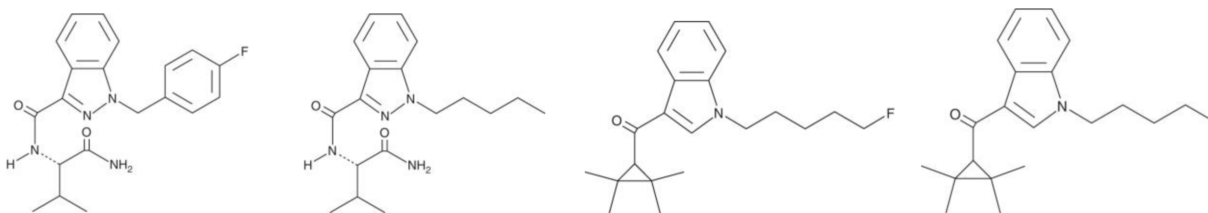


Figure 3. AB-Fubinaca , AB-Pinaca, XLR-11, and UR-144 (left to right, respectively) Cayman Chem. (2014)

Shanks and Terrell (2012) have been successful at detecting synthetic cannabinoid compounds in blood. In their work, detailed experimental conditions and extraction techniques for isolating JWH-018 and JWH-073 in post-mortem whole blood were documented. Drug stability in postmortem matrices poses an added level of difficulty in detection and quantification, due to post-mortem byproducts produced in the autolysis and putrefaction processes of death. Blood samples present a significant number of artifacts besides the drug or analyte of interest. For example, blood samples contain cholesterol as well as various proteins and metabolites that can alter the recovery of other analytes of interest. Dresen et al. (2010) validated a method for LC-MS/MS analysis of synthetic cannabinoids. Their study also incorporated stability studies, analyzing whole blood and serum in the assessment of freeze-thaw stability and long-term stability. They found that the stabilities of the analytes stored at room temperature in glass and polypropylene tubes were not dependent upon the material of the container. For all of the tested compounds, refrigeration proved to be most effective storage conditions, compared to freeze-thaw cycle stability. Room temperature suffered a slight degradation after the 72-hour period. Shanks et al. (2012) validated their procedure for work on an Ultra Performance Liquid Chromatograph tandem Mass Spectrometer (UPLC-MS/MS), which is the instrumentation used in the current project.

Kneisel and Auwater (2012) analyzed 30 synthetic cannabinoids in serum after liquid-liquid extraction followed by LC-MS/MS analysis. Constructed similarly to Dresen et al. (2010), freeze and thaw stability and long-term stability (-20°C) was examined. Their study was configured as a stability study, using a specific time-schedule for analysis, individual temperature or environment conditions, and a spiked sample with the analytes of interest.

The stability of analytes of interest to forensic chemists and toxicologists in biological samples was addressed in a review published in the journal Analytical Bioanalytical Chemistry by Peter (2007). Definitions and specific types of stability were consistent with those reported later in Clarke's Analytical Forensic Toxicology (2013). Peter (2007) found that stability must also be regarded as a process where the result depends on two pathways: degradation of the analyte and its potential *in vitro* formation from precursor and related compounds. Another key aspect of stability studies is comparing the results of quality-control samples analyzed before and with the stability samples after being exposed to the conditions of interest.

Stability studies begin with a designated set of concentrations in the test medium, whether blood or serum, so that a concentration calibration curve can be generated. Then a blood or biological matrix sample will be spiked with the analytes of interest. These known concentrations are then tracked according to a preset schedule with the rate of analyte degradation being assessed.

As mentioned in the previous example, some synthetic cannabinoid compounds have been studied by forensic scientists during the development of extraction techniques and compound profiles while monitoring the evolving drug market. Some stability studies have been conducted to get an idea about how these compounds may degrade over time in evidentiary

samples, but no literature is available examining the stability of AB-Pinaca, AB-Fubinaca, XLR-11, and UR-144, the most- recently observed and prevalent synthetic cannabinoids seen in casework for forensic laboratories at the present time (June 2014). Stability data for these compounds would be extremely useful to not only gain more insight into synthetic cannabinoid stability in biological matrices overall, but for the expected appearance of structurally related variants.

Purpose of Study

The goal of this study was to establish degradation data for four synthetic cannabinoid compounds, specifically AB-Pinaca, AB-Fubinaca, XLR-11, and UR-144, in whole blood. Variables such as time and temperature were explored for the conditions commonly encountered under which these samples are collected and stored before testing in forensic science laboratories. In collaboration with the Office of the Chief Medical Examiner (OCME) Toxicology Laboratory, the impact of these variables in storage conditions were analyzed to generate chemical stability and degradation data for synthetic cannabinoids. Due to the increasing popularity of these drugs and the growing inventory of compounds, an understanding of how these molecules degrade in biological matrices for interpretational purposes is vital information for forensic science laboratories, in particular as it relates to case prioritization schemes. The information was relevant for evidentiary purposes to know how long blood samples containing controlled substances are viable and testable.

Scope of Study

The scope of this study was bounded by the following areas:

1. The purpose of this study was to establish stability and degradation for synthetic cannabinoids under a set of given storage conditions. Specific freeze-thaw stability was not determined, but instead correlated with data from ambient, refrigerated, and frozen temperatures.

2. The study established degradation for four specific synthetic cannabinoids. While these are the most recent cannabinoids observed in casework, the scope of the study pertains only to the specific cannabinoids mentioned and cannot easily be applied to all synthetic cannabinoids.

3. It was not the goal of this research to suggest that all or none of the synthetic cannabinoids suffer from degradation when left in appropriate storage conditions. Generalizations would not be appropriate to state affirmatively all or none suffer from stability issues. Further research will need to be conducted to apply findings to other certain synthetic cannabinoid compounds. However, this study may suggest initial parameters for study of related compounds, as well as, similar structural components following similar degradation profiles.

Significance to the Field

Presently, there is no data published regarding the stability to the four specific synthetic cannabinoids being monitored in the study. This information, however, would be extremely useful to forensic chemists and toxicologists across the globe. Synthetic cannabinoids are an

international problem for law enforcement and for laboratory personnel. Knowing approximate drug stability before significant degraded loss occurred loss would be pertinent for all those involved in these cases. For instance, the earlier proposed DUI situation could quickly be handled and turned over to the proper lab personnel if law enforcement were aware of potential sample degradation. In return, forensic chemists and toxicologists would also know optimal possible storage conditions and time frame under which testing would need to be completed in order to achieve accurate and reliable results. The synthetic cannabinoid market is vast and quickly evolving. Trying to understand the stability of these species in biological matrices provides the best possible solution to maintaining evidentiary integrity, also making the difference between answering the cause of death question for a grieving family member when making a drug identification, or facing an “unknown” possible drug overdose cause of death certificate. Not only will the project provide a validated method for whole blood testing, but it will also gain insight into the stability of the four synthetic cannabinoids evaluated in the present study.

Literature Review

The literature review will address the areas of related research that pertain to the analysis of degradation in synthetic cannabinoids in blood. The first section addresses the evolution of the synthetic cannabinoid market to gain a better understanding behind these compounds. The second brief section contains further information into the pharmacology of synthetic cannabinoids. The third section deals solely with the analysis types for synthetic cannabinoids for methodology practices. Finally, the last section highlights the impact of degradation studies in similar study areas and their typical study design.

History of Synthetic Cannabinoid Evolution

Synthetic cannabinoids are typically sold at head shops and smoke shops across the country. They can be marketed as “Incense” or “Spice” and all usually come with the same warning, “Not for Human Consumption.” This label serves as a warning to deter unknowing users who are unfamiliar with the leafy material contained in the Mylar package, and to bypass requirements of the Controlled Substance Analogue Act enacted by the United States Congress in 1986. The seemingly harmless packages go by euphemistic names such as “K2, Mr. Nice Guy, Space, Barely Legal, Bombay Blue” and others. The packages contain a leafy plant material, sometimes fragrant, that is ostensibly sold for incense use, but instead is utilized as a smoking agent to reach a “legal” high. The sensation of an altered state of awareness is somewhat legal because the chemicals used to produce these are not all controlled by the DEA. The popularity of these drugs expanded as availability increased and effects were noticed to be similar to marijuana (Logan et al., 2012). However, the United States wasn’t the only country experiencing the growing popularity of these drugs. Countries such as Germany, The Netherlands, Japan, and Russia have attracted the attention of enforcement agencies such as the UK Advisory Council on the Misuse of Drugs (ACMD) to study these major compounds to help classify the structures and hopefully control their distribution (Ernst et al., 2012). The ACMD is a British public body that was established under the Misuse of Drugs Act of 1971 in the United Kingdom. It serves as a council to restrict availability of dangerous drugs, promotes research, and educates the public about the dangers of illicit drugs.

JWH-018 is one of the first and most infamous of the synthetic cannabinoid compounds. 1-pentyl-3-(1-naphthoyl) indole, also known simply as JWH-018, was first synthesized by John W. Huffman. JWH-018 is a potent cannabinoid agonist, especially for the CB_1 receptor in a

fashion similar to tetrahydrocannabinol (THC), the active ingredient of marijuana. The CB_1 receptor is mainly expressed in the brain, and is more importantly in charge of the central nervous system response to synthetic cannabinoid compounds. In March of 2010, JWH-018 became a Schedule 1 controlled substance in the U.S., along with its other similar derivatives JWH-073, JWH-200, CP-47, and CP-497 (Dunham, Hooker, & Hyde, 2012).

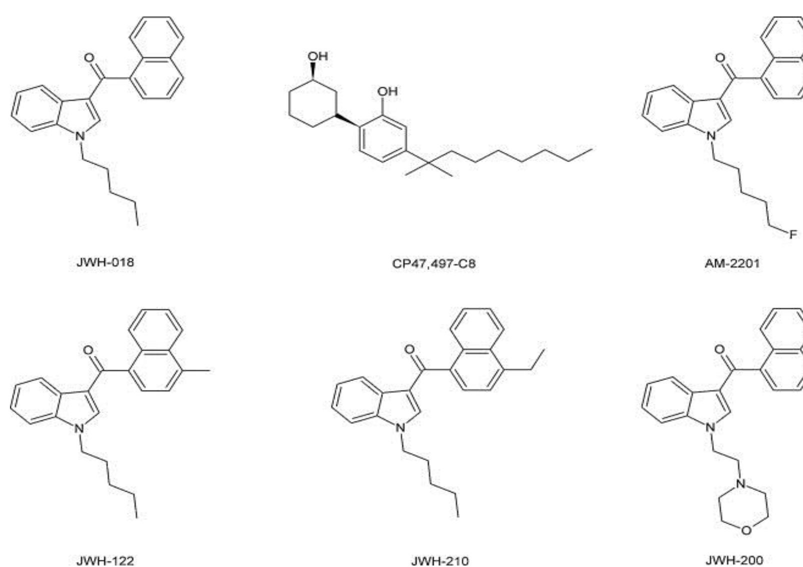


Figure 4. Molecular structures studied by Dunham. (2012)

This ban halted the legal possession and consumption of these substances, but synthetic cannabinoids continued to be produced and synthesized as structural isomers and chemical analogs of Schedule 1 compounds.

The new isomeric compounds were given short names like JWH-018: JWH-412, JWH-122, JWH-210, AM-2201, CP-47, 497. Most of these compounds belong to the aminoalkylindole family, and express a similar binding affinity for the CB_1 and CB_2 receptors.

JWH-018 has been a Schedule I controlled substance since March 1st, 2011. Many other compounds followed, being continually added to the DEA controlled substance list as they

appeared on the synthetic cannabinoid drug market. However, as mentioned by Ernst et al. (2012), JWH-018 has reappeared on the market in Germany, and this is true for the United States as well. The European market saw the appearance of a new compound, known as JWH-307, as well as the reappearance of JWH-018. Since JWH-018 has become controlled, chemists and drug manufacturers in charge of making these substances started creating new isomers and compounds that produce the same or similar binding affinity for the CB_1 receptor in the brain. In Germany, JWH-307 was the new structurally similar compound introduced in 2012. While it has been tested for its receptor binding affinities, not much spectroscopic information is known about this compound.

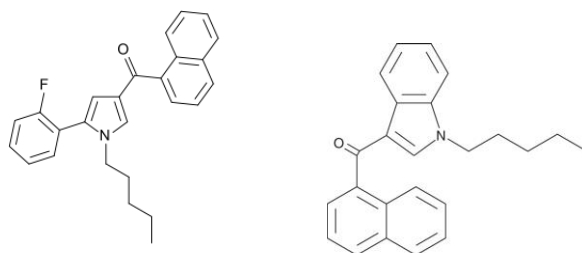


Figure 5. Molecular structures of JWH-307 and JWH-018 from Cayman Chemical (2014)

For the European authorities, as well as worldwide, having little spectroscopic information is a problem because JWH-307 is the first synthetic cannabinoid of the naphthoylpyrrole class, which means that the popular indole group that is typically used in synthetic cannabinoid compounds is substituted by a phenyl-pyrrole. As demonstrated by JWH-307, synthesis of these compounds usually involves the movement or altering of functional groups on the naphthoylindole structure (Ernst et al. 2012).

Researchers in Germany discovered the napthoylindole alteration issue when they were introduced to a new product that was proving to be very dangerous to many teenage users having

purchased “Lava Red.” In the study conducted by Ernst et al. (2011), JWH-122 was introduced as the new synthetic cannabinoid ingredient comprising the “Lava Red” product.

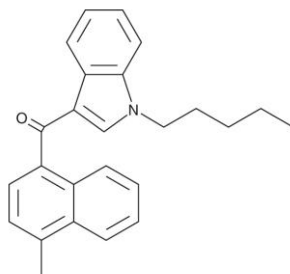


Figure 6. Molecular structure of JWH-122 from Cayman Chemical (2014)

This ingredient was shown to be (N-alkyl-3-(1-naphthoyl) indole, which was previously detected in only a few instances in Germany. Because JWH-122 was an emerging threat, the need to determine and characterize molecular structure, composition, and binding affinity became apparent. Analyses were run for the “Lava Red” component, and *in vitro* experiments concluded that JWH-122 to be a very potent CB_1 agonist with a low affinity constant, just like its predecessor JWH-018. However, when compared to JWH-018, the K_i binding constant of JWH-122 is lower by a factor of 10, therefore having a stronger binding affinity. K_i is an indication of how potent an inhibitor is; it is essentially the concentration required to produce half maximum inhibition. Ernst et al. (2011) discussed how the development of JWH-122 and the lower binding affinity demonstrates the impact of JWH-018 becoming a controlled substance on the supply chain for drug analogs. Specifically, suppliers are finding other ways to create the same drug, with even stronger physiological effects. This can lead to overdose cases as witnessed by German authorities with “Lava Red” (Ernst et al., 2011).

Information about JWH substances can also be found in many locations online, often containing manufacturing information and reported personal mixtures that are dangerous to the user and the public. JWH compounds, such as the aforementioned JWH-122 and JWH-307, are

only growing stronger in terms of CB binding abilities than the original JWH-018 substance, and are growing more popular with the increasing synthetic cannabinoid market worldwide. These are two common JWH compounds found in "Spice" mixtures.

In a publication by Gurney et al. (2014), synthetic cannabinoid compounds are suggested to have a life cycle of about 12-24 months after which they are replaced by the next compound in succession. In 2011, the DEA scheduled its first synthetic cannabinoids adding five compounds to Schedule I, the highest level of control and threat to public health. The most recently added synthetic cannabinoids to the DEA emergency Schedule I controlled substances list in 2013 included XLR-11 and UR-144, among many other synthetic cannabinoids (Gurney et al., 2014).

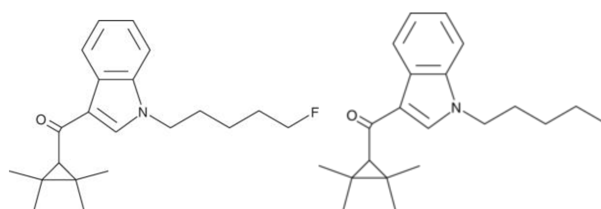


Figure 7. Molecular structures of XLR-11and UR-144 from Cayman Chemical (2014)

Pharmacology of Synthetic Cannabinoids

In a very recent publication from Gurney et al. (2014), the pharmacology and toxicology of synthetic cannabinoids was studied in great depth, since a lack of information had been available about the pharmacology of these compounds. The CB_1 and CB_2 receptors are the main receptor of the endocannabinoid system. The CB_1 receptor has been shown to be responsible for most of the psychotropic effects of *Cannabis*. The CB_2 receptor serves as an immune modulator and the main target for therapeutic agents. Halogen-substituted synthetic cannabinoids, including XLR-11, often experience an increased binding affinity due to the halogenation of the synthetic cannabinoid side chain structure. For example, XLR-11 (5-fluoro-pentyl-UR-144) is the

fluorinated version of UR-144. Using *in vivo* animal studies, potency, spontaneous activity, among other characteristics were studied to understand how chemical structure, halogenated compounds versus non-halogenated, causes increases or decreases to the binding affinity of synthetic cannabinoids. In the study, UR-144 showed full agonist effects for both CB_1 and CB_2 receptors, producing dose-dependent effects such as antinociception, hypothermia, catalepsy and suppression of locomotor activity. Results for XLR-11 proved to be similar. However, also reported was that the tetramethylcyclopropyl group confers selectivity for the CB_2 receptor over the central CB_1 receptor (Cayman Chemical, 2014).

An in-depth report for the World Health Organization (WHO) Expert Committee on Drug Dependence critically reviewed UR-144 and its chemistry, pharmacology, and toxicology. Drafted by Pennings along with other members of the World Health Organization (2014), binding affinities for UR-144 were generated by Abbott Laboratories for the WHO research effort. UR-144 showed a high binding affinity for both CB_1 and CB_2 receptors, being a highly CB_2-selective ligand. Dosage-dependent effects were produced as a full agonist, which were blocked by the cannabinoid antagonist rimonabant in mice. A two-fold lower affinity of UR-144 to the CB_1 receptor as compared to THC may imply that UR-144 is less psychoactive than herbal *Cannabis*. This could lead to increased dosages by the user, and therefore unexpected side effects accompanying use of synthetic cannabinoids.

In a presentation for the New Drugs 2014 conference in Rome, Italy, Moosmann et al. (2014) from the Institute of Forensic Medicine presented on the current known toxicity for synthetic cannabinoid receptor agonists. In particular, the researchers focused on many groups of synthetic cannabinoids containing the cyclopropylindoles, which UR-144 and XLR-11 belong to, as well as the indazole derivatives which contain AB-Pinaca and AB-Fubinaca. Moosmann et al.

(2014) provided information regarding the different instrumentation types and known receptor affinities for synthetic compounds, in comparison to natural *Cannabis* containing THC. Overall, lower doses of synthetic cannabinoids have stronger effects than THC, also showing strong maximum effects for CB receptor bindings. They have higher affinities and higher efficacies than *Cannabis* for CB receptor binding.

Analysis of Synthetic Cannabinoids

Analytical efforts directed at synthetic cannabinoids have resulted in fundamental findings as to the chemical structure and binding affinities of these compounds. In most of the JWH, CP, and AM series components, members differ mainly in the movement of one functional group or other small isomeric change to the chemical structure, but still result in the same or similar physiological effects. Isomers as well as new molecules are being encountered in crime labs commonly, so many so that it is becoming difficult to keep up with the associated identification process. The constantly evolving drug market finds new blends to manufacture while still being considered "legal." In the expansive investigation by Logan et al. (2012), synthetic cannabinoid compounds were explored using several analytical techniques in an effort to determine which was the most effective. Liquid chromatography time-of-flight mass spectrometry proved to be the most useful technique, but is fairly new and is not usually utilized by all crime labs. Gas chromatography tandem mass spectrometry was utilized by almost every study in the literature to determine the molecular weights of the involved compounds. The literature discusses the difficulties faced by the forensic science field if more mass spectral data is not generated in response to the rapidly evolving field of synthetic cannabinoids. From Logan's analysis, the packages analyzed contained either one or two compounds, both of which were identifiable using the aforementioned analytical techniques. However, new compounds are

continuously introduced in an effort to stay ahead of regulators. This evolution creates time constraint difficulties for forensic chemists in elucidating the structures of these compounds for the associated regulatory bodies.

How synthetic cannabinoid plant material specimens are prepped for chemical analysis varies by the type of analysis to be undertaken, and the preferences and protocols of the laboratories conducting the analyses. In most instances, since the physical appearance of a synthetic cannabinoid product is a green, leafy, dry plant material. Samples are often vortexed after the addition of an organic solvent, and then the chemical component containing the synthetic cannabinoid compounds is extracted into the organic solvent. This method is a simple and a typical preparation for follow-on analysis using gas chromatography tandem mass spectrometry. The use of deuterated internal standards is important in validating the procedure for proper comparisons of chromatographic data. The four options given when using chromatographic techniques are to use no internal standard, a structurally related compound, or a structurally similar compound or a stable isotope labeled compound, the latter three as internal standards. Internal standards are used to demonstrate that the extraction process from the biological sample was successful, as well as addressing any variations encountered in individual samples. Depending on the type of analysis and the degree of sample clean-up, an optimal internal standard can be selected. Using past chromatographic data are also useful for comparing synthetic cannabinoid data, since the market is quickly evolving, standards and measurements are difficult to keep present.

Synthetic cannabinoid compounds are detectable in blood, as determined in a study by Shanks, Dahn and Terrell (2012). In “Determination of JWH-018 and JWH-073 by UPLC-MS-MS in Postmortem Whole Blood Casework,” detailed experimental conditions are critical to note

for this research project (Shanks, Dahn & Terrell, 2012). The instrumental analysis and liquid-liquid organic extraction procedure was imperative, since the study was performed on post-mortem blood samples. Forensic toxicology labs are commonly given post-mortem samples to work with in an attempt to answer questions concerning cause of death, and they can often be more difficult than ante-mortem samples to work with due to clotting and other post-mortem effects such as putrefaction and microbial production. The study also validates their method for further applications of blood analysis of synthetic cannabinoids using LC-MS-MS. Not only did the researchers validate their own methods, but they also analyzed three post-mortem cases. It was noted that peak concentration of synthetic cannabinoids in the blood stream is five minutes after administration of the drug, often inhalation through smoking, which poses the question to how long it will take these compounds to metabolize and degrade in biological samples, such as blood. Also, this was one of the first few studies to detail post-mortem blood work for synthetic cannabinoid substances. Shanks and Terrell were able to develop and validate a precise method that can identify JWH-018 and JWH-073 in human blood post-mortem.

Dresen et al. (2011) were able to validate a method for LC-MS/MS analysis of synthetic cannabinoids in serum. This method utilized an alkaline liquid-liquid extraction analyzing for ten synthetic cannabinoids of interest. Freeze-and-thaw stability as well as long-term stability were performed with fortified serum samples, each of which were analyzed a week after initial fortification. Room temperature stability was analyzed using glass and polypropylene tubes to determine analyte stability in storage container conditions. Incorporating the liquid-liquid extraction with hexane and ethyl acetate proved to be the best extraction method to reduce matrix effects, which can often be problematic for electrospray ionization with LC-MS/MS. Results for stability showed that neither temperature condition affected the stability of the analytes for the

study’s one-week period. It was also shown that the stability of the analytes at room temperature in polypropylene and glass did not depend on the nature of the container. However, since all analytes showed stability at frozen conditions, it was recommended that samples should be shipped as well under these conditions. This study also encompassed a forensic aspect, using known serum samples from patients of known synthetic cannabinoid use to determine the most prevalent parent compounds at the time of the study for forensic casework (Dresen et al., 2011).

Kacinko et al. (2011) were able to validate a procedure for the identification and quantitation of synthetic cannabinoid compounds of interest in human whole blood. JWH-018, JWH-073, JWH-019, and JWH-250 were analyzed after spiking a whole blood with analytes and deuterated internal standards. Not only did the study validate a specific LC-MS/MS method, but also compiled a short stability study as a function of the storage conditions, chemical properties of the analyte, and the storage container. The stability timeline spanned 30 days and consisted of room temperatures, refrigerator, frozen and repeated freeze-thaw cycles. Ultimately, it was found that all analytes were stable for a minimum of 30 days under all conditions. Interferences, linearity, LOD, LOQ, matrix effects, and dilution integrity were all also assessed to ensure the validity of the methods developed. Along with validating a method for the analysis of synthetic cannabinoids via LC-MS/MS, proof of the method was also established by testing the blood of a known spice abuser to demonstrate applicability to forensic case work.

Shanks, Dahn, and Terrell (2012) were able to develop and validate a method pertaining to synthetic cannabinoid analysis, and apply it to postmortem forensic casework. Applying a liquid-liquid extraction to postmortem blood is more difficult than extracting antemortem blood. The decomposition process can contribute to matrix issues associated with biological extraction processes. Decomposition contributes microbial contaminants and putrefaction products during

the breakdown processes. At the time of publishing, this was the first analytical method of its type, utilizing a liquid-liquid extraction including a hexane-ethyl acetate organic mixture. The use of a sodium carbonate buffer and the hexane-ethyl acetate mixture helped to stabilize the endogenous compounds produced in the decomposition process, such as metabolism products and biological matrix components that can often contaminate samples. This method was also applied to three cases encountered in laboratory work to quantitate JWH compounds prevalent at the time.

In 2014, the above mentioned research group of Shanks et al. updated their extraction method to look for newer synthetic cannabinoid compounds. Using the same liquid-liquid extraction procedure from 2012, AB-Fubinaca, ADB-Pinaca, and PB-22 were quantitated in postmortem forensic samples. Due to their prevalence and emergency scheduling by the DEA, these newer compounds were added to the current method used at the time. This method was validated for the new compounds, and then applied to forensic casework as well using postmortem blood and serum. A substantial noted difference provided in the research was that newer compounds such as 5-F-PB-22 contain ester linkages, as compared to earlier compounds

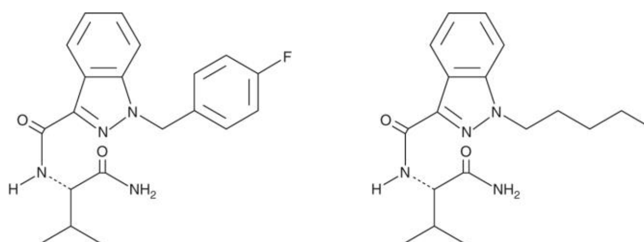


Figure 8. Molecular structures of AB-Fubinaca and AB-Pinaca from Cayman Chemical (2014)

such as JWH-018 and XLR-11 that contain ketone linkages between the indole moiety and naphthoyl groups. It is suspected that the ester bond may be susceptible to *in vivo* hydrolysis

reactions. This could cause accumulation of the metabolites producing toxic effects; however, more research would need to be done to further support these findings (Shanks et al. 2014).

Degradation Studies and the Impact on Toxicology

From Clarke's Analytical Forensic Toxicology (2013), drug stability in postmortem matrices poses an added level of difficulty in detection and quantification due to the decomposition products produced, as mentioned previously. Blood specimens add a significant amount of other compounds besides the drug or analyte of interest. Negrusz, Cooper and Clarke (2013) suggest that drug stability in blood should be evaluated in multiple schemes: long-term stability in the matrix of interest, the effect of freeze-thaw cycles, and short-term stability under multiple storage conditions (Negrusz, Cooper & Clarke, 2013). To mimic the multiple storage conditions of evidentiary samples, the latter short-term stability monitoring will be explored for the duration of the present project. Stability data on natural cannabinoids is also provided, comprising the information that cannabinoid concentrations in refrigerated as well as frozen conditions suffer from oxidative losses. Since synthetic cannabinoids are made to mimic the structural effects of natural cannabinoids and thus often share structural similarities, this information could possibly also be applied to the synthetic cannabinoids. Storage containers and conditions were extremely important when containing THC-cannabinoid samples as were housed for long periods of time.

The question of stability of analytes in biological samples being a large issue for forensic toxicology was raised in Peter's review for the journal Analytical Bioanalytical Chemistty (2007). To correctly determine the answer to this question, definitions and types of stability were clearly defined, that were compatible with the definitions set in Clarke's Analytical

Forensic Toxicology. An important point from Peter (2007) was that stability must also be regarded as a process where the result depends on two conditions: degradation of the analyte and its potential formation from precursor compounds (Peter, 2007). Another key component to stability studies is comparing the results of quality-control samples analyzed before and after (the stability samples) being exposed to the conditions of interest. Once again, cannabinoids were demonstrated to be susceptible to concentration losses due to oxidation and lipophilic binding to the storage container. This will be important information to keep in mind for the duration of the project.

In a study by Giorgi and Meeker (1995), illicit drug stability was studied in blood samples. The time period studied in this experiment was up to 5 years and included many drug compounds such as cocaine, benzoylecgonine, morphine, codeine, methamphetamine, amphetamine and phencyclidine. While cannabinoids were not explored in this study as previously mentioned in the literature by Levine and Smith (1990), this study is integral to understanding the methodology and relevance of toxicological testing of the degradation issue faced when testing biological samples for illicit drugs. In the forensic science lab these analyses can be performed using GC-MS, but currently with the growing popularity of liquid chromatography tandem mass spectrometry (LC-MS/MS), better sensitivity to analytes in biological specimens can be achieved.

Liquid chromatography tandem mass spectrometry has been explored for drug analysis in many studies since its emergence on the analytical analysis market roughly ten years ago. In the publication by Sauvage et al. (2008), many relevant and helpful methods such as using stable-isotope internal standards, establishing relative retention times, and using two transitions or more per compound were suggested when using LC-MS/MS for analysis. Their experiments explored

previous false-positive tests and different methods of analysis for the best possible identification modes for drug analyses. False-positive results are often noticed during toxicological analysis because of the metabolites or xenobiotics that are created after the body's efforts to degrade such molecules. Details of the results and keys to success using LC-MS/MS were clearly indicated in the work of Sauvage et al., but the importance of using the proper ion-monitoring mode was crucial to ensuring accurate results, rather than false-positive conclusions. This study also demonstrates the importance of finding the best possible technique that is suited to the different cases of drug analysis. As the researchers discovered, the high sensitivity of LC-MS/MS requires different monitoring modes (selected-reaction versus general unknown screening) for differing compounds. Although the study analyzed atropine and lysergide compounds and their metabolites, and not synthetic cannabinoids, their analytical methods, suggestions and references are important to understanding LC-MS/MS methodology.

Method validation for synthetic cannabinoid stability has not been studied in depth for liquid chromatography-tandem mass spectrometry using biological specimens, in specific whole blood and serum. Dresen et al. (2010) performed freeze-thaw stability and long-term stability using spiked serum samples. Overall, they found that the stability of the analytes stored at room temperature in glass and polypropylene tubes did not depend on the material of the container. Also, all of the tested compounds proved to be most stable under frozen conditions (-20°C). Specimens stored at room temperature suffered a slight degradation after a 72 hour period. In Kneisel and Auwater's (2012) analysis using liquid chromatography-electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS), they analyzed for 30 synthetic cannabinoids in serum after using a liquid-liquid extraction technique. Similar to Dresen's (2010) study, freeze and thaw stability and long-term stability were tested. Their study was constructed in the same

fashion most stability studies are, using a specific time-schedule for analysis, individual temperature or environment conditions, and a spiked sample with the analytes of interest.

Stability studies begin with a designated set of analyte concentrations in the selected test medium, whether blood or serum, so that a concentration calibration curve can be generated. Then a known sample will be fortified with the analytes of interest. The known concentration will be evaluated according to the schedule to track the stability or instability of the compounds due to analyte degradation. Kacinko et al. (2010) were also able to develop a method for LC-MS/MS identification and quantification of four JWH compounds using whole blood. They found that analyte stability is a function of storage conditions, chemical properties of the analyte, the matrix and storage container. A short and long term comparison was used for their study; however, their four JWH compounds analyzed were stable at room, refrigerated and frozen temperatures for 30 days.

Summary

Having established a foundation for understanding the field of synthetic cannabinoids and how stability studies are designed, more information is needed on the stability of recently-encountered synthetic cannabinoids. With the quickly-evolving market and turnover of new compounds, keeping up with these compounds is a difficult task in the laboratory setting. Few studies have been done to establish the stability of synthetic cannabinoid compounds. Furthermore, having an understanding as to how long these compounds are stable under various storage conditions would prove extremely useful for the forensic laboratory setting and the forensic chemistry and toxicology fields as a whole.

Materials and Methods

Reagents and Standards

All solvents and substances were at least of HPLC grade. EMD Omnisolve high purity HPLC-grade acetonitrile was purchased from VWR International (Radnor, Pennsylvania), while Optima LC/MS-grade Formic Acid, LCMS-grade hexanes, HPLC grade ethyl acetate and HPLC grade water were purchased from Fisher Scientific (Hampton, New Hampshire). ACS-grade sodium bicarbonate and anhydrous sodium carbonate were also purchased from Fisher Scientific (Houston, TX) for sodium carbonate buffer preparation.

One hundred µg/mL of AB-Fubinaca, XLR-11, and UR-144 in methanol were obtained from Cerilliant Corporation (Round Rock, TX). One hundred µg/mL of XLR-11-d_5 in methanol, as well as solid preparations of 1.0 mg of AB-Pinaca and AB-Pinaca-d_9 were purchased from Cayman Chemical (Ann Arbor, MI).

The sodium carbonate buffer, pH 10.2, was prepared by adding 1.87 g of sodium bicarbonate and 0.29 g of sodium carbonate buffer salts to 250 mL of deionized (DI) water and adjusting the pH to 10.2 with 0.1M sodium hydroxide. Mobile phase A (0.1% formic acid in DI water) was prepared by adding 1.00 mL of concentrated optima formic acid to 1L of DI water. Mobile phase B (0.1% formic acid in acetonitrile) was prepared by adding 1.00 mL of formic acid to 1L of acetonitrile.

Preparation of stock solutions

Stock solutions (10 µg/mL) of each analyte of interest were prepared in acetonitrile from their 100 ug/mL original prepared concentrations.

Specimen collection

Date-expired human donor blood was donated to the OCME laboratory by the Oklahoma Blood Institute (OBI) and used in this study. Routine toxicological analyses for blood screening included immunoassay (ELISA) drug screen, alkaline drug screen and acid-neutral drug screen by an Agilent 5895C GC/MS to ensure absence of possible contaminants. A composite blood sample was stored under refrigerated conditions (4°C) until initial aliquots were made for stability test Day 0. Whole blood was preserved by the addition of sodium fluoride.

The control whole blood sample was spiked by adding 100 µL of each of the 10 µg/mL drug stock standards to 200 mL of the control whole blood. This resulted in 5.0 ng/mL of each analyte of interest. The spiked sample was then aliquotted out individually for three storage conditions, containing ten tubes of approximately 5 mL of the composite blood sample.

Preparation of working solutions

Working solutions were made for calibration curves ranging from 0.1 ng/mL to 10.0 ng/mL. A 50 ng/mL internal standard working solution was prepared consisting of XLR-11-d_5 and AB-Pinaca-d_9 in acetonitrile. A seven point calibration curve was prepared using the following concentrations: 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, and 10.0 ng/mL. Low-and high-concentration quality control samples were prepared containing all of the 4 compounds. Quality control working stocks were prepared at 5 ng/mL and 50 ng/mL in acetonitrile. Sample preparation involved adding 100 µL of each QC working solution to 1.0 mL of blood, yielding extracted concentrations of 0.5 ng/mL and 5.0 ng/mL. Linearity, accuracy, bias, and precision were determined from QC and calibration curve data.

Sample preparation and extraction

For analysis, 100 µL of deuterated internal standards were pipetted into the bottom of a 15 mL screw cap tube. One milliliter of sample blood was transferred to the tube. One milliliter of the pH 10.2 sodium carbonate buffer (pH 10.2) was added, then vortexed well. Following this, 5.0 mL of an 80:20 (v/v) hexanes and ethyl acetate mixture were added to the sample. Specimens were vigorously shaken by hand 20 times. They were then placed on a rotor-extractor for 10 minutes. The specimens were then centrifuged at 3,000 rpm for 10 minutes. Subsequently, the organic supernatant was transferred to a 7.0 mL glass conical vial and evaporated to dryness under a gentle stream of dry nitrogen at 40°C. The dry study specimens were reconstituted with 200 µL of a 50:50 (v/v) 0.1% formic acid in water and 0.1% formic acid in acetonitrile mixture. Samples were then vortexed, syringe filtered through a 0.2 µm Titan filter, and transferred to glass auto-sampler vials.

Liquid chromatography-electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS)

The LC-ESI-MS/MS system employed in this study consisted of an Agilent 6420 triple-quadrupole mass spectrometer paired with an Agilent 1290 Infinity series liquid chromatography system, which used a G4226A auto-sampler, G4204A quaternary pump, and a G1316A thermostat-regulated column compartment.

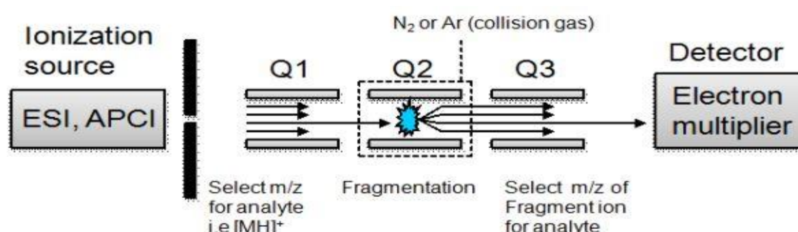


Figure 9. Fragmentation Schematic of LC/MS/MS based on Ni and Rowe (2012)

Figure 10. Agilent LC/MS/MS system used in study

A Restek Ultra-C18 5µm, 50x2.1 mm HPLC column was utilized because of its common acceptance and usage with polar mobile phases. Three ion transitions were monitored using a multiple-reaction monitoring (MRM) method. The parent and qualifier ions used for monitoring are listed in Table 1.

Instrument Conditions

An 8.5 minute run time with a post time of 1.4 minutes utilized positive (+) Electrospray Ionization and a multiple reaction monitoring scanning mode. A 10 µL injection with a 10 mL/min flow of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B); the column compartment was regulated at 40°C while source gas was kept at 350°C. Capillary voltage was at 400 volts. Gradient control is stated in Table 2.

Table 1

Gradient Flow LC Conditions in study

Time (min)	% Mobile Phase A	% Mobile Phase B
0.0	70%	30%
1.5	40%	60%
7.0	10%	90%
8.5	70%	30%

Scan Segments along with other experimental instrumental controls are included in Table 2.

Table 2

Fragmentation Patterns and Instrumental Conditions in study

Compound Name	ISTD	Precursor Ion	Product Ion	Dwell Time (s)*	Fragment (V)**	CE (V)***	Cell Acc (V) ****	Polarity
AB-Fubinaca	No	369.2	324.1	50	95	10	7	Positive
AB-Fubinaca	No	369.2	253	50	95	22	7	Positive
AB-Fubinaca	No	369.2	109	50	95	50	7	Positive
AB-Pinaca-d9	Yes	340.3	295.2	50	100	14	7	Positive
AB-Pinaca-d9	Yes	340.3	224.1	50	100	26	7	Positive
AB-Pinaca-d9	Yes	340.3	146	50	100	38	7	Positive
XLR-11-d5	Yes	335.3	236.7	50	154	26	7	Positive
XLR-11-d5	Yes	335.3	125	50	154	22	7	Positive
XLR-11-d5	Yes	335.3	55.1	50	154	46	7	Positive
AB-Pinaca	No	331.2	286.1	50	100	10	7	Positive
AB-Pinaca	No	331.2	215.1	50	100	22	7	Positive
AB-Pinaca	No	331.2	145	50	100	46	7	Positive
XLR-11	No	330.2	232	50	149	22	7	Positive
XLR-11	No	330.2	125	50	149	22	7	Positive
XLR-11	No	330.2	55.1	50	149	46	7	Positive
UR-144	No	312.2	214	50	159	22	7	Positive
UR-144	No	312.2	125	50	159	22	7	Positive
UR-144	No	312.2	55.1	50	159	42	7	Positive

*Dwell time is the amount of time required for the MS to analyze a single MRM transition
**Fragmentor voltage the energy required to fragment precursor ions into product/qualifier ions
***Collision energy is the amount of energy that precursor ions receive as they are accelerated to the collision cell, where they collide with gas molecules and fragment
****Cell Acceleration the electric potential that excels ions through the collision quadrupole

LOD/LOQ, Carryover, Precision, and Bias Studies

Studies were performed to determine limit of detection (LOD), limit of quantitation (LOQ), carryover, precision and bias. LOD and LOQ were evaluated making serial dilutions with concentrations ranging from 0.0125 to 0.10 ng/mL. These concentrations are lower than those used for the calibrators. The samples were extracted from control blood using the same liquid-liquid extraction technique for the study specimens. Requirements for validation criteria are included in the respective results section for each validation area.

Specificity testing

Specificity analysis was done by spiking control blood with drugs that expected to cause interferences. These drugs would be of similar class and/or of similar structural nature. The test included 20 alkaline drugs commonly detected, 9 acidic or neutral drugs, as well as using 3 different synthetic cannabinoid mixes purchased from Cerilliant Corporation (Round Rock, TX). Ten nanograms of each mix was extracted after fortifying control blood with the following compounds contained in Table 4.

Table 3

Alkaline drugs and Acidic/Neutral drugs Tested

Alkaline drugs	Acidic/Neutral Drugs
Methamphetamine	Valproate
Pseudoephedrine	Ibuprofen
α- PVP	Acetaminophen
Fluoxetine	Butalbital
Diphenhydramine	Hexobarbital
Tramadol	Carisoprodol
Venlafaxine	Phenobarbital
Methadone	Phenytoin
Cocaine	Lamotrigine
Amitriptyline	
Cyclobenzaprine	
Promethazine	
Citalopram	
Diazepam	
Hydrocodone	
Oxycodone	
Paroxetine	
Olanzapine	
Anileridine	
Trazodone	

Table 4

Synthetic Cannabinoid Test Mixes Purchased from Cerillant Co. (2014)

Synthetic Mix #1	Synthetic Mix #2	Synthetic Mix #3
JWH-250	JWH-019	JWH-015
JWH-200	JWH-122	JWH-203
HU-211	JWH-081	JWH-210
(+) CP-47, 497	AM-2201	AM-2233
(-) CP-47, 497	RCS-4	
C8 Homologue	RCS-8	

Matrix Effects/ Ion Supression or Enhancement

Matrix effects (ion suppression or ion enhancement) were studied using 10 synthetic cannabinoid negative, post-mortem bloods stored for method development purposes. The analyses were performed by adding 100 µL of the 50.0 ng/mL of the working quality control solution as well as 100 µL of the 50.0 ng/mL of the internal standard working solution to whole blood blank vials, following the extraction process. These samples were compared to the "neat" vial, which contained only internal standard and 5.0 ng/mL of QC that was evaporated to dryness, and reconstituted. The matrix recovery and efficiency was calculated based upon the pre-extraction spiking recovery and the post-extraction spiking recovery.

Benchtop Stability

For validation purposes, benchtop stability was assessed to test for any possible contaminants or possible quality control degradation. This was achieved by using one set of

QC's from a previous run, allowing said QC's to sit at ambient temperature for 72 hours, then analyzing the same QC's with a new calibration curve. No stability or degradation issues were detected in the quality controls over the 72 hour period.

Stability Timeline and Preparation

Study timeline was set to span twelve weeks. Samples were stored under three temperature conditions, ambient temperature (~22°C), refrigerator temperature (4°C), and freezer conditions (-20°C). On study day 0, 200 mL of a human, whole blood sample was spiked with 100 µL of each drug standard, for a final concentration of 5.0 ng/mL for each analyte of interest. Specimens for the three temperature conditions were aliquotted out for the 11 proposed study days, plus an additional three tubes for safety, equaling 42 total test tubes. Fourteen individual 16x100 mm borosilicate glass test tubes, secured with polypropylene screw caps, for each temperature condition. Stability test was performed on Study days: Day 0, 3, 7, 14, 21, 28, 35, 42, 56, 70, and 84.

Data Analysis and Statistics

The descriptive statistics used were means, standard deviation, and coefficient of variation. Data plots were used to determine the estimated rate of decay, as well as to calculate the half-life of the analyte based on the equation of the line.

Results

Validation Data

Linearity, precision between and within runs, ion suppression/enhancement, and bias were calculated by using 5 consecutive runs of calibration curves, low, and high concentration quality control samples. Validation procedures and requirements come from those established by the Scientific Working Group for Toxicology (SWGTOX, 2013) and are also required by the American Board of Forensic Toxicology (ABFT).

Limit of Detection/Limit of Quantitation

Limit of quantitation was established at 0.1 ng/mL after running test calibration curves for all compounds to determine how low concentrations could range and still meet the required criteria. LOQ requires integration of a parent and daughter ion, as well as a signal to noise ratio of 10:1. LOD was determined by diluting several curve points below the established LOQ. This was found to be 0.025 ng/mL for all compounds. LOD requires the integration of one parent and one daughter ion, as well as a signal to noise ratio of 3:1.

Linearity

Five independent calibration curve runs were all pooled and averaged, as well as calculating the percent coefficient of variation for each analyte. For linearity, R^2 values for curve fit required at least a 0.99 value, as well as a coefficient of variation (CV) value less than 10%. It is also required that each calibration curve point for each analyte can not deviate beyond 20% from the targeted value. All four drugs analyzed during the validation period met the linearity requirements.

Table 5

Linearity Data for AB-Fubinaca

	9/16/2014	9/23/2014	9/30/2014	10/7/2014	10/21/2014			
Concentration	Run 1	Run 2	Run 3	Run 4	Run 5	**Average**	**SD**	**CV**
0.10	0.11	0.11	0.11	0.12	0.12	**0.11**	0.01	4.8%
0.25	0.24	0.26	0.25	0.25	0.24	**0.25**	0.01	3.4%
0.50	0.50	0.47	0.50	0.45	0.46	**0.48**	0.02	4.8%
1.00	0.95	0.91	0.91	0.89	0.90	**0.91**	0.02	2.5%
2.50	2.43	2.40	2.53	2.53	2.41	**2.46**	0.06	2.6%
5.00	5.14	5.29	5.20	4.95	5.28	**5.17**	0.14	2.7%
10.00	9.99	9.91	9.85	10.16	9.93	**9.97**	0.12	1.2%

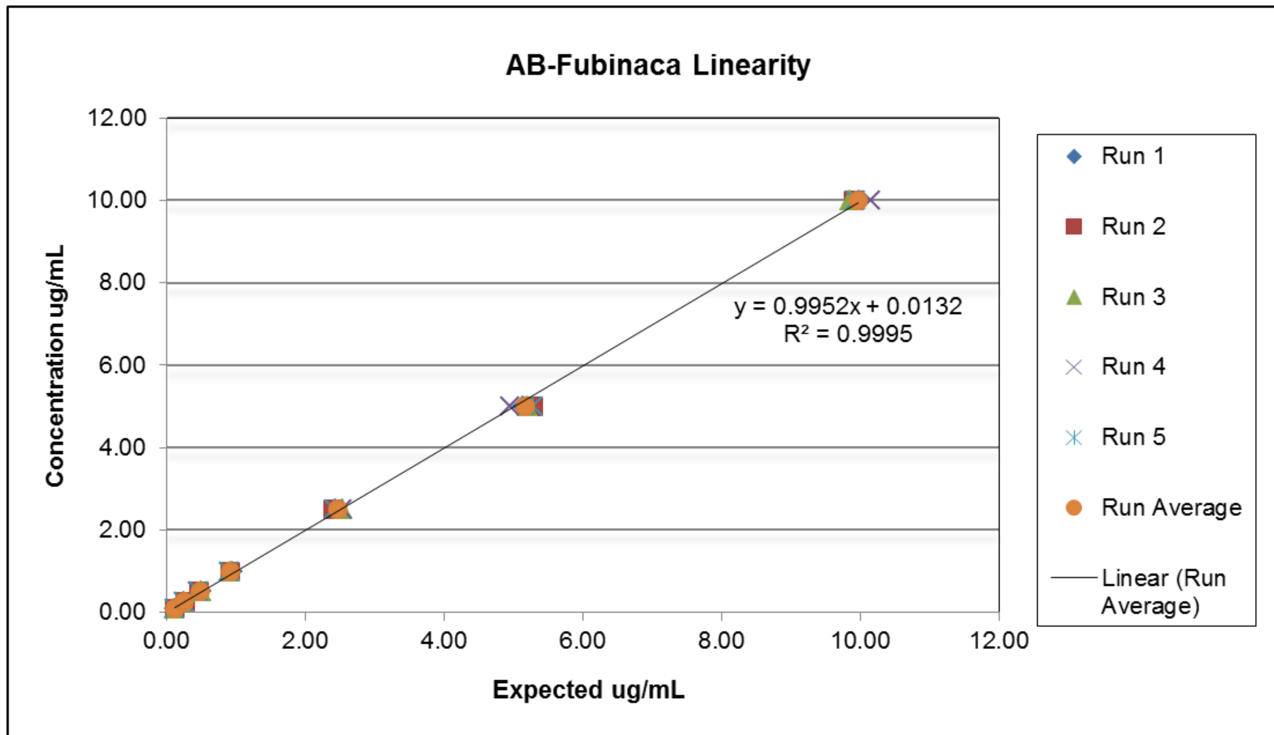


Figure 11. Linearity Plot for AB-Fubinaca (All runs included)

Table 6

Linearity Data collected for AB-Pinaca

	9/16/14	9/23/14	9/30/14	10/7/2014	10/21/14			
Concentration	Run 1	Run 2	Run 3	Run 4	Run 5	**Average**	**SD**	**CV**
0.10	0.12	0.11	0.12	0.12	0.12	**0.12**	0.00	3.8%
0.25	0.24	0.27	0.25	0.24	0.25	**0.25**	0.01	4.9%
0.50	0.46	0.46	0.46	0.45	0.44	**0.45**	0.01	2.0%
1.00	0.96	0.98	0.92	0.92	0.96	**0.95**	0.03	2.8%
2.50	2.45	2.35	2.39	2.57	2.40	**2.43**	0.08	3.5%
5.00	5.01	5.11	5.07	4.97	4.95	**5.02**	0.07	1.3%
10.00	10.12	10.08	10.15	10.08	10.23	**10.13**	0.06	0.6%

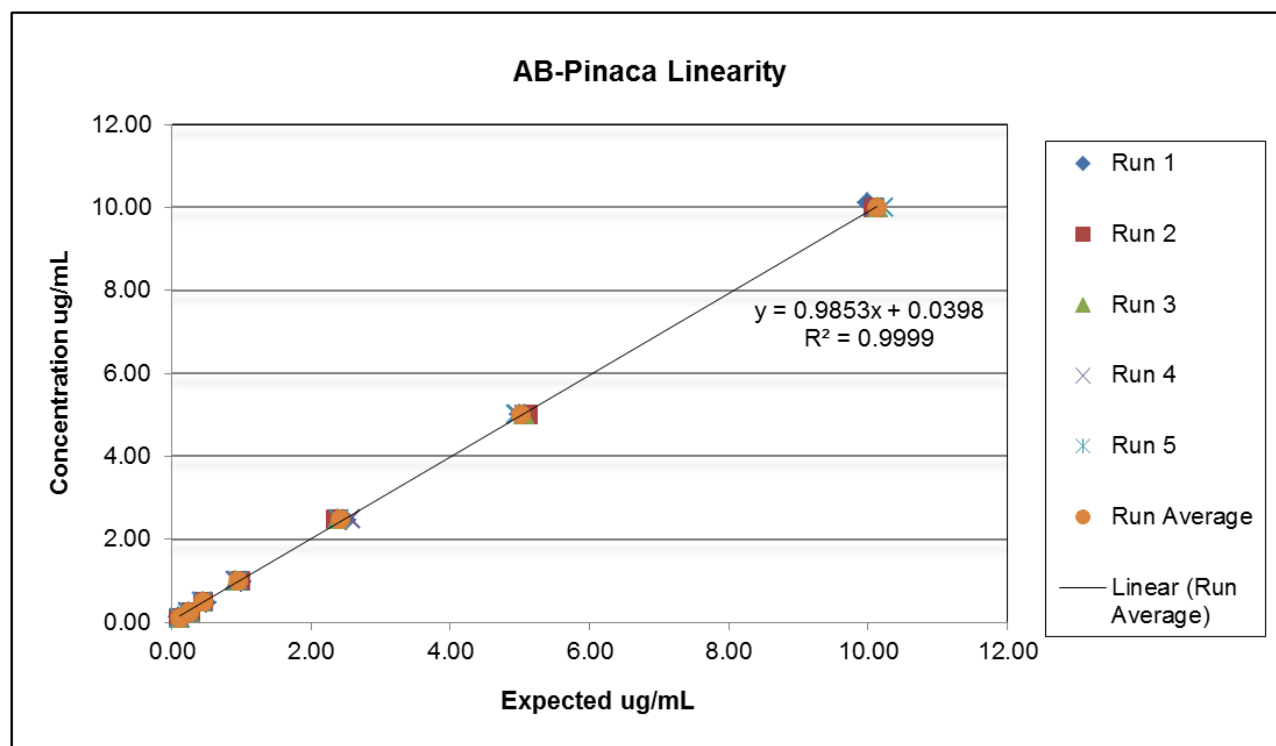


Figure 12. Linearity Plot for AB-Pinaca (All runs included)

Table 7

Linearity Data collected for UR-144

	9/16/14	9/23/14	9/30/14	10/7/14	10/21/14			
Concentration	Run 1	Run 2	Run 3	Run 4	Run 5	**Average**	**SD**	**CV**
0.10	0.12	0.11	0.12	0.10	0.12	**0.11**	0.01	7.8%
0.25	0.23	0.25	0.24	0.24	0.24	**0.24**	0.01	2.9%
0.50	0.44	0.49	0.48	0.51	0.47	**0.48**	0.03	5.4%
1.00	0.99	0.91	0.90	0.98	0.93	**0.94**	0.04	4.3%
2.50	2.47	2.49	2.43	2.60	2.42	**2.48**	0.07	2.9%
5.00	5.15	5.03	4.98	4.79	5.03	**5.00**	0.13	2.6%
10.00	9.95	10.08	10.21	10.13	10.14	**10.10**	0.10	1.0%

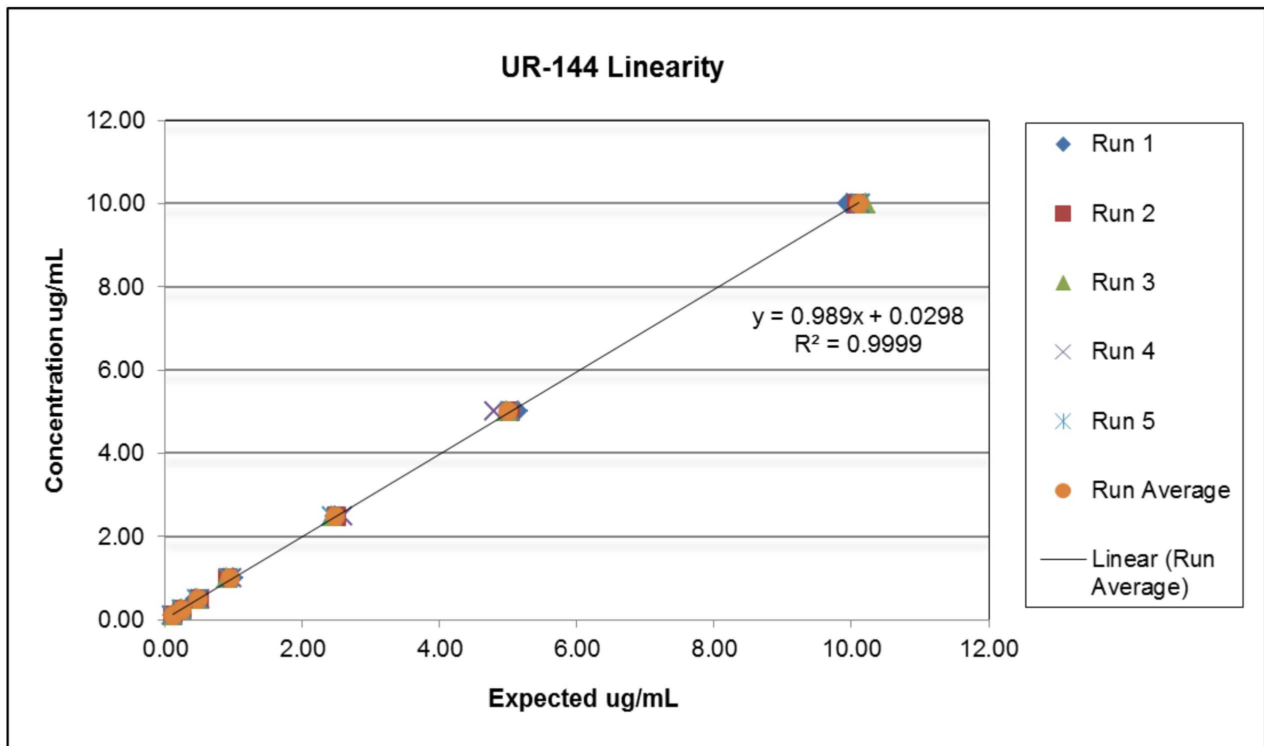


Figure 13. Linearity Plot for UR-144 (All runs included)

Table 8

Linearity Data Collected for XLR-11

	9/16/14	9/23/14	9/30/14	10/7/14	10/21/14			
Concentration	Run 1	Run 2	Run 3	Run 4	Run 5	**Average**	**SD**	**CV**
0.10	0.12	0.11	0.12	0.12	0.12	**0.12**	0.00	3.8%
0.25	0.23	0.25	0.24	0.23	0.24	**0.24**	0.01	3.5%
0.50	0.49	0.49	0.47	0.49	0.45	**0.48**	0.02	3.7%
1.00	0.95	0.93	0.94	0.91	0.99	**0.94**	0.03	3.1%
2.50	2.49	2.43	2.39	2.46	2.37	**2.43**	0.05	2.0%
5.00	5.01	4.90	5.02	5.01	5.04	**5.00**	0.06	1.1%
10.00	10.07	10.24	10.17	10.13	10.14	**10.15**	0.06	0.6%

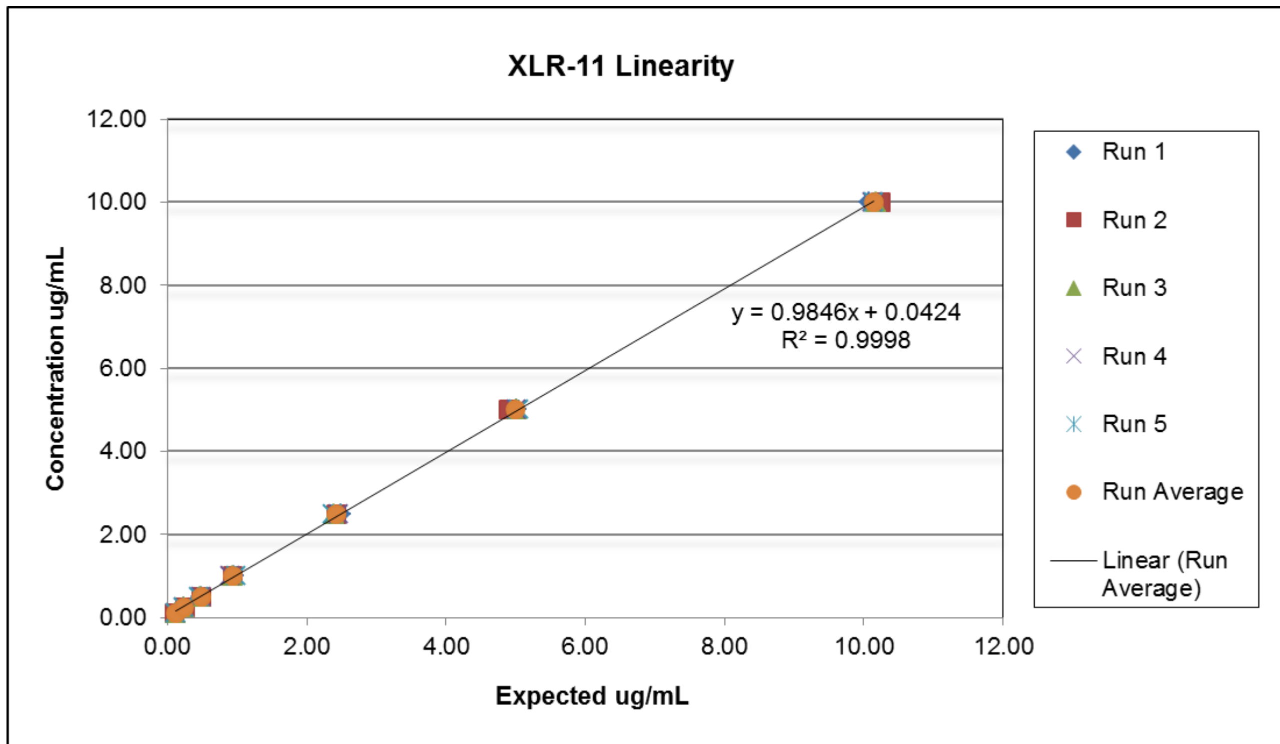


Figure 14. Linearity Plot for XLR-11 (All runs included)

Precision

Precision was calculated and compared by using the two extracted levels of QC, 0.5 ng/mL and 5.0 ng/mL, and calculating the mean, standard deviations, and percentage of coefficient variation from the rough data for five runs. The percent coefficient variation limit was cutoff at 10%. Precision was calculated between run and within run for two levels of QC. Within-run precision is calculated for each concentration separately for each of the five runs using each triplicate. Between-run is calculated from the combined data from all replicates of each concentration level. The coefficients of variation calculated for precision must be less than 10%. Between-Run data collected is included in Table 9 (a-d), within-run data is included in Appendix C.

Table 9 (a-d)

Precision Data and Bias Percentages Between-Run for all compounds

AB-Fubinaca (a)

Replicate #	Level I (0.5 ng/mL)	Level II (5.0 ng/mL)
1	0.42	4.54
2	0.47	4.39
3	0.40	4.31
4	0.42	4.70
5	0.41	4.61
6	0.41	4.47
7	0.44	4.77
8	0.41	4.28
9	0.48	4.78
10	0.40	4.38
11	0.42	4.52
12	0.39	4.55
13	0.42	4.84
14	0.44	4.59
15	0.48	4.33
Expected	0.50	5.00
Mean	0.43	4.54
SD	0.03	0.18
% CV	6.76%	3.96%
% CV Limit	<10 %	<10 %
% Bias (Avg) Level I	-14.53%	
% Bias Limit	<20 %	
% Bias (Avg) Level 2	-9.25%	
% Bias Limit	<20 %	

AB-Pinaca (b)

Replicate #	Level I (0.5 ng/mL)	Level II (5.0 ng/mL)
1	0.37	3.61
2	0.37	3.64
3	0.36	3.46
4	0.37	3.75
5	0.37	3.62
6	0.38	3.53
7	0.37	3.99
8	0.38	3.65
9	0.39	3.81
10	0.34	3.76
11	0.31	3.78
12	0.34	3.88
13	0.36	3.87
14	0.34	3.91
15	0.35	3.67
Expected	0.50	5.00
Mean	0.36	3.73
SD	0.02	0.15
% CV	5.75%	3.99%
% CV Limit	<10 %	<10 %
% Bias (Avg) Level I	-28.00%	
% Bias Limit	<20 %	
% Bias (Avg) Level 2	-25.43%	
% Bias Limit	<20 %	

UR-144 (c)

Replicate #	Level I (0.5 ng/mL)	Level II (5.0 ng/mL)
1	0.44	4.51
2	0.47	4.68
3	0.45	4.44
4	0.48	5.03
5	0.44	4.35
6	0.46	4.53
7	0.44	4.82
8	0.45	4.36
9	0.45	5.27
10	0.40	4.94
11	0.42	4.52
12	0.43	4.65
13	0.32	4.55
14	0.40	4.76
15	0.40	4.38
Expected	0.50	5.00
Mean	0.43	4.65
SD	0.04	0.27
% CV	9.09%	5.76%
% CV Limit	<10 %	<10 %
% Bias (Avg) Level I	-14.00%	
% Bias Limit	<20 %	
% Bias (Avg) Level 2	-6.95%	
% Bias Limit	<20 %	

XLR-11 (d)

Replicate #	Level I (0.5 ng/mL)	Level II (5.0 ng/mL)
1	0.43	4.42
2	0.44	4.42
3	0.45	4.17
4	0.42	4.69
5	0.44	4.33
6	0.42	4.08
7	0.44	4.50
8	0.45	4.33
9	0.44	4.52
10	0.38	4.38
11	0.42	4.54
12	0.44	4.39
13	0.42	4.47
14	0.41	4.49
15	0.39	4.25
Expected	0.50	5.00
Mean	0.43	4.40
SD	0.02	0.15
% CV	4.84%	3.49%
% CV Limit	<10 %	<10 %
% Bias (Avg) Level I	-14.80%	
% Bias Limit	<20 %	
% Bias (Avg) Level 2	-12.03%	
% Bias Limit	<20 %	

Within run precision was also calculated by taking each replicate for every drug, and calculating the highest %CV along with descriptive statistics. All % CV's were within the required limit for the method validation.

Bias

Bias percentage was calculated for each level of quality controls for each drug, requiring that bias must be less than 20%. This calculation was done by comparing the calculated value to the expected level of the control. All bias percentages were less than 20%, except for AB-Pinaca, which at the current time is an unexplained phenomenon. A secondary set of quality controls was freshly prepared after this discovery and compared to the previous used QC's in an attempt to eliminate bias. However, the calculated results for the new QC's were still on the lower range of values, similar to the previous QC. While the bias issue has been identified, due to the fact that there are no other outside sources to verify this quantification problem, the compound may still be screened using the established method. AB-Pinaca cannot be further validated for

quantification purposes due to the bias percentage lying outside of the required range. These validation criteria are based on the criteria and purposes of validation for the Office of the Chief Medical Examiner.

Carryover

Carryover was assessed by spiking a control blood sample with 100 ng of each analyte of interest, which is a ten-fold increase from the used highest calibrator sample. Defined as the appearance of unintended analyte signal in subsequent samples following a positive, a blank sample was run following the carryover spike to detect any possible contaminants. No carryover was detected on following samples or blanks.

Matrix Effects/ Ion Suppression or Enhancement

Matrix effects (ion suppression or ion enhancement) were studied using 10 synthetic cannabinoid negative, post-mortem bloods stored for method development purposes. This analysis monitors for any direct or indirect interference in the instrument response. The analyses were performed by adding 100 µL containing 50.0 ng of the working quality control as well as 100 µL containing 50.0 ng of working internal standard to whole blood blank vials, following the extraction process. These samples were compared to the "neat" vial, which contained only internal standard and 5.0 ng QC that was evaporated to dryness, and reconstituted. The matrix recovery and efficiency was calculated based upon the pre-extraction spiking recovery and the post-extraction spiking recovery. Overall, all analytes demonstrated good recovery and matrix percentages; however, UR-144 suffered from unexplainable matrix effects. The quantification ion (parent ion) as well as two qualifier ions was monitored for matrix effects when compared to the "neat" vial for pre- and post-extraction additions. Data is presented in Tables 10-13.

Table 10

AB-Fubinaca Ion Suppression/Enhancement Data for Quant and Qualifier Ions

	AB-FUBINACA (Quant)					
	Neat	**Pre-Extraction**	**Post-Extraction**	**Matrix**	**Recovery**	**Efficiency**
	44994					
Blood 1	44994	28824	36223	80.51%	79.57%	64.06%
Blood 2	44994	32569	30442	67.66%	106.99%	72.39%
Blood 3	44994	34501	33258	73.92%	103.74%	76.68%
Blood 4	44994	32639	40736	90.54%	80.12%	72.54%
Blood 5	44994	31126	41435	92.09%	75.12%	69.18%
Blood 6	44994	26694	46471	103.28%	57.44%	59.33%
Blood 7	44994	30124	48107	106.92%	62.62%	66.95%
Blood 8	44994	31346	45408	100.92%	69.03%	69.67%
Blood 9	44994	33192	41354	91.91%	80.26%	73.77%
Blood 10	44994	34870	38007	84.47%	91.75%	77.50%
			Average	89.22%	80.66%	70.21%
			Standard Dev.	12.75%	16.26%	5.64%
			% CV	14.29%	20.16%	8.03%

	AB-FUBINACA (Qual1)					
	Neat	**Pre-Extraction**	**Post-Extraction**	**Matrix**	**Recovery**	**Efficiency**
	40456					
Blood 1	40456	26125	32193	79.58%	81.15%	64.58%
Blood 2	40456	34309	29193	72.16%	117.52%	84.81%
Blood 3	40456	32607	30740	75.98%	106.07%	80.60%
Blood 4	40456	28800	36247	89.60%	79.45%	71.19%
Blood 5	40456	28699	37257	92.09%	77.03%	70.94%
Blood 6	40456	24240	43368	107.20%	55.89%	59.92%
Blood 7	40456	27351	43228	106.85%	63.27%	67.61%
Blood 8	40456	29090	41807	103.34%	69.58%	71.91%
Blood 9	40456	30150	38427	94.98%	78.46%	74.53%
Blood 10	40456	30355	35261	87.16%	86.09%	75.03%
			Average	90.89%	81.45%	72.11%
			Standard Dev.	12.52%	18.52%	7.27%
			% CV	13.77%	22.73%	10.08%

	AB-FUBINACA (Qual2)					
	Neat	**Pre-Extraction**	**Post-Extraction**	**Matrix**	**Recovery**	**Efficiency**
	48825					
Blood 1	48825	30505	37167	76.12%	82.08%	62.48%
Blood 2	48825	34309	32567	66.70%	105.35%	70.27%
Blood 3	48825	37158	35267	72.23%	105.36%	76.10%
Blood 4	48825	33891	41958	85.94%	80.77%	69.41%
Blood 5	48825	34635	41997	86.02%	82.47%	70.94%
Blood 6	48825	29166	48329	98.98%	60.35%	59.74%
Blood 7	48825	31144	50289	103.00%	61.93%	63.79%
Blood 8	48825	32779	47956	98.22%	68.35%	67.14%
Blood 9	48825	34310	42935	87.94%	79.91%	70.27%
Blood 10	48825	35957	38784	79.43%	92.71%	73.64%
			Average	85.46%	81.93%	68.38%
			Standard Dev.	12.07%	15.88%	5.10%
			% CV	14.12%	19.38%	7.46%

Table 11

AB-Pinaca Ion Suppression/Enhancement Data for Quant and Qualifier Ions

	AB-PINACA (Quant)					
	Neat	Pre-Extraction	Post-Extraction	Matrix	Recovery	Efficiency
	78984					
Blood 1	78984	54427	53922	68.27%	100.94%	68.91%
Blood 2	78984	55992	46539	58.92%	120.31%	70.89%
Blood 3	78984	62250	47675	60.36%	130.57%	78.81%
Blood 4	78984	58495	62483	79.11%	93.62%	74.06%
Blood 5	78984	50986	64946	82.23%	78.51%	64.55%
Blood 6	78984	55525	78982	100.00%	70.30%	70.30%
Blood 7	78984	51506	74605	94.46%	69.04%	65.21%
Blood 8	78984	55731	73666	93.27%	75.65%	70.56%
Blood 9	78984	60283	66626	84.35%	90.48%	76.32%
Blood 10	78984	55812	57813	73.20%	96.54%	70.66%
			Average	79.42%	92.60%	71.03%
			Standard Dev.	14.22%	20.65%	4.46%
			% CV	17.91%	22.30%	6.28%

	AB-PINACA (Qual1)					
	Neat	Pre-Extraction	Post-Extraction	Matrix	Recovery	Efficiency
	46519					
Blood 1	46519	35604	34026	73.14%	104.64%	76.54%
Blood 2	46519	35548	28842	62.00%	123.25%	76.42%
Blood 3	46519	40064	31029	66.70%	129.12%	86.12%
Blood 4	46519	37101	38529	82.82%	96.29%	79.75%
Blood 5	46519	32850	39283	84.45%	83.62%	70.62%
Blood 6	46519	64547	49937	107.35%	129.26%	138.75%
Blood 7	46519	32209	47274	101.62%	68.13%	69.24%
Blood 8	46519	34995	47618	102.36%	73.49%	75.23%
Blood 9	46519	36782	40731	87.56%	90.30%	79.07%
Blood 10	46519	36623	36321	78.08%	100.83%	78.73%
			Average	84.61%	99.89%	83.05%
			Standard Dev.	15.42%	22.01%	20.14%
			% CV	18.22%	22.04%	24.25%

	AB-PINACA (Qual2)					
	Neat	Pre-Extraction	Post-Extraction	Matrix	Recovery	Efficiency
	42768					
Blood 1	42768	31910	31721	74.17%	100.60%	74.61%
Blood 2	42768	32722	27224	63.66%	120.20%	76.51%
Blood 3	42768	35735	28540	66.73%	125.21%	83.56%
Blood 4	42768	34209	35723	83.53%	95.76%	79.99%
Blood 5	42768	29848	35937	84.03%	83.06%	69.79%
Blood 6	42768	31261	45623	106.68%	68.52%	73.09%
Blood 7	42768	30062	42999	100.54%	69.91%	70.29%
Blood 8	42768	31259	43374	101.42%	72.07%	73.09%
Blood 9	42768	33238	37699	88.15%	88.17%	77.72%
Blood 10	42768	32475	33213	77.66%	97.78%	75.93%
			Average	84.66%	92.13%	75.46%
			Standard Dev.	14.73%	19.86%	4.26%
			% CV	17.40%	21.56%	5.64%

Table 12

UR-144 Ion Suppression/Enhancement Data for Quant and Qualifier Ions

	UR144 (Quant)					
	Neat	Pre-Extraction	Post-Extraction	Matrix	Recovery	Efficiency
	193829					
Blood 1	193829	65296	48775	25.16%	133.87%	33.69%
Blood 2	193829	67550	56433	29.11%	119.70%	34.85%
Blood 3	193829	103523	51217	26.42%	202.13%	53.41%
Blood 4	193829	77520	63267	32.64%	122.53%	39.99%
Blood 5	193829	69771	95235	49.13%	73.26%	36.00%
Blood 6	193829	96877	110932	57.23%	87.33%	49.98%
Blood 7	193829	71089	64800	33.43%	109.71%	36.68%
Blood 8	193829	121453	107662	55.54%	112.81%	62.66%
Blood 9	193829	79405	43520	22.45%	182.46%	40.97%
Blood 10	193829	116206	75354	38.88%	154.21%	59.95%
			Average	37.00%	129.80%	44.82%
			Standard Dev.	12.74%	40.09%	10.82%
			% CV	34.44%	30.88%	24.14%

	UR144 (Qual1)					
	Neat	Pre-Extraction	Post-Extraction	Matrix	Recovery	Efficiency
	38732					
Blood 1	38732	20231	10514	27.15%	192.42%	52.23%
Blood 2	38732	14001	12242	31.61%	114.37%	36.15%
Blood 3	38732	21266	10758	27.78%	197.68%	54.91%
Blood 4	38732	15975	13033	33.65%	122.57%	41.24%
Blood 5	38732	14411	20289	52.38%	71.03%	37.21%
Blood 6	38732	20926	21940	56.65%	95.38%	54.03%
Blood 7	38732	14449	13255	34.22%	109.01%	37.31%
Blood 8	38732	25227	21432	55.33%	117.71%	65.13%
Blood 9	38732	16958	9846	25.42%	172.23%	43.78%
Blood 10	38732	23187	15223	39.30%	152.32%	59.87%
			Average	38.35%	134.47%	48.19%
			Standard Dev.	12.07%	42.33%	10.39%
			% CV	31.46%	31.48%	21.55%

	UR144 (Qual2)					
	Neat	Pre-Extraction	Post-Extraction	Matrix	Recovery	Efficiency
	77337					
Blood 1	77337	39247	20502	26.51%	191.43%	50.75%
Blood 2	77337	27333	24125	31.19%	113.30%	35.34%
Blood 3	77337	39631	22835	29.53%	173.55%	51.24%
Blood 4	77337	30734	27131	35.08%	113.28%	39.74%
Blood 5	77337	27877	39111	50.57%	71.28%	36.05%
Blood 6	77337	39160	45597	58.96%	85.88%	50.64%
Blood 7	77337	28625	28308	36.60%	101.12%	37.01%
Blood 8	77337	46910	44664	57.75%	105.03%	60.66%
Blood 9	77337	31728	20055	25.93%	158.20%	41.03%
Blood 10	77337	46411	32905	42.55%	141.05%	60.01%
			Average	39.47%	125.41%	46.25%
			Standard Dev.	12.44%	39.14%	9.66%
			% CV	31.52%	31.21%	20.88%

Table 13

XLR-11 Ion Suppression/Enhancement Data for Quant and Qualifier Ions

	XLR11 (Quant)					
	Neat	**Pre-Extraction**	**Post-Extraction**	**Matrix**	**Recovery**	**Efficiency**
	116343					
Blood 1	116343	84507	54306	46.68%	155.61%	72.64%
Blood 2	116343	84427	60954	52.39%	138.51%	72.57%
Blood 3	116343	105881	52826	45.41%	200.43%	91.01%
Blood 4	116343	86527	66976	57.57%	129.19%	74.37%
Blood 5	116343	80782	82277	70.72%	98.18%	69.43%
Blood 6	116343	93234	104862	90.13%	88.91%	80.14%
Blood 7	116343	78247	74020	63.62%	105.71%	67.26%
Blood 8	116343	100216	97612	83.90%	102.67%	86.14%
Blood 9	116343	81482	54223	46.61%	150.27%	70.04%
Blood 10	116343	103022	77335	66.47%	133.22%	88.55%
			Average	62.35%	130.27%	77.21%
			Standard Dev.	15.73%	33.58%	8.62%
			% CV	25.23%	25.78%	11.16%

	XLR11 (Qual1)					
	Neat	**Pre-Extraction**	**Post-Extraction**	**Matrix**	**Recovery**	**Efficiency**
	36520					
Blood 1	36520	28518	17410	47.67%	163.80%	78.09%
Blood 2	36520	27254	19561	53.56%	139.33%	74.63%
Blood 3	36520	34339	17797	48.73%	192.95%	94.03%
Blood 4	36520	28799	22029	60.32%	130.73%	78.86%
Blood 5	36520	26376	25927	70.99%	101.73%	72.22%
Blood 6	36520	29539	34972	95.76%	84.46%	80.88%
Blood 7	36520	24759	23589	64.59%	104.96%	67.80%
Blood 8	36520	32327	30347	83.10%	106.52%	88.52%
Blood 9	36520	24926	17909	49.04%	139.18%	68.25%
Blood 10	36520	32827	23213	63.56%	141.42%	89.89%
			Average	63.73%	130.51%	79.32%
			Standard Dev.	15.88%	32.41%	9.09%
			% CV	24.91%	24.84%	11.47%

	XLR11 (Qual2)					
	Neat	**Pre-Extraction**	**Post-Extraction**	**Matrix**	**Recovery**	**Efficiency**
	46415					
Blood 1	46415	34204	22077	47.56%	154.93%	73.69%
Blood 2	46415	33010	24778	53.38%	133.22%	71.12%
Blood 3	46415	42392	21488	46.30%	197.28%	91.33%
Blood 4	46415	34744	27496	59.24%	126.36%	74.86%
Blood 5	46415	32174	33140	71.40%	97.09%	69.32%
Blood 6	46415	36998	43372	93.44%	85.30%	79.71%
Blood 7	46415	31438	30799	66.36%	102.07%	67.73%
Blood 8	46415	40547	40031	86.25%	101.29%	87.36%
Blood 9	46415	33019	22210	47.85%	148.67%	71.14%
Blood 10	46415	41626	30231	65.13%	137.69%	89.68%
			Average	63.69%	128.39%	77.59%
			Standard Dev.	16.34%	33.73%	8.86%
			% CV	25.65%	26.27%	11.42%

Stability and Degradation Results

Stability samples were run on each of the study days for the three temperature conditions in triplicate. The data was then pooled and descriptive statistics such as mean, standard deviation, and the coefficient of variation was calculated for each drug in the appropriate condition. These results were graphed to assess degradation trending, so that the order of kinetic degradation along with half-life or "shelf life" could be determined for each drug. The percentage of degradation was also calculated based on the initial concentrations of each analyte from the Study Day 0 findings. Below is an example MRM chromatogram of the 10.0 ng/mL standard to demonstrate peak shapes and elution times, as well as ion monitoring modes for AB-Fubinaca, AB-Pinaca, XLR-11, and UR-144, along with the two deuterated internal standards. Deuterated internal standards, AB-Pinaca-d_9 and XLR-11-d_5, will elute at the same or close retention time to their similar compounds. Because of the highly specific and sensitive monitoring of the specific ionization ranges for each compound, there is a small window for retention time for each compound.

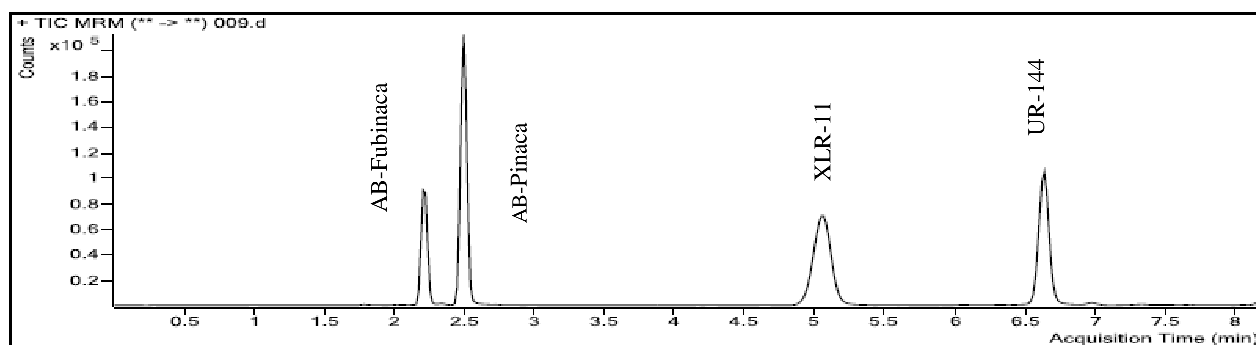
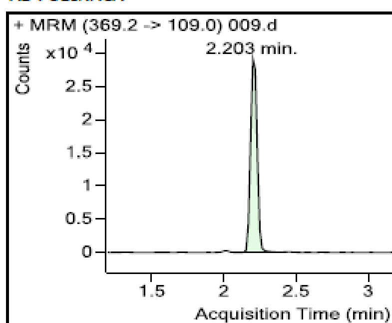
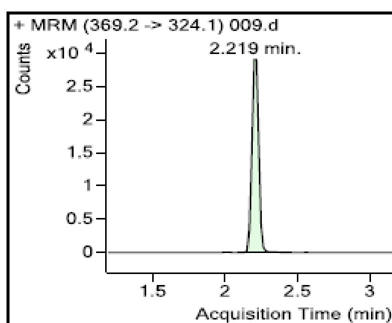
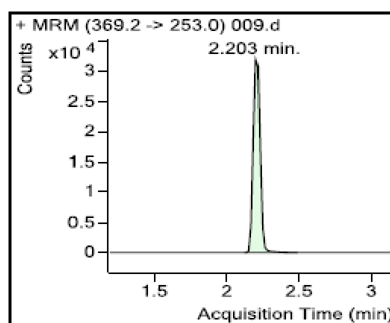


Figure 15. Total Ion Chromatogram for the 10.0 ng/mL standard mixture

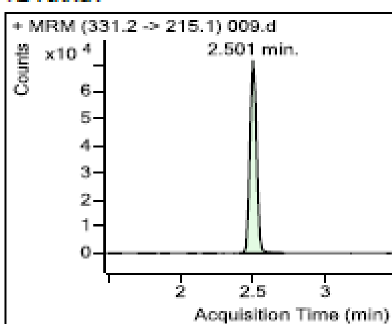
AB-FUBINACA

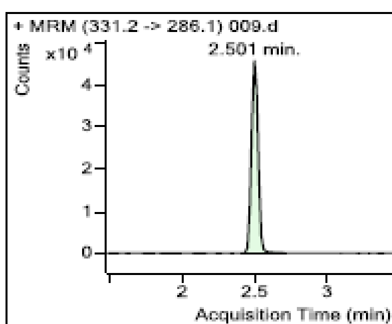


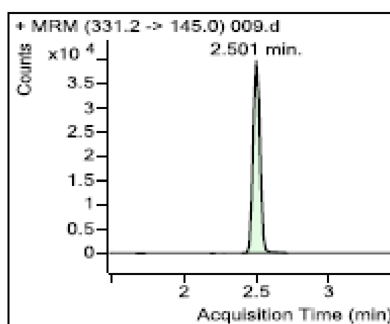




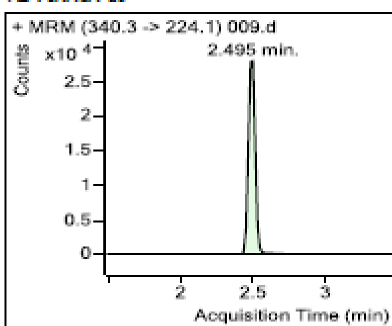
AB-PINACA

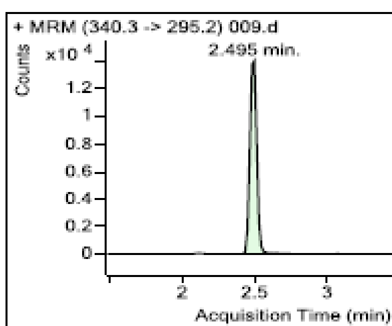


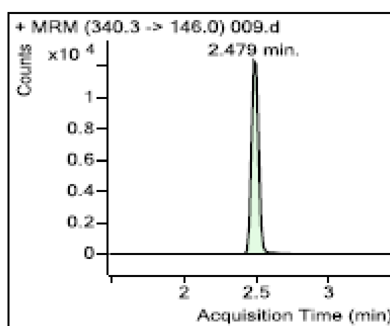




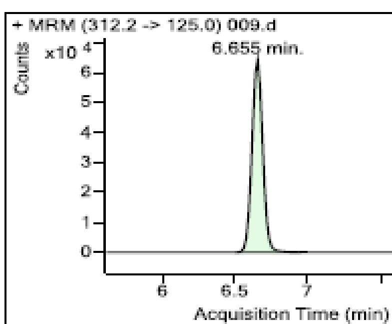
AB-PINACA-d9

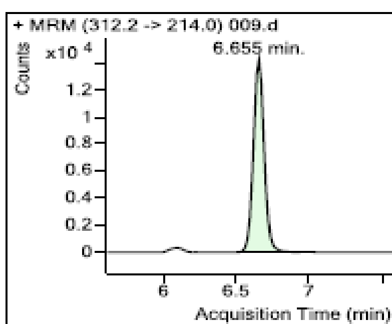


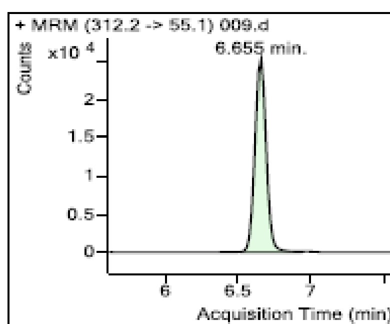




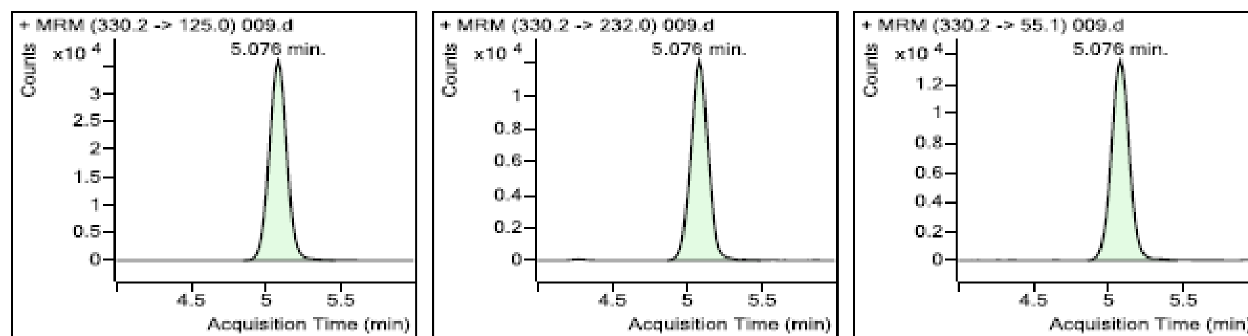
UR144







XLR11



XLR11-d5

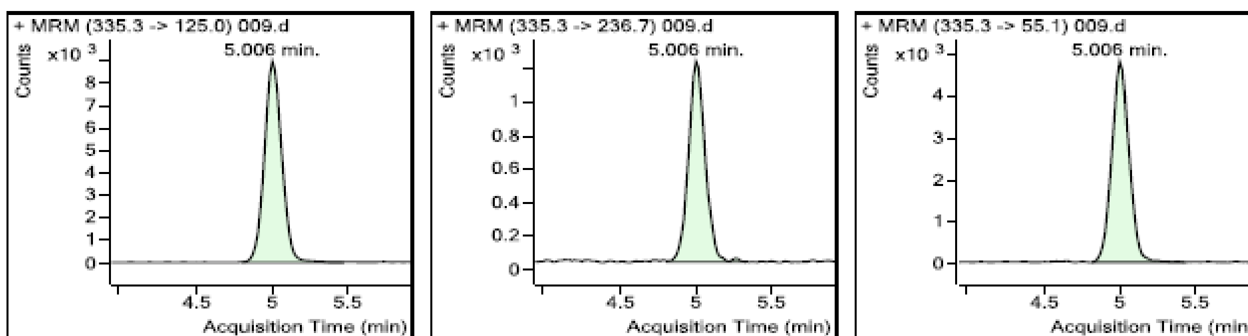


Figure 16. Parent and daughter ion peaks for 10.0 ng Standard for all MRM

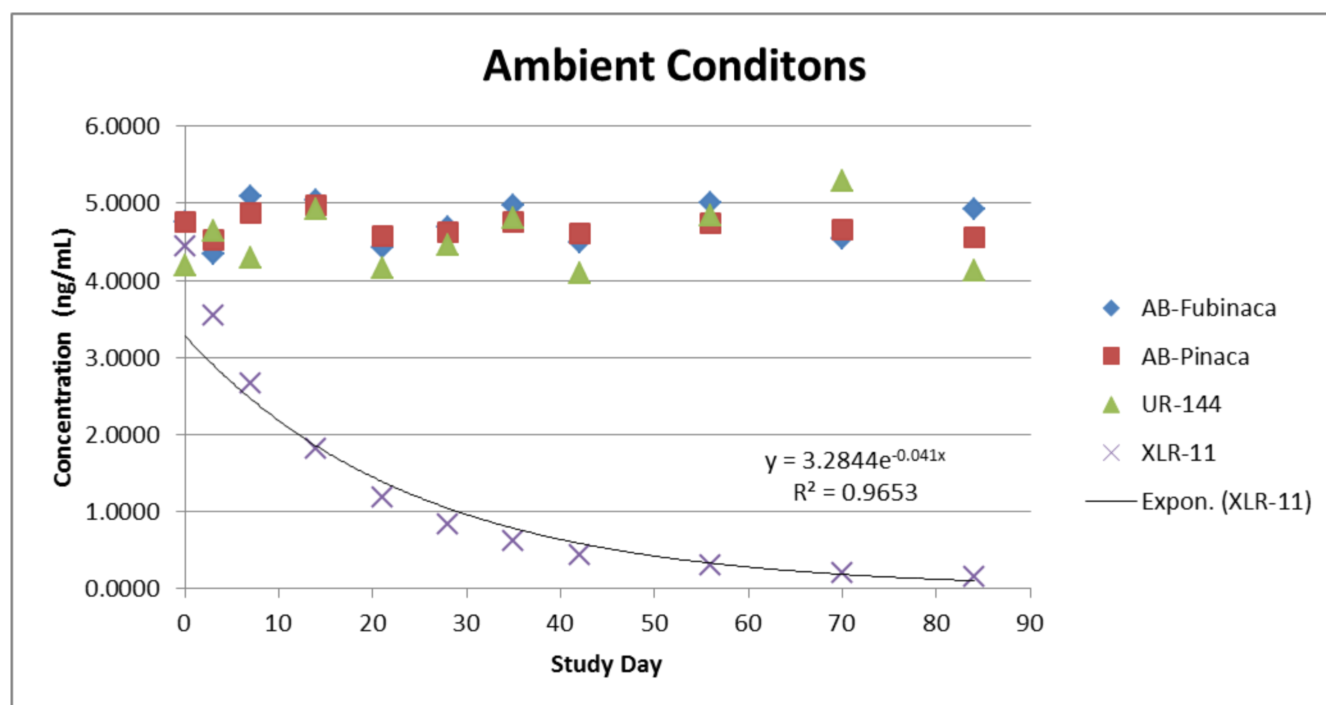


Figure 17. Ambient Temperature Storage Data

From the data plot above for ambient conditions (22°C), it can be noted that the trendline for XLR-11 follows a first-order decay scheme based on the exponential trendline. The equation for the line given can be used to extrapolate the approximate day that XLR-11 would reach the LOQ established for the study. Based on this knowledge, the time it would take for XLR-11 to reach 0.10 ng/mL, or the established LOQ for the study method would be 85.16 days, just shortly after the end of study. All of the drugs showed to be relatively stable in ambient conditions. XLR-11 was the only drug that showed significant degradation at ambient conditions. First-order reaction rates do not vary with increasing or decreasing reactant concentrations. The rate of the degradation reaction is equal to the constant of the reaction. An approximate half-life or "shelf-life" can also be calculated from the degradation generated using the equation: $N_t = N_0(1/2)^{t/t1/2}$. The equation variables indicate that N_t is the quantity remaning after a time (t), N_0 is the initial quantity of analyte, and $t_{½}$ is the half life. XLR-11 in ambient conditions has an approximate

half-life of 17.19 days under these conditions until half of the initial concentration is eliminated. This calculation is based on the initial concentration of analyte, the final concentration, and the time point since the initial, which was the 84th final study day.

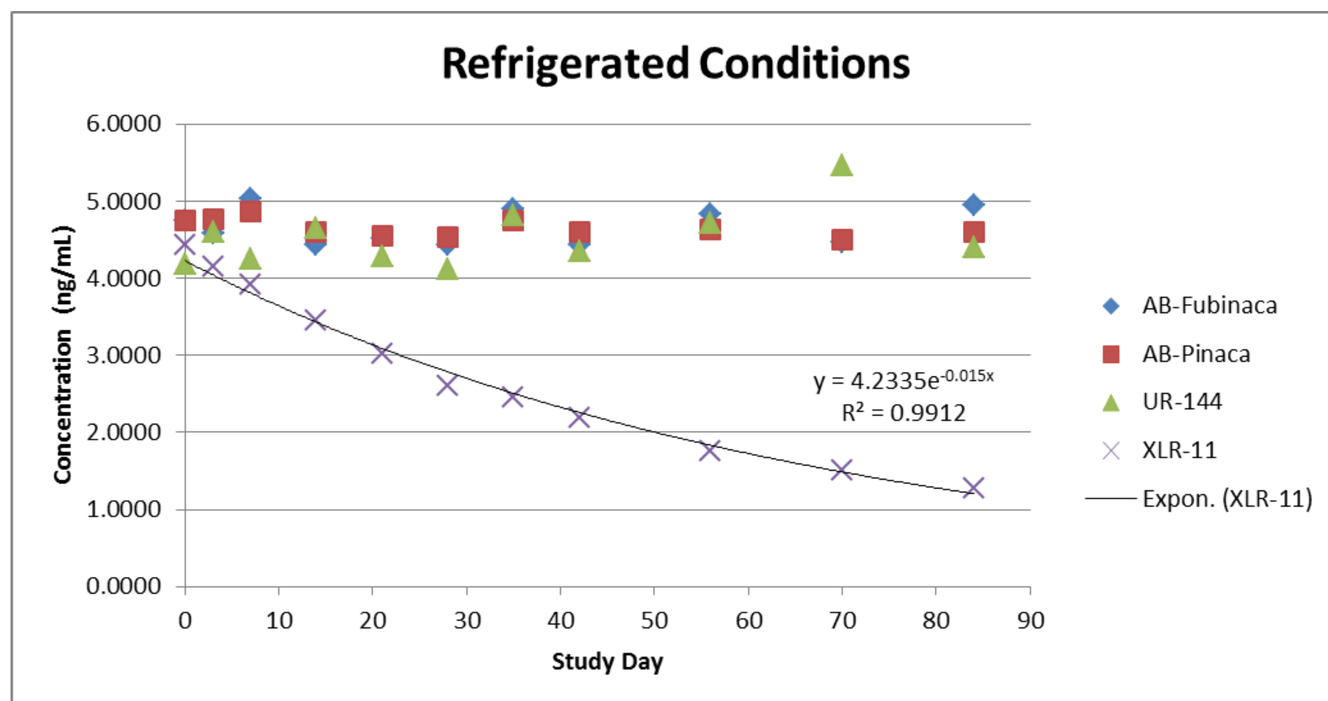


Figure 18. Refrigerated Storage Condition Data

Refrigerated conditions suffered from less degradative effects, however XLR-11 was still degrading at refrigerated conditions (4°C). Based on the exponential equation of the trendline for XLR-11 data in refrigerated conditions, the approximate time to reach the LOQ was calculated. The time required to reach 0.10 ng/mL would be 249.71 days. The approximated half-life calculated for XLR-11 under refrigerated conditions would be 26.99 days, longer than ambient conditions.

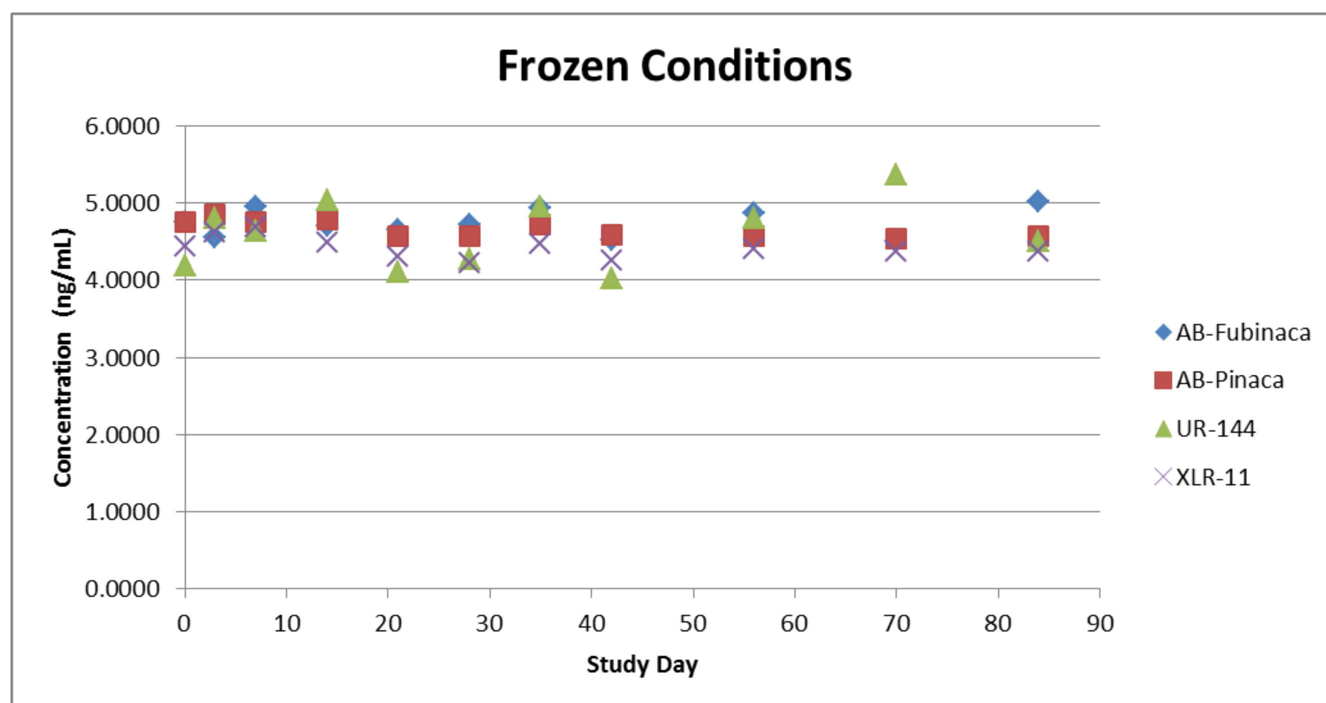


Figure 19. Frozen Storage Condition Data

Frozen conditions (-20°C) showed stabilization of all analytes, which results in it being the suggested medium for storage of biological samples containing suspected synthetic cannabinoids.

The percentages of degradative loss were calculated based on the initial Day 0 ambient concentrations for all of the monitored compounds. These calculations were based on the averaged concentrations, per analyte, for each condition at the 3, 6, and 12 week time points. The percentages of degradative loss are insignificant for all analytes except XLR-11, which clearly suffers from large degradative loss percentages early in the study at ambient and refrigerated temperatures.

Table 14.

Percent Loss Calculated Table for all compounds of interest

	% Loss		Week 3		Week 6		Week 12
AB-Fubinaca	Ambient		6.92%		5.55%		-3.42%
	Refrigerated		5.03%		6.82%		-3.93%
	Frozen		2.16%		4.97%		-5.55%
AB-Pinaca	Ambient		4.09%		3.18%		4.18%
	Refrigerated		4.19%		3.43%		3.28%
	Frozen		3.97%		3.71%		3.91%
UR-144	Ambient		1.01%		2.28%		1.63%
	Refrigerated		-2.34%		-3.70%		-4.92%
	Frozen		1.91%		4.23%		-7.50%
XLR-11	Ambient		**73.07%**		**89.95%**		**96.61%**
	Refrigerated		**31.76%**		**50.59%**		**71.12%**
	Frozen		**2.91%**		**4.11%**		**1.27%**

Discussion

Stability studies have been used for a great deal of time to determine shelf life for prescription or illicit drugs, proving to be advantageous for the chemist or toxicologist dealing with stability of samples. In further detail, drug stability can be altered or affected by many different conditions which include characteristics of the matrix, physicochemical properties, metabolism pathways, container selection, addition of preservatives, and storage temperature. Furthermore, drug stability can suffer even more in biological matrices, especially when post-mortem processes due to putrefaction and microbial invasion interfere. However, most forensic laboratories deal with ante-mortem or post-mortem samples, leaving the biological properties a common factor when understanding how drugs or analytes degrade in the specimen. Controlling the highest number of possible variables is crucial to conducting stability studies. Storage conditions are usually the most crucial variable, and are commonly used for drug stability studies. Understanding the stability or instability of analytes and how they are impacted by temperature or storage condition, provides the chemist or toxicologist with knowledge as to how

specimens should be treated, under what conditions, and what time window they have to work with, before detrimental losses to the analyte are encountered.

The four compounds chosen for the project were recently noticed in forensic physical evidence sampling at the Office of the Chief Medical Examiner, and were also lacking of published data regarding their stability; therefore, AB-Fubinaca, AB-Pinaca, XLR-11, and UR-144 were chosen to be monitored. One of the outcomes to this project, along with the stability data generated, was the validated procedure used to extract and identify the four investigated synthetic cannabinoid compounds. All of the compounds were tested and analyzed in the areas of linearity, bias, precision, matrix effects, LOD/LOQ, bench top stability, specificity, and carryover. Establishing and validating the extraction method used for this project was necessary due to the fact that previous extraction methods used did not provide good recovery for all analytes. A forward Foerster-Mason alkaline extraction was used first in experiment validation exploration. While this method facilitated extraction of the four synthetic cannabinoids monitored in the project with good recovery, in our extraction, when analyzing post-mortem samples provided by the OCME laboratory that were a year or older suffered greatly on recovery due to intrinsic extraction process. We feel this is due to poor specimen quality and an extraction solvent not conducible for all compounds.

A different extraction technique was selected for use in the present study after the publication of a recent article by Shanks et al. (2014). They used a sodium carbonate buffer and 98:2 hexane: ethyl acetate as the organic phase, while also using post-mortem whole blood in their experimental work. Since the compounds they studied are similar to AB-Fubinaca and ADB-Pinaca, it was hoped that this method would also work for the project analytes of interest. When analyzing for matrix effects in the validation process, it was found that only UR-144

suffered slightly from matrix effects, so the hexane: ethyl acetate ratio was changed to 80:20 to accommodate polarity differences. Using a hexane and ethyl acetate organic solvent involves a non-polar and polar organic phase. The established 80:20 ratio created a solvent that solves polarity differences between the four compounds at different ratios. All recovery data was shown to be highly effective for all four compounds monitored. This demonstrates the efficacy of the new optimized extraction method as well as its efficiency.

Specificity testing, also known as interference, was conducted by taking multiple analytes through the optimized extraction process to see if any interferences caused problems in the ability to detect, identify, or quantitate a targeted analyte. This specificity testing was done by spiking whole blood samples with a selected series of twenty alkaline drugs and nine acidic neutral drugs (Table 3), as well as 15 other synthetic cannabinoid compounds including: JWH-250, JWH-200; HU-211, (±)-CP 47,497, (±)-CP 47,497 C8 homologue, JWH-019, JWH-122, JWH-081, AM-2201, RCS-4, RCS-8, JWH-015, JWH-203, JWH-210, and AM2233. No interferences were detected for any of the tested drugs with the ion monitoring modes used with the LC-MS/MS method.

Ionization suppression or enhancement studies were applicable to the method used for the research effort since the technique utilized specific ionization technology typically used in LC-MS/MS analyses. These effects were studied by contrasting pre- and post-extraction recoveries for the three ions monitored in the experimental method. Pre- and post-extraction recoveries were compared to a “neat” vial. The “neat” vial was an un-extracted pure sample containing no blood matrix, only 5.0 ng/mL of QC and 5.0 ng/mL of internal standards, evaporated to dryness, then reconstituted. From this comparison, matrix percentage, recovery percentage and efficiency percentage were calculated. All drug matrix, recovery, and efficiency percentages were within

OCME required limits as required for validation data except UR-144. The matrix percentage was extremely low, which therefore impacted the recovery percentage. While it is unknown why UR-144 suffered from matrix effects more greatly than the other analytes of interest, it should be explored further for method validation purposes.

Limit of detection (LOD) as well as the limit of quantitation (LOQ) was assessed after establishing the calibration model. The LOD is the lowest concentration of an analyte that can be differentiated from a blank matrix, whereas the LOQ is the lowest concentration that can be measured within an acceptable bias and precision. The calibration model used was a1/x weighted linear calibration, with concentrations ranging from 0.1 to 10 ng/mL. A low concentration range calibration curve was used instead of the commonly used drugs of abuse curves reported at micrograms per milliliter; further reasoning being that often synthetic cannabinoids are quantitated in blood specimens at low quantitated amounts such as 1 ng/mL or less. Showing degradation at lower level limits (0.1-10 ng/mL) is more sensitive and applicable to current forensic casework. With the established limit of quantitation being 0.1 ng/mL, a lower limit was explored for the limit of detection. It was found that the limit of detection was 0.025 ng/mL, meeting the criteria that at least one parent ion and one daughter or qualifier ion integrated properly, as well as having a signal to noise ratio of 3:1. The LOD and LOQ criteria are set by SWGTOX and required at the OCME Laboratory.

Linearity is based upon the calibration model established at the research project initiation. This model requires at least six different non-zero concentrations to establish the model, and also requires a minimum of five replicates per concentration. Linearity was calculated based on the equation of the line and requires an R^2 value greater than 0.900. Linearity was greater than 0.999 for all of the quantitated analyte compounds of interest.

Precision is defined as the measure of closeness of agreement between a series of measurements obtained from multiple samplings of the same homogenous sample. There are two differing precision calculations, those that are within-run and between-run. Within-run precision is calculated for each concentration separately for each of the five runs using each triplicate used. Between-run is calculated from the combined data from all replicates of each concentration level. It is required that the coefficients of variation for all precision data be lower than 10%. All compounds of interest met the coefficient of variation validation criteria, having a % CV less than 10%.

Bias studies must be carried out for all quantitative models, as required by SWGTOX. These are usually partnered along with precision, contrasting the levels of control with their respective expected concentration. Bias validation requires a coefficient of variation less than 20%. For AB-Fubinaca, XLR-11, and UR-144 this criteria was sufficiently met; however, AB-Pinaca suffered from bias differences despite meeting precision criteria. The bias percentages for the two levels of control were greater than the required <20%. This is currently an unexplained phenomenon and will need to be investigated further for future studies involving AB-Pinaca.

For the stability portion of the study, at initial 5 ng/mL fortified concentrations, the ambient temperature condition proved to be the most variable for all compounds, with XLR-11 suffering from the most stability fluctuations and degradative losses. Other compounds were stable at ambient and refrigerated conditions, while XLR-11 suffered from first-order degradation. The frozen storage condition was the most stable condition overall, with all analytes proving to be stable at their initial fortified 5 ng/mL concentration. Frozen storage conditions, for blood in specific, would be encouraged for all synthetic cannabinoid compounds for ultimate stability.

AB-Fubinaca was relatively stable throughout the entire study. Suffering from only slight degradative losses, frozen conditions remained the most stable throughout the twelve week period. Ambient and refrigerated conditions experienced an approximate 7% decrease or fluctuation in concentration, while frozen samples experienced a 5% loss overall. This is a small percentage, but does demonstrate that samples of this synthetic cannabinoid preserve well at ambient and refrigerated conditions, and best overall at frozen conditions.

While being structurally similar to AB-Fubinaca, AB-Pinaca remained even more stable under all storage conditions. Throughout the first six week period, after which this analyte dropped below the acceptable bias parameter, AB-Pinaca only suffered less than 5% of degradative loss. Being the most stable in frozen conditions, AB-Pinaca was very stable in all temperature conditions throughout the entire study while also having the smallest replicate coefficient of variation. This demonstrates that AB-Pinaca is extremely stable in biological specimens, especially blood, for twelve weeks and in multiple storage conditions.

UR-144 remained relatively stable throughout the twelve weeks of testing. Overall, the UR-144 compound had very small percentages of degradative loss throughout the study, but suffered from the most concentration fluctuation over time. While stability remained around the initial established 4.2 ng value, UR-144 showed changes in concentration over the time period, which is reflected in the coefficients of variation calculated for each analysis time point. However, the stability of UR-144 remained constant throughout the entirety of the study, favoring the storage conditions of refrigerated or frozen temperatures. No significant degradative losses were encountered in UR-144 concentrations for the three storage conditions, and fluctuations in concentration could be contributed to the matrix effects encountered in the validation portion of study.

Fluctuations in overall concentration can be observed for all of the studied compounds. However, these fluctuations are parallel for each compound, suggesting the effects that human handling variability adds to the procedure. Human error, pipetting discrepancies, etc. can all add to variability for the overall concentration of the analyte. However, these fluctuations were not detrimental to the experiment as a whole or statistically significant in magnitude.

XLR-11 was the most unstable compound under ambient and refrigerated temperature conditions. While Kacinko (2011) showed that four independent JWH compounds were relatively stable for 30 days under ambient conditions, XLR-11 suffered approximately 73% degradative loss in the first three weeks of analysis in the ambient temperature conditions, demonstrating that not all synthetic cannabinoid compounds are relatable. Further so, 32% of the initial compound was lost under refrigerated conditions during the first three- week period. Therefore, it cannot be easily concluded that all synthetic cannabinoid compounds are relatively stable for thirty days. Even further, after a six week period, XLR-11 suffered dropout at ambient and refrigerated conditions of 90% and 51%, respectively. At the final twelve week time point, nearly 97% of the initial ambient XLR-11 was lost, 71% being lost in the refrigerated sample. Frozen conditions proved to be the only way for storage of biological specimens containing XLR-11 for optimal preservation. Frozen conditions suffered less than 5% of degradative losses. From the data plots, it is observed that XLR-11 follows first-order pharmacokinetic degradation. First-order reactions proceed at a rate that depends on the concentration of one reactant. These reactions follow exponential decay, which was expressed by XLR-11. The results of this study suggest that any specimens possibly containing XLR-11 should be frozen upon receipt, since refrigerated conditions resulted in significant losses.

The calculated values for XLR-11 half-life and time until LOQ reached are very useful to determine the shelf-life of XLR-11 at different temperature conditions, as well as determining approximately how long until the bottom of the concentration level is reached before dropping beneath the quantitation limit. The half-life of XLR-11 in ambient conditions was determined to be approximately 17 days. This is the time until half of the initial concentration has been lost. This is an unusually short amount of time to process forensic casework before the possible evidentiary concentration is affected significantly. The time until the LOQ was reached was calculated to be 85 days, just one day after the completion of the stability timeline. Evidence samples stored under ambient conditions must be processed in a timely manner upon receipt so that concentrations are not detrimentally affected. Samples stored under refrigerated conditions fared better when compared to ambient conditions, but significant loss could still be encountered since the estimated half-life under refrigerated conditions is 27 days. It would take 250 days for XLR-11 to reach the LOQ, which is significantly longer than ambient conditions; however, it would still be recommended for biological samples containing XLR-11 to be stored under frozen conditions.

The stability of XLR-11 compared to UR-144 was originally hypothesized to be relatively comparable due to their structural similarities; however, this was not the case. While UR-144 proved to be stable under multiple storage conditions, XLR-11 suffered significant compound degradation. While these two compounds are somewhat structurally similar, both containing the tetramethylcyclopropyl group, XLR-11 exists as the halogenated form of UR-144, adding the 5-fluoropentyl chain to the aminoalkylindole backbone. No current published data to support whether the addition of the fluoropentyl chain would influence stability is known to this study.

An interesting trend may have been observed in this study in the area of halogenated compounds versus non-halogenated compounds. AB-Fubinaca and AB-Pinaca both exist with the same similar indazole backbone, but differ with the addition of contrasting functional groups. AB-Fubinaca includes the addition of the fluorobenzene moiety, whereas AB-Pinaca includes the carboxamide moiety. While AB-Fubinaca did not suffer from large stability effects, it did degrade more than AB-Pinaca. These degradative effects were not statistically significant or detrimental to the overall compound stability, but it is something that could be investigated further. This is especially true for contrasting XLR-11 and UR-144, which also involves the comparison of halogenated and non-halogenated compounds. XLR-11 clearly suffered from degradation, while UR-144 remained stable. As previously mentioned, XLR-11 is a halogenated compound containing the 5-fluoropentyl chain whereas UR-144 contains no halogen in this position. Considering these two compounds are so closely related also, it is intriguing to know whether the halogenation alters the stability of these compounds in blood, or if another interference or effect is contributing to the stability losses and effects of XLR-11.

Summarizing, AB-Pinaca, AB-Fubinaca, and UR-144 remain fairly stable in whole blood for extended amounts of time regardless of storage condition. XLR-11 is extremely sensitive to temperature conditions and degradative effects and must therefore be stored in appropriate conditions for optimal preservation. All compounds suffered from increased fluctuations at ambient temperatures, which is why it should be suggested that biological samples suspected of containing synthetic cannabinoids be stored, at least, in refrigerated or preferably in frozen conditions. Frozen conditions preserved XLR-11 and kept this compound stable from degradation. The underlying issue addressed in this study is that the stability of the synthetic cannabinoids studied can be time sensitive. Forensic testing and analysis should be done in a

timely manner, due to the fact that XLR-11 is extremely sensitive to degradative losses and can be lost completely from evidentiary samples if not handled properly.

Limitations

When evaluating the results of this project, there are several things to keep in mind that influence the research and its interpretation. Firstly, the parameters of the project included only four synthetic cannabinoid compounds, when in actuality there are hundreds that have been discovered. Unpublished data from the OCME and OSBI laboratories indicate that these four compounds are the most recently encountered in forensic casework and were therefore selected as the most relevant to be studied. The stability of other synthetic cannabinoids is largely unknown, aside from those mentioned over the course of this narrative.

Another limitation of this study is that the monitoring period was 12 weeks. This time period was longer than other previously published stability studies associated with synthetic cannabinoids; however, stability beyond twelve weeks was not monitored. Due to the relevance of applying the findings to the fields of forensic chemistry and toxicology, case prioritization schemes would hopefully be analyzed within the 12 week period monitored in the study. According to 2009 statistics provided from the U.S. Department of Justice (2012), publically-funded forensic crime laboratories in the toxicology section have backlog cases (cases not completed after 30 days) of less than 3%. Thus whether significant degradative losses subsequently occurred after the testing period was completed is not known.

This project also only dealt with the processing and extracting of human blood. Often in forensic toxicology or chemistry laboratories, other specimens such as serum or plasma, urine, or

tissues are submitted for analysis. This study only validates and details a method for the analysis of the four mentioned synthetic cannabinoids in human whole blood.

Suggestions for Further Research

While the synthetic cannabinoid is quickly evolving, it is hoped that soon these drugs will no longer be the “trending” synthetic drug market. As with other drugs throughout history, drug popularity changes over time. However, the growing market of compounds for synthetic cannabinoids cannot be ignored. As new compounds are detected in forensic casework, degradation information may be useful in particular for forensic caseload prioritization. With that being said, similar studies to this one will undoubtedly need to be conducted related to the new compounds.

The pursuit of chemical structure differences and how they may or may not influence stability could also be suggested. As seen in this study, halogenated compounds suffered from more degradation than their non-halogenated related compounds. While there hasn’t been any evidence of this in other publications, synthetic cannabinoid stability in biological matrices has not been studied in depth. Greater understanding of the field of illicit synthetic drug chemistry would be of benefit to forensic science.

Conclusion

This study has demonstrated the analytical issues that can be faced when encountering synthetic drugs in the forensic laboratory. An analytical method was developed and validated for analyzing synthetic cannabinoid compounds in human whole blood, as well as generating vital stability data for the four studied synthetic cannabinoid compounds. The validation phase of the project was necessary not only for method development, but also for OCME usage to establish a

working method for analysis of these synthetic cannabinoids. Validation also demonstrates the efficiency and accuracy of the method for the three validated compounds, AB-Fubinaca, XLR-11, and UR-144. The validation criteria for AB-Pinaca were all met with the exception of bias; however, any further exploration into this issue was beyond the scope of this project. Stability projects are vital for forensic chemists and toxicologists for understanding analyte stability in different variable conditions. Due to the lack of stability data published for synthetic cannabinoids, the information generated will be useful for those analyzing these compounds. We found that out of the four synthetic cannabinoid compounds studied, AB-Fubinaca, AB-Pinaca, and UR-144 demonstrated good stability in all temperature conditions. XLR-11 was found to have accelerated degradation in ambient conditions and refrigerated conditions, suffering from 97% loss and 71% loss respectively for each condition. By demonstrating the relatively rapid degradation of XLR-11, analysis for synthetic cannabinoids can be time sensitive. Due to storage temperature conditions and molecular differences in structure, significant degradation can be encountered when analyzing for synthetic cannabinoids in biological samples. Because of the prevalence of these drugs in the synthetic drug market and across the globe, this information will be imperative to those in the laboratory analyzing for these compounds and also for law enforcement handling possible biological evidence samples. It is clear that biological samples containing synthetic cannabinoids should be refrigerated, if not frozen, upon receipt and analyzed quickly to prevent any possible degradative losses and to ultimately preserve the evidentiary integrity of that specimen.

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Appendix A –Quality Control Data and Descriptive Statistics

	8/22/2014		8/26/2014		8/29/2014		9/2/2014		9/9/2014		9/16/2014	
	0.5ng	5.0 ng	0.5ng	5.0 ng	0.5ng	5.0 ng	0.5ng	5.0 ng	0.5ng	5.0 ng	0.5ng	5.0 ng
AB-Fubinaca	0.40	4.58	0.42	4.56	0.45	4.79	0.44	5.22	0.44	4.69	0.42	4.54
	0.45	4.40	0.44	4.44	0.46	4.52	0.43	4.91	0.39	4.65	0.47	4.39
	0.40	4.22	0.38	4.62	0.47	4.70	0.43	4.88	0.40	4.20	0.40	4.31
AB-Pinaca	0.41	3.94	0.38	3.88	0.38	4.07	0.37	4.16	0.36	3.96	0.37	3.61
	0.36	3.85	0.35	3.78	0.38	3.93	0.39	3.85	0.35	3.98	0.37	3.64
	0.37	3.62	0.38	3.77	0.39	3.91	0.37	3.97	0.34	3.67	0.36	3.46
UR-144	0.45	4.73	0.44	4.87	0.44	5.23	0.45	5.16	0.42	6.30	0.44	4.51
	0.46	4.67	0.45	4.80	0.46	5.39	0.45	5.08	0.52	6.44	0.47	4.68
	0.46	4.79	0.47	4.70	0.45	5.17	0.48	5.12	0.52	6.32	0.45	4.44
XLR-11	0.46	4.39	0.41	4.43	0.44	4.73	0.44	4.87	0.42	4.28	0.43	4.42
	0.43	4.39	0.45	4.56	0.44	4.78	0.43	4.73	0.44	4.46	0.44	4.42
	0.42	4.24	0.43	4.47	0.46	4.65	0.44	4.66	0.43	4.23	0.45	4.17

STATISTICS	8/22/2014		8/26/2014		8/29/2014		9/2/2014		9/9/2014		9/16/2014	
Average	0.42	4.40	0.41	4.54	0.46	4.67	0.43	5.00	0.41	4.51	0.43	4.41
SD	0.03	0.18	0.03	0.09	0.01	0.14	0.01	0.19	0.03	0.27	0.04	0.12
CV	6.93%	4.09%	7.39%	2.02%	2.17%	2.94%	1.33%	3.76%	6.45%	6.03%	8.39%	2.65%
Average	0.38	3.80	0.37	3.81	0.38	3.97	0.38	3.99	0.35	3.87	0.37	3.57
SD	0.03	0.17	0.02	0.06	0.01	0.09	0.01	0.16	0.01	0.17	0.01	0.10
CV	6.96%	4.34%	4.68%	1.60%	1.51%	2.20%	3.07%	3.91%	2.86%	4.48%	1.57%	2.70%
Average	0.46	4.73	0.45	4.79	0.45	5.26	0.46	5.12	0.49	6.35	0.45	4.54
SD	0.01	0.06	0.02	0.09	0.01	0.11	0.02	0.04	0.06	0.08	0.02	0.12
CV	1.26%	1.27%	3.37%	1.78%	2.22%	2.16%	3.77%	0.78%	11.86%	1.19%	3.37%	2.72%
Average	0.44	4.34	0.43	4.49	0.45	4.72	0.44	4.75	0.43	4.32	0.44	4.34
SD	0.02	0.09	0.02	0.07	0.01	0.07	0.01	0.11	0.01	0.12	0.01	0.14
CV	4.77%	2.00%	4.65%	1.48%	2.59%	1.39%	1.32%	2.25%	2.33%	2.80%	2.27%	3.33%

	9/23/2014		9/30/2014		10/8/2014		10/21/2014		11/4/2014		11/18/2014	
	0.5ng	5.0 ng	0.5ng	5.0 ng	0.5ng	5.0 ng	0.5ng	5.0 ng	0.5ng	5.0 ng	0.5ng	5.0 ng
AB-Fubinaca	0.42	4.70	0.44	4.77	0.40	4.38	0.42	4.84	0.47	4.32	0.47	4.75
	0.41	4.61	0.41	4.28	0.42	4.52	0.44	4.59	0.41	4.25	0.47	4.81
	0.41	4.47	0.48	4.78	0.39	4.55	0.48	4.33	0.45	4.07	0.41	4.61
AB-Pinaca	0.37	3.75	0.37	3.99	0.34	3.76	0.36	3.87	0.36	3.70	0.36	3.80
	0.37	3.62	0.38	3.65	0.31	3.78	0.34	3.91	0.33	3.67	0.36	3.66
	0.38	3.53	0.39	3.81	0.34	3.88	0.35	3.67	0.36	3.64	0.38	3.68
UR-144	0.48	5.03	0.44	4.82	0.40	4.94	0.32	4.55	0.30	5.98	0.46	4.77
	0.44	4.35	0.45	4.36	0.42	4.52	0.40	4.76	0.40	5.75	0.48	5.12
	0.46	4.53	0.45	5.27	0.43	4.65	0.40	4.38	0.44	5.19	0.47	4.73
XLR-11	0.42	4.69	0.44	4.50	0.38	4.38	0.42	4.47	0.44	4.30	0.43	4.59
	0.44	4.33	0.45	4.33	0.42	4.54	0.41	4.49	0.40	4.45	0.43	4.52
	0.42	4.08	0.44	4.52	0.44	4.39	0.39	4.25	0.41	4.30	0.44	4.42

STATISTICS	9/23/2014		9/30/2014		10/8/2014		10/21/2014		11/4/2014		11/18/2014	
Average	0.41	4.59	0.44	4.61	0.40	4.48	0.45	4.59	0.44	4.21	0.45	4.72
SD	0.01	0.12	0.04	0.29	0.02	0.09	0.03	0.26	0.03	0.13	0.03	0.10
CV	1.40%	2.52%	7.92%	6.20%	3.79%	2.02%	6.84%	5.56%	6.89%	3.06%	7.70%	2.17%
Average	0.37	3.63	0.38	3.82	0.33	3.81	0.35	3.82	0.35	3.67	0.37	3.71
SD	0.01	0.11	0.01	0.17	0.02	0.06	0.01	0.13	0.02	0.03	0.01	0.08
CV	1.55%	3.04%	2.63%	4.46%	5.25%	1.69%	2.86%	3.37%	4.95%	0.82%	3.15%	2.04%
Average	0.46	4.64	0.45	4.82	0.42	4.70	0.37	4.56	0.38	5.64	0.47	4.87
SD	0.02	0.35	0.01	0.46	0.02	0.22	0.05	0.19	0.07	0.41	0.01	0.21
CV	4.35%	7.60%	1.29%	9.45%	3.67%	4.57%	12.37%	4.17%	18.98%	7.20%	2.13%	4.40%
Average	0.43	4.37	0.44	4.45	0.41	4.44	0.41	4.40	0.42	4.35	0.43	4.51
SD	0.01	0.31	0.01	0.10	0.03	0.09	0.02	0.13	0.02	0.09	0.01	0.09
CV	2.71%	7.02%	1.30%	2.35%	7.39%	2.02%	3.76%	3.02%	5.00%	1.99%	1.33%	1.89%

Appendix B- Baseline QC Stability Data

Baseline QC Stability	8/29/14 analyzed 9/2/2014	
	0.5ng	5.0 ng
AB-Fubinaca	0.45	4.16
	0.42	4.34
	0.45	4.42
AB-Pinaca	0.4	3.67
	0.38	3.81
	0.37	3.9
UR-144	0.47	5.1
	0.45	4.92
	0.44	4.8
XLR-11	0.44	4.48
	0.44	4.55
	0.44	4.37

STATISTICS	8/29/14 analyzed 9/2/2014	
Average	0.44	4.31
SD	0.02	0.13
CV	3.94%	3.09%
Average	0.38	3.79
SD	0.02	0.12
CV	3.98%	3.06%
Average	0.45	4.94
SD	0.02	0.15
CV	3.37%	3.06%
Average	0.44	4.47
SD	0.00	0.09
CV	0.00%	2.03%

Appendix C- Within Run QC Validation Data

AB-Fubinaca

Replicate #	Level I (0.5 ng/mL)	Level II (5.0 ng/mL)
1	0.42	4.54
2	0.47	4.39
3	0.40	4.31
Expected	0.50	5.00
Mean	0.43	4.41
SD	0.04	0.12
% CV	8.39%	2.65%
4	0.42	4.70
5	0.41	4.61
6	0.41	4.47
Expected	0.50	5.00
Mean	0.41	4.59
SD	0.01	0.12
% CV	1.40%	2.52%
7	0.44	4.77
8	0.41	4.28
9	0.48	4.78
Expected	0.50	5.00
Mean	0.44	4.61
SD	0.04	0.29
% CV	7.92%	6.20%
10	0.40	4.38
11	0.42	4.52
12	0.39	4.55
Expected	0.50	5.00
Mean	0.40	4.48
SD	0.02	0.09
% CV	3.79%	2.02%
13	0.42	4.84
14	0.44	4.59
15	0.48	4.33
Expected	0.50	5.00
Mean	0.45	4.59
SD	0.03	0.26
% CV	6.84%	5.56%
Highest % CV	8.4%	6.2%
% CV Limit	<10 %	<10 %

AB-Pinaca

Replicate #	Level I (0.5 ng/mL)	Level II (5.0 ng/mL)
1	0.37	3.61
2	0.37	3.64
3	0.36	3.46
Expected	0.50	5.00
Mean	0.37	3.57
SD	0.01	0.10
% CV	1.57%	2.70%
4	0.37	3.75
5	0.37	3.62
6	0.38	3.53
Expected	0.50	5.00
Mean	0.37	3.63
SD	0.01	0.11
% CV	1.55%	3.04%
7	0.37	3.99
8	0.38	3.65
9	0.39	3.81
Expected	0.50	5.00
Mean	0.38	3.82
SD	0.01	0.17
% CV	2.63%	4.46%
10	0.34	3.76
11	0.31	3.78
12	0.34	3.88
Expected	0.50	5.00
Mean	0.33	3.81
SD	0.02	0.06
% CV	5.25%	1.69%
13	0.36	3.87
14	0.34	3.91
15	0.35	3.67
Expected	0.50	5.00
Mean	0.35	3.82
SD	0.01	0.13
% CV	2.86%	3.37%
Highest % CV	5.2%	4.5%
% CV Limit	<10 %	<10 %

UR-144

Replicate #	Level I (0.5 ng/mL)	Level II (5.0 ng/mL)
1	0.44	4.51
2	0.47	4.68
3	0.45	4.44
Expected	0.50	5.00
Mean	0.45	4.54
SD	0.02	0.12
% CV	3.37%	2.72%
4	0.48	5.03
5	0.44	4.35
6	0.46	4.53
Expected	0.50	5.00
Mean	0.46	4.64
SD	0.02	0.35
% CV	4.35%	7.60%
7	0.44	4.82
8	0.45	4.36
9	0.45	5.27
Expected	0.50	5.00
Mean	0.45	4.82
SD	0.01	0.46
% CV	1.29%	9.45%
10	0.40	4.94
11	0.42	4.52
12	0.43	4.65
Expected	0.50	5.00
Mean	0.42	4.70
SD	0.02	0.22
% CV	3.67%	4.57%
13	0.32	4.55
14	0.40	4.76
15	0.40	4.38
Expected	0.50	5.00
Mean	0.37	4.56
SD	0.05	0.19
% CV	12.37%	4.17%
Highest % CV	12.4%	9.4%
% CV Limit	<10 %	<10 %

XLR-11

Replicate #	Level I (0.5 ng/mL)	Level II (5.0 ng/mL)
1	0.43	4.42
2	0.44	4.42
3	0.45	4.17
Expected	0.50	5.00
Mean	0.44	4.34
SD	0.01	0.14
% CV	2.27%	3.33%
4	0.42	4.69
5	0.44	4.33
6	0.42	4.08
Expected	0.50	5.00
Mean	0.43	4.37
SD	0.01	0.31
% CV	2.71%	7.02%
7	0.44	4.5
8	0.45	4.33
9	0.44	4.52
Expected	0.50	5.00
Mean	0.44	4.45
SD	0.01	0.10
% CV	1.30%	2.35%
10	0.38	4.38
11	0.42	4.54
12	0.44	4.39
Expected	0.50	5.00
Mean	0.41	4.44
SD	0.03	0.09
% CV	7.39%	2.02%
13	0.42	4.47
14	0.41	4.49
15	0.39	4.25
Expected	0.50	5.00
Mean	0.41	4.40
SD	0.02	0.13
% CV	3.76%	3.02%
Highest % CV	7.4%	7.0%
% CV Limit	<10 %	<10 %

Appendix D- Raw Stability Data

	Temperature	Day-0 8/26/14	Day-3 8/29/14	Day-7 9/2/14	Day-14 9/9/14	Day-21 9/16/14	Day-28 9/23/14	Day-35 9/30/14	Day-42 10/7/14	Day-56 10/21/14	Day-70 11/4/14	Day-84 11/18/14
AB-Fubinaca	Ambient	4.6033	4.2799	5.0610	4.8939	4.3931	4.6581	5.0606	4.5822	4.9470	4.5768	4.9533
		4.6214	4.3116	5.1753	5.1701	4.4270	4.6047	5.1045	4.3933	4.8738	4.5251	4.8932
		5.0626	4.4188	5.0210	5.0529	4.4787	4.8104	4.7587	4.5186	5.2033	4.5016	4.9292
	Refrigerator	-	4.5559	4.9241	4.4198	4.4714	4.2152	5.1878	4.5079	4.9746	4.4652	4.8335
		-	4.5808	5.1789	4.2025	4.4990	4.6296	4.8246	4.4644	4.7056	4.4554	5.0859
		-	4.6162	5.0164	4.6840	4.5983	4.4594	4.6985	4.3409	4.8556	4.4774	4.9300
	Freezer	-	4.6180	4.9331	5.0223	4.6348	4.9708	5.2285	4.5925	5.0481	4.5718	5.1933
		-	4.6196	4.9800	4.5722	4.7343	4.7120	4.7569	4.4911	4.8370	4.5272	4.9148
		-	4.4504	4.9346	4.5020	4.6089	4.5016	4.8109	4.4941	4.7481	4.3966	4.9715
AB-Pinaca	Ambient	4.3885	4.5016	4.8833	4.9652	4.6398	4.6546	4.8697	4.6096	4.6532	4.6976	4.6485
		4.7906	4.4314	4.8831	5.0855	4.5190	4.6020	4.8436	4.6353	4.7752	4.7066	4.5216
		5.1030	4.6337	4.8610	4.8436	4.5398	4.6242	4.5794	4.5827	4.8024	4.5714	4.5145
	Refrigerator	-	4.7678	4.7775	4.6562	4.5682	4.5599	4.9895	4.6404	4.6771	4.4839	4.4943
		-	4.6957	5.0020	4.3374	4.5152	4.6703	4.6771	4.6153	4.5463	4.5299	4.7160
		-	4.8629	4.8199	4.8132	4.6001	4.4080	4.5907	4.5365	4.6681	4.4997	4.6027
	Freezer	-	4.9224	4.7542	5.0160	4.3804	4.7873	4.9034	4.5756	4.6171	4.5566	4.6800
		-	4.9025	4.7515	4.56.27	4.7515	4.6271	4.6979	4.6254	4.5332	4.5712	4.4972
		-	4.7188	4.7615	4.5503	4.5832	4.3078	4.5814	4.5509	4.5564	4.5078	4.5468
UR-144	Ambient	3.9361	4.6348	4.2130	5.3422	4.2429	4.4146	4.9160	4.0435	5.0248	5.1590	4.1785
		4.2384	4.6486	4.5050	4.8409	4.3227	4.3566	4.9832	4.1659	4.7050	5.4446	3.9775
		4.4096	4.6637	4.1470	4.5805	3.8911	4.6046	4.5374	4.0872	4.7894	5.2592	4.2231
	Refrigerator	-	4.5702	4.1657	4.7671	4.1166	3.8903	4.9693	4.3460	4.6789	5.3989	4.5071
		-	4.4029	4.1707	4.5149	4.4793	4.2938	4.7016	4.5771	4.7705	5.3865	4.3135
		-	4.8349	4.4094	4.6951	4.2824	4.1801	4.8004	4.1265	4.7222	5.6000	4.3823
	Freezer	-	4.9872	4.4696	4.8498	4.0921	4.3560	4.6091	4.0113	4.9499	5.6249	4.8765
		-	4.8486	4.8988	4.8885	4.2730	4.1651	5.2685	4.0436	4.7794	5.2422	4.3861
		-	4.5880	4.5291	5.3994	3.9791	4.3167	4.9952	3.9967	4.6855	5.2685	4.2648
XLR-11	Ambient	4.2136	3.4961	2.6305	1.8192	1.2420	0.8303	0.5985	0.4422	0.3083	0.2141	0.1518
		4.3619	3.5645	2.6983	1.8552	1.1739	0.8347	0.6667	0.4530	0.3144	0.1922	0.1508
		4.7288	3.5886	2.6712	1.8024	1.1674	0.8546	0.6226	0.4413	0.2988	0.1889	0.1478
	Refrigerator	-	4.1441	3.9954	3.5371	3.0320	2.6652	2.4928	2.2300	1.8007	1.5123	1.2807
		-	4.0614	3.8304	3.3388	3.0499	2.6366	2.4743	2.1658	1.7816	1.5420	1.2708
		-	4.2515	3.9259	3.4832	2.9971	2.5143	2.3809	2.1774	1.7137	1.4873	1.2905
	Freezer	-	4.7980	4.6586	4.6743	4.2033	4.3092	4.5909	4.2384	4.3768	4.4230	4.5341
		-	4.6152	4.7268	4.3821	4.3910	4.1684	4.3957	4.2754	4.2895	4.4162	4.2481
		-	4.4615	4.6808	4.4296	4.3229	4.2027	4.4507	4.2431	4.5329	4.2675	4.3531

Appendix D Continued

STATISTICS		Day-0 8/26/14	Day-3 8/29/14	Day-7 9/2/14	Day-14 9/9/14	Day-21 9/16/14	Day-28 9/23/14	Day-35 9/30/14	Day-42 10/7/14	Day-56 10/21/14	Day-70 11/4/14	Day-84 11/18/14
AB-Fubinaca	Ambient AVG	4.7624	4.3368	5.0858	5.0390	4.4329	4.6911	4.9746	4.4980	5.0080	4.5345	4.9252
	SD	0.2601	0.0728	0.0801	0.1386	0.0431	0.1067	0.1883	0.0961	0.1730	0.0385	0.0302
	CV	5.46%	1.68%	1.57%	2.75%	0.97%	2.28%	3.78%	2.14%	3.45%	0.85%	0.61%
	Refrigerator AVG	-	4.5843	5.0398	4.4354	4.5229	4.4347	4.9036	4.4377	4.8453	4.4660	4.9498
	SD	-	0.0303	0.1290	0.2411	0.0667	0.2083	0.2540	0.0866	0.1348	0.0110	0.1274
	CV	-	0.66%	2.56%	5.44%	1.48%	4.70%	5.18%	1.95%	2.78%	0.25%	2.57%
	Freezer AVG	-	4.5627	4.9492	4.6988	4.6593	4.7281	4.9321	4.5259	4.8777	4.4985	5.0265
	SD	-	0.0972	0.0267	0.2823	0.0662	0.2350	0.2581	0.0577	0.1541	0.0910	0.1472
	CV	-	2.13%	0.54%	6.01%	1.42%	4.97%	5.23%	1.27%	3.16%	2.02%	2.93%
AB-Pinaca	Ambient AVG	4.7607	4.5222	4.8758	4.9648	4.5662	4.6269	4.7642	4.6092	4.7436	4.6585	4.5615
	SD	0.3582	0.1027	0.0128	0.1210	0.0646	0.0264	0.1606	0.0263	0.0795	0.0756	0.0754
	CV	7.52%	2.27%	0.26%	2.44%	1.41%	0.57%	3.37%	0.57%	1.68%	1.62%	1.65%
	Refrigerator AVG	-	4.7755	4.8665	4.6023	4.5612	4.5461	4.7524	4.5974	4.6305	4.5045	4.6043
	SD	-	0.0839	0.1193	0.2424	0.0429	0.1317	0.2098	0.0542	0.0731	0.0234	0.1109
	CV	-	1.76%	2.45%	5.27%	0.94%	2.90%	4.41%	1.18%	1.58%	0.52%	2.41%
	Freezer AVG	-	4.8479	4.7557	4.7832	4.5717	4.5741	4.7276	4.5840	4.5689	4.5452	4.5747
	SD	-	0.1122	0.0052	0.3293	0.1858	0.2441	0.1630	0.0379	0.0433	0.0332	0.0945
	CV	-	2.32%	0.11%	6.88%	4.06%	5.34%	3.45%	0.83%	0.95%	0.73%	2.07%
UR-144	Ambient AVG	4.1947	4.6490	4.2883	4.9212	4.1522	4.4586	4.8122	4.0989	4.8397	5.2876	4.1264
	SD	0.2398	0.0145	0.1905	0.3871	0.2296	0.1297	0.2403	0.0620	0.1657	0.1449	0.1308
	CV	5.72%	0.31%	4.44%	7.87%	5.53%	2.91%	4.99%	1.51%	3.42%	2.74%	3.17%
	Refrigerator AVG	-	4.6027	4.2486	4.6590	4.2928	4.1214	4.8238	4.3499	4.7239	5.4618	4.4010
	SD	-	0.2178	0.1393	0.1299	0.1816	0.2081	0.1354	0.2253	0.0458	0.1198	0.0981
	CV	-	4.73%	3.28%	2.79%	4.23%	5.05%	2.81%	5.18%	0.97%	2.19%	2.23%
	Freezer AVG	-	4.8079	4.6325	5.0459	4.1147	4.2793	4.9576	4.0172	4.8049	5.3785	4.5091
	SD	-	0.2027	0.2325	0.3068	0.1483	0.1008	0.3313	0.0240	0.1340	0.2138	0.3239
	CV	-	4.22%	5.02%	6.08%	3.60%	2.36%	6.68%	0.60%	2.79%	3.97%	7.18%
XLR-11	Ambient AVG	4.4348	3.5497	2.6667	1.8256	1.1944	0.8399	0.6293	0.4455	0.3072	0.1984	0.1501
	SD	0.2652	0.0480	0.0341	0.0270	0.0413	0.0129	0.0346	0.0065	0.0079	0.0137	0.0021
	CV	5.98%	1.35%	1.28%	1.48%	3.46%	1.54%	5.50%	1.46%	2.56%	6.90%	1.39%
	Refrigerator AVG	-	4.1523	3.9172	3.4530	3.0263	2.6054	2.4493	2.1911	1.7653	1.5139	1.2807
	SD	-	0.0953	0.0828	0.1025	0.0269	0.0802	0.0600	0.0342	0.0457	0.0274	0.0099
	CV	-	2.30%	2.11%	2.97%	0.89%	3.08%	2.45%	1.56%	2.59%	1.81%	0.77%
	Freezer AVG	-	4.6249	4.6887	4.4953	4.3057	4.2268	4.4791	4.2523	4.3997	4.3689	4.3784
	SD	-	0.1685	0.0348	0.1568	0.0950	0.0734	0.1007	0.0201	0.1233	0.0879	0.1447
	CV	-	3.64%	0.74%	3.49%	2.21%	1.74%	2.25%	0.47%	2.80%	2.01%	3.30%