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Title: **Retrospective Identification of Synthetic Cannabinoids in Forensic Toxicology Casework using Archived High Resolution Mass Spectrometry Data**

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Abstract

Since 2008, synthetic cannabinoids have continued to proliferate and challenge the forensic science community due to rapid appearance and diverse chemistry. To address these concerns, an optimized method using high resolution mass spectrometry was developed that allowed for high throughput analysis of biological samples and extracts for the presence of synthetic cannabinoids and/or their metabolites. Analysis was preformed using a Sciex TripleTOF® 5600+ quadrupole time-of-flight mass spectrometer with SWATH® Acquisition. To complement the analysis, a comprehensive library was developed containing more than 250 parent compounds, metabolites, and internal standards. The library database was regularly updated with emerging synthetic cannabinoids identified by monitoring various intelligence streams.

The net result of this approach was the analysis of more than 6,000 sample extracts over the course of eighteen months, which in turn helped generate six quarterly trend reports, eight new identification drug monographs (5F-MDMB-PICA, APP-BINACA, and MDMB-4en-PINACA), and one public health alert (4F-MDMB-BINACA). These reports involve synthetic cannabinoids that were not included in the original scope of testing at the time of reporting. Collectively, these reports provided timely data to forensic science professionals about the current landscape of synthetic cannabinoids in the United States, information which previously had not existed. Using the described approach, our scientists monitored the rise and fall of synthetic cannabinoids and provided intelligence about how quickly newly identified synthetic cannabinoids were being implicated in forensic toxicology casework. The end goal was to provide laboratories with information about how frequently their scope of testing needed to be updated and specifically with which new analytes.

In addition, through *in vivo* metabolic studies, the most appropriate biomarkers for 4F-MDMB-BINACA and APP-BINACA were characterized and reported. Studies performed on stability of FUB-AMB (MMB-FUBINACA), 5F-ADB (5F-MDMB-PINACA), and 5F-MDMB-PICA led to the conclusion that methyl ester synthetic cannabinoids are unstable in blood when stored in the refrigerator, but the butanoic acid metabolites proved to be good biomarkers in both blood and urine to identify use. Both studies provided relevant and useful information to forensic science practitioners to improve the capability of detecting and reporting synthetic cannabinoid intoxication in casework.

This research project has shown that the synthetic cannabinoid market continues to diversify and expand. Surveillance of the changing synthetic cannabinoid market should be continued using the model developed herein, where sample mining and data mining are applied to various population types to provide public health and safety stakeholders with the most up to date data. With the completion of this funding, there is no other surveillance system currently operating in the United States to continue providing this intelligence. Based on our findings, it is strongly recommended that the scope of testing for synthetic cannabinoids in forensic laboratories be re-evaluated and/or updated on a quarterly basis to include the most prevalent analytes. Synthetic cannabinoids continue to impact morbidity and mortality in the United States and continued research efforts should be conducted as the body of knowledge needs to continue expanding.

1. Introduction

In late 2008, a botanist at United States (US) Customs and Border Protection noticed a steady stream of “herbal incense” being express-shipped into the US (1). Subsequent laboratory testing identified a synthetic cannabinoid receptor agonist known as HU-210, originally synthesized in the 1980’s at Hebrew University (2). Historically, these substances were created by researchers, such as John W. Huffman (Clemson University, SC), Alexandros Makriyannis (Northeastern University, MA), and pharmaceutical companies, such as Pfizer (New York City, NY), as tools to investigate the cannabinoid receptor system and their use as potential therapeutic agents (3). However, in recent years, scientific manuscripts and patents published on the synthesis and potential activity of these cannabinoids have provided a blueprint to entrepreneurial chemists and drug users looking for non-scheduled alternatives to marijuana.

Synthetic cannabinoids have subsequently proliferated, and there are now over 175 synthetic cannabinoids as reported by the European Monitoring Centre for Drugs and Drug and Drug Addiction (EMCDDA), reported between 2008 and 2018 with additional compounds being reported each month (4). In the US between 2009 and 2015, 84 synthetic cannabinoids were reported to the National Forensic Laboratory Information System (NFLIS) (5). Synthetic cannabinoids accounted for 1% of all drugs reported in 2018 with 21,925 reports (6). This class of drugs is of forensic significance due to an emerging understanding of toxicity, as reflected by increasing numbers of hospitalizations, impaired driving arrests, mass poisoning outbreaks, and fatalities (7–10). Synthetic cannabinoids are now reported as the major drug class used as currency within correctional institutions in the Europe (11), United Kingdom (12) and Canada (13); however, there is currently no systematic monitoring of synthetic cannabinoid use in US correctional systems.

The chemistry of synthetic cannabinoids has been discussed in detail (14–16) and several websites now provide tools to help identify synthetic cannabinoids based on their chemical structure (17, 18). In general, synthetic cannabinoids were originally classified based on a structure consisting of head, core, and tail sections. This complex and extensively variable chemistry is what contributes to the continued appearance of new substances on the market.

The analytical challenges of keeping current with synthetic cannabinoids include the diversity of compounds and chemistries in the drug class, the large number of potential analogs and configurations, delays in obtaining analytical standards for addition to toxicological mass spectral databases, and having a consistent and universally agreed upon system for nomenclature. This typically means that by the time the standard is available and added to an analytical scope, months of drug positive cases could have been missed and that the synthetic cannabinoid in question is potentially no longer used within the drug using populations of interest.

The objective of this research was to develop and employ a novel analytical approach utilizing a high-resolution mass spectrometry (HRMS) method for the analysis of synthetic cannabinoids, incorporating a comprehensive data-independent acquisition technique called SWATH[®] acquisition (Sciex, Framingham, MA) (19–21). This approach allows for real-time sample mining, as well as retrospective data mining of previously acquired human blood and urine drug testing data. The process of data mining has gained interest in recent years as a tool for reprocessing of data to discover additional findings based on new knowledge (22–24). In addition, our laboratory has subsequently coined the term “sample mining”, to complement data mining, as a real-time approach for NPS discovery and up-to-date trend reporting. Sample mining enables current and real-time identifications of synthetic cannabinoids that were not part of the scope of testing at the time of original analysis using a targeted screen and/or confirmation

approach, but requires re-analysis of the samples on a more advanced analytical platform. Data mining, is performed by simply reprocessing and querying the data, as previously described in the literature (22), without incurring the time and cost of re-extraction and re-analysis of the biological samples.

SWATH[®] acquisition is a non-targeted data acquisition technique (or data independent acquisition [DIA] mode) available on Sciex instrumentation, including the TripleTOF[®] 5600+ high resolution quadrupole time-of-flight mass spectrometer (QTOF). SWATH[®] acquisition collects comprehensive accurate mass data of precursor and product ions (19–21). Precursor ions are isolated in the quadrupole using variable mass filter windows, passing only a range of masses at a given time. Product ions are generated using rapid cycling of low, medium, and high collisions energies between two set points (also known as a collision energy spread), allowing library search capabilities of comprehensive and detailed mass spectral data, which adds an additional level of specificity. This information is extremely valuable during data review for definitive analyte identification of novel compounds and their metabolites. This acquisition mode and specificity can frequently allow preliminary structural elucidation without standard reference materials (25), until the reference material becomes available. It is important to note that this type of DIA (e.g. MS/MS^{ALL} or similar) is available on other instrument platforms; specifically, ThermoScientific[™] offers DIA with an inclusion list on Orbitrap[™] systems (26) and Agilent offers quadrupole-resolved all ions MS/MS on its newer QTOF systems (27).

SWATH[®] acquisition has previously been employed for the detection of synthetic cannabinoid metabolites in biological specimens (28). In a study by Scheidweiler et al., a SWATH[®] acquisition method was developed for the quantitation of 47 synthetic cannabinoids in human urine and a set list of metabolites was used for targeted data processing; the work did not

focus on other synthetic cannabinoid metabolites possibly present in the samples. Contrary to this and other studies that have used the acquisition mode in a targeted and/or quantitative/semi-quantitative manner, our approach focused on the non-targeted power of SWATH[®] acquisition for drug-discovery.

Using SWATH[®] acquisition, it was hypothesized that the appearance and disappearance of novel synthetic cannabinoids in drug markets could be monitored more efficiently, from the time they were first encountered in drug-using populations through their decline in popularity, and replacement by another analogue. This hypothesis was made based on the greater analytical power of HRMS over traditional quadrupole or ion trap mass spectrometry techniques.

Currently, the ability to monitor these changes in the drug market in a timely manner is not possible due to substances not being identified or incorporated into testing procedures early in their life cycle, and the current screening methods being targeted only to the currently known compounds. The approach developed here provides more comprehensive information about the prevalence and frequency of synthetic cannabinoid use than has been possible using other approaches, specifically (i) for discovery of drugs in case samples that could potential go unreported and (ii) for development of trend monitoring or reporting for surveillance purposes.

2. Method Development and Validation

2.1 Sample Acquisition

De-identified biological sample extracts (n=6,008) from medicolegal death investigations (MDI), driving under the influence of drugs (DUID), and clinical casework were de-identified and discarded from NMS Labs Toxicology Laboratory (Horsham, PA, USA) and received at the Center for Forensic Science Research and Education (CFSRE) between March 2018 and June

2019 to conduct “sample mining” using expanded LC-QTOF-MS testing. The extracts were obtained from biological samples submitted for directed forensic analysis for synthetic cannabinoids. No personal identifying information was received with the extracts; therefore, this protocol was deemed by the National Institute of Justice Institutional Review Board to be exempt because the extracts had already been collected for another purpose and were de-identified prior to inclusion in this study. Basic demographic information (i.e. age, sex, state, date of receipt) was provided, along with any initially reported analytical findings.

De-identified urine samples (n=570) collected as part of standard urine drug testing procedures were discarded from two prison facilities associated with the Pennsylvania Department of Corrections near Philadelphia, PA. Urine samples were received at the Center for Forensic Science Research and Education (CFSRE) between March 2019 and July 2019. Urine samples were extracted for analysis of synthetic cannabinoids. No personal identifying information was received with the urine samples; therefore, this protocol was also deemed by the National Institute of Justice Institutional Review Board to be exempt, because the samples had already been collected for another purpose and were de-identified. The subject’s age was provided.

De-identified urine samples (n=167) collected as part of clinical investigations were received in collaboration with the National Drug Early Warning System (NDEWS) and the Center for Substance Abuse Research (CESAR). Urine samples were received at the Center for Forensic Science Research and Education (CFSRE) between February 2019 and August 2019. Urine samples were extracted for analysis of synthetic cannabinoids. The collection of the urine samples was conducted in accordance with Institutional Review Boards of the participating institutions.

2.2 Sample Preparation and Extraction

Blood and urine samples were prepared in accordance with previously described liquid-liquid extraction (LLE) protocol (29) and a solid phase extraction (SPE) protocol described below.

Blood samples (0.5 mL), fortified with parent internal standards (50 μ L of a 0.2 ng/ μ L), were basified with TRIS HCl buffer (1.0 M, pH 10.2). Methyl tert-butyl ether (MTBE, 3 mL) was added as an extraction solvent. Resulting sample mixtures were rotated for 15 minutes prior to centrifugation at 4600 rpm for 10 minutes. The aqueous layer was frozen using a -80 °C freezer and the supernatant was transferred for dry down at 35 °C for roughly 25 minutes.

Urine samples (1 mL), fortified with metabolite internal standards (50 μ L of a 0.2 ng/ μ L), were initially hydrolyzed using rapid hydrolysis buffer (50 μ L) and IMCSzyme (40 μ L) prior to incubation at 55 °C for one hour. Ammonium carbonate (1 mL, pH 9.3) was added and all samples were vortex mixed. Agilent Bond Elut Plexa PAX (60 mg, 3 mL) SPE cartridges were conditioned for extraction using 2 mL of methanol and 2 mL of deionized water. Samples were then applied to the cartridges, and the cartridges were washed using 2 mL of deionized water, 2 mL of ammonium carbonate buffer, and 2 mL of methanol. Analytes were eluted using two separation additions of 1 mL of 5% formic acid in methanol. The eluent was dried to completion at 55 °C.

Samples from both procedures were reconstituted (200 μ L) in initial mobile phase conditions (95:5) and subsequently analyzed via liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF).

2.3 LC-QTOF-MS Method

Sample extracts were analyzed on the previously described 7-minute method using a Sciex (Framingham, MA) TripleTOF® 5600+ QTOF coupled with a Shimadzu (Shimadzu, Kyoto, Japan) Nexera UHPLC, A reverse phase gradient was employed using ammonium formate (A, 10mM, pH 3) and methanol/acetonitrile (B, 50:50) to achieve chromatographic separation. A Phenomenex® Kinetex C18 analytical column (50mm x 3.0mm, 2.6µm) was used, as well as a flow rate of 0.5 mL/min and injection volume of 20 µL. The gradient conditions are shown in Table 1.

Table 1: LC Gradient Conditions

Time (min)	%A	%B
0	95	5
0.5	95	5
4	5	95
6	5	95
6.1	95	5
7	95	5

Analytes were ionized via positive electrospray ionization (ESI+). The source conditions were as follows: ion source gas one 40 psi, ion source gas two 75 psi, curtain gas 45 psi, temperature 600 °C, and IonSpray Voltage Floating (ISVF) 4000 V. Precursor ions were first acquired by TOF MS scan from 100-550 Da. Precursor ions were then isolated in the quadrupole (Q1) based on overlapping SWATH® acquisition windows; Q1 isolation segments spanned the entirety of the TOF MS range, as previously described. Fragment occurred using a collision energy spread (35±15eV) and the resulting fragment (MSMS) ions were acquired from 50-550 Da. The total cycle time was calculated to be 0.91 seconds.

Acquired datafiles were processed using a three tiered approach, including targeted, non-targeted, and manual processing strategies; but for purposes of this manuscript, only the targeted

data processing approach will be discussed. All data processing was conducted using PeakView® (Version 2.2) and MasterView™ (Version 1.1) (Sciex, Framingham, MA). Created during method development following analysis of all standard reference material acquired, an extracted ion chromatogram (XIC) list was generated containing more than 250 synthetic cannabinoid parent compounds, metabolites, and internal standards. This XIC list contained information relating to compound name, formula, adduct (H⁺), calculated precursor exact mass, retention time, and the accurate masses of five fragment ions. Datafiles were processed based on pre-established criteria (Table 2), and the generated MSMS data was compared to the developed accurate mass library database. Pre-established criteria (e.g. mass error, library score) were determined based on mass spectrometry industry standards (30) and the evaluation of data generated in-house. Additional criteria for positive identification included acceptable chromatography, acceptable chromatographic and mass spectral peak shape, acceptable library spectrum, and control (blank) comparison; all aspects that were visually evaluated by the analyst in accordance with industry standards.

Table 2: Data Processing Criteria

Criteria	Pass	Additional Review	Fail
Mass Error (ppm)	<5	<10	>10
Retention Time Error (min)	<0.25	<0.35	>0.35
Isotope Ratio (% Difference)	<30	<100	>100
Library Score	>70	>50	<50
Signal-to-Noise Ratio	>10	-	<10
Peak Intensity (counts)	>800	-	<800

2.3 Method Validation

Results from this section have been published in the peer review literature: Krotulski et al. [“Emerging Synthetic Cannabinoids: Development and Validation of a Novel Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry Assay for Real-Time Detection”](#) published in the Journal of Analytical Toxicology (2019) – in production.

The described methods were qualitatively validated using a fit-for-purpose protocol as described below derived from the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology (31). Performance characteristics evaluated included: precision/accuracy, limits of detection, interferences, processed sample stability, and carryover.

Precision/accuracy was defined as the accurate identification of analytes with precise measurements within and between runs. Measurements evaluated included ppm error, retention time error, isotope difference, library score, and peak area. The percent coefficient of variation (%CV) of these values was required to be less than 20% over the course of the validation study. Serial dilutions were prepared in blood or urine to monitor limit of detection (LOD) from 0.2 ng/mL down to 0.0125 ng/mL. The LOD was then determined by evaluating all the above parameters for positive analyte identification, as well as signal-to-noise ratio, which was required to be greater than 10. Interferences were evaluated from negative matrix types, as well as mixes of other therapeutic, abused, and emerging drugs. More than 250 other compounds were evaluated to determine whether any of these common drugs would result in a false positive result. Processed sample stability was evaluated by re-analyzing previously prepared samples, using the same criteria as for determining precision. Carryover was evaluated by analyzing blank

injections following analysis of parent (50 ng/mL) and metabolite (varying concentrations) positive samples.

For the blood validation, 19 synthetic cannabinoid parent compounds were evaluated, spanning a range of generations and chemistries (Table 3). All parent compounds were prepared at 1 ng/mL in blank blood matrices. For the urine validation, 19 synthetic cannabinoid metabolites were evaluated, also spanning a range of generations and chemistries based on standard availability and metabolite prevalence (Table 4). Metabolites were evaluated at varying concentrations based on spiking mixes previously prepared. For this study, these 38 compounds were determined to be representative and adequate for qualitative validation purposes.

Table 3: Between-Run Blood Validation Results (%CV, n=15)

Analyte	[M+H] ⁺ (Da)	RT (min)	Conc. (ng/mL)	Mass (Da)	ppm Error	RT (min)	Isotope (% Diff.)	Library Score	Peak Area	LOD (ng/mL)	Stability (Days)
UR-144	312.2322	5.21	1	312.2322	0.18	5.22 (0.11%)	12.9	99.7 (0.2%)	1058 (63.2%)	1	8
XLR-11	330.2228	4.92	1	330.2232	1.38	4.93 (0.16%)	22.2	80.3 (26.2%)	912 (54.3%)	1	8 (-33%)
JWH-018	342.1852	5.06	1	342.1849	-1.03	5.06 (0.05%)	15.9	99.9 (0.2%)	670 (53.9%)	1	8
AB-CHMINACA	357.2285	4.66	1	357.2286	0.26	4.65 (0.16%)	18.2	98.2 (2.7%)	1152 (34.3%)	1	8
AM-2201	360.1758	4.76	1	360.1762	1.16	4.75 (0.13%)	8.2	100.0 (0.0%)	1629 (34.6%)	0.2	8
4-cyano CUMYL- BUTINACA	361.2023	4.45	1	361.2022	-0.10	4.42 (0.08%)	3.3	69.3 (21.8%)	3209 (24.3%)	0.05	8
AKB-48 (APINACA)	366.2540	5.60	1	366.2542	0.51	5.56 (0.12%)	9.2	98.1 (1.5%)	1860 (44.0%)	1	8
MMB-CHMICA	371.2329	4.87	1	371.2326	-0.78	4.87 (0.10%)	15.6	99.6 (1.7%)	894 (30.7%)	1	8
5F-PB-22 (5-fluoro QUPIC)	377.1660	4.63	1	377.1659	-0.34	4.61 (0.09%)	7.8	100.0 (0.0%)	857 (24.7%)	0.2	8
5F-MDMB-PICA	377.2235	4.61	1	377.2234	-0.20	4.59 (0.11%)	6.7	100.0 (0.0%)	1955 (15.0%)	0.1	8
5F-7-QUPAIC	378.1612	4.47	1	378.1614	0.39	4.46 (0.43%)	8.1	99.7 (1.3%)	2372 (44.3%)	0.1	8
5F-NPB-22	378.1612	4.56	1	378.1610	-0.59	4.54 (0.13%)	5.4	58.0 (19.2%)	1601 (37.6%)	0.1	8
5F-MDMB- PINACA	378.2188	4.73	1	378.2190	0.71	4.72 (0.12%)	3.8	94.5 (6.1%)	3148 (24.8%)	0.1	8
5F-AEB		4.72						98.1 (3.4%)			
ADB-FUBINACA	383.1878	4.39	1	383.1877	-0.21	4.38 (0.15%)	10.8	100.0 (0.0%)	876 (17.8%)	0.2	8
MMB-FUBINACA	384.1718	4.65	1	384.1737	5.03	4.64 (2.66%)	20.7	95.3 (12.6%)	360 (55.1%)	1	8
5F-EDMB- PINACA	392.2344	4.85	1	392.2344	0.11	4.85 (0.11%)	18.4	93.7 (11.4%)	825 (34.2%)	0.2	8
FUB-AKB-48	404.2133	5.23	1	404.2136	0.92	5.22 (0.12%)	13.2	95.5 (5.0%)	385 (50.8%)	1	8
MDMB- CHMCZCA	435.2642	5.27	1	435.2640	-0.48	5.28 (0.09%)	30.4	98.7 (1.0%)	714 (47.8%)	1	8

Table 4: Between-Run Urine Validation Results (%CV, n=15)

Analyte	[M+H] ⁺ (Da)	RT (min)	Conc. (ng/mL)	Mass (Da)	ppm Error	RT (min)	Isotope (% Diff.)	Library Score	Peak Area	LOD (ng/mL)	Stability (Days)
PB-22 3-Carboxyindole	232.1332	4.44	5	232.1336	1.58	4.42 (0.14%)	2.7	100.0 (0.0%)	7145 (19.4%)	0.5	8
5F-PB-22 3-Carboxyindole	250.1238	4.08	0.2	250.1239	0.22	4.05 (0.16%)	49.4	98.8 (4.4%)	5029 (18.4%)	0.2	8
BB-22 3-Carboxyindole	258.1489	4.65	5	258.1493	1.62	4.61 (0.10%)	2.3	99.3 (1.0%)	5503 (22.5%)	0.5	8
UR-144 N-Pentanoic Acid	342.2064	4.60	0.5	342.2067	0.95	4.57 (0.17%)	5.9	100.0 (0.0%)	4140 (23.3%)	0.05	8
5F-AMB 3-Methylbutanoic Acid	350.1875	4.32	0.2	350.1882	2.10	4.30 (0.15%)	42.0	99.7 (0.8%)	2058 (29.5%)	0.2	8
AB-CHMINACA 3- Methylbutanoic Acid	358.2125	4.84	2.5	358.213	1.40	4.81 (0.17%)	4.0	100.0 (0.0%)	15836 (21.5%)	0.125	8
AB-PINACA N-Pentanoic Acid	361.1870	3.63	5	361.187	-0.09	3.62 (0.16%)	19.6	100.0 (0.0%)	11293 (10.6%)	0.5	8
5F-MDMB-PICA 3,3- Dimethylbutanoic Acid	363.2079	4.38	0.2	363.2106	7.55	4.35 (0.14%)	13.8	100.0 (0.0%)	3005 (27.2%)	0.2	8
5F-ADB 3,3-Dimethylbutanoic Acid	364.2031	4.48	0.2	364.2049	4.86	4.45 (0.14%)	21.4	99.6 (1.7%)	2324 (26.5%)	0.2	8
4-cyano CUMYL-BUTINACA N-Butanoic Acid	366.1812	4.28	0.2	366.1811	-0.31	4.25 (0.14%)	24.2	99.0 (1.6%)	1903 (25.0%)	0.2	8
MMB-FUBINACA 3- Methylbutanoic Acid	370.1562	4.40	0.5	370.1567	1.27	4.38 (0.23%)	51.0	92.0 (10.6%)	1892 (20.8%)	0.5	8
JWH-018 N-Pentanoic Acid	372.1594	4.38	0.2	372.1593	-0.39	4.37 (0.11%)	19.3	99.8 (0.4%)	1189 (19.8%)	0.2	8
MAB-CHMINACA 3,3- Dimethylbutanoic Acid	372.2282	4.96	0.2	372.2281	-0.21	4.94 (0.16%)	13.9	100.0 (0.0%)	820 (23.0%)	0.2	8
ADBICA N-Pentanoic Acid	374.2074	3.80	5	374.2073	-0.38	3.78 (0.11%)	36.7	Pass*	9044 (20.4%)	5	8
ADB-PINACA N-Pentanoic Acid	375.2027	3.84	5	375.2033	1.48	3.82 (0.16%)	6.2	100.0 (0.0%)	13593 (10.9%)	1	8
MDMB-FUBICA 3,3- Dimethylbutanoic Acid	383.1766	4.45	0.2	383.1781	3.87	4.42 (0.09%)	29.3	100.0 (0.0%)	1139 (30.8%)	0.2	8
MDMB-FUBINACA 3,3- Dimethylbutanoic Acid	384.1718	4.56	0.5	384.1723	1.41	4.53 (0.11%)	11.7	100.0 (0.0%)	2337 (25.2%)	0.1	8
AKB-48 N-Pentanoic Acid	396.2282	4.80	0.5	396.2283	0.35	4.78 (0.16%)	5.4	100.0 (0.0%)	5353 (20.5%)	0.05	8
AB-FUBINACA Oxobutanoic Acid	399.1463	3.92	2.5	399.1464	0.28	3.90 (0.13%)	11.3	100.0 (0.0%)	3584 (19.3%)	0.5	8

For qualitative validation purposes, five samples were prepared each day and over three days (total n=15 per matrix) to evaluate precision/accuracy. LOD samples were prepared in duplicate (total n=6 per concentration, per matrix). Interference mixes (n=4) were prepared and analyzed on all three days. Carryover was evaluated each day for parent compounds and metabolites.

3. Synthetic Cannabinoid Discovery and Prevalence

Results from this section have been published online at www.NPSDiscovery.org in the form of new drug monographs, trend reports, and public health alerts.

3.2 New Identifications

Throughout the course of this funded research, eight emergent synthetic cannabinoids were identified for the first time, some of which were identified for the first time in forensic toxicology samples or for the first time in any type of forensic sample. On average, one to two new synthetic cannabinoids were discovered each quarter, some of which in turn were identified with increasing frequency within the sample set, and others were only identified in a few samples. These analytes include 5F-MDMB-PICA, 5Cl-AKB-48, 4-cyano-CUMYL-BINACA, 5F-EDMB-PINACA, 4F-MDMB-BINACA, APP-BINACA, ACHMINACA, and MDMB-4en-PINACA (Figure 1).

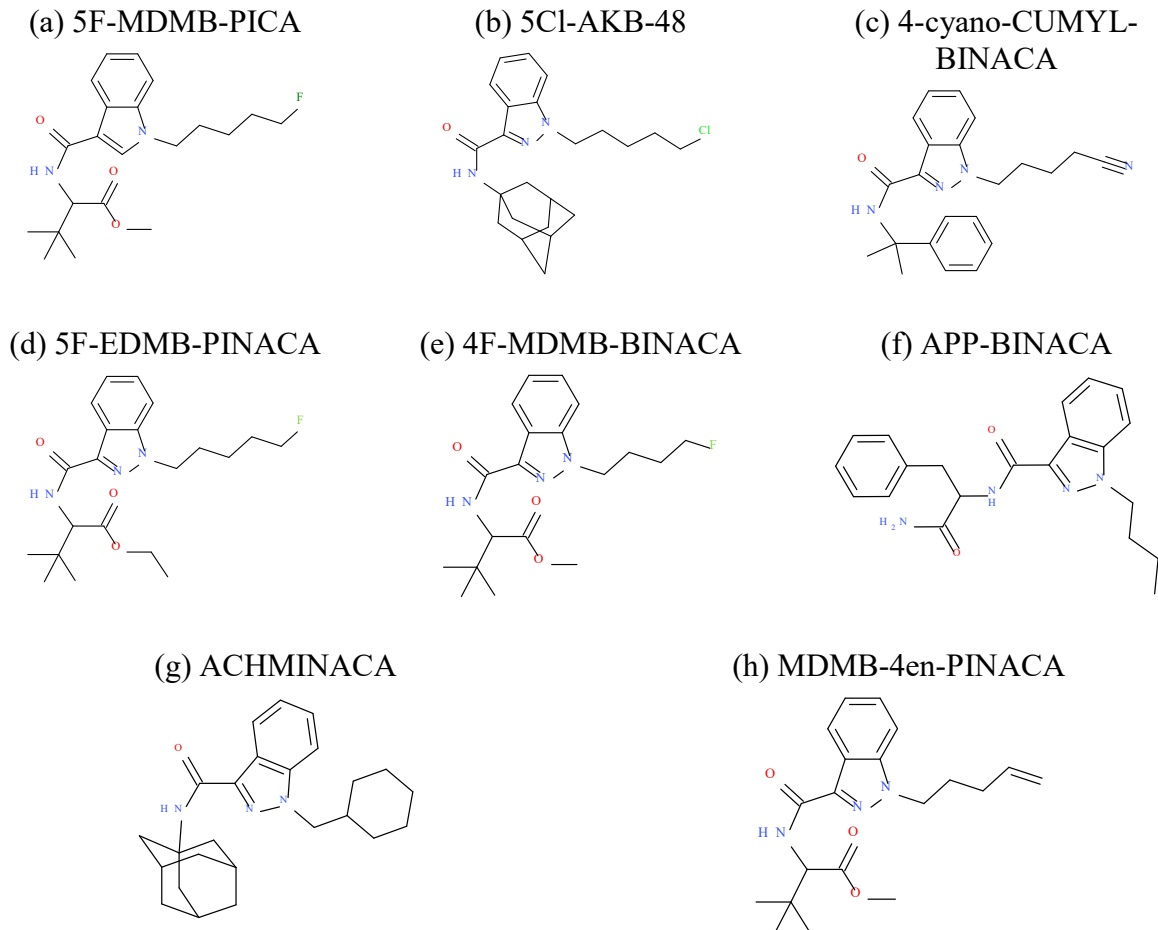


Figure 1: Structures of emergent synthetic cannabinoids identified

5F-MDMB-PICA was first substance identified during extract analysis in Q2 2018. This identification came from a postmortem blood sample. The earliest identification of 5F-MDMB-PICA in the United States dates back to November 2017, as reported by NMS Labs and CFSRE (32). 5F-MDMB-PICA has been identified in toxicology samples, as part of this research funding, and seized drug material analysis. The identification of 5F-MDMB-PICA in Q2 2018 was only the beginning in terms of positivity and prevalence, as described below. In 2019, 5F-MDMB-PICA became the most prevalent synthetic cannabinoid in the United States.

5Cl-AKB-48 was first identified during extract analysis in Q3 2018 alongside 5F-ADB (5F-MDMB-PINACA), the most prevalent synthetic cannabinoid at the time. This identification came from a postmortem blood sample. The earliest identification of 5Cl-AKB-48 in the United States dates back to April 2018 (33). 5Cl-AKB-48 has been identified in toxicology samples, as part of this research funding, and seized drug material analysis. 5Cl-AKB-48 was only identified twice during this research, the second time coming in Q4 2018 alongside FUB-AMB (MMB-FUBINACA).

4-cyano-CUMYL-BINACA was first identified in the United States during extract analysis in Q3 2018 alongside ADB-FUBINACA. This identification came from a postmortem blood sample. The earliest identification of 4-cyano-CUMYL-BINACA internationally dates back to May 2016 (34). 4-cyano-CUMYL-BINACA has been identified in toxicology samples and seized drug materials around the world. During this research, 4-cyano-CUMYL-BINACA was identified in six blood sample extracts, with two identifications alongside its metabolite 4-cyano CUMYL-BUTINACA N-butanoic acid. Although its prevalence and positivity never took off in the United States, the popularity of 4-cyano-CUMYL-BINACA internationally was high. This phenomenon showcases the distinct synthetic cannabinoid trends among the United States, Europe, and other world countries may differ.

5F-EDMB-PINACA was first identified during extract analysis in Q3 2018 alongside 5F-MDMB-PINACA (its methyl ester counterpart), MMB-FUBINACA, and ADB-FUBINACA. This identification came from a postmortem blood sample. The earliest identification of 5F-EDMB-PINACA in the United States dates back to May 2018 (35). 5F-EDMB-PINACA has been identified in toxicology samples, as part of this funded research, and seized drug material.

During this research, 5F-EDMB-PINACA was identified in six blood sample extracts, the latest of which came during Q4 2018.

4F-MDMB-BINACA was first identified during extract analysis in Q1 2019, but data mining revealed additional positives in Q4 2018. 4F-MDMB-BINACA was identified alongside 5F-MDMB-PICA, the most prevalent synthetic cannabinoid at the time. This identification came from a postmortem blood sample. The earliest identification of 4F-MDMB-BINACA in the United States dates back to November 2018 (36). 4F-MDMB-BINACA has been identified in toxicology samples, as part of this funded research, and seized drug material. Like 5F-MDMB-PICA, the first identifications of 4F-MDMB-BINACA in Q4 2018 and Q1 2019 were only the beginning in terms of positivity and prevalence, as described below. In 2019, 4F-MDMB-BINACA became the second most prevalent synthetic cannabinoid in the United States.

APP-BINACA was first identified during extract analysis in Q1 2019 as the lone synthetic cannabinoid. This identification came from a postmortem blood sample and marks the earliest identification of APP-BINACA in the United States. APP-BINACA was identified in 13 blood sample extracts during this research and commonly found in combination with 4F-MDMB-BINACA. The latest identification of APP-BINACA was during Q3 2019.

ACHMINACA was first reported following seized drug analysis in May 2018 (37). After this notification, ACHMINACA was added to the library database for processing of extracts. Not until Q3 2019, over one year later, was ACHMINACA first identified during extract analysis. This identification came from a postmortem blood sample. Since Q3 2019, ACHMINACA has been identified three times, each time in combination with 5F-MDMB-PICA and/or 4F-MDMB-BINACA.

MDMB-4en-PINACA was first identified during extract analysis in Q3 2019 through discovery of its metabolite, MDMB-4en-PINACA N,N-dimethylbutanoic acid, in a urine extract associated with a clinical intoxication case. The parent compound was later confirmed in a postmortem blood specimen from another subject a few weeks later. To our knowledge, this marks the earliest identification of MDMB-4en-PINACA in toxicology specimens in the United States or internationally. These identifications of MDMB-4en-PINACA in Q3 2019 appear to be the beginning of an upward trend in terms of positivity and prevalence. To date, MDMB-4en-PINACA and/or its metabolite have been identified in 18 total cases.

3.3 Trends and Prevalence

Between Q2 2018 and Q3 2019, 6,008 sample extracts from NMS Labs were analyzed using the described LC-QTOF-MS method. In total, 38 different synthetic cannabinoid related analytes were detected (25 parent compounds and 13 metabolites) which correlated to at least 27 unique synthetic cannabinoids. Table 5 details the positivity of all analytes detected. 5F-MDMB-PICA was the most prevalent analyte identified during this funded research, followed by 5F-MDMB-PINACA, MMB-FUBINACA, and 4F-MDMB-BINACA.

Table 5: Synthetic cannabinoid positivity

Synthetic Cannabinoid	Positive Samples	% Pos. (n=6,008)
5F-MDMB-PICA	225	3.7%
5F-ADB (5F-MDMB-PINACA)	149	2.5%
MMB-FUBINACA 3-Methylbutanoic Acid	118	2.0%
5F-ADB 3,3-Dimethylbutanoic Acid	117	1.9%
4F-MDMB-BINACA	104	1.7%
5F-MDMB-PICA 3,3-Dimethylbutanoic Acid	65	1.1%
MMB-FUBINACA (FUB-AMB)	50	0.8%
4F-MDMB-BINACA 3,3-Dimethylbutanoic Acid	37	0.6%
ADB-FUBINACA	23	0.4%
APP-BINACA	12	0.2%
4-cyano CUMYL-BUTINACA	6	0.1%

5F-EDMB-PINACA	6	0.1%
AB-FUBINACA	5	0.08%
FUB-AKB-48	5	0.08%
ADB-PINACA N-Pentanoic Acid	4	0.07%
MDMB-4en-PINACA	4	0.07%
MDMB-FUBICA 3,3-Dimethylbutanoic Acid	4	0.07%
AB-CHMINACA	3	0.05%
MAB-CHMINACA	3	0.05%
4-cyano CUMYL-BUTINACA N-Butanoic Acid	2	0.03%
5CI-AKB-48	2	0.03%
5F-AB-PINACA	2	0.03%
5F-ADBICA	2	0.03%
5F-AMB	2	0.03%
5F-PB-22 3-Carboxyindole	2	0.03%
AB-PINACA	2	0.03%
MDMB-4en-PINACA 3,3-Dimethylbutanoic Acid	2	0.03%
4OH-MDMB-BINACA	1	0.02%
5CI-AB-PINACA	1	0.02%
5F-ADB-PINACA	1	0.02%
5F-AMB 3-Methylbutanoic Acid	1	0.02%
5F-NPB-22 3-Carboxyindazole	1	0.02%
5F-PB-22	1	0.02%
ACHMINACA	1	0.02%
HU-331	1	0.02%
MDMB-FUBINACA	1	0.02%
MDMB-FUBINACA 3,3-Dimethylbutanoic Acid	1	0.02%
MMB-CHMICA	1	0.02%

Figure 2 displays the trajectory of positive over time for the top four analytes. Beginning in Q2 2019, 5F-MDMB-PINACA and MMB-FUBINACA were the two most prevalent analytes identified, mimicking national trends in seized drug and toxicology casework. The dominance of these two synthetic cannabinoids persisted through 2018 until noticeable decline in positive in 2019. 5F-MDMB-PICA was first identified in Q2 2018, increasing in positivity each quarter through Q3 2019. 5F-MDMB-PICA became the most prevalent synthetic cannabinoid in Q1 2019 and has remained the top analyte since that time. 4F-MDMB-BINACA was first identified in Q1 2019 (although data mining resulted in identification in Q4 2018). Since that time, the

positivity of 4F-MDMB-BINACA has continued to increase; however, not as significantly as the proliferation of 5F-MDMB-PICA. 4F-MDMB-BINACA remains the second most prevalent analyte at the end of 2019.

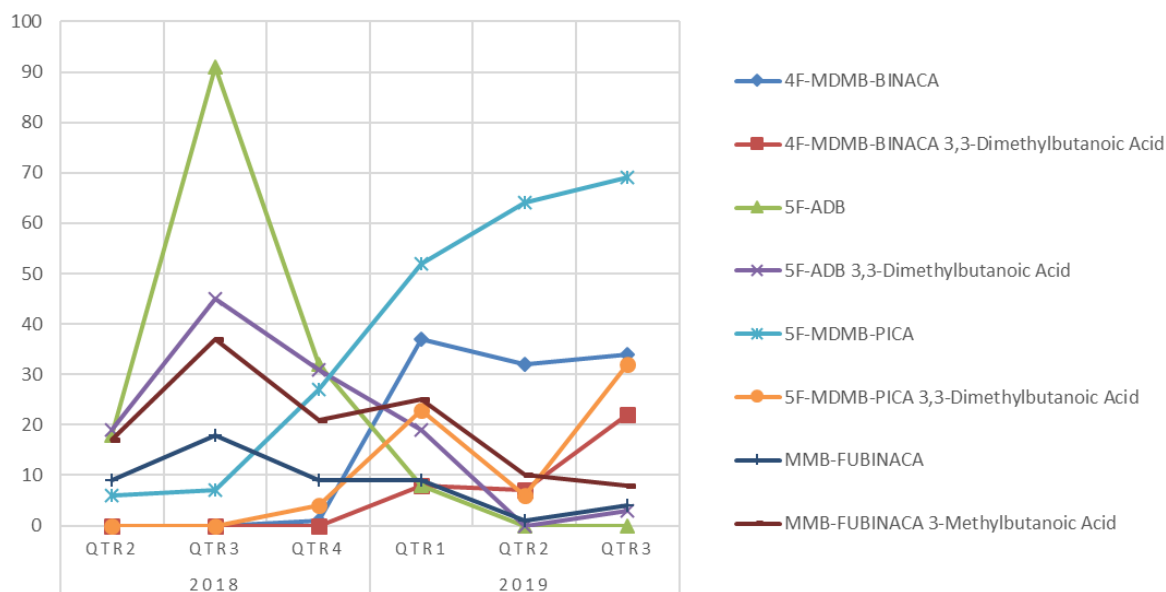


Figure 2: Synthetic cannabinoid positivity by quarter (top 4 analytes)

When examining the future of synthetic cannabinoid trends in the United States, it appears the positivity of 5F-MDMB-PICA will likely decline over the next year. While 4F-MDMB-BINACA could be the next analyte to take the top spot in terms of prevalence, its emergence and proliferation resembles that of 5F-MDMB-PICA, so if this trend follows prior patterns, it is likely that a new as yet unknown or recently emergent drug could become the next most popular within the first few months of 2020. MDMB-4en-PINACA appears to be increasing in prevalence and popularity; however, it is difficult to predict the analyte which will dominate.

When evaluating synthetic cannabinoid positivity based on submitting agency information and demographics, the majority of samples were associated with MDI casework,

followed by DUID investigations (Figure 3), and were primarily submitted as blood samples (Figure 4). The majority of individuals were male (Figure 5), and age ranged from 10 to 71 (Figure 6). When examining geographical distribution, samples were submitted from all areas of the country (Figure 7); however, increased positivity from state to state was not a consistent and accurate reflection of synthetic cannabinoid distribution in the United States and is rather linked to clients submitting samples to NMS Labs.

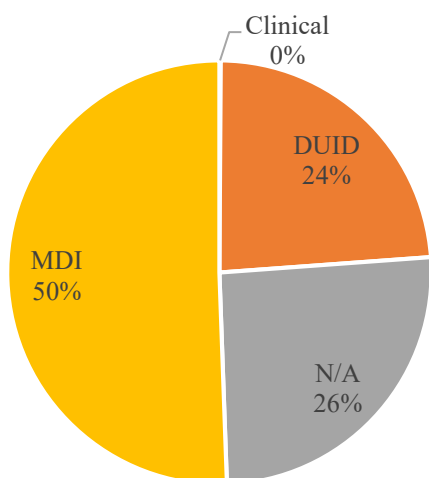


Figure 3: Case type

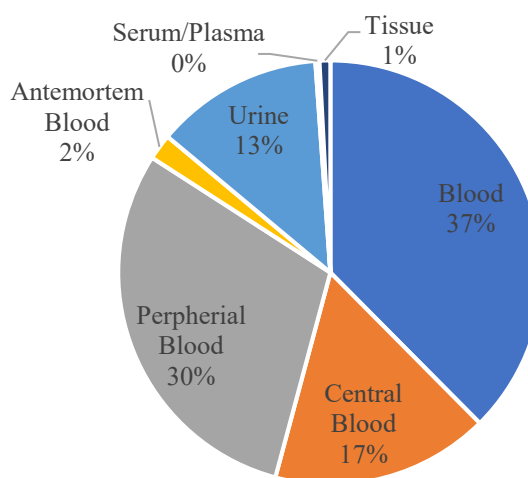


Figure 4: Sample type

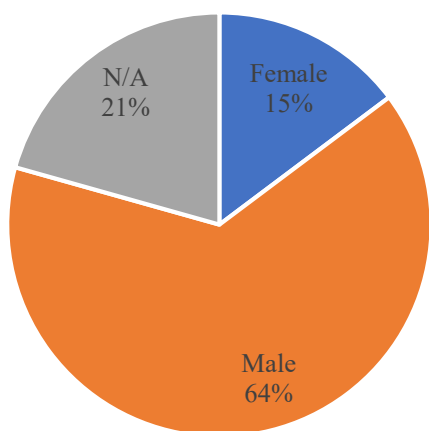


Figure 5: Sex

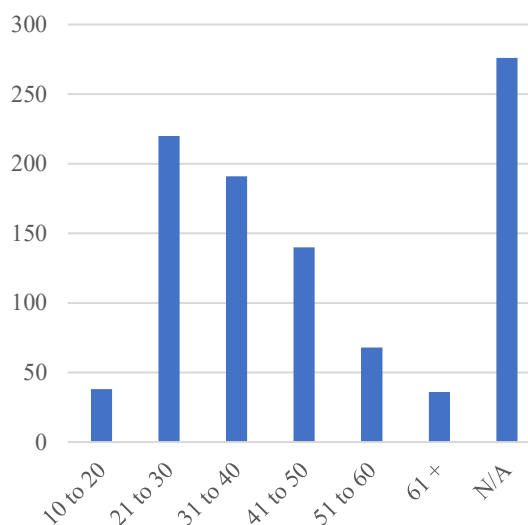


Figure 6: Age

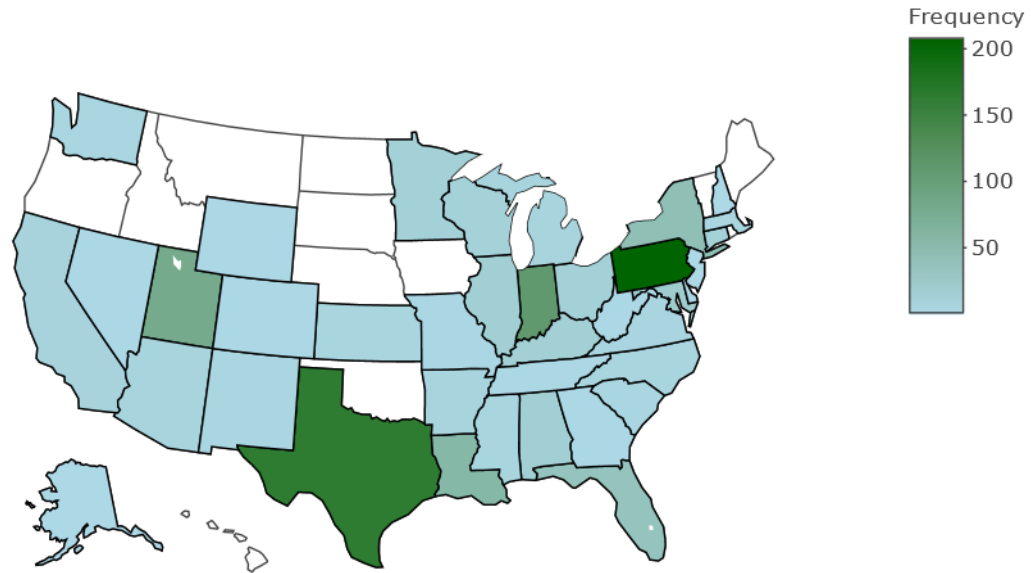


Figure 7: Heat map of synthetic cannabinoid positivity

3.4 Synthetic Cannabinoids in Prisons

Analysis of urine specimens (n=570) collected at two correctional facilities yielded positivity of 2.4%; comparatively other reports reflect higher use of synthetic cannabinoids in prison populations in other parts of the United States and internationally (11, 12, 38–40). Fourteen samples were positive for synthetic cannabinoids, 11 (1.9%) of which were positive for 5F-MDMB-PICA 3,3-dimethylbutanoic acid and three for 4F-MDMB-BINACA 3,3-dimethylbutanoic acid (0.5%). Demographics associated with the positive samples included an average age of 31($\pm$ 3) years old and median age of 30. Ten of the positive samples were from individuals identified as black or African American, and four were identified as Hispanic. Drug positivity rates reported by the Pennsylvania Department of Corrections between October 2018 and September 2019 ranged between 0.3% and 0.9% (41). The increase in positivity among those testing positive for synthetic cannabinoids could be explained by the use of a comprehensive testing methodology and a regularly updated library database. For example, 4F-

MDMB-BINACA was the newest synthetic cannabinoid to emerge around the time of testing and incorporation of this new analyte into the database yielded positive results that would otherwise be missed without vigorous and novel protocols.

3.5 Synthetic Cannabinoids in Clinical Populations

Analysis of urine specimens (n=167) collected from emergency room patients suspected of being under the influence of synthetic cannabinoid use and/or synthetic drug use yielded positivity of 24.6% (n=41); however, there were distinct differences between the sets of samples collected at different locations. In the first set of samples collected (n=127), only three individuals tested positive (2.4%). Positive results correlated only to the metabolites of FUB-AMB (MMB-FUBINACA). In the second set of samples (n=40), 38 individuals tested positive (95%). Contrarily, the metabolites of at least five synthetic cannabinoids were identified in the second collection of samples, including 5F-MDMB-PINACA 3,3-dimethylbutanoic acid, MMB-FUBINACA 3-methylbutanoic acid, 5F-MDMB-PICA 3,3-dimethylbutanoic acid, MDMB-FUBINACA 3,3-dimethylbutanoic acid, 4F-MDMB-BINACA 3,3-dimethylbutanoic acid. No demographic information was available for these urine samples.

4. Metabolism of Synthetic Cannabinoids

Results from this section have been published in the peer review literature: Krotulski et al. “[4F-MDMB-BINACA: A New Synthetic Cannabinoid Widely Implicated in Forensic Casework](#)” published in the Journal of Forensic Science (2019) and Krotulski et al. “[Detection and characterization of the new synthetic cannabinoid APP-BINACA in forensic casework](#)” published in Drug Testing and Analysis (2019).

4.1 Methods

Due to the nature of the LC-QTOF-MS non-target acquisition workflow, comprehensive data acquisition allowed for the study of synthetic cannabinoid drug metabolism without the need for *in vivo* human or animal studies and/or *in vitro* experiments. LC-QTOF-MS data independent acquisition (DIA) allowed for detection of all theoretical masses and fragment ions during the run (within 100-550 Da). This afforded the opportunity to data mine the QTOF data files for suspected metabolites, based on known patterns for metabolism in other members of similar synthetic cannabinoid sub-classes. This process has been used successfully in the investigation of other members of this NPS class (42, 43). Datafiles from analysis of MDI and DUID populations (the largest sample size) were data mined for potential metabolites associated with specific synthetic cannabinoids (e.g. 4F-MDMB-BINACA and APP-BINACA). Results were compiled and, if applicable, reference standards were ordered for the appropriate metabolites.

The metabolism of 4F-MDMB-BINACA and APP-BINACA had not previously been reported; however, the reports on metabolism of structurally similar synthetic cannabinoids are available in the literature. Theoretical metabolites of 4-MDMB-BINACA were formulated based on knowledge about the metabolism of 5F-MDMB-PINACA and 5F-MDMB-PICA (Figure 8) (44, 45). Theoretical metabolites of APP-BINACA were formulated based on knowledge about the metabolism of the PX series (Figure 8) (42, 46, 47, 44, 45). Theoretical metabolites included products of ester hydrolysis, amide hydrolysis, hydroxylation, de-fluorination, de-alkylation, etc. The resulting theoretical metabolites were converted to formulae (and exact mass) and added to a targeted screening program. Based on the proposed points of metabolism, reference exact mass

fragment ions were formulated and recorded. Data processing and analysis were conducted using PeakView[®] (SCIEX, Version 2.2), and MasterView[™] (SCIEX, Version 1.1).

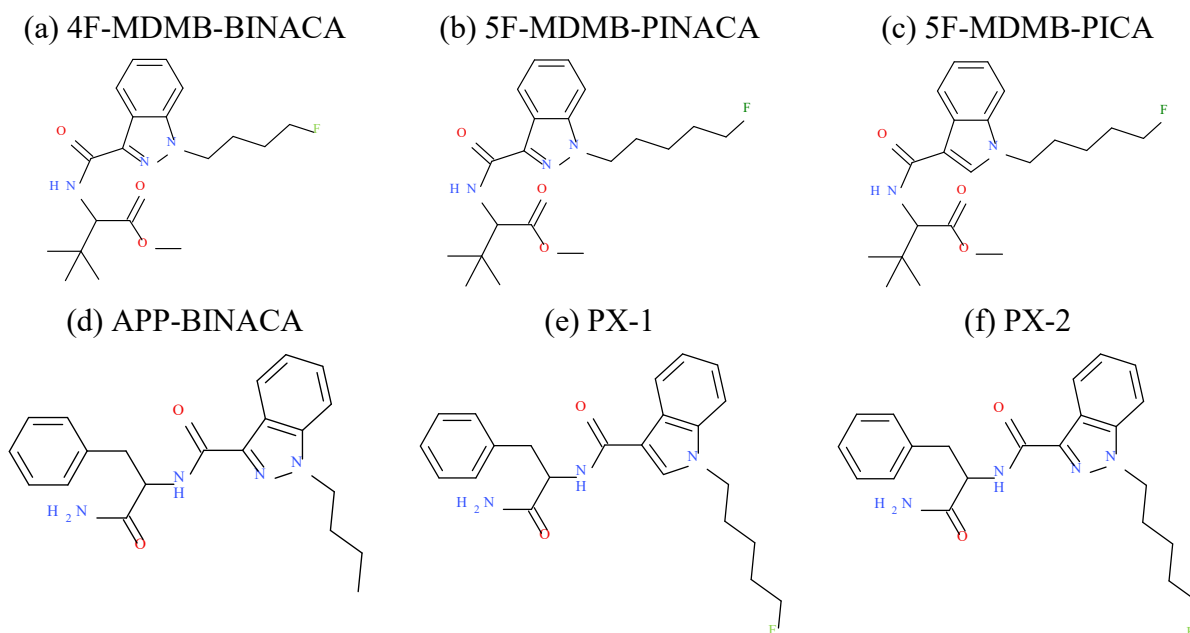


Figure 8: Comparison of synthetic cannabinoid structures

Positive identification criteria for metabolites using this approach were identical to those used for determining positive synthetic cannabinoid results in samples; however, retention time error and library score could not be evaluated as this was a true new discovery approach, and no standard reference materials were available. A minimum of three fragment ions were used for structural elucidation to determine the site of biotransformation. The identification results reported are qualitative.

4.2 Results for 4F-MDMB-BINACA

Processing of datafiles associated with blood (n=4) and urine (n=4) positive cases for 4F-MDMB-BINACA (retention time 4.48 mins) resulted in the identification of 9 metabolites

(Figure 9, Table 6), two of which proved to be valuable biomarkers for monitoring 4F-MDMB-BINACA ingestion (4F-MDMB-BINACA 3,3-dimethylbutanoic acid and 4-OH-MDMB-BINACA).

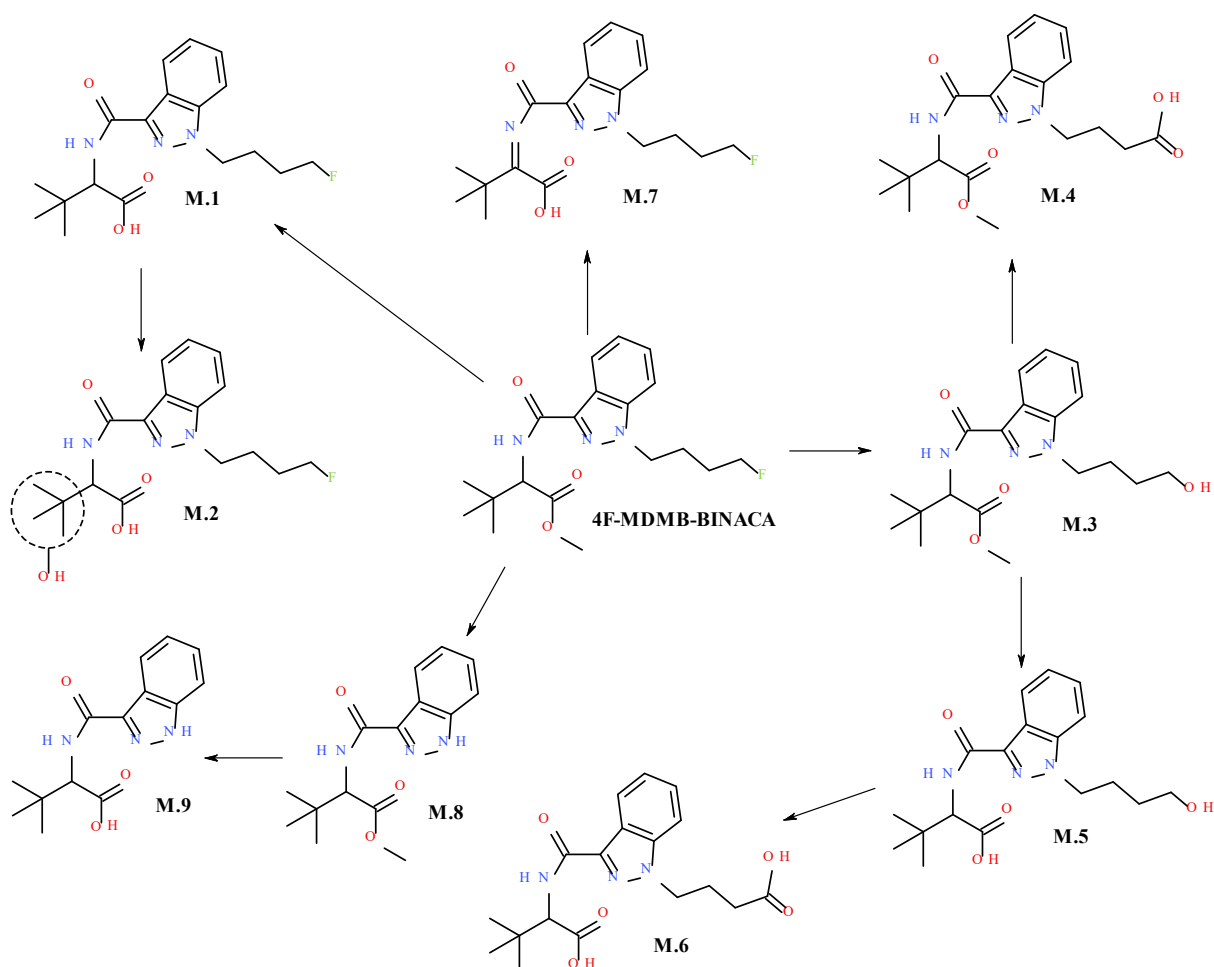


Figure 9: *In-vivo* 4F-MDMB-BINACA metabolism

Table 6: *In-vivo* 4F-MDMB-BINACA metabolites identified (4 blood and 4 urine samples)

ID	Biotransformation	RT (min)	Formula	[M+H] ⁺	Error (ppm)	Product Ions	Area (Blood)	Area (Urine)
P.0	4F-MDMB-BINACA	4.48	C ₁₉ H ₂₆ FN ₃ O ₃	364.2032	0.4	219.0934, 304.1825, 332.1775, 145.0397	-	-
M.1	Ester hydrolysis	4.29	C ₁₈ H ₂₄ FN ₃ O ₃	350.1876	0.5	219.0934, 304.1825, 332.1775, 145.0397	5,097 (n=4)	558,282 (n=4)
M.2	Ester hydrolysis, Hydroxylation	3.85	C ₁₈ H ₂₄ FN ₃ O ₄	366.1821	-0.8	219.0934, 348.1723, 320.1774	-	23,892 (n=1)
M.3	Hydrolytic defluorination	4.08	C ₁₉ H ₂₇ N ₃ O ₃	362.2079	1.3	217.0981, 302.1869, 330.1819	11,866 (n=4)	-
M.4	Pentatonic acid	4.18	C ₁₉ H ₂₅ N ₃ O ₅	376.1865	-0.6	330.1818, 316.1661, 231.0770	-	145,233 (n=2)
M.5	Ester hydrolysis, Hydrolytic defluorination	3.88	C ₁₈ H ₂₅ N ₃ O ₄	348.1919	0.4	217.0977, 302.1876, 145.0397	-	14,321 (n=1)
M.6	Ester hydrolysis, Pentatonic acid	3.88	C ₁₈ H ₂₃ N ₃ O ₅	362.1709	-0.4	231.0770, 316.1661, 145.0397	-	112,783 (n=2)
M.7	Ester hydrolysis, -H ₂	4.13	C ₁₈ H ₂₂ FN ₃ O ₃	348.1721	0.9	219.0934, 330.1618, 145.0397	2,045 (n=4)	48,561 (n=1)
M.8	<i>N</i> -dealkylation	4.03	C ₁₅ H ₁₉ N ₃ O ₃	290.1497	-0.7	230.1293, 145.0402	336 (n=1)	-
M.9	Ester hydrolysis, <i>N</i> -dealkylation	3.76	C ₁₄ H ₁₇ N ₃ O ₃	276.1346	1.3	145.0402, 230.1293	-	19,797 (n=2)

The most prominent metabolite identified was 4F-MDMB-BINACA 3,3-dimethylbutanoic acid (M.1, 4.29 mins), identified in all blood and urine specimens of this subset. Its presence has been confirmed following synthesis and addition of a standard to the library database. Eight positive results (from 7 cases: 4 urine, 4 blood) for 4F-MDMB-BINACA 3,3-dimethylbutanoic acid have been identified to date in the overall sample population. In addition, 4-OH-MDMB-BINACA (M.3, 4.08 mins) was identified as a useful biomarker in blood, identified in all blood samples of this subset containing 4F-MDMB-BINACA. Likewise, it was added to the library using standard reference material and its presence has subsequently

been confirmed in 14 blood samples overall ($n > 3,000$). In four urine cases, metabolites (e.g. 4F-MDMB-BINACA 3,3-dimethylbutanoic acid) were identified without the parent compound; these represent positive case findings that would have been missed without the incorporation of these metabolites into the workflow methodology.

4.1 Results for APP-BINACA

Five proposed metabolites of APP-BINACA were identified from data mining of one blood extract and one urine extract (Table 7, Figure 10). The two metabolites found in urine were APP-BINACA 3-phenylpropanoic acid (M.1) and 4-HO-APP-BINACA 3-phenylpropanoic acid M.3. The most prominent metabolite in blood (based on peak area) was 4-HO-APP-BINACA (M.2). Datafiles from urine samples acquired during this study ($n=312$) were subjected to data mining for all APP-BINACA metabolites discovered; one positive urine sample was identified.

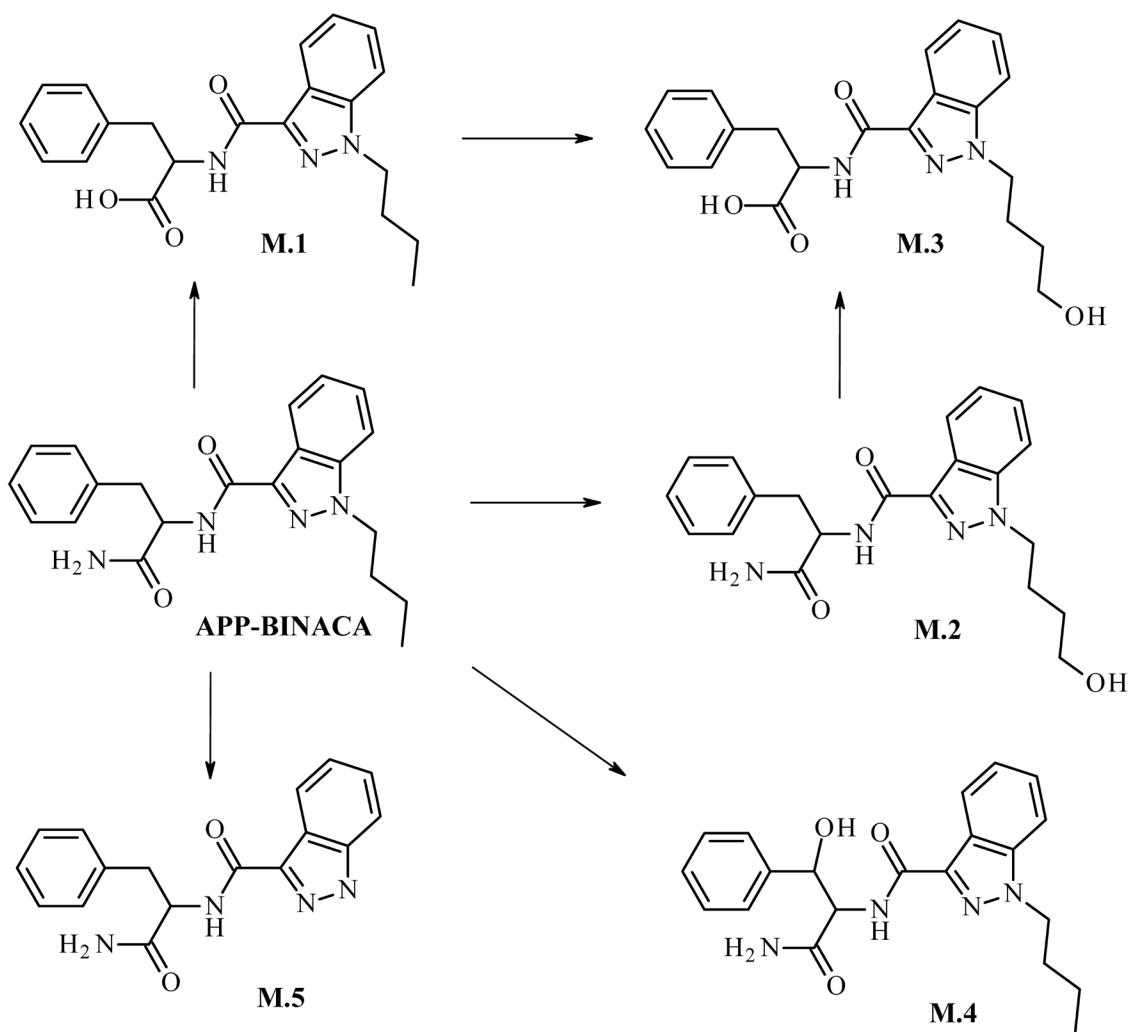


Figure 10: Observed *In Vivo* Metabolism of APP-BINACA

Table 7: *In Vivo* Metabolites of APP-BINACA Observed

ID	Biotransformation	RT (min) [±0.35]*	Formula	Exact [M+H] ⁺	Mass Error (ppm)	Product Ions (Mass Error [†])	Matrix	Area
P.0	APP-BINACA	4.25	C ₂₁ H ₂₄ N ₄ O ₂	365.1972	-0.8	348.1701 (-1.4) 320.1749 (-2.5) 201.1016 (-3.0) 145.0395 (-0.7)	Blood	205,568
M.1	Amide hydrolysis [APP-BINACA 3-phenylpropanoic acid]	4.51	C ₂₁ H ₂₃ N ₃ O ₃	366.1812	-1.9	320.1749 (-2.5) 201.1026 (2.0) 145.0406 (6.9)	Urine	5,164
M.2	Hydroxylation (butyl chain) [4-HO-APP-BINACA]	3.68	C ₂₁ H ₂₄ N ₄ O ₃	381.1921	0.2	364.1639 (-4.7) 336.1699 (-2.1) 217.0973 (0.5) 145.0394 (-1.4)	Blood	13,164
M.3	Amide hydrolysis + hydroxylation (butyl chain) [4-HO-APP-BINACA 3-phenylpropanoic acid]	3.98	C ₂₁ H ₂₃ N ₃ O ₄	382.1761	1.0	364.1636 (-5.5) 336.1693 (-3.9) 217.0961 (-5.1) 145.0396 (0.0)	Urine	5,306
M.4	Hydroxylation (benzyl, α-carbon)	4.05	C ₂₁ H ₂₄ N ₄ O ₃	381.1921	0.9	364.1643 (-3.6) 346.1511 (-11.3) 336.1679 (-8.0) 258.1216 (-8.1) 201.0999 (12.4) 145.0373 (-15.8)	Blood	4,292
M.5	Dealkylation	3.53	C ₁₇ H ₁₆ N ₄ O ₂	309.1346	3.5	292.1055 (-8.6) 264.1122 (-3.4) 145.0395 (-0.7)	Blood	413

*Analytes were detected among differing analytical runs (e.g. column age, column status); therefore, there may be deviations of RT in comparison to that of a standard reference material. [†]Fragment ions were acquired via high sensitivity mode vs. high resolution mode which can lead to increased mass error (±20 ppm was considered acceptable).

Frequently with synthetic cannabinoids, common metabolites can correlate to structurally similar parent compounds. In this report, it is important to note that the 3-phenylpropanoic acid metabolite of APP-BINACA could theoretically be produced by biotransformation of other synthetic cannabinoids: for example, the methyl ester MPP-BINACA [methyl 2-[(1-butylindazole-3-carbonyl)amino]-3-phenyl-propanoate]. However, MPP-BINACA has not been reported nationally or internationally.

5. Stability of Synthetic Cannabinoids

Results from this section have been published in the peer review literature: Krotulski et al. "[Evaluation of Synthetic Cannabinoid Metabolites in Human Blood in the Absence of Parent Compounds](#)" published in the Journal of Analytical Toxicology (2019) – currently under review.

5.1 Methods

Stability of synthetic cannabinoid parent compounds and metabolites was evaluated in human blood during this study. Blank human blood was spiked with MMB-FUBINACA, 5F-MDMB-PINACA, 5F-MDMB-PICA, and ADB-FUBINACA at 10 ng/mL. In a separate pool, blank human blood was spiked with MMB-FUBINACA 3-methylbutanoic acid, 5F-MDMB-PINACA 3,3-dimethylbutanoic acid, and 5F-MDMB-PICA 3,3-dimethylbutanoic acid at 10 ng/mL. These bulk blood supplies were then separately aliquoted (0.5 mL) into clean glass test tubes for a total of 138 individual samples (Table 2). Forty-two samples (or 21 x 2) were stored on the bench at room temperature (approx. 22 °C), 42 were stored in the refrigerator (approx. 4 °C), 42 were stored in the freezer (approx. -20 °C), and six were immediately analyzed on the date of preparation (Day 0).

At defined time intervals (i.e. Day 1, Day 2, Day 3, Day 7, Day 14, Day 21, and Day 35), three samples from each analyte type (i.e. parent and metabolite) and from each storage temperature (i.e. room temperature, refrigerator, and freezer) were removed and prepared for analysis. Samples were prepared using the acidic extraction method described below. In total, 18 samples were analyzed each day. Following extraction, samples were analyzed via the developed LC-QTOF-MS method.

Analyte stability was determined based on the change in average peak area ratio (PAR) of the triplicate samples over time and monitored over the course of roughly one month. For parent

compounds, the presence of the respective butanoic acid metabolites was monitored. Even though deuterated analogues were not available for use as internal standards during this study, it was necessary to monitor stability via PAR due to large matrix effects observed over time, especially for samples stored at room temperature. These matrix effects were noted by decrease of internal standard peak area over time, declining from an area of 12,113 at Day 0 to 5,147 at Day 35 for AB-FUBINACA-D4.

In addition to matrix stability, autosampler stability was conducted over 14 days to determine stability of all compounds reconstituted in mobile phase and stored at 10 °C (the autosampler temperature). Sample extracts prepared at Day 0 remained in the autosampler for two weeks and were reinjected with each new batch of matrix stability samples. Autosampler stability was determined in the same manner as matrix stability.

All experimental blood samples were prepared alongside blank and control samples. For preparation by LLE, blood was aliquoted (0.5 mL) into clean test tubes, fortified with internal standard (AB-FUBINACA-D4 at 5 ng/mL), and then vortexed for homogeneity. One milliliter of phosphoric acid in water (5%, v:v) was then added to all samples, followed by additional brief vortex. Three milliliters of extraction solvent (80:10:10, hexane:ethyl acetate:MTBE, v:v) was added. Samples were capped and rotated for 15 minutes, followed by centrifugation at 4600 rpm for 10 minutes. The supernatant was removed by freezing the aqueous layer and then evaporating to dryness at 35 °C. Following dry down, all samples were reconstituted in 200 µL of initial chromatographic conditions (95% A, 5% B).

Samples were analyzed using a previously validated LC-QTOF-MS analytical method. Due to the nature of this study, a non-targeted analytical workflow was selected, consisting of

generic chromatographic separation and data independent acquisition. This approach allowed for breakdown product discovery, if necessary.

To determine peak area, the protonated precursor ion associated with each analyte and internal standard were extracted from the TOF MS data and the resulting chromatographic peaks were used. To determine PAR, the peak area of each analyte was divided by the peak area of the internal standard (AB-FUBINACA-D4). Several internal standards were evaluated during the development of this research study (e.g. JWH-018-D8, XLR-11-D5, AM-2201-D5), but it was determined that AB-FUBINACA-D4 was most chemically equivalent to the synthetic cannabinoid parent compounds and metabolites studied based on structure and experimental data.

5.2 Parent Compound Stability

Over the course of this study, the stability experiments described here were replicated three times, each time concluding similar results (e.g. instability and formation of butanoic acid metabolites). Two sources of human blood were evaluated, as well as gray and lavender top blood collection tubes. The results presented herein represent the final study using preserved (sodium fluoride and potassium oxalate) human whole blood, as this set was evaluated concurrently with metabolite stability.

Figures 11, 12, and 13 show the relative abundance of MMB-FUBINACA, 5F-MDMB-PINACA, 5F-MDMB-PICA, ADB-FUBINACA, as well as their respective metabolites over time when stored at room temperature, in the refrigerator, and in the freezer, respectively.

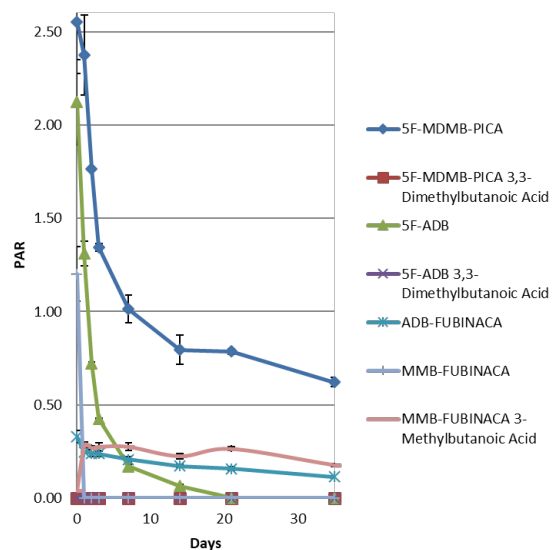


Figure 11: Room temperature stability for parent compounds

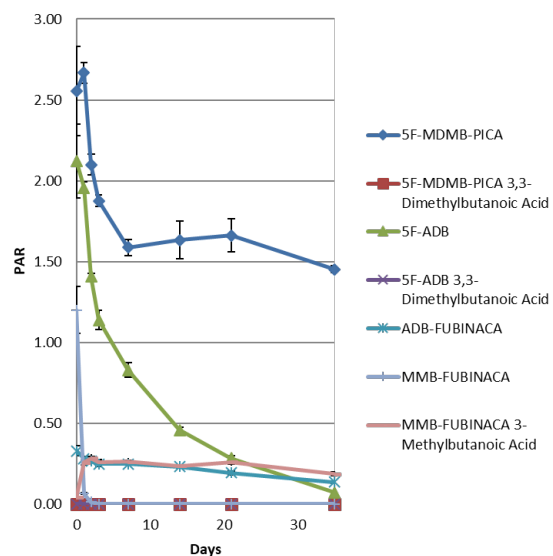


Figure 12: Refrigerated stability for parent compounds

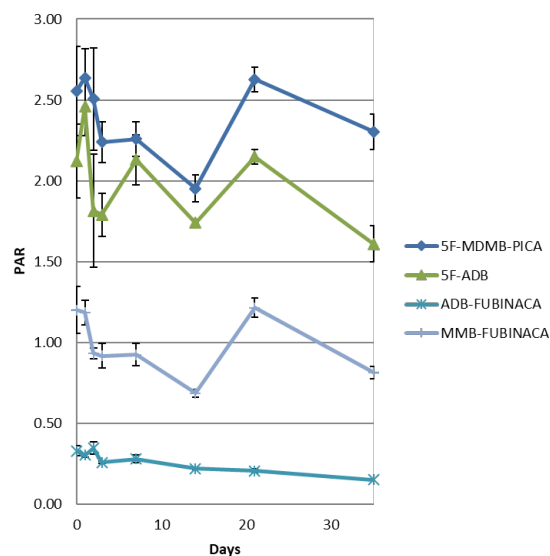


Figure 13: Frozen stability for parent compounds

The greatest stability was noticed with all analytes when stored in the freezer, which was expected based on other drug stability studies and what is known about temperature effects on analyte degradation. During these experiments, ADB-FUBINACA 3,3-dimethylbutanoic acid was not detected in any blood sample, irrespective of storage temperature. All other metabolites were detected in at least one blood sample, during one of the replicated experiments.

MMB-FUBINACA was found to be extremely unstable in blood when stored at room temperature and in the refrigerator. In fact, MMB-FUBINACA was not detectable in blood specimens even after 1 day stored at room temperature and after 3 days stored refrigerated. These results are of interest based on typical best practices for blood storage conditions (e.g. refrigerator) and typical time between sample collection and analysis (10-30 days). MMB-FUBINACA was detected in all blood samples stored frozen with no noticeable decline in abundance.

With the degradation of MMB-FUBINACA, this study found the increase of metabolite (or breakdown product) MMB-FUBINACA 3-methylbutanoic acid. MMB-FUBINACA 3-methylbutanoic acid was detected as early as 1 day after preparation and storage at both room temperature and refrigerated. MMB-FUBINACA 3-methylbutanoic acid was not detected in the blood specimens stored frozen. Of note, the average peak area of the MMB-FUBINACA 3-methylbutanoic acid identified in samples prepared with MMB-FUBINACA was the same as the average peak area in samples prepared with MMB-FUBINACA 3-methylbutanoic acid (identical experimental conditions). Both analytes were prepared at a concentration of 10 ng/mL, leading one to believe this degradation from MMB-FUBINACA to MMB-FUBINACA 3-methylbutanoic acid was nearly complete.

5F-MDMB-PINACA was also found to be highly unstable in blood when stored at room temperature and in the refrigerator, losing nearly 90% after only 7 days. Furthermore, the parent compound was undetectable in all blood samples analyzed after three weeks at room temperature; but when store refrigerated or frozen, there was no time point where the parent was undetectable. 5F-MDMB-PINACA 3,3-dimethylbutanoic acid was detected in blood samples stored at room temperature after one month during the first iteration of this study, but not during the final iteration, likely due to high matrix effects. This metabolite was undetected at all other storage temperatures and lengths of time tested. Inconsistency in the detection of 5F-MDMB-PINACA 3,3-dimethylbutanoic acid over the time points could be attributed to ionization efficiency difference and limit of detection. 5F-MDMB-PINACA was detected in all blood samples stored frozen with no noticeable deviation in abundance.

5F-MDMB-PICA was found to be unstable in blood with a 75 % loss at room temperature after one month of storage, but in comparison to MMB-FUBINACA and 5F-

MDMB-PINACA, 5F-MDMB-PICA was overall more stable. Parent 5F-MDMB-PICA was detectable at all time points and storage conditions studied. 5F-MDMB-PICA 3,3-dimethylbutanoic acid was detected in blood samples stored at room temperature after 30 days during the first iteration of this study only, and undetected at all other storage temperatures and lengths of time tested.

ADB-FUBINACA was found to be stable during this study, serving as an appropriate internal control. ADB-FUBINACA was detected in all blood samples, regardless of storage condition and length, with no noticeable deviation in abundance.

Detection of butanoic acid synthetic cannabinoid metabolites, specifically those formed from terminal esters on the head region, proves to be useful for the accurate characterization of historic synthetic cannabinoid ingestion. Specifically relating to MMB-FUBINACA and 5F-MDMB-PINACA, the detection of these metabolites in blood could be more useful from an analytical perspective and increase the detection windows for suspected synthetic cannabinoid use, a finding contradictory to traditional synthetic cannabinoid blood testing procedures.

As mentioned previously, the detection of synthetic cannabinoid metabolites in blood was found to be source dependent and prone to matrix effects. 5F-MDMB-PINACA 3,3-dimethylbutanoic acid and 5F-MDMB-PICA 3,3-dimethylbutanoic acid were only detected at room temperature during the first iteration of the experiments using a different human blood source. Irrespective of this issue, these butanoic acid metabolites continue to be identified in postmortem forensic toxicology casework (48).

While this study examined three terminal methyl esters, it is hypothesized that instability of ethyl esters (e.g. 5F-EDMB-PINACA) and other methyl esters (e.g. 4F-MDMB-BINACA) would lead to similar results. Due to similarities in metabolism (e.g. ester dealkylation) (44, 45),

degradation of ethyl esters would produce identical breakdown products to the methyl esters, complicating toxicological interpretation. Nonetheless, the incorporation of these potential common breakdown species (or common metabolites) into analytical methods would greatly impact utility and effectiveness in identifications.

5.3 Metabolite Stability

Figures 14, 15, and 16 show the relative abundance of MMB-FUBINACA 3-methylbutanoic acid, 5F-MDMB-PINACA 3,3-dimethylbutanoic acid, and 5F-MDMB-PICA 3,3-dimethylbutanoic acid over time when stored at room temperature, in the refrigerator, and in the freezer, respectively. All three metabolites were found to be stable in blood regardless of storage condition, unlike their parent counterparts. No significant loss in abundance was observed.

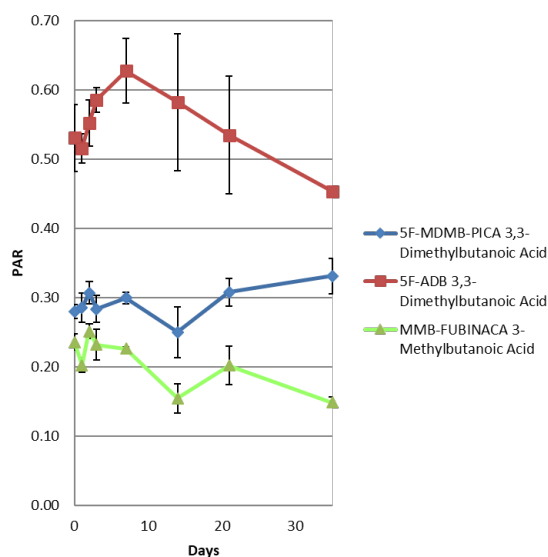


Figure 14: Room temperature stability for metabolites

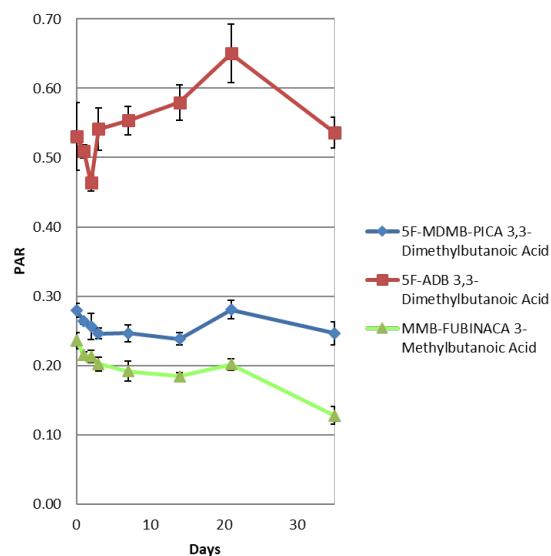


Figure 15: Refrigerated stability for metabolites

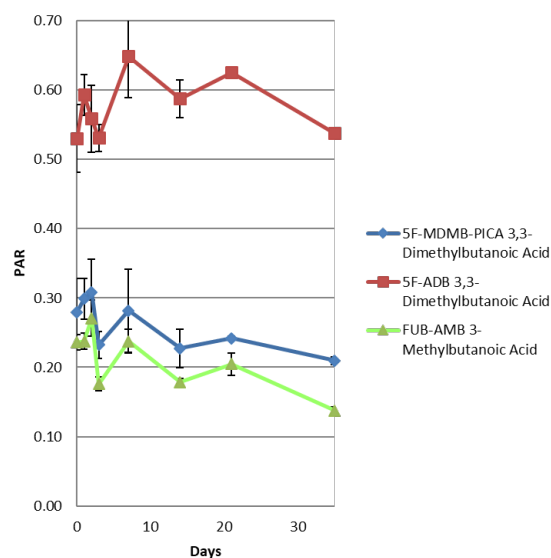


Figure 16: Frozen stability for metabolites

5.4 Autosampler Stability

Figure 17 shows the relative abundance for MMB-FUBINACA, MMB-FUBINACA 3-methylbutanoic acid, 5F-MDMB-PINACA, 5F-MDMB-PINACA 3,3-dimethylbutanoic acid, 5F-

MDMB-PICA, 5F-MDMB-PICA 3,3-dimethylbutanoic acid, and ADB-FUBINACA when stored in the autosampler. All analytes were found to be stable in extract form when stored at 10 °C for at least two weeks. No significant loss in abundance was observed for any analyte. These results show that the instability of the parent compounds displayed during this study is the result of matrix instability rather than processed sample instability.

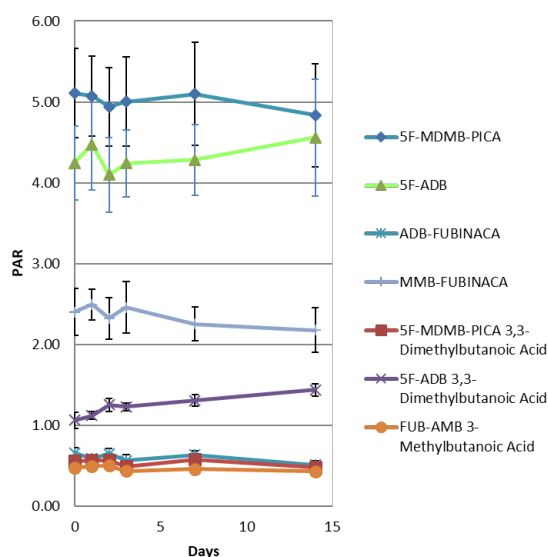


Figure 17: Autosampler stability for parent compounds and metabolites

5.5 Discussion

In 2018 and 2019, MMB-FUBINACA, 5F-MDMB-PINACA, and 5F-MDMB-PICA were the most prevalent synthetic cannabinoids identified in forensic casework. Through evaluation of stability in human blood, we found that these three compounds degrade to their butanoic acid metabolites under standard storage conditions. These findings are of great value as these metabolites can be used as biomarkers of synthetic cannabinoid use, but caution should be used with respect to analysis and interpretation.

Analytically, synthetic cannabinoids differ from other NPS classes due to basicity of parent compounds and acidity of metabolites, often requiring separate extraction methods. Herein, we present a workflow that allows for characterization of parent compounds and metabolites within a single extraction protocol and single instrumental method, although further evaluation of this extraction protocol is needed (e.g. full validation) for full implementation in forensic casework. Laboratory scientists and toxicologists should be aware of synthetic cannabinoid instability and should develop methods to remedy the issue in forensic samples for more accurate identification of synthetic cannabinoids ingestion.

The absence of synthetic cannabinoid parent compound in blood presents great interpretative challenges for forensic toxicologists. Toxicological interpretation of synthetic cannabinoid positive cases has historically been challenging due to unknown toxicity and lack of correlation among reference data, if available. While the results of this study may seem to complicate this matter, it is important to understand the link between synthetic cannabinoid metabolite positivity in blood. Forensic toxicologists should consider this case report during future interpretation of synthetic cannabinoid metabolite only blood results.

Storage of blood specimens bound for synthetic cannabinoid testing should be stored in optimal conditions, depending on available storage conditions and length of time between collection and analysis. In forensic toxicology, samples are often analyzed more the 3-4 weeks from time of collection; therefore, we suggest storage of blood specimens in the freezer. As is the case with most postmortem casework, the use of collection tubes with preservatives should continue to be used; however, there was no significant impact of preservative on the analytes studied.

6. Conclusions

In the United States, synthetic cannabinoids pose significant challenges for public health and public safety agencies. New synthetic analogues that target endogenous cannabinoid receptors continue to appear on recreational drug markets, sometimes increasing in potency and toxicity in comparison to previous generations. Synthetic cannabinoid positivity and prevalence has changed over the last ten years since the emergence of the first compounds in 2008. Current trends suggest that new synthetic cannabinoids appear on a monthly basis, but typically only one (or two) analyte(s) will proliferate and dominate the market for roughly one year in time. When this research was funded, 5F-MDMB-PINACA and MMB-FUBINACA were the most prevalent synthetic cannabinoids in the United States. It was the goal of this project to overcome the analytical challenges associated with synthetic cannabinoid detection to identify the next wave(s).

During this funded research, a comprehensive workflow for the identification and characterization of synthetic cannabinoids was developed, validated, and implemented for forensic toxicology testing. The primary challenge was overcoming the complexity of detecting older generations of compounds alongside the newest (and yet to be known) generations with high accuracy. In order to accomplish this task, LC-QTOF-MS was selected as the appropriate analytical platform, allowing for the use of a non-targeted acquisition method which could be all-inclusive within a given mass range. Acquisition of HRMS data allowed for greater certainty with respect to positive analyte identifications, and incorporation of fragment ion spectra proved to be the most useful aspect for successful sample mining and data mining. In addition, a large library database was developed using available standard reference material totaling more than 250 synthetic cannabinoid parent compounds, metabolites, and internal standards. In the end,

more than 6,000 discard sample extracts were analyzed using the developed workflow, which in turn allowed for generation of trend reports and other important documentation to track changes among synthetic cannabinoids in the United States.

Throughout the course of this research, changes in the rise and fall of specific synthetic cannabinoids were monitored and noted. By the beginning of 2019, 5F-MDMB-PINACA and MMB-FUBINACA were dethroned as the most prevalent compounds and replaced by 5F-MDMB-PICA. The prevalence and positivity of 5F-MDMB-PICA continued to increase throughout 2019 and it is unclear whether this analyte will continue to proliferate or begin to fall off in positivity over the coming months. Mirroring the rise of 5F-MDMB-PICA, 4F-MDMB-BINACA emerged and increased significantly in prevalence and positivity in 2019. Other synthetic cannabinoids were also discovered over the course of this research, of which MDMB-4en-PINACA deserves close monitoring in 2020. It is important to note that all of the synthetic cannabinoids identified during this research were found in postmortem samples and associated with death investigations, alluding to the severity of their public health and public safety impacts.

A primary aim for this research was to identify emerging synthetic cannabinoids in a timely manner and disseminate information to the forensic science community for swift action and implementation. Several new synthetic cannabinoids were identified for the first time in forensic toxicology casework during this research, some of which were identified for the first time in any forensic casework (e.g. APP-BINACA, MDMB-4en-PINACA). New drug monographs, containing basic drug information, brief description, and analytical data, were produced and disseminated for all new synthetic cannabinoids identified during this study. While it is difficult to truly examine their timeliness and impact, it is believed that these reports were released three to six months sooner than they would have been without this dedicated research.

In addition, a public health alert was released for 4F-MDMB-BINACA to increase awareness among clinical professionals, medical examiners and coroners, and public health communities, specifically due to the rapidly increasing number of death investigations possibly being unreported or underreported (i.e. reported as “no drug findings”) due to testing protocols not incorporating this new analyte at the beginning of 2019.

Several interesting phenomena were documented during this study that should be considered for future investigations involving synthetic cannabinoids. First, the discovery of an emerging synthetic cannabinoid was often linked to combinations with other prevalent analytes. For example, 5F-MDMB-PICA was first discovered in combination with 5F-MDMB-PINACA, and 4F-MDMB-BINACA was first discovered with 5F-MDMB-PICA. These findings are significant because they validate the workflow and efficacy of testing discarded sample extracts but also may allow for predictive modeling and/or prevalence forecasting. Second, analysis of prison urine samples showed that emerging synthetic cannabinoids were infiltrating prison supplies, rather than prison supplies remaining stagnant or intruded by older generation compounds. 4F-MDMB-BINACA was detected in urine samples from prison populations around the same time it emerged within postmortem casework. This requires laboratories testing urine specimens from prison to remain at the forefront of synthetic cannabinoid trends and discovery. Third, it is critical to incorporate analytical testing for synthetic cannabinoids with clinical populations where adverse events are associated and/or reported. A variety of synthetic cannabinoids were found among individuals presenting to emergency departments; however, clinicians should be aware that the specific synthetic cannabinoid(s) present within their municipality could vary based on location or could be in combination based on individual prevalence.

To conclude, given the success of this project in identifying novel synthetic cannabinoids in the United States (some of which for the first time prior to elevation to the most prevalent drugs in this class), it is unfortunate that this project comes to completion at the end of 2019. There is currently no funded initiative to continue this sentinel drug monitoring work. It is likely that forensic science and criminal justice communities will return to a state of being surprised by new, potent, and toxic synthetic cannabinoids (and NPS in general) only after their continued appearance in emergency rooms and autopsy suites, as detections may be missed or go undiscovered for several months. Moreover, there is no system that will provide real time reports on the identification of these substances or emerging trends for dissemination to the forensic science and criminal justice communities. NPS intelligence, surveillance, and accurate reporting are of great importance as the United States enters a poly-drug crisis, and the need for further non-targeted, comprehensive testing of biological samples for NPS is of substantial value to all.

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