Challenges of screening and identifying NPS in the forensic laboratory

An interview with Dr Alex Krutolski, Research Scientist at the Center for Forensic Science Research and Education (CFSRE)

Left: A sneak peak into Alex's laboratory and working environment. Right: SCIEX TripleTOF® 6600 LC-MS/MS System for non-targeted screening of NPS.

The prevalence of novel psychoactive substances (NPS) has increased over the last few decades. The challenges relating to NPS screening and identification are impacting scientists globally.

Dr. Alex J. Krotulski serves as a Research Scientist at the Center for Forensic Science Research and Education (CFSRE) and the Program Manager for NPS Discovery – which is a collaborative flagship program for the identification of new synthetic drugs and the dissemination of information surrounding their impact. His current research and casework focus heavily on aspects related to the detection and characterization of NPS, including studies that examine NPS positivity, trends, metabolism, and effects through intelligence, surveillance, monitoring, and response efforts.

In this interview, Alex shares his insights on the scope of the global NPS issue, the challenges associated with NPS screening and detection and the work that is being conducted in his laboratory to overcome these challenges.

Q: Can you provide some context as to why designer drugs and NPS are an issue and why is it important to detect these substances?

A: Novel psychoactive substances (NPS) (sometimes referred to as designer drugs, synthetic drugs, or research chemicals), are chemical substances that are specifically designed to act like traditional drugs of abuse by targeting endogenous receptor systems within the body. There are several reasons why different or new NPS can emerge, such as the desire for an increase in favorable effects or a decrease in adverse effects, the evasion of laws based on new drug legislature or scheduling actions, or simply drug user curiosity. These factors lead to the emergence of new NPS on a weekly to monthly basis. This can be very challenging for analytical chemists and forensic scientists who are trying to remain up-to-date with scopes of testing and other associated information (e.g. concentrations, combinations, metabolism).

The history of specific NPS differs based on the origin of their discovery. Some NPS were previously synthesized and studied by pharmaceutical companies or academic researchers, resulting in the availability of peer-reviewed literature or patent filing that can serve as road maps for their synthesis in clandestine (or more sophisticated) laboratories. When studying these substances in the past, often in the 60s, 70s, or 80s, information about activity and potency may have been generated and published – this is

desirable for those intending to produce, sell, or use the substance since they know it will create an effect, whether desirable or, unknowingly, undesirable.

NPS that do not have a historical record are often modified based on the chemical structure of previously described or prevalent substances and, in turn, their activity or potency is assumed based on those comparisons. However, there are truly no accurate ways to evaluate the toxicity of a new synthetic substance without performing experimental studies, either *in vitro* or *in vivo*. The risks associated with NPS use that lead to morbidity and mortality consider all of these factors.

Emerging NPS can be more potent and more toxic compared to the last generation of the substance, leading to an increased risk of drug overdose or death. In addition, emerging NPS can have different effects on the body that are uncharacterized or unstudied, which can complicate aspects of interpretation, whether by scientists, medical professionals, law enforcement, etc. Based on their effects on the body, NPS are often detected among forensic investigations (i.e. postmortem/death, driving under the influence of drugs (DUID)) and clinical investigations (i.e. non-fatal overdoses, emergency department admissions, poison center calls).

The majority of these investigations will include testing of biological samples to confirm the presence of an intoxicating agent (e.g. NPS), however, the issue becomes "is this NPS in the scope of testing". It has become crucial for laboratories to maintain testing protocols that allow for the detection and discovery of NPS. Resolution of these investigations is often contingent on the identification and confirmation of the substance. If NPS and specifically, the newest and emerging NPS, are not incorporated into testing workflows, results could be reported as "negative." This can lead to inaccurate or under reporting, which can have downstream effects such as a lack of connection between an impairment and the presence of a drug, inconsistent autopsy findings in comparison with toxicology testing, public health reporting of drug use or death statistics.

Q: What NPS emerging or recurring trends has your laboratory observed over the years?

A: The emergence of NPS in the United States began around 2008. Since then, the landscape of NPS has evolved differently based on specific classes. Typically, NPS are subdivided into categories including opioid, cannabinoid, benzodiazepine, stimulant, and hallucinogen.

Fentanyl (a drug patented under pharmaceutical development and widely used among current medical practices) was the first major player to take over the NPS opioid landscape. Prior to this time, other fentanyl analogues had emerged – causing considerable numbers of deaths in areas nationally and internationally – but these are largely considered isolated incidences prior to fentanyl's emergence under the current NPS era. Once fentanyl took over as the dominant NPS opioid, clandestine chemists began looking for ways to increase overall output or impact. This ultimately led to the emergence (or re-emergence) of fentanyl analogues. These drugs were largely simple modifications of the basic fentanyl scaffold, substituting or adding atoms or functional groups. This process had differing effects on activity, potency, and overall toxicity. Several fentanyl analogues proliferated nationally, resulting in hundreds to thousands of deaths, which can be accounted for among the rise in opioid deaths during what is currently considered to be an opioid

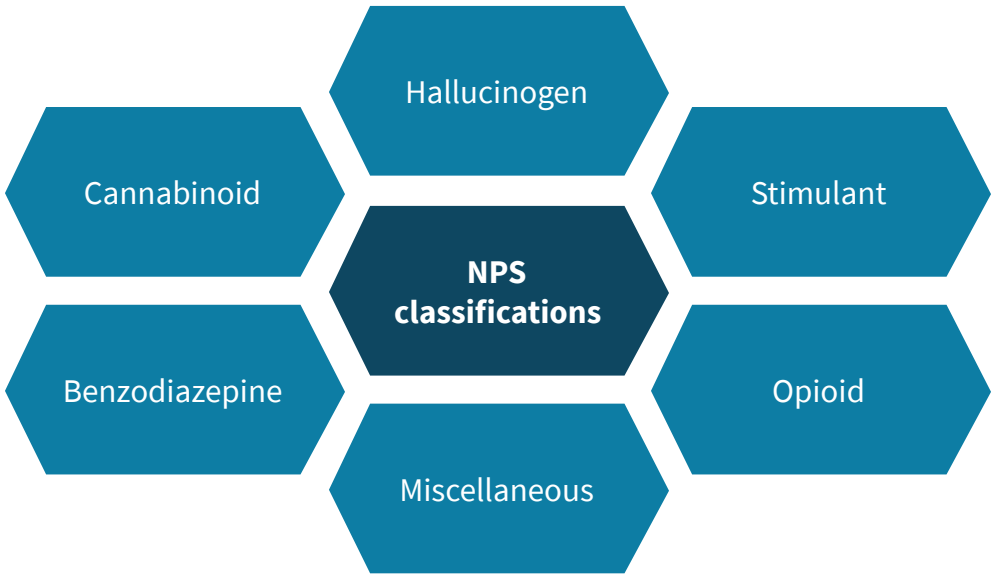

Figure 1: *A diagram showing the seven different NPS classifications*

epidemic. Key players at this time were furanylfentanyl, 3-methylfentanyl, and carfentanil (notorious due to its reported relative potency). During this time, other NPS opioids were also present and prevalent, notably U-47700, a non-fentanyl derived substance (which was also patented by a pharmaceutical company during drug development).

Due to the staggering number of fentanyl analogue deaths, scientists, in collaboration with law enforcement, devised a plan for core structure scheduling of the fentanyl class. Beginning in 2016, this meant that fentanyl analogues were all Schedule I substances, the highest ranking within drug scheduling. As intended, this legislative action resulted in the sharp decline in the number of positive testsfor these substances. Now, in 2020, fentanyl analogues are rare occurrences among the NPS landscape, replaced by new NPS opioids which look structurally different. Fentanyl continues to dominate in this space, but new and emergent NPS opioids continue to appear on at least a monthly basis. This shift has created new challenges for scientists, as many of the new NPS opioids have limited or no available pharmacological data available (where it was previously assumed that the fentanyl analogues retained activity and had similar/increased potency). The current NPS opioid landscape continues to be quite dynamic.

The NPS synthetic cannabinoid landscape largely started with the emergence of new substances that were pirated from academic research and pharmaceutical drug discovery. The most notable substance was JWH-018. The synthetic cannabinoids class historically is the most chemically diverse and analytically challenging – this can somewhat be imagined by the nomenclature used for these substances. Turnover among the trends within this class are often referred to as “generations”, which is a term linked originally to structural representations. Synthetic cannabinoid positivity, like many of the classes, is directly linked to scheduling actions – as a substance is scheduled, a new substance emerges. Through this process, certain structural features have remained or become common, providing insight into preferential synthetic pathways or patterns of use. The most common drugs among this class recently are 5F-MDMB-PINACA (5F-ADB), 5F-MDMB-PICA, 4F-MDMB-BINACA, and MDMB-4en-PINACA.

With respect to NPS benzodiazepines, this class is typically comprised of the fewest structural variations. These substances retained the fused benzene (or other aromatic) ring and diazepine ring, with or without the addition of the triazole ring. Common variations include the addition of halogens (e.g. fluorine, chlorine, bromine). Many of these substances were developed for medicinal purposes, so literature regarding their activity and potency may be available. One challenge among this class is the different uses of NPS benzodiazepines internationally – some of these substances can be prescribed in one country and be emerging or abused in another country. There does not appear to be an overall trend with respect to the next substance to emerge – like other classes, this is usually related to drug scheduling or user preference or availability.

Depending on location, NPS stimulants can be the most commonly encountered NPS class, and this class has seen many new synthetic variants over the years. NPS stimulants are mostly developed to mimic the effects and/or structure of amphetamine, MDMA, and cathinone at their core. To complicate matters, there are several NPS stimulant subclassifications, of which the most commonly encountered substances belong to the beta-keto-methylenedioxyamphetamine category. The first substance from this category was methylone (the beta-keto version of MDMA). Since methylone, several homologues have emerged, including ethylone and butylone, and the series continues over several analogues with elongated carbon tails and amine substitutions. While the variations here seem endless, there is a limit to chain length that dictates effects. Other common NPS stimulants belong to amphetamine and beta-keto-amphetamine categories, including compounds like fluoro-amphetamine and mephedrone, respectively. Trends among this class continue to see the emergence of new substances that are structurally related but differ based on simple function group variations (i.e. adding a methyl group, adding a halogen).

NPS hallucinogens are the least commonly encountered class, and, like other classes, the most commonly encountered substances are often structure related to traditional hallucinogen (e.g. ketamine, PCP, LSD, tryptamine). The rate of turnover among this class can be rapid, but with very few positives – a certain challenge for analytical chemists. Trends among NPS hallucinogens also vary geographically (i.e. East vs. West coast).

Q: How can mass spectrometry be used to detect designer drugs and NPS, and what are its advantages over other screening approaches?

A: Mass spectrometry (MS) is one of the most useful analytical tools for detecting small molecules, such as drugs and NPS. MS allows for the detection of mass characteristics for both intact (or precursor) molecules and their fragments, which can serve as a chemical fingerprint for the identification or structural elucidation purposes. Paired with chromatographic separation, MS has become the gold standard for drug detection in forensic chemistry and forensic toxicology. Increased sensitivity and good specificity have allowed MS to become the go-to analytical technique over others. Due to the ability to separate species among the mass filters, mass spectrometers allow for the analysis of complex sample matrices (i.e. drugs in blood samples, or drugs in a powder that has been cut or diluted with other drugs) – of course, chromatography helps the notion or need for separation. All of these factors together make MS an accurate, reliable, and preferred means for drug identification.

Q: Can you talk us through some of the challenges associated with the various methods for screening and detecting NPS and designer drugs?

A: Like other analytical platforms, mass spectrometers come in many shape and sizes, often due to their capabilities and internal hardware (i.e. mass filters). Mass filters make a mass spectrometer unique, differentiating their abilities to generate specific information among their close relatives. For example, mass spectrometers with quadrupole mass filter only allow for nominal mass measurements, and as such, these instruments are often used for comparative purposes (i.e. library searching, confirmation, quantitation, etc.). Some structural information can be gained by the use of quadrupoles alone, however, better and more accurate structural information is acquired via the use of high resolution mass spectrometry (HRMS) mass analyzers, such as time-of-fight (TOF) or orbitrap. TOF MS generates accurate mass measures which can be compared to the theoretical exact mass of a compound, and within certain constraints, a scientist can determine the chemical formula of a detected species. This information becomes extremely useful when discovering new synthetic drugs, but also has great utility for screening purposes. TOF analyzers placed in parallel with quadrupole analyzers allows for the generation of accurate mass fragment data, which can be used for more reliable structural elucidation (another great benefit).

Quadrupole time-of-flight (QTOF) MS is an expanding field in drug detection and has proved to be the most valuable tool for drug discovery and the most accurate tool for drug screening or identification. However, QTOF systems are very complex platforms and there is no standard method of operation. Due to the variability among mass analyzer operation and vender configurations, QTOF systems can be operated in numerous manners, which can be referred to as acquisition modes. These acquisition modes define how the mass analyzers function, and more specifically how the quadrupole is operated. Examples include MS^2, MS^e (or MS^{ALL}), and MS/MS^{ALL}. MS/MS^{ALL} (or SWATH® Acquisition, as referred to by SCIEX) is the middle of the road option between MS^2 (or information dependent acquisition [IDA], a targeted acquisition approach) and MS^e (or data independent acquisition [DIA]), a non-targeted acquisition approach. SWATH Acquisition is a DIA, non-targeted approach. SWATH Acquisition combines the powers of accuracy and specificity to provide a complete picture of the drugs within a sample while alleviating any of the worry that pertinent information will not be collected. In short, SWATH Acquisition utilizes the quadrupole as a segmented mass filter, meaning it allows only a range of masses to pass through Q1 at a given time (MS^2 allows only one mass to pass at a given time, MS^e allows all masses to pass at a given time). This results in higher specificity among fragment ions produced (compared to MS^e), and increased accuracy when conducting tasks such as structural elucidation.

Some of the most impactful challenges associated with these acquisition modes and NPS detection involve the ability to distinguish isobaric species and to accurately perform structural elucidation. SWATH Acquisition alleviates some of the challenges presented with respect to structural

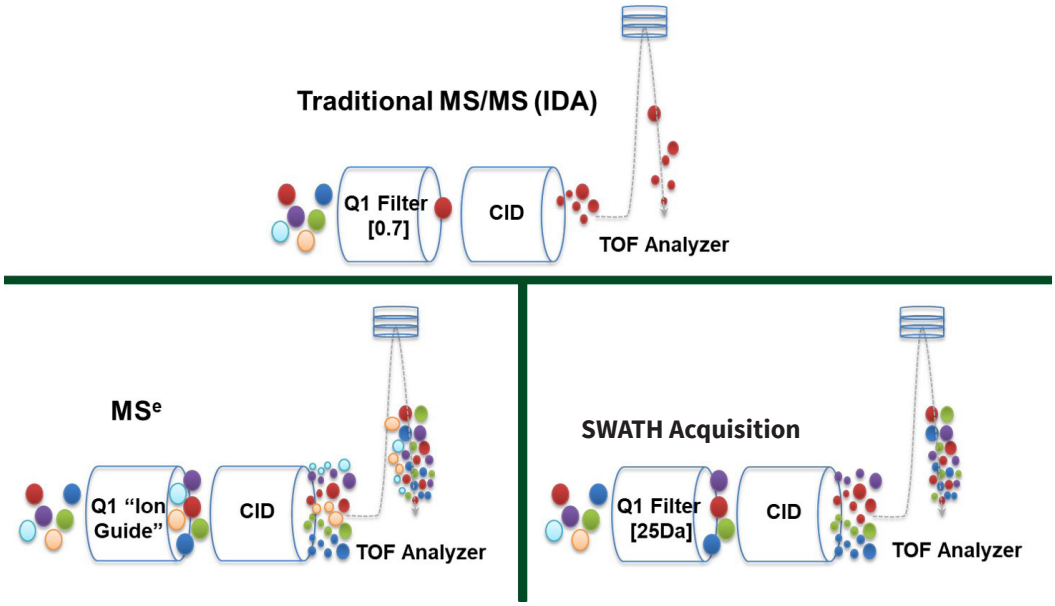

Figure 2: *A schematic view of a traditional QTOF MS depiciting the different acqusition modes: MS2, MSe and SWATH Acquisition. The function of the quadrupole (Q1) dictates what masses make it through to the colliusion cell (CID) and TOF analyzer.*

elucidation because there is certainty that the fragment (or MSMS) data will be available for review. In addition, formula finder searching can be performed on accurate mass fragment data (like those of the precursor ion, or TOF MS data), which allows the scientist to determine the formula of a given fragment. Structural elucidation is a difficult science and requires specific expertise; however, acquisition using the technique described positively impacts the interpretation. Isobaric species, and specifically positional isomers, are a great challenge among all aspects of forensic chemistry and forensic toxicology. Accurate determination of structural isomers is extremely important, especially when the isomer pair can have differing potency or toxicity. The use of HRMS, and specially QTOF MS, can assist with distinguishing isomers, from a mass spectrometer standpoint alone. Like traditional GC-EI-MS data processing, QTOF-MS fragment ion spectra can be compared to a library generated from the analysis of standard reference materials. This links back to the notion that the instruments acquire chemical fingerprints for drugs. This is an added benefit to using accurate mass fragment data to distinguish isobaric species, increasing confidence. However, it should still be noted that certain isomers (specifically several fentanyl analogues) cannot be distinguished by MS methods alone – this remains a great challenge analytically.

Q: What strategies have your lab been using for NPS early identification and discovery? What tools do you have in place to streamline the process?

A: Early on in our program, our laboratory developed and validated two LC-QTOF-MS methods for the detection and discovery of NPS. Both of these methods employ SWATH Acquisition and we have had a lot of success using these methods. We have made it a priority to maintain up-to-date libraries, often incorporating the newest reference standards to become available. This has led to our library database growing to more than 800 compounds, all of which we can accurately identify (this means they include fragment spectra – this is not just a suspect screen).

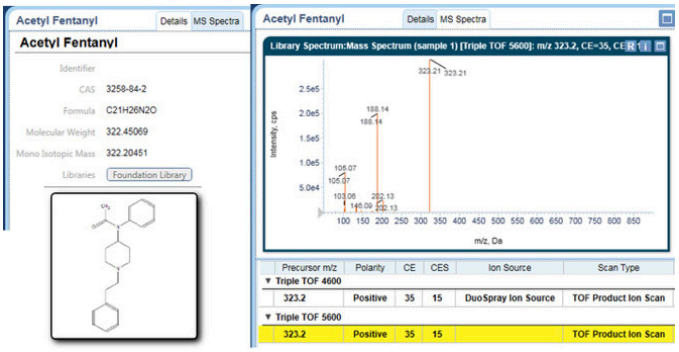

Figure 3: *The library view of a QTOF-MS fragment spectra compared to a library generated from the analysis of standard reference materials.*

While the upfront work to get these methods off the ground was no small task, this is not where the work ends. In order to develop an accurate and timely workflow for the discovery of NPS, a laboratory needs to identify which sample populations they will begin

Figure 4: *Data processing with MasterView™ Software for TOF MS and MSMS data*

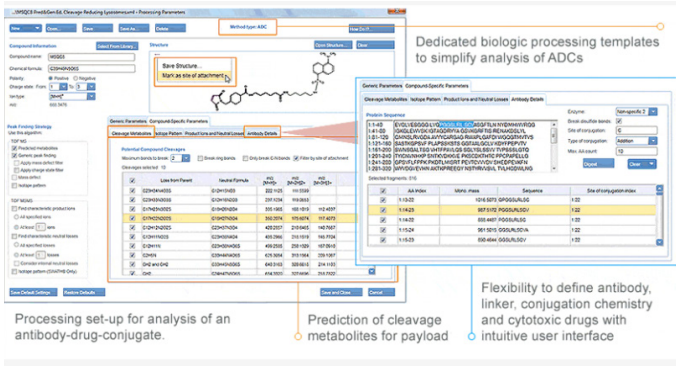

Figure 5: *MetabolitePilot™ Software which has structural drawing features, can be used to piece together a tentative structure of an unknown compound*

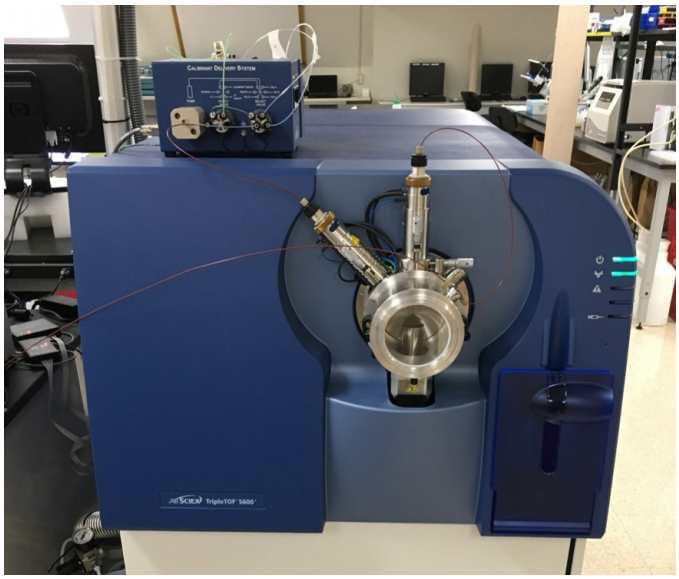

Figure 6: *A front view of the SCIEX TripleTOF® 5600+ LC-MS/MS System used for NPS identifications.*

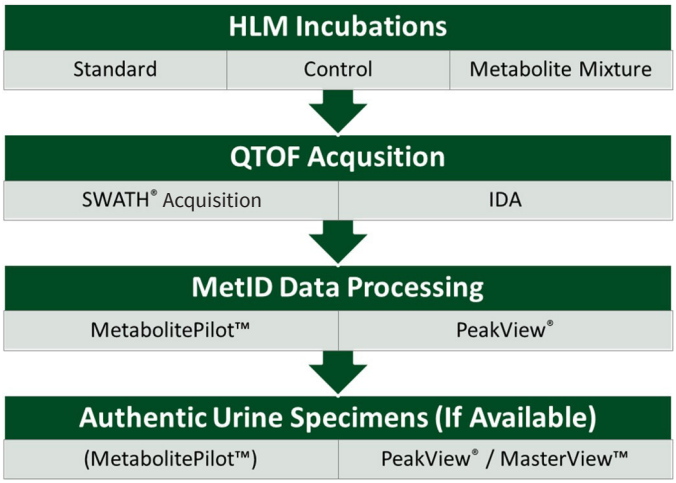

Figure 7: *The customized workflow used for metabolite identifications for new and emerging NPS. Experiments begin with HLM incubations and lead to analysis of authentic urine samples, if available.*

to test or monitor. We began implementing our SWATH Acquisition methods for the detection of emerging synthetic drugs among seized drug materials and toxicology samples. We created partnerships with federal laboratories to test powders entering the country through the mail. We work with state and local partners to test seized street level samples and/or toxicology samples. And finally, for our largest population, we partner with a forensic toxicology laboratory to receive and test discarded sample vial extracts from authentic forensic casework where NPS use is suspected. Through all these avenues, and paired with our non-targeted SWATH Acquisition methods, we are positioned to detect and characterize NPS at their first incidence, or as close as possible to their first incidence, among the drug supply.

For identification purposes, we use SCIEX PeakView® Software and MasterView™ Software to process data and view TOF MS and MSMS data, comparing acquired mass spectra with those that are expected or within the library database. For true unknown identifications of NPS, we use SCIEX PeakView Software and MetabolitePilot™ Software (which has great structural drawing features) to piece together a tentative structure, based on our expertise and what we have seen before with other drugs or NPS.

Q: Can you expand on the work your laboratory has done over the past couple years (more specifically with the work around NPS Discovery) for NPS early identification and discovery?

A: Our laboratory has broken NPS identification and discovery into three main areas surveillance, monitoring, and response.

Under our surveillance initiatives (as described above), we spend a lot of time and effort to discover new NPS as they emerge within the drug supply or as they emerge with death investigation casework. This process can be the most time

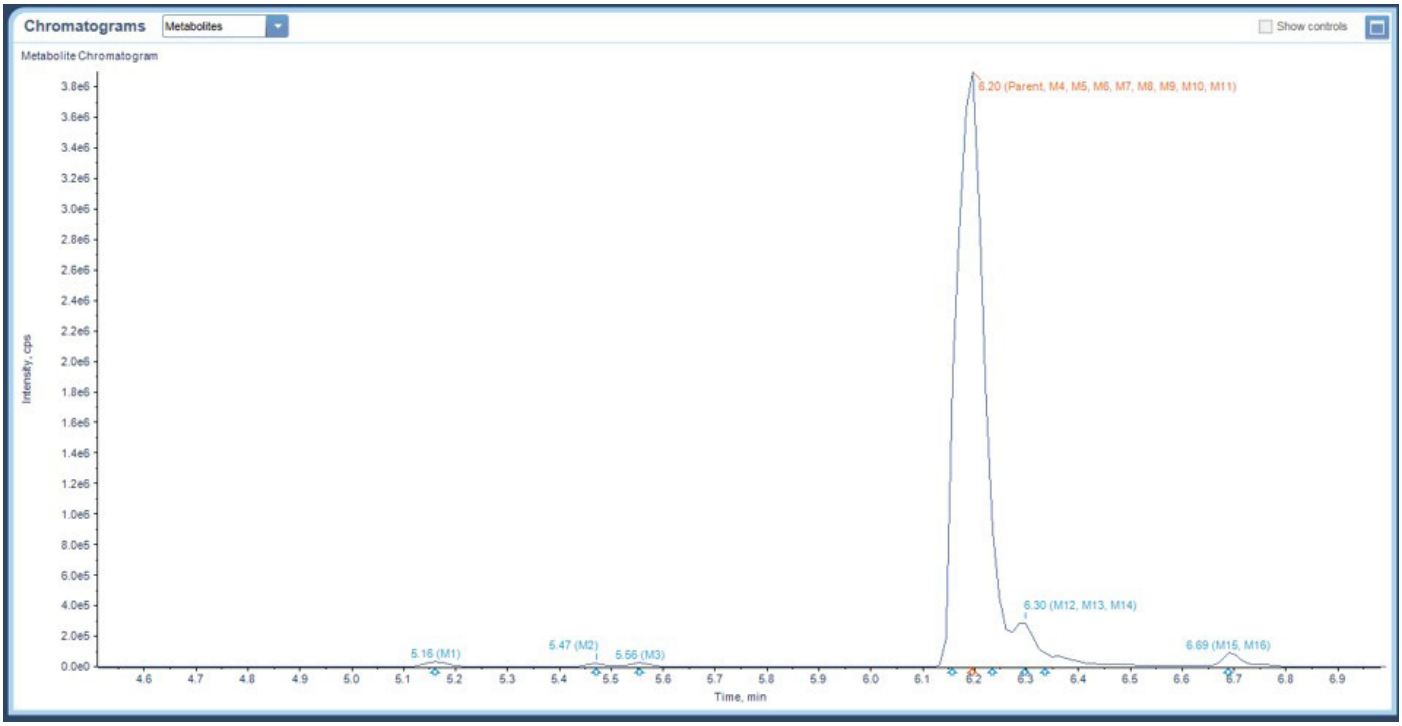

Figure 8: *An example of a MetID chromatogram showing the presence of the parent compound (6.20 min), primary metabolite (6.20 mins, closely eluting), and other minor metabolites (5.16-5.55 mins).*

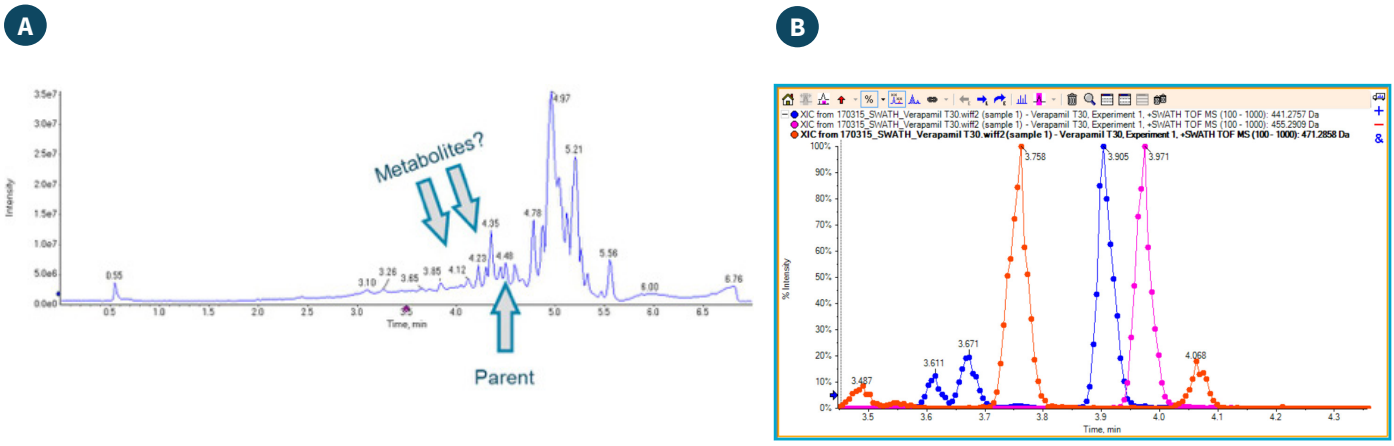

Figure 9: *A) An example of a MetID chromatogram, which allows the parent compound and its associated metabolites to be distinguished. B) Accurate metabolite ID at UHPLC timescales with ultra-fast acquisition capabilities without sacrificing resolution.*

and labor intensive, but it is the initiator for the rest of the work we do – we cannot initiate work with a certain NPS if we do not know that drug exists or if we do not have a good understanding of how to detect it.

Once a new NPS is discovered through our surveillance, we begin monitoring for this substance in all of our other populations, including additional seized drug materials, forensic toxicology samples, and clinical nonfatal overdose samples. This monitoring allows us to determine what substances are most prevalent and are having the greatest impact on the drug market. In reality, not every new NPS we discover will be identified in a toxicology case or will go on to become the next "most popular" substance. With that in mind, it is important for our laboratory to determine what the most prevalent substances are, so we can do further work with these substances to create the best opportunity for scientific impact.

There is often not enough time and resources to study all aspects of all emergent NPS, so we must pick and choose which substances are the most important to study. This leads to our response efforts, which entail work related to confirmation, quantitation, and metabolism. Once we see a notable increase in NPS prevalence among a certain population, we move to create confirmatory methods for those substances so we can get a better idea of the drug's characteristics (and also we must develop confirmatory methods to report our findings among forensic casework).

The confirmatory methods are often quantitative in nature, so we are able to gather information about how much drug was in a person's system when the incident occurred (e.g. overdose, death, accident, etc.). This can help us understand the potency or toxicity of a drug, from a toxicological viewpoint, depending on the information we receive from a case history, autopsy report, and other drugs present. Another important aspect of our response involves metabolite identifications (MetID) and discovery. From a forensic toxicology perspective, it can be vastly important to study metabolism, as the results can help prolong detection windows, help further understand toxicity or effects, and help determine what the most appropriate biomarker is for future method development. For example, synthetic cannabinoids metabolize extensively in the body, typically resulting in little to no parent compound excreted in the urine. This means scientists must perform MetID studies to determine what biomarker to look for in urine samples associated with synthetic cannabinoid use – this initial uncertainty can make this drug class very challenging. Discovery of active metabolites can also be extremely important (think, for example, of heroin → 6-MAM → morphine). MetID studies can help shed light in this area, which can in turn assist with toxicologist's interpretations and/or future analytical method design.

Q: New NPS and designer drugs emerge often into the market, posing a risk to public health. How do you disseminate information to other laboratories and agencies to ensure people have access to the most up-to-date information? In that regard, what approaches is your laboratory taking in terms of sharing the information and intelligence you are gathering on NPS?

A: Our motto has always been simple – rapid and far-spread information sharing to all interested stakeholders. Or in other words, our work is an "open book." It is not beneficial to our colleagues at large if we generate certain information or make certain discoveries and do not share the information as rapidly and widely as possible.

In this space, we have worked hard to create vast networks of stakeholders to whom the information is disseminated.

Our distribution list includes many federal, state, and local agencies, as well as numerous international agencies, with public health, public safety, and scientific interests. Our distribution list is open and easy to join (**npsdiscovery@cfsre.org**), and we welcome any individuals who have an interest in the information we are distributing.

Our initial dissemination strategy involves direct communication to stakeholders via email, where individuals get a firsthand look at our newest discoveries or trending data. These reports and emails are then secondarily distributed by the recipients to other colleagues or organizations where our information is posted to websites, social media platforms, etc. Dissemination at scientific meetings, conferences, and gatherings is also an integral part of our strategy, as these forums often allow for Q&A or feedback from other colleagues and jurisdictions.

In addition, all of the information we generate for NPS is archived on our website (**www.npsdiscovery.org**) where individuals can access any reports free of charge, including additional access to resources such as recent publications, presentations, and an electronic GC-EI-MS library database.

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