Increasing Safety, Speed, Sensitivity, and Selectivity of Controlled Substance Analysis

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Final Research Report

Principal Investigator:
Amber Burns
Forensic Chemistry Manager
Maryland State Police, Forensic Sciences Division
221 Milford Mill Road
Pikesville, MD 21208
amber.burns@maryland.gov
(443)357-1391

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U.S. Department of Justice
National Institute of Justice
Office of Justice Programs
810 Seventh Street NW
Washington, DC 20531

Award Organization:
Maryland State Police, Forensic Sciences Division
221 Milford Mill Road
Pikesville, MD 21208

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Summary of Project

Statement of the Problem & Project Overview

The field of drug chemistry has been changing drastically and is continuing to change due to the increased prevalence of synthetic opioids, novel psychoactive substances (NPSs), and other emerging drugs. Like many laboratories throughout the country, the Maryland State Police – Forensic Sciences Division (MSP-FSD) has faced challenges in keeping up with these emerging drug trends. As the laboratory continues to see an increasing number of NPSs submitted, current screening methods, such as color tests and gas chromatography flame ionization detection (GC-FID), are becoming less effective due to the complexities of samples being submitted. Increased complexity of cases is causing climbing backlogs within the Controlled Dangerous Substance (CDS) Unit – from 2015 to 2019 there was a 2,500 % increase in the backlog.

While color tests work well on simple samples containing traditional drugs of abuse like cocaine and heroin, they do not provide much information about samples containing fentanyls and other NPSs. These compounds will either not elicit a response or be present at such small amounts that the response is either masked by other chemicals present or is too faint to reliably detect. Color tests may require more material than is submitted. Additionally, the chemicals used in these tests represent additional safety concerns for the analyst.

GC-FID can detect the majority of drugs, including NPSs and emerging drugs, but it is not particularly effective at resolving drugs with similar chemical structures. Another disadvantage of GC-FID is that it is not a very efficient method - with each sample requiring approximately 15 minutes of instrument time. Another issue is the high potency of fentanyls. Fentanyl compounds have been detected by MSP-FSD in samples both in pure form and at very low concentrations. For some of the more potent analogs, or in cases where multiple analogs are present, the concentration of the individual compounds is likely lower than 5 % by weight and has been shown to be below the level that our current instrumentation can confidently detect.

Interference due the presence of cutting agents, which can mask the presence of compounds at low concentrations, is also a concern.

One technique that could address many of these limitations is direct analysis in real time mass spectrometry (DART-MS). The effectiveness of DART-MS for seized drug analysis has been extensively demonstrated elsewhere[1]. DART-MS provides a more information rich, more sensitive analysis that is less hindered by cutting agents, and is better able to distinguish between similar chemical structures than color tests and GC-FID.

The ability to use an information-rich screening tool like DART-MS opens the door for other approaches for confirmation. Currently at MSP-FSD, general-purpose gas chromatography mass spectrometry (GC-MS) methods are used for confirmation. While they are sufficient for the identification of a number of common drugs, they present a number of analytical challenges as well. Differentiation of isomeric or isobaric compound pairs based on retention times can be difficult and oftentimes the mass spectra of the two compounds are too similar to distinguish one from another. Co-elution of some compound pairs, such as acetyl fentanyl and FIBF (*para*-fluoroisobutyryl fentanyl) or quinine and carfentanil, can challenge confirmation when they are co-presented in a sample. Also, for a subset of samples, the current methods had inadequate sensitivity, precluding detection of compounds when they are present in mixtures at low weight percentages.

The overall goal of this project was to re-envision the traditional workflow for the analysis of controlled dangerous substances at MSP-FSD to address the current analytical challenges and provide a safer, more rapid, more sensitive, and more selective approach. This project aimed to evaluate the benefits and weaknesses of replacing current screening protocols with DART-MS and current confirmation protocols with GC-MS methods developed for specific drug classes (Figure 1). This project evaluated the efficacy of the two workflows on the analysis of powders containing synthetic cannabinoids, synthetic cathinones, and opioids.

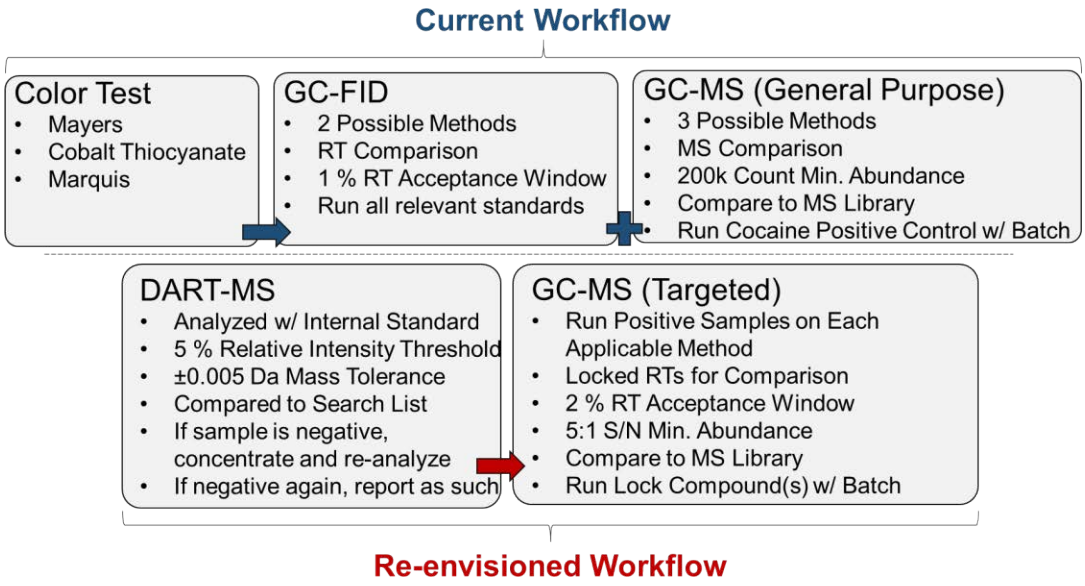

Figure 1. Current and re-envisioned workflows. Figure adapted from Sisco *et al.*[9] The abbreviation "RT" stands for retention time.

Major Goals and Objectives

Goal 1 – Development, Optimization, and Validation of a DART-MS Screening Method

The first component of this study was to develop the methods for the re-envisioned workflow. For DART-MS, method development relied on literature to provide a baseline for method optimization. Once optimized, the method was validated by examining the following components: (1) accuracy and precision, (2) reproducibility, (3) specificity, (4) sensitivity, (5) environmental effects (solvent effects), (6) non-probative casework, and (7) method robustness.

In the process of method development and validation, it was decided that the use of an internal standard would assist in data processing and reduce the number of false positives presented by negative samples. Significant efforts were therefore put in to establish an appropriate internal standard and a data interpretation workflow that reduced analysis time.

A deliverable of this goal is the DART-MS documentary package that consists of templates of documents laboratories need to bring new technology online (standard operating procedures, validation plan,

maintenance manuals, etc.). These documents have been made freely available to the community to help minimize the barrier for adoption of DART-MS or any other ambient ionization mass spectrometry platform.

Goal 2 – Creation of Targeted GC-MS Confirmation Methods

A large component of this project focused on the development of targeted GC-MS confirmation methods. To create these methods a stepwise, data-driven framework was developed. The framework provided ways to both develop and evaluate methods with the goals of maximizing retention time differences between compounds of interest in a reasonable amount of time and understanding the role common GC-MS parameters played in regard to sensitivity and reproducibility. Incorporation of retention time locking into the methods was completed, and a new test was developed that allowed us to objectively identify instances where the targeted methods would fail to confirm the presence of a compound based on similar retention times and/or mass spectra. For this project, targeted methods were created for synthetic cannabinoids, synthetic cathinones, and opioids.

Goal 3 - Implementation and Evaluation of Re-Envisioned Workflow

Once the methods were established for the re-envisioned workflow, the current workflow and the re-envisioned workflow were compared. Four chemists within MSP-FSD were trained to use DART-MS and the targeted GC-MS methods. The chemists were then given a set of 50 samples and asked to analyze half of each with the current workflow and half with the re-envisioned workflow. Analysts documented and timed all steps required for analyses they completed and interpreted the resulting data. This allowed for the efficacy of the two workflows, using a variety of metrics, to be compared. To assist in data comparison of the non-similar screening techniques (color tests and DART-MS), a scoring system was developed and implemented as means to see differences in the methods.

Research Questions

This project attempted to answer the following research questions:

1) Could thermal desorption (TD)-DART-MS be a reliable tool for seized drug analysis or is traditional DART-MS better suited for this application?
2) Is there an objective, data-driven way to develop targeted GC-MS methods?
3) Could targeted GC-MS methods outperform general-purpose GC-MS methods for seized drug confirmation?
4) Does the re-envisioned workflow provide higher quality data than the current workflow?
5) Does the re-envisioned workflow provide any time savings over the current workflow?
6) Are there any other potential benefits or drawbacks of the re-envisioned workflow, compared to the current workflow?

Research Design, Methods, Analytical and Data Analysis Techniques

Goal 1 – Development, Optimization, and Validation of a DART-MS Screening Method

Once the DART-MS system was installed at the laboratory, validation of the system was completed. The validation process involved seven separate studies: (1) accuracy and precision, (2) reproducibility, (3) specificity, (4) sensitivity, (5) environmental effects (solvent effects), (6) non-probative casework, and (7) method robustness. These studies, except for (6) non-probative casework, were completed in both positive and negative ionization mode. For accuracy and precision, a 15-component test solution (Table 1) and 3-component test solution (Table 2) were run in positive and negative ionization mode, respectively, ten times over the span of one day. The ability of the system to produce expected *m/z* values for peaks of interest in the low orifice 1 energy spectrum within the given tolerance (±0.005 Da) was measured. Solutions containing the individual components of Table 1 and Table 2 were also run and the ability to reproducibly obtain accurate base peaks in mass spectra from each of four orifice 1 voltages (±20 V, ±30 V, ±60 V, and

±90 V) was measured. Data was processed using a combination of msAxel (to extract mass spectra) and MassMountaineer (to search mass spectra against a peak table).

Table 1. List of compounds used for the 15-component DART-MS validation solution for positive ionization mode.

Compound	Formula	Molecular Mass (Da)	DART-MS Base Peak (*m/z*)
Methamphetamine	$C_{10}H_{15}N$	149.120	150.128
α-Pyrrolidinobutiophenone	$C_{14}H_{19}NO$	217.147	218.154
Butylone	$C_{12}H_{15}NO_3$	221.105	222.113
Ethylone	$C_{12}H_{15}NO_3$	221.105	222.113
α -Pyrrolidinovalerophenone	$C_{15}H_{21}NO$	231.162	232.170
Phencyclidine	$C_{17}H_{25}N$	243.199	244.207
Tenocyclidine	$C_{15}H_{23}NS$	249.155	250.163
Nandrolone Decanoate	$C_{28}H_{44}O_3$	428.328	429.336
Cocaine	$C_{17}H_{21}NO_4$	303.147	304.155
Alprazolam	$C_{17}H_{13}ClN_4$	308.083	309.091
Stanozolol	$C_{21}H_{32}N_2O$	328.251	329.259
Heroin	$C_{21}H_{23}NO_5$	369.158	370.165
Furanyl Fentanyl	$C_{24}H_{26}N_2O_2$	374.199	375.207
Furanyl Fentanyl 3-Furancarboxamide	$C_{24}H_{26}N_2O_2$	374.199	375.207
5-Fluoro ADB	$C_{20}H_{28}FN_3O_3$	377.211	378.219

Table 2. List of compounds used for the 3-component DART-MS validation solution for negative ionization mode.

Compound	Formula	Molecular Mass (Da)	DART-MS Base Peak (*m/z*)
Gamma Hydroxy-Butyrate (GHB)	$C_4H_8O_3$	104.047	103.039
Secobarbital	$C_{12}H_{18}N_2O_3$	238.132	237.124
AB-FUBINACA	$C_{20}H_{21}FN_4O_2$	368.165	367.157

For the reproducibility study, the multi-component test solutions were analyzed five times a day on seven different days and the ability to obtain peaks with accurate *m/z* values was measured. Carryover between samples was measured (carryover was defined as peaks of interest produced in the MassMountaineer search of a blank spectrum using a 5 % relative intensity threshold). The ability to successfully calibrate, or mass drift compensate, the system each day was also measured.

Specificity was measured through two studies for each ionization mode. In the first study, the mass spectra at all four orifice 1 voltages for each of the compounds in Tables 1 and 2 were searched against the NIST DART Forensics Library[5,10] to see how well the spectra matched library spectra. This was completed using NIST MS Search software. For the second study, the identical process was completed but this time using commonly seen isomer sets, shown in Table 3.

Table 3. Isomer sets used for the specificity component of the DART-MS validation.

Positive Mode Sets		
Set 1	**Set 3**	**Set 5**
Methamphetamine	Cyclopropyl Fentanyl	6-APDB
Phentermine	Crotonyl Fentanyl	5-APDB
	Methacryl Fentanyl	Buphedrone
		Dimethylcathinone
Set 2	**Set 4**	Ethcathinone
Butylone	m-FBF	Mephedrone
Dimethylone	o-FBF	2-MMC
Ethylone	p-FBF	MMAI
3,4-EDMC	m-FiBF	
3,4-MDPA	o-FiBF	
	p-FiBF	
Negative Mode Set		
AB-FUBINACA	AB-FUBINACA 2'-indazole isomer	AB-FUBINACA 2-fluorobenzyl isomer
AB-7-FUBAICA	AB-FUBINACA isomer 1	

For sensitivity studies, the limits of detection for the compounds listed in Table 1 and Table 2 were calculated in accordance with the procedures outlined in ASTM E2677[11]. To do so, a calibration curve ranging from 0.5 µg/mL to 25 µg/mL was created, gravimetrically, for each compound. Ten replicates of 1 µL aliquots of each solution, along with ten replicates of a methanol blank, were run, and the peak areas of the base peak were obtained from the extracted ion chronogram. The peak areas, and corresponding concentrations, were inputted into the ASTM E2677 LOD calculator[11] using a confidence limit of 0.10 (90 % confidence).

For environmental studies, solutions containing the compounds in Tables 1 and 2 were prepared in acetone, chloroform, and hexane, instead of methanol. The solutions were analyzed, in triplicate, using the DART-MS method. Peak areas and *m/z* values of the notable peaks in the spectra were then obtained using msAxel and MassMountaineer and compared to the results from the methanolic solutions. For non-probative casework, a suite of 50 case samples was analyzed using the DART-MS method and the extracted mass spectra were searched against an in-house peak list in MassMountaineer using a threshold of 5 % relative intensity and a mass tolerance of ±0.005 Da. The results were compared to the previously obtained GC-MS results.

For the method robustness study, the reproducibility study (2) was completed with a second chemist. As with that study, the 15-component and 3-component test solutions were analyzed five times per day over the span of seven different days. Accuracy of the *m/z* values and the presence of carryover was measured.

To establish an appropriate internal standard for DART-MS analysis, a series of studies were completed. The first of which identified tetracaine as an appropriate internal standard candidate. The appropriate concentration of tetracaine was established by first measuring the average reporting limits for current GC-MS analyses at MSP-FSD and finding the tetracaine concentration that allowed for detection of a suite of drugs at a 2.5 % to 5 % relative intensity to tetracaine. To understand the competitive ionization effect, if any, that tetracaine had on the suite of drugs, the peak areas of the compounds when analyzed with and without tetracaine were compared. Finally, a set of 60 mock or adjudicated case samples were analyzed both with and without internal standard to see if the presence of the internal standard caused any detection challenges.

The two instruments used for all DART-MS work consisted of an IonSense DART-SVP source coupled with JEOL AccuTOF 4G LC-Plus mass spectrometer. All analyses used helium as the DART gas with a gas stream temperature of 400 ºC. For positive ionization mode, a DART grid voltage of +150 V was employed. Mass spectrometer settings included an *m/z* scan range from *m/z* 100 to *m/z* 600 at 0.4 s per scan. An orifice 1 temperature of 120 ºC, ring lens voltage of +5 V, orifice 2 voltage of +5 V and ion guide

voltage of +1000 V were used. Orifice 1 was cycled, using the parameter switching, between +20 V, +30 V, +60 V, and +90 V at 0.4 s per voltage. The negative mode method was identical to the positive mode, aside from the voltage polarity and the use of a -900 V ion guide voltage. For validation efforts, aside from the non-probative casework, data acquisition was set to run for up to 180 min but was stopped whenever all samples for a particular study were collected. PEG-600 was sampled at the beginning and end of every run as well as approximately every 10 min throughout the run.

For the non-probative casework portion, and the efforts to develop an internal standard, only positive mode ionization was used, and the method was slightly modified. Instead of cycling the orifice 1 voltage, a method with an orifice 1 voltage of +30 V and another with +60 V were used. In addition, instead of a 180 min acquisition time, the method was shortened to 1 min and data was collected using the Sequence Table instead of the Single Run option, which was used for all other studies. A single non-probative case sample was analyzed in a single 1 min run along with a tetracaine positive control. A +30 V orifice 1 datafile was collected for each sample, and a +60 V datafile was also collected when needed to assist in compound identification. When applicable, mass drift compensation was automatically completed at the end of the sequence, using the protonated molecule of the internal standard, tetracaine (*m/z* 265.1916).

Goal 2 – Creation of Targeted GC-MS Confirmation Methods

Creation of the targeted GC-MS confirmation methods followed a six-step, data-driven framework for method development and evaluation, which is summarized in Figure 2, and discussed in more detail elsewhere[12]. To create the methods, multi-component test solutions for each drug class were created that spanned the range of volatilities and included difficult to distinguish compounds. The goal of the development process was to maximize the retention time differences between components of the test solution during a reasonable run time while also understanding the effects of different parameters on sensitivity and reproducibility. To achieve the first goal, an appropriate stationary phase was established by analyzing the test solution on a suite of different GC columns, keeping all other parameters the same (Step

1). The percent retention time difference (%RTD, Eqn. 1), peaks areas, mass spectral similarity scores, and peak purities were measured to establish the best column.

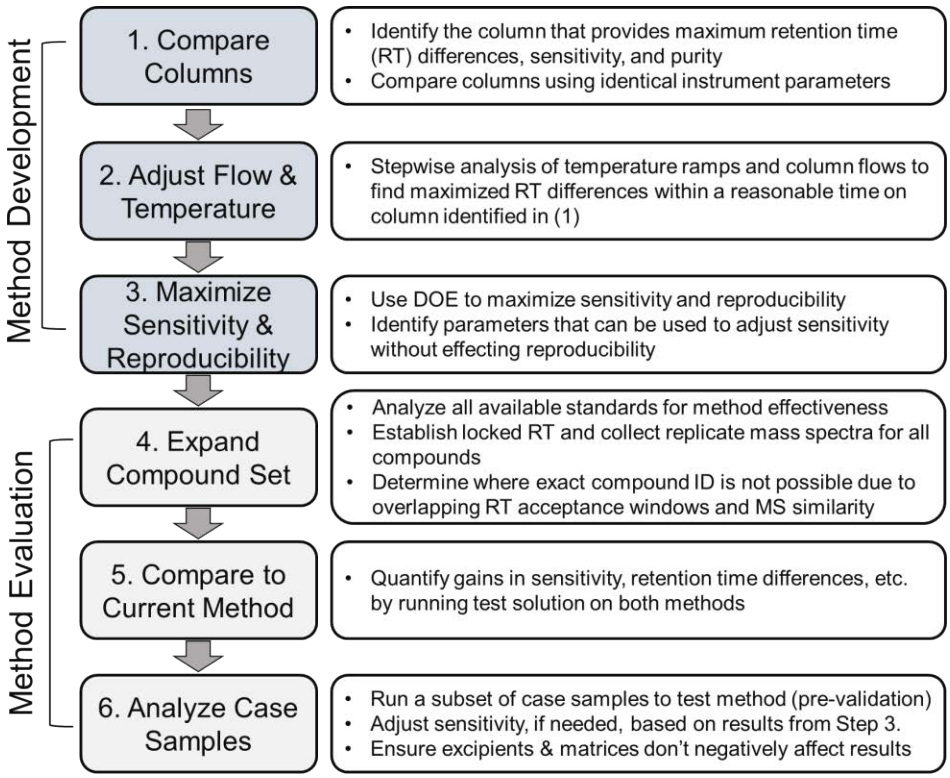

Figure 2. Summary of the method development and evaluation framework. Figure adapted from Sisco *et al*, 2020[12].

$$\textbf{Eqn. 1}\ \%RTD = \frac{|(Retention\ Time\ Compound\ 2) - (Retention\ Time\ Compound\ 1)|}{Retention\ Time\ Compound\ 1}$$

Once a column was established, the flow and temperature programs were varied to establish parameters that maximized retention time differences within a reasonable runtime (Step 2). A design of experiments (DOE) was then used to understand the effect of inlet temperature, split ratio, source temperature, injection volume, and tune type on the reproducibility and sensitivity of the method (Step 3). The results of this step provided guidance as to how to alter the method to achieve the desired sensitivity in Step 6. At the end of Step 3, a preliminary targeted method was developed.

Once established, the targeted method was then evaluated through an additional three studies. In the first study (Step 4), the compound list was expanded beyond those compounds in the test solution to include all

compounds of interest (50 cannabinoids, 55 cathinones, and 222 opioids). This allowed for the identification of compound pairs that could not be differentiated – defined as compounds with similar mass spectra that had retention times within ±2 % of one another. To accomplish this, all compounds of interest were first analyzed with the targeted method to determine if adjustments to the method needed to be made (*i.e.* extending the method an additional minute for late eluting compounds). The method was then retention time locked to a lock compound and each compound was analyzed three (opioids) or ten (cannabinoids and cathinones) times. The average locked retention times and the mass spectra for each replicate of each analysis were then extracted using Mass Hunter. A list of all compound pairs with overlapping acceptance windows (pairs with %RTDs of ≤2 %) was generated and the mass spectra of the two compounds were objectively compared using a newly developed, objective, comparison test called the *min-max test*.

The *min-max test* is described in more detail elsewhere[12], but, briefly, the difference between the minimum match factor computed between replicate spectra of the same compounds and the maximum match factor computed between all spectra of the two different compounds is computed as the *min-max index*. The *min-max index* employs identity match factors, a numerical estimate of similarity between a pair of mass spectra. Confidence that the two compounds are distinguishable via their mass spectra grows as min-max indices increase, on a scale from -999 to 999. Comparisons with min-max indices greater than zero have, objectively, different mass spectra, while comparisons with indices less than zero indicate that the mass spectra of the two compounds are indistinguishable.

In Step 5, the targeted methods were compared to the existing method by analyzing the test solution, and dilutions of the test solution, on both methods. The same suite of measurements used in Step 1 was used to quantify gains offered by the methods, along with calculation of approximate limits of detection for each of the compounds. In Step 6, a suite of mock or adjudicated case extracts was evaluated using the targeted method to establish usability for casework prior to validation. During this step, changes to the sensitivity of the method were made, if needed, using the results from Step 3.

All analyses were completed on an Agilent 7890 / 5977-B GC-MS (Agilent Technologies) system using ultra-pure helium as the carrier gas. A total of six different columns (DB-1UI, DB-5, DB-5UI, DB-35, DB-200, and VF-1701ms) were evaluated, all of which had dimensions of 30 m x 0.25 mm x 0.25 µm and were purchased from Agilent Technologies. All columns were conditioned according to the manufacturer's suggested protocols. The MS was tuned daily using the standard spectra tune (stune), unless otherwise noted. Relevant MS parameters, that were kept constant throughout the method development, include an MS scan range of m/z 40 to m/z 550, a threshold of 150 counts, and a scan speed of N = 2. Additional method parameters are provided throughout the text.

Goal 3 - Implementation and Evaluation of Re-Envisioned Workflow

The final goal of this project was to evaluate the re-envisioned workflow against the current workflow. To complete this, 50 mock samples were given to four different chemists who were asked to analyze 25 with their current workflow and 25 with the re-envisioned workflow. Samples were grouped into batches of five to simplify the time recording and data collection process. For the current workflow, chemists completed color tests, GC-FID, and general-purpose GC-MS analyses on all samples, noting the time required from sample preparation, sample analysis, and data interpretation for each step. They were also asked to provide interpretation of the results after each step. The same recording process was used for the re-envisioned workflow where chemists analyzed samples with DART-MS and targeted GC-MS methods.

Comparison of the two workflows involved looking at the quality of the information at each step as well as the time required, amount of sample consumed, and any safety considerations involved. Comparison of GC-FID and GC-MS results was done by looking at what compounds could and could not be identified using the criteria outlined in Figure 1. To compare the screening results, a scoring scheme was created, and the result of each analysis was given a score from -1 to 4, based on the quality and specificity of data, as shown in Table 4. The distributions and average scores for each technique were then compared. Additional comparisons, such as the amount of material required for analysis and any health and safety concerns at each step were also made.

Table 4. Scoring system used for comparison of color tests and DART-MS. Table adapted from Sisco *et al*[9].

Score	Results
-1	Incorrect result
0	Inconclusive result
1	Correct identification of compound class for at least one compound
2	Correct identification of *at least* the sub-class or isomer group identified for *at least* one compound (mixtures only)
3	Correct identification of *at least* the sub-class or isomer group for all compounds
4	Correct identification of all compounds identified *OR* correct identification of a negative sample as negative for controlled substances

Expected Applicability of the Research

The results of this research have had an impact on the current and future operations of the CDS unit at MSP-FSD and have the ability to further impact the broader seized drug community. At MSP-FSD, the procurement of DART-MS technology will enable the CDS unit to better screen samples and has proven an invaluable tool for unknown analysis. The use of the internal standard and sequence-based analysis approach will lead to broader adoption of the technology throughout the lab due to simplified data processing and simplified data interpretation. The targeted GC-MS methods are currently being validated for use in casework and will provide the capability to address several measurement challenges that currently exist with the general-purpose methods.

The results of this project benefit not only MSP-FSD but the community in general. Creation of the DART-MS documentary package and the framework for GC-MS method development provides the community with tools to lower the barrier of adoption of these approaches. Adoption of targeted methods by other laboratories could provide increased data sharing capabilities if retention time locking procedures are standardized. The results of the workflow comparison study can be used by laboratories as examples of gains that could be expected by changing their analytical workflows. While the results would not be directly applicable, since laboratories have differing workflows, it does provide insight into the magnitude of gains that could be expected.

Participants & Other Collaborating Organizations

Completion of this project required a number of participants both within MSP-FSD and outside of the agency. Within MSP-FSD Amber Burns, Elizabeth Schneider, Charles R. Miller, Laurel Hardy, Ikeanna Ikpeama (allied chemist for Saint Mary's and Calvert counties), and Wanda Kuperus worked on various components of the project or management of the project. Researchers from NIST, Edward Sisco and Arun Moorthy, also assisted in various stages of the research and assisted in publication writing. Chip Cody, from JEOL, was invaluable in providing assistance with developing the sequence-based DART-MS analysis approach.

Changes in Approach

The major change in approach for this project was the utilization of DART-MS for sample screening instead of TD-DART-MS. It was identified early-on in the project that use of TD-DART-MS for bulk drug analysis was not going to be a suitable approach because the thermal desorption unit is easily saturated. With TD-DART-MS, chemists must be extremely mindful of analyte concentration, otherwise you risk long bakeout times to achieve low background signal from introducing too much sample. Because traditional DART-MS is open air, not confined like TD-DART-MS, these concerns are much lower. Other minor changes included use of an internal standard in the DART-MS sampling scheme, the framework for GC-MS method optimization, and the development of the *min-max test* for GC-MS method evaluation.

Outcomes

Activities & Accomplishments

The following are a list of the activities and accomplishments that occurred for each of the three goals of the project.

<u>Goal 1 – Development, Optimization, and Validation of a DART-MS Screening Method</u>

- DART-MS system was procured, installed, and validated at MSP-FSD main laboratory

    - A second system was also procured using unrelated funding during this time for one of the satellite laboratories and has also been installed and validated
- Developed an analysis scheme that incorporates an internal standard and a sequence-based data collection approach along with automated mass drift compensation and centroided to significantly reduce the time required for data workup and interpretation
- DART-MS documentary package was created which other laboratories can use to assist in implementation efforts
- Draft publications regarding the use of internal standard for DART-MS and the validation efforts are now undergoing review

Goal 2 – Creation of Targeted GC-MS Confirmation Methods

- Created a data-driven framework for method development and evaluation
- Created a new mass spectral comparison test, the *min-max test*, to objectively measure differences in the mass spectra of two compounds
- Developed targeted GC-MS methods for synthetic cannabinoids, synthetic cathinones, and opioids
- Published manuscripts on the framework, the synthetic cannabinoid method, and the synthetic cathinone method
    - Drafted a publication on the opioid method which is now undergoing review
- Currently working on validating the targeted methods for casework at MSP-FSD

Goal 3 - Implementation and Evaluation of Re-Envisioned Workflow

- Trained four chemists at MSP-FSD on the re-envisioned workflow
- Completed workflow comparison study to measure the strengths and weaknesses of the current and re-envisioned workflow
- Drafted a publication on the study which is currently under review

Results and Findings

Goal 1 – Development, Optimization, and Validation of a DART-MS Screening Method

Validation of the DART-MS systems was successful, and results were largely what was expected. A summary of some of the key results are presented here since a full description of the results can be found elsewhere[13]. Accuracy and reproducibility of the method, defined as the ability to produce peaks within ±0.005 Da of the expected value, was achieved, as shown in Figure 3. This was found to be true in both positive and negative ionization modes and across the four orifice 1 voltages studied. For the reproducibility studies, standard deviations of ±0.0005 Da to ±0.0012 Da were found along with coefficients of variation of less than 0.001 %.

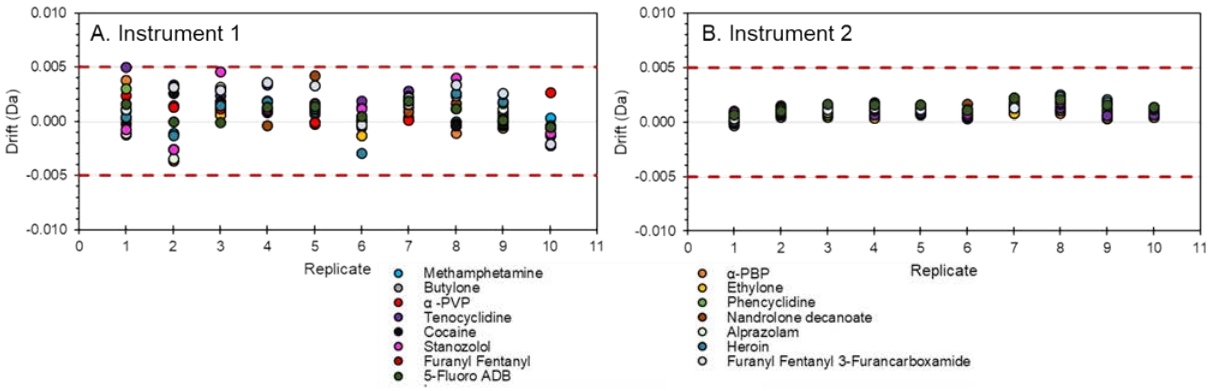

Figure 3. Results from the positive mode accuracy and precision study (Study 1) for Instrument 1 (A., left) and Instrument 2 (B., right). The red dotted lines indicate the high and low bounds of the allowable drift from the theoretical m/z values. Figure adapted from Sisco *et al.*[13]

For the specificity studies, as expected, isomer differentiation – namely differentiation of butylone and ethylone or furanyl fentanyl and furanyl fentanyl 3-furancarboxamide – was not always possible, especially at low (±20 V and ±30 V) orifice 1 voltages. This is a well-known, and well documented limitation of DART-MS analysis that utilizes a time-of-flight mass spectrometer[1]. In positive mode, six of the fifteen compounds had at least one other isomer or related compound that produced a hit in MS Search with a higher score than the compound, though the actual compound was always within the top five hits.

Similarly, in the second specificity study, isomer differentiation was not always possible, especially at low (±20 V and ±30 V) fragmentation voltages. For isomer set 1, differentiation of methamphetamine and phentermine was not possible at low orifice 1 voltages but was possible at high voltages, though methamphetamine and other amphetamines produce similar spectra at high voltages. For isomer Set #2 and isomer Set #5, the synthetic cathinones, differentiation was not possible at low orifice 1 voltages, as expected, since the spectra were dominated by the molecular ion. Spectra from higher orifice 1 voltages, however, did allow for a greater degree of differentiation than expected. This trend was also observed for the synthetic cannabinoid set in negative mode. For the fentanyl isomer sets, Set #3 and Set #4, differentiation across the voltages was not possible due to both identical molecular ions and similar fragmentation spectra for each set. The results of these studies highlighted that leveraging the higher orifice 1 voltages may assist in isomer differentiation for some cases. While this is true for pure compounds, the added benefit of the higher fragmentation spectra may not be realized for multi-component mixtures unless advanced search algorithms are employed.

Sensitivities of the DART-MS systems were found to be in the single to sub-nanogram range for all compounds analyzed. The limits of detection ranged from a maximum of 4.41 ng for ethylone to a minimum of 0.12 ng for butylone. In terms of environmental effects, acetone and chloroform were found to be largely detrimental to all compounds examined, though they did not cause the formation of new or additional ions or adducts. Methanol, generally, produced analyte peaks with the highest abundance. For the method robustness component, results were found to be in line with those obtained from the reproducibility study.

During the non-probative casework study, several things were identified that led to modifications in the analytical protocols. The first modification was brought about because it was discovered that the mass spectra obtained using the +20 V and +30 V orifice 1 voltages were nearly identical except for an increased dimer presence in the +20 V spectra. To minimize the dimer contribution, the +30 V orifice 1 spectra were used for analysis of case samples. Second, to minimize issues with the false identification in low intensity spectra, the use of an internal standard was incorporated, details of which are provided below. Inclusion of

tetracaine as an internal standard ensured that the 5 % relative abundance threshold did not cause false identification of noise peaks, which was observed in Study 2, and provided a mass calibration check standard in each sample. For a sample to have a positive identification, the peak corresponding to tetracaine had to also be within the ±0.005 Da tolerance and present at or above 5 % relative intensity in addition to compounds identified in the search process.

To assist in the identification process, a series of fragment ion search lists were created for instances where differentiation was possible and were employed in this portion of the study, where appropriate. For some compounds this required the acquisition and searching of the +60 V spectra, in addition to the +30 V spectra, to obtain the necessary fragment ion. A total of 50 samples were analyzed for this component of the study. In 39 of the 43 samples containing a controlled substance, DART-MS was able to correctly identify all substances that were identified by GC-MS. Of the four cases where not all controlled substances were detected, three produced results for at least some of the controlled substances and only one case produced a false negative result. For the three incomplete results (3, 14, and 42), detection of low-concentration compounds that had poorer ionization efficiencies were not obtained at the 5 % threshold. Given the lack of chromatography in DART-MS, competitive ionization can prohibit detection of low concentration compounds when those compounds are more poorly ionized than the major constituents. For the case where a false negative was obtained, insufficient sample amount was found to be the likely cause of the missed compound identification. The case was analyzed as if it was a powder but the sample actually originated from a counterfeit pharmaceutical tablet. Because of the small amount of material sampled (<5 mg) given it was a tablet, the controlled substances were likely below the detection limit.

In all seven cases where the sample did not contain a controlled substance, no controlled substance was detected by DART-MS. Use of tetracaine as an internal standard was found to assist in correctly identifying negative samples and eliminate false identification of background or noise peaks. As expected, limitations due to the inability to differentiate isomers precluded definitive identification by DART-MS in some

instances. This does not present any limitations in the overall analysis but instead highlights the complementarity of data obtained by DART-MS and GC-MS.

One of the challenges identified with traditional DART-MS analysis was that some negative samples (*i.e.*, inorganics, starch, etc.) did not produce spectra with a dominant peak, and therefore when a relative intensity threshold was used for peak searching, noise peaks could be falsely identified as compounds of interest. To eliminate this concern, an internal standard was incorporated into the DART-MS analysis scheme to ensure that a dominant peak was present in all samples. The internal standard also acted as a mass calibration check and could be used to tune the sensitivity of the result by varying its concentration.

Evaluation of an internal standard for incorporation into DART-MS analysis is discussed in detail elsewhere[14], but a brief summary of the results is presented here. A number of criteria were established for what an internal standard for DART-MS should be, including: soluble in methanol, not a common diluent in drug samples, having a molecular formula that is not identical to commonly seen compounds of interest, cost-effective, and having a molecular mass in the 200 Da to 400 Da range. Tetracaine was identified as a compound that met these criteria and therefore was studied further. It was found to predominantly produce a molecular ion peak (*m/z* 265.1910, $[C_{15}H_{24}N_2O_2+H]^+$) in addition to small fragment peak at *m/z* 176.1075, as shown in Figure 4.

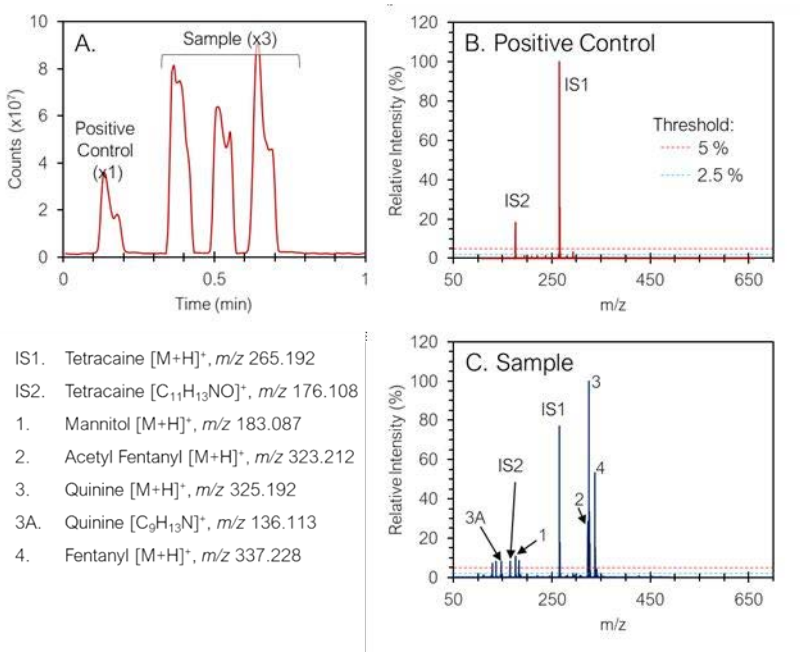

Figure 4. Representative chronogram (A.) and mass spectra (B. and C.) for data collection using the sequence-based approach for an example case containing acetyl fentanyl, fentanyl, mannitol, and quinine. Example chronogram (A.) shows the single analysis of the tetracaine positive control as well as the triplicate analysis of the sample. Extracted mass spectra of the tetracaine positive control (B.) and sample (C.) are also shown with peak labeling associated with the assignments in the bottom left quadrant. The dotted lines in (B.) and (C.) represent the 5 % (red) and 2.5 % (blue) relative intensity thresholds. Figure adapted from Sisco, *et al.*[14]

After the candidate compound was chosen, the appropriate concentration was established. Since the goal was to tune the sensitivity of the DART-MS to be similar to that of the GC-MS methods used at MSP-FSD, the reporting limits of the GC-MS systems were first measured for cocaine, methamphetamine, and fentanyl. The reporting limits, defined as the concentration which elicited a chromatographic peak with an abundance of 200,000 counts, were found to be around 100 µg/mL, on average, with values as low as 10 µg/mL depending on the GC-MS and method used. A series of experiments were then completed measuring the response of six drugs (alprazolam, cocaine, fentanyl, heroin, methamphetamine, and MPP-PICA) at a concentration of roughly 10 µg/mL in the presence of differing levels of tetracaine to identify a tetracaine concentration that allowed for the peaks of the drugs to be at least 5 % relative to the peak height of the internal standard. A tetracaine concentration of 0.1 mg/mL (100 µg/mL) was found to produce this response. Once chosen, an additional set of experiments (Figure 5) were completed to identify the relative intensity of the six previously studied drugs as a function of increasing concentration using the tetracaine

concentration of 0.1 mg/mL. All drugs were found to increase in relative intensity as a function of increasing concentration, as expected. Two of the drugs, cocaine and fentanyl, reliably produced peaks above the 5 % relative intensity threshold at 10 µg/mL while the remaining four produced peaks above the 2.5 % threshold at that concentration.

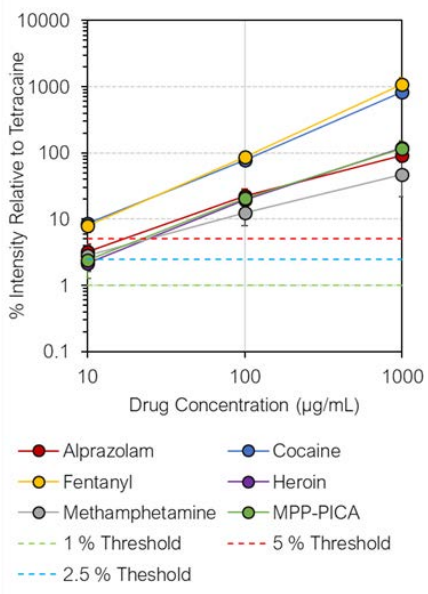

Figure 5. Peak intensity, relative to tetracaine, for six drugs. Three commonly used thresholds (1 %, 2.5 %, and 5 %) are also provided. Uncertainties represent the standard deviation of five replicates. Points above 100 % show that the drug peak was more intense than the tetracaine peak. Figure adapted from Sisco, *et al.*[14]

A potential concern for including an internal standard in a DART-MS analysis, where there is no chromatographic separation, is the fact the internal standard may cause competitive ionization that inhibits detection of the compound(s) of interest. To evaluate this, the six drugs were analyzed at 10 µg/mL, 100 µg/mL, and 1000 µg/mL both with and without internal standard and the ratio of the two responses was plotted. The results, shown in Figure 6, show that competitive ionization (points below the red dotted line indicating the signal is higher for the drug without the internal standard present) did occur for four of the six compounds at varying degrees. Methamphetamine was the most impacted by the presence of tetracaine, where the signal was reduced by 90 % when present with tetracaine. Methamphetamine was also the only drug to see competitive ionization effects at all concentration levels. Alprazolam, heroin, and MPP-PICA saw roughly a 50 % reduction in signal when present with tetracaine at 10 µg/mL, with slight improvements

at the 100 µg/mL level and no competitive ionization observed at the 1000 µg/mL level. Cocaine and fentanyl did not have any competitive ionization effects, which was expected given the high proton affinity of these two compounds. The key takeaway of this study was that while competitive ionization did occur, detection of all compounds was possible at all levels and the respective peaks were consistently present at or above a 2.5 % relative intensity threshold.

The final component of the study looked at the ability to use the internal standard in case samples by analyzing a suite of 60 case extracts with and without internal standards. Spectra for each extract were then searched using a 5 % and 2.5 % relative intensity threshold. The spectra from extracts with and without internal standard were largely identical. Slight differences between the GC-MS results and one or both the DART-MS results were observed for eight of the sixty samples. Of the extracts containing internal standard, there was one instance where identification of all six drugs in the mixture was not achieved. Noscapine, which is poorly ionized by DART, and cocaine, which was present at an insufficient amount for reporting by GC-MS, were not identified. The remaining four controlled substances (acetyl fentanyl, fentanyl, FIBF, and heroin) were readily identified. For the extracts containing the internal standard, the relative intensities of the peaks corresponding to controlled substances were above 5 % for all but two instances of heroin and one instance of ketamine, all of which were present at a low concentration. Heroin can also be poorly ionized when present with high proton affinity compounds. All of these peaks were, however, present above 2.5 % relative intensity. Importantly, there were no false-positive identifications of controlled substances in any of the samples, including the eight cases that were negative.

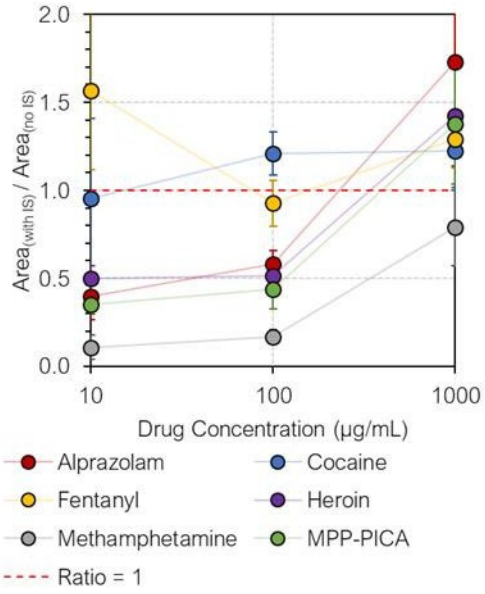

Figure 6. Ratio of the integrated peak area from the protonated molecule of the drug with and without the presence of the internal standard as a function of concentration. A ratio of 1 (red dotted line) indicates no competitive ionization. Points below a ratio of 1 indicate competitive ionization occurred in the presence of tetracaine while points above a ratio of 1 indicate an increase in drug signal due to the presence of tetracaine. Uncertainties represent the standard deviation of five replicates. Figure adapted from Sisco, *et al.*[14]

For the extracts created in methanol without internal standard, there were no instances where identification of a controlled substance was missed. In four instances, controlled substances in the sample were identified but were between 2.5 % and 5 % relative intensity. Unlike the extracts containing internal standard, there were ten instances where a hit for a controlled substance was obtained but was not identified by GC-MS (false-positive). For two of these, identifications of a false positive occurred in the presence of actual controlled substances and in one instance the peak was below 5 % relative intensity. The remaining four cases contained no controlled substances and the hit occurred because noise peaks were present above the 2.5 % or 5 % threshold that fell within the threshold of a compound in the search list. While it is simple to explain the presence of these false-positive peaks, and confirmatory analysis will further demonstrate that they are not attributable to actual analytes, the use of an internal standard provides a dominant peak that ensures low intensity noise peaks are not above the threshold in the searching process.

Goal 2 – Creation of Targeted GC-MS Confirmation Methods

Using the framework discussed above (Figure 2), targeted GC-MS methods were created for synthetic cannabinoids, synthetic cathinones, and opioids. The compositions of the test solutions used for these methods are provided in Table 5. Detailed results for these methods are provided elsewhere[12,15,16] and are briefly discussed here.

Table 5. Compounds included in the test solutions used for targeted method development. All compounds were present at a nominal concentration of 0.1 mg/mL in methanol.

Cannabinoids	Cathinones	Opioids
FUB-AMB	Phentermine	m-FIBF
MDMB-FUBINACA	Methamphetamine	p-FIBF
EMB-FUBINACA	Dimethylone	Cyclopropyl Fentanyl
MMB2201	Butylone	Crotonyl Fentanyl
ADB-FUBINACA	Ethylone	Carfentanil
AB-FUBINACA	Dibutylone	Methoxyacetyl Fentanyl
5F-ADBICA	Pentylone	Furanyl Fentanyl
5F-ABICA	Dimethylpentylone	Etizolam
	Ethylpentylone	Noscapine
		Benzodioxole Fentanyl

In the first step of the process, the test solutions were analyzed on a suite of six columns (DB-1UI, DB-5, DB-5UI, DB-35, DB-200, and VF-1701ms) while all other parameters were kept constant. From these results, the effect of different columns on the ability to separate and detect test solution compounds was established. An example of the results from this type of analysis, using the opioid test solution, is shown in Figure 7. The ability to separate compounds is shown in Figure 7A which plots the %RTD between pairs of neighboring peaks. For the opioid test solution, more polar columns (DB-35, DB-200, and VF-1701ms) provided better separation than the less polar compounds, using the same parameters. Sensitivity and peak purity were also measured and are shown on the right-hand side of Figure 7. For the synthetic cannabinoids and the opioids, the DB-200 column was chosen for further development while for the synthetic cathinones the DB-5 column was chosen.

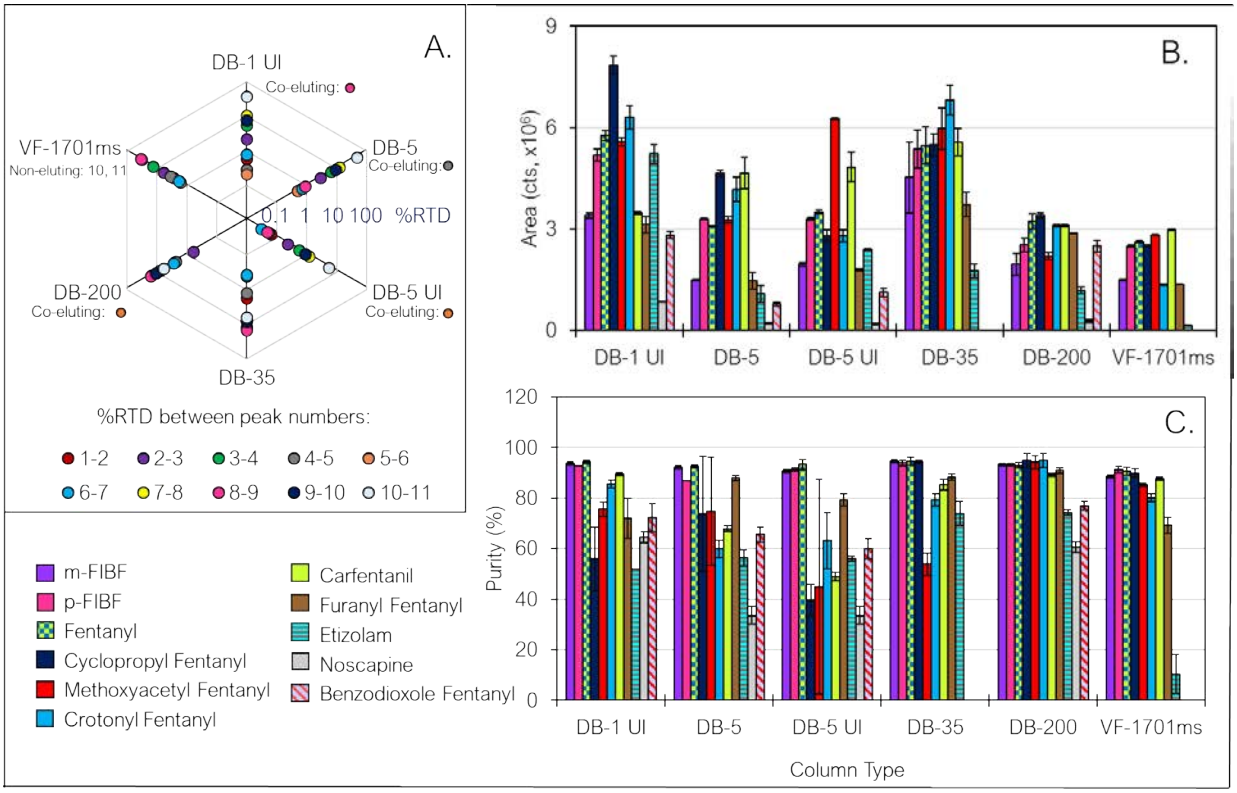

Figure 7. Results of the column comparison study (Step 1). The radar plot (A.) shows the percent retention time difference (%RTD) for neighboring peaks in the test mixture (sequentially numbered 1 through 11 because of differences in elution order). Points further out on the web indicate better separation. Note the plot is log scale. Average peak areas (B.) and peak purities (C.) for each compound analyzed on each column are also shown. Uncertainties represent the standard deviation of triplicate measurements. Compounds are listed in the elution order when using a DB-5 column. For the DB-35 column, detection of noscapine and benzodioxole fentanyl was only possible using extracted ion chromatograms and therefore they are not included in (B.) or (C.). Figure adapted from Sisco *et al.*[16]

Once a column was chosen, the temperature and flow programs were altered to try to minimize runtime while maximizing the average %RTD between neighboring pairs of compounds. For these studies, a minimum 1 %RTD between pairs was required, but typically %RTDs were substantially higher. The DOE was then completed to establish the effects of the remaining major parameters of sensitivity and reproducibility of the method. The results of these steps provided the targeted methods, which are shown in Table 6.

Once the methods were developed, they were evaluated. In the first step of the evaluation procedure, the panels for each method were expanded to include all relevant standards available at MSP-FSD which totaled 50 cannabinoids, 55 cathinones, and 222 opioids and related compounds. The compounds were measured

on their appropriate methods and then the retention times compared to see which pairs had %RTDs of 2 % or less. A total of 27, 35, and 599 pairs of compounds fell within 2 % of one another for the three methods, respectively, and were subjected to the *min-max test* to see if any were not differentiable based on their mass spectra. All cannabinoids were found to be differentiable, indicating that there were no limitations that would prohibit identification of any of the 50 compounds in the panel. For the cathinones, there was one pair (3-chloroethcathinone | 4-chloroethcathinone) that was found to have a negative min-max index and therefore would not be able to be differentiated using this method. For the opioid method, four pairs of compounds (m-methyl Cyclopropyl fentanyl | o-methyl Cyclopropyl Fentanyl, p-fluoro Furanyl Fentanyl 3-furancarboxamide | p-fluoro Furanyl Fentanyl, m-Methylfentanyl | o-Methylfentanyl, and Reminfentanil | Remifentanil acid) were found to not be differentiable.

Table 6. Parameters of the three targeted methods that were developed.

Compound Class	Cannabinoids	Cathinones	Opioids
Lock Compound	AB-FUBINACA	Butylone	Fentanyl
Column	DB-200 30 m x 0.25 mm x 0.25 µm	DB-5 30 m x 0.25 mm x 0.25 µm	DB-200 30 m x 0.25 mm x 0.25 µm
Temperature Program	Isothermal at 290 °C	190 °C for 0.5 min Ramp 5 °C/min to 210 °C Ramp at 30 °C/min to 255 °C Hold 1.5 min	230 °C for 0.0 min Ramp at 2 °C/min to 290 °C Hold 5.0 min
Flow Rate	1.2 mL/min	1.9 mL/min	1.2 mL/min
Injection Volume	1.0 µL	1.0 µL	1.0 µL
Inlet Temperature	300 °C	300 °C	300 °C
Split Ratio	30:1	30:1	20:1
Transfer Line	300 °C	300 °C	300 °C
Quad Temperature	150 °C	150 °C	150 °C
Source Temperature	280 °C	280 °C	280 °C
Tune Mode	stune	stune	stune
Solvent Delay	1.4 min	1.15 min	1.3 min
Mass Scan Range	*m/z* 40 – *m/z* 550	*m/z* 40 – *m/z* 550	*m/z* 40 – *m/z* 550
Threshold	150 counts	150	150
Scan Speed	N = 2 [≈4 scan s^{-1}]	N = 2 [≈4 scans s^{-1}]	N = 2
Total Run Time	12.0 min	7.5 min	35.0 min

The methods were then compared to the general GC-MS method used at MSP-FSD. Representative chromatograms of the comparisons are shown in Figures 8, 9, and 10. In all instances, separation of compounds in the test solution was better with the targeted method than the general method. For two of the

compound classes, this was achieved with methods that were shorter than the general method currently in use. The opioid method was substantially longer but had marked gains in the ability to separate many of the compound pairs that are challenging using the current methods. Upon analysis of case extracts for the final step of method evaluation, higher than desired peak heights were obtained for all three methods. To achieve similar peak heights to current analyses, the split ratio of the methods was lowered. Given the results of DOEs (Step 3), this was achievable with no impact on the reproducibility of the method.

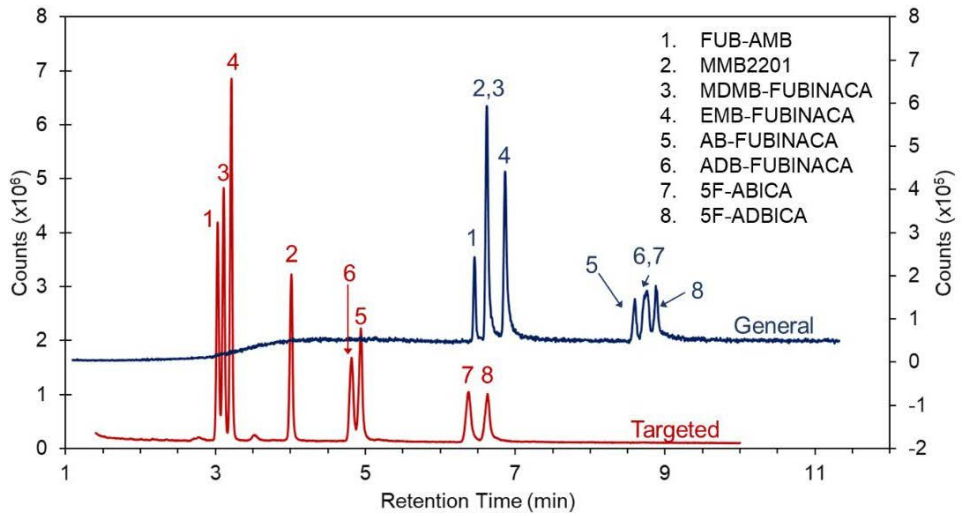

Figure 8. Representative chromatograms of the synthetic cannabinoid test solution analyzed on the general method (blue) and targeted GC-MS method (red). Note the secondary y-axis for the general method. The elution order is different for the two runs due to the use of different stationary phases. Figure adapted from Sisco *et al.*, 2021[12].

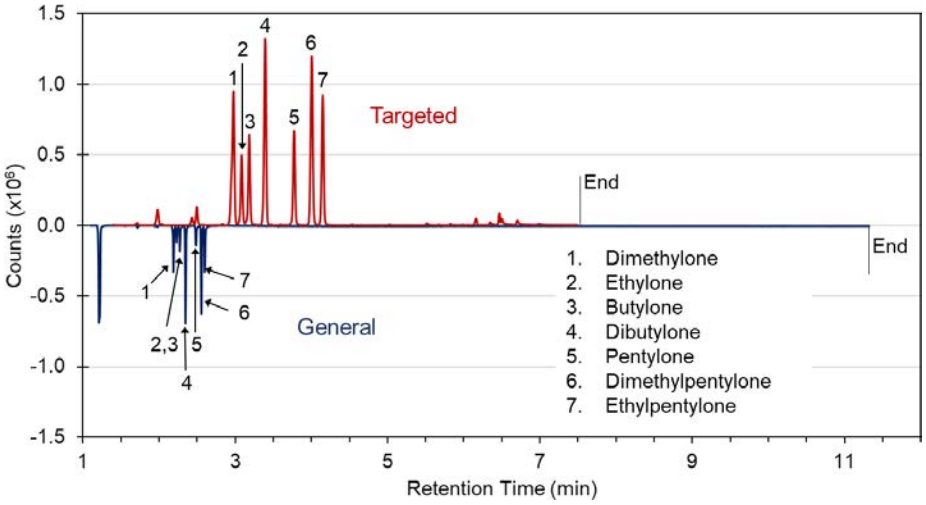

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Figure 9. Representative chromatograms of the synthetic cathinone test solution analyzed on the general method (blue) and targeted GC-MS method (red). Figure adapted from Sisco *et al.*, 2021[15].

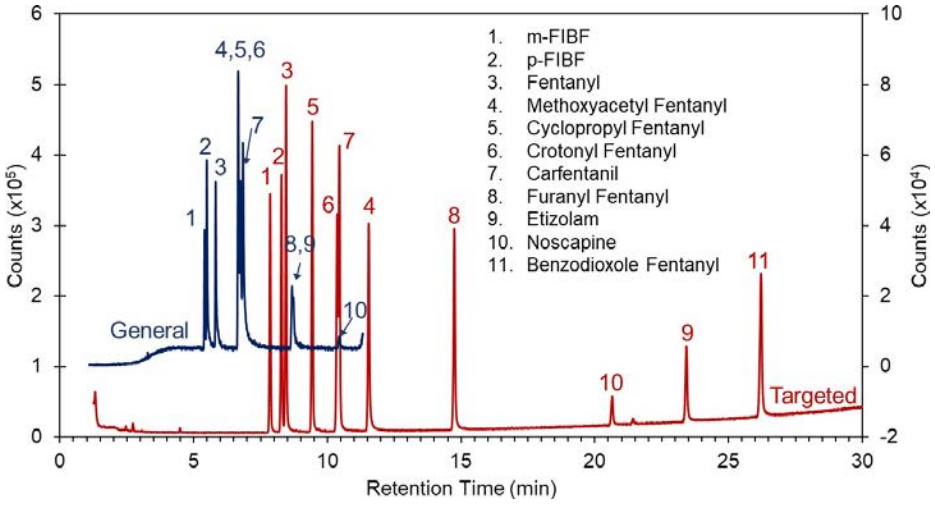

Figure 10. Representative chromatograms of the opioid test solution analyzed on the general method (blue) and targeted GC-MS method (red). Note the secondary y-axis for the general method. The elution order is different for the two runs due to the use of different stationary phases. Figure adapted from Sisco *et al.*[16]

Goal 3 - Implementation and Evaluation of Re-Envisioned Workflow

The final goal of this work was to use the developed DART-MS and targeted GC-MS methods in a casework setting and evaluate how well they compared to the current workflow used at MSP-FSD. Four chemists were trained to use the re-envisioned workflow and were asked to analyze the same 50 mock case samples, identities of which are provided in Table 7. Each chemist analyzed 25 of the samples using the current workflow and 25 using the re-envisioned workflow. Between the four chemists, two analyzed each sample on each workflow. To simplify the process, cases were batched into groups of five. Chemists documented the time and the results for each batch at each step in the analytical process.

Table 7. List of samples used in this study. Non-controlled substances that were known to be in the samples are also listed, in italics. Sample numbers with a dagger (†) were created using one or more adjudicated case samples and sample numbers with an asterisk (*) were created using standards. Some samples were created using a mixture of both. Compound names with a double dagger (‡) are compounds that when analyzed for the actual case, were found to be at concentrations low enough to preclude confirmation.

Sample #	Contents	Sample #	Contents
1†	No Controlled Substance *Pill Binder*	26†	Eutylone *Caffeine*
2†	Methamphetamine	27*	No Controlled Substance *Caffeine*
3†	Heroin, MDMA	28†	4-Methylethcathinone

	Mannitol, Quinine		
4†	Fentanyl, Tramadol‡ *Levamisole, Mannitol, N-Phenylpropanamide, Procaine*	29†*	5-Fluoro-AKB48, α-PBP *Mannitol*
5†	MPHP *Dextrorphan*	30*	Dibutylone, Fentanyl, JWH-250
6†	MDMA	31†	Tramadol *Dextromethorphan*
7†	No Controlled Substance *Mannitol*	32†	JWH-250
8†	Heroin *Papaverine*	33†	Fentanyl, Heroin, Acetyl Fentanyl‡, FIBF‡ *Caffeine, Quinine*
9†	Methyl Norfentanyl	34†	Eutylone
10†	4-Ethylmethcathinone	35†	Fentanyl, Tramadol‡ *Caffeine, Levamisole, Mannitol, N-Phenylpropanamide, Procaine*
11†*	Dibutylone *Caffeine*	36†	Methyl-AP-237
12†*	4-Ethylmethcathinone, Fentanyl, 4-Me-α-ethylaminopentiophenone	37†	Heroin
13†	FUB-AMB	38†	JWH-250, α-Methyl Fentanyl
14†	Cyclopropyl Fentanyl, Heroin, Phenyl Fentanyl *Caffeine, Mannitol*	39†	Fentanyl *Caffeine, Quinine, Xylazine*
15*	AB-FUBINACA 2-fluorobenzyl isomer	40†*	4-Chloroethcathinone, Cyclopropyl Fentanyl
16†	No Controlled Substance *Inorganic Compound*	41†	No Controlled Substance *Mannitol*
17†	Dibutylone	42†	Heroin, Acetyl Fentanyl‡, Cocaine‡, Fentanyl‡, FIBF‡, Noscapine‡ *Caffeine, Quinine*
18†	Acetyl Fentanyl, Fentanyl *Mannitol, Quinine*	43†	Methylone
19†	Heroin, Acetyl Fentanyl‡, Fentanyl‡, FIBF‡ *Caffeine, Lidocaine, Mannitol, Quinine*	44†	N-methyl Cyclopropyl norfentanyl
20*	No Controlled Substance *Guaifenesin, Quinine*	45*	No Controlled Substance *Lidocaine, Quinine*
21*	No Controlled Substance *Acetaminophen, Citric Acid, Xylitol*	46†	4-Methylethcathinone
22†*	Fentanyl, XLR11	47†	JWH-018, 3,4-MDPV
23†	JWH-250	48†	N-Ethyl Pentylone
24†	JWH-018	49*	FUB-AMB
25†	α-PVP	50†*	α-PVP *Sodium Bicarbonate*

In order to compare the information obtained from the screening steps of each workflow, the scoring system defined in Table 4 was used. As expected, results obtained by DART-MS were found to score, on average, significantly higher than color tests (Figure 11) due to the ability to detect all or most of the compounds

present in mixtures and the ability to obtain more specific information about a sample. In no instance were there any incorrect identifications with DART-MS. The technique provided class-level information or better for all compounds present for all but two samples, which received a score of two. In Sample 3, heroin was not detected above the 5 % relative intensity threshold and in Sample 42, cocaine, FIBF, and noscapine were not detected due to low concentration. The average score for the DART-MS results was 3.4 (±0.6) compared to 1.2 (±1.6) for color tests.

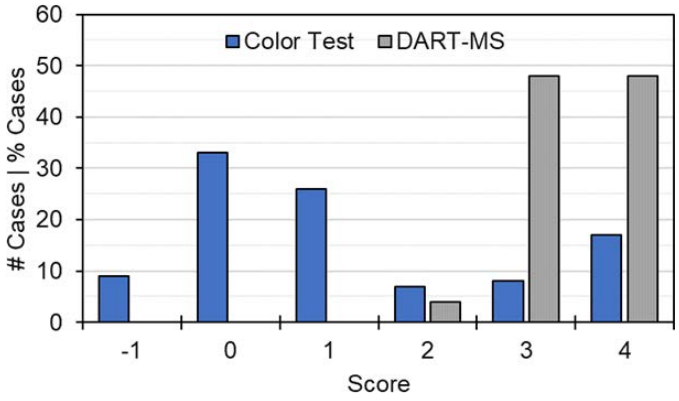

Figure 11. Histogram showing the distribution of scores for the color tests (blue, n = 100) and the DART-MS (grey, n = 100) results. Figure adapted from Sisco *et al.*[9]

The color tests were less effective on this set of samples than expected, with a third of the results being inconclusive and nine of the results being incorrect (defined as a response that was interpreted as a class of compound not in a sample). Additionally, 20 % of samples produced different results when analyzed by different chemists, resulting in different scores for the same sample. Of the incorrect results, four of the nine instances were traced to samples that did not contain a controlled substance but did contain quinine, which elicited a result similar to an opiate. A third sample that contained JWH-018 produced a response similar to a cathinone. Even though there are limitations with the color tests, the use of a general screening method with GC-FID and GC-MS allow for detection of all CDS present in the samples.

For both screening approaches, the time required for analysis was quite similar. Color tests took on average 18.6 min to analyze a batch of five samples while DART-MS took 20 min on average (broken out into 5 min for sample prep, 2 min for sequence prep, 5 min for analysis, and 8 min for data workup). Both

approaches required similar amounts of material, though color tests required slightly more (5 mg versus 2 mg). From a safety standpoint, DART-MS presented a lower overall risk as handling of bulk powder was limited to only one transfer of material. DART-MS required only methanol in addition to the sample, while color tests required the use of other, more hazardous, chemicals like formaldehyde and concentrated acids. While DART-MS provided a more information-rich, possibly safer, analysis in roughly the same amount of time as color tests, it does require a large upfront investment in the technology, which could be a barrier for adoption by some laboratories. The lack of class-specific indications and the high frequency of inconclusive results with the color tests means that this screening approach would be ill-suited for inclusion in a workflow that utilized targeted confirmation methods.

The second piece of the analysis scheme was the confirmation step which, for the existing workflow confirmation, was completed in a two-step process. Under this workflow, the mass spectra for all peaks in the GC-MS chromatogram of an unknown were searched against a library of reference spectra to identify compounds in the sample. Only peaks with chromatographic abundances above 200,000 counts and mass spectra that contained the molecular ion were confirmable, provided the GC-FID data also passed. Peaks with abundances below 200,000 counts were labeled insufficient for identification. To better represent current protocols, a cocaine standard was also run for every method used within a batch as a positive control sample. Second, the retention time of the peak(s) in the sample GC-FID chromatogram were compared to the retention time(s) of standards collected on the same method. Retention times between the sample and the standard needed to be within 1 % of one another and if the compound was an isomer or had compounds that eluted at a similar retention time, standards for all related compounds must be run. To better represent current protocols at MSP-FSD, where standards need to be run on a weekly basis, a standard only needed to be run once per batch (i.e., if multiple samples in a batch contained fentanyl, the fentanyl standard was only run once in that batch). For both GC-FID and GC-MS, chemists had the option to choose between multiple methods, depending on the laboratory where they were completing the analysis and the level of chromatographic separation that was desired.

Confirmation with the experimental workflow was accomplished using the targeted GC-MS methods. With the targeted methods, retention time and mass spectral similarity measurements were made from the same analytical run, unlike in the existing workflow where GC-FID and GC-MS were required. The use of locked retention times eliminated the need for running multiple standards with each batch of samples, like is done for GC-FID in the existing workflow. For this approach the lock compound for each of the targeted methods used in a batch was run as a positive control. Because of the use of high polarity columns, retention time agreement between the previously run standard and a peak in the sample chromatogram needed to be within 2 % of one another. The chromatographic peak had to have a signal to noise ratio of greater than 5:1 and the mass spectral similarity score, compared to the reference spectra, needed to be 85 a.u. or greater.

Overall, as expected, the confirmation results obtained from the existing workflow and the experimental workflow were largely similar. Because of differences in confirmation criteria between the two approaches, there were some differences regarding which compounds could be confirmed versus which compounds were insufficient for identification. For both the existing and experimental workflows, there were analytical limitations which were identified. For the existing workflow, there were several instances where co-elution precluded the ability to make an identification – namely where acetyl fentanyl and FIBF were present. These compounds were not sufficiently separated on the GC-FID method and did not provide sufficient separation to obtain clean mass spectra with GC-MS. With the targeted method, developed specifically for opioid analysis, detection of the two compounds was readily achieved. In one other instance, co-elution of tramadol and mannitol caused difficulty for both of the workflows.

The other limitation was in the identification of dibutylone on the existing method. Dibutylone and many of its isomers elute well within 1 % of one another on the general analysis methods, and, therefore, confirmation of dibutylone could not be achieved. Using the targeted methods, sufficient separation for confirmation of dibutylone was obtained. The 200,000 count threshold cutoff for confirmation using the existing workflow also precluded identification of compounds in six samples. Finally, in two instances, compounds were present in the sample that were not part of the panels for any of the targeted methods. This

limitation can be addressed by adding compounds to the panel, though this process does require some time due to the need to complete replicate measurements of standards.

The biggest difference between the two confirmatory approaches came from comparing the time for analysis, summarized in Table 8. As expected, sample preparation for each of the instrumental techniques was largely identical, with GC-FID, general GC-MS, and targeted GC-MS all requiring approximately 10 min per batch. However, because the current workflow requires GC-FID and GC-MS, the net time for sample preparation per batch is roughly twice as long, compared to the experimental workflow. Instrument time was drastically different for the workflows, with the current workflow requiring 7728.8 min (128.8 hours) for all analyses while the re-envisioned workflow required only 2853.5 min (47.6 hours) for all analyses. These numbers include the running of all samples, standards, and controls. A major driver of this difference is the large number of standards that were required for GC-FID analysis using the existing workflow. As shown in Table 8, the existing workflow required an average of 25.5 analyses per batch, 19.0 of which, on average, came from GC-FID. GC-FID accounted for 68 % of the instrument runtime for the current workflow.

In terms of data analysis, the general GC-MS analysis and targeted GC-MS analysis required similar amounts of analyst time, though the targeted method analysis was slightly faster. This is likely due to the use of a locked retention time lookup table that allowed examiners to enter the retention time of a peak in a sample, and the possible compound(s) that fell within 2 % of that time were shown. Adding in the need to manually compare retention times to standards using GC-FID, the data interpretation component for the existing workflow was found to be almost twice as long as the experimental workflow.

In terms of the amount of sample consumed and the safety risks to chemists, both confirmatory workflows are nearly identical. The existing workflow does require slightly more sample, since separate samples are created for GC-FID and GC-MS, but this difference is likely negligible for almost all cases. One potential challenge with the targeted method approach is that it requires two different column phases (DB-200 and DB-5) which means laboratories would need at least two instruments to leverage this approach.

Table 8. Metrics for the GC-FID and GC-MS measurements for both workflows. Table adapted from Sisco *et al.*[9]

	Existing Workflow			Experimental Workflow
	GC-FID	**General GC-MS**	**GC-FID + GC-MS**	**Targeted GC-MS**
Average Sample Preparation per Batch (min)	9.0 (±2.0)	13.6 (±4.0)	22.6 (±6.0)	8.8 (±1.3)
Average Data Interpretation per Batch (min)	8.2 (±5.4)	22.7 (±10.4)	30.9 (±15.8)	16.5 (±1.5)
Average Instrument Time per Batch (min)	264.3 (±108.9)	116.3 (±43.2)	386.4 (±116.3)	142.7 (±50.0)
Cumulative Average Time per Batch (min)	281.5 (±116.3)	152.6 (±57.6)	439.9 (±138.1)	168 (±52.8)
# Runs per Batch (Samples + Standards)	19.0	6.5	25.5	7.4
Total Instrument Time (min)	5286.3	2442.5	7728.8	2853.5

Limitations

The results of this project demonstrate the possible qualitative and quantitative gains that can be achieved by altering a seized drug workflow. Given the two workflows used here, it was found that screening of samples using color tests and DART-MS required approximately the same amount of time but the quality of the data obtained by DART-MS far exceeded that of color tests. The use of DART-MS also eliminated false positives within the samples studied, eliminated the need for toxic chemicals and acids, and provided documentable data. Though DART-MS was studied in combination with targeted GC-MS methods, the improved data quality and results it offers could benefit the confirmation process of the current workflow as well. While implementation of DART-MS has obvious advantages, the initial and recurring costs as well as the time required to implement the technique needs to be weighed.

In terms of the confirmation processes studied, major improvements in analysis time were observed alongside some notable gains in analytical capabilities. Temporal benefits were largely driven by the use of a single confirmation tool (targeted GC-MS) in the re-envisioned workflow instead of a dual-technique confirmation. The use of locked retention times provided further instrument time reductions along with

reduced consumption of standards. Other approaches to reduce the frequency of running standards, such as relative retention times or retention indices could also prove fruitful and may have their own benefits. Interestingly, even with the need to analyze a sample on multiple targeted methods, instrument time was not substantially greater than the existing workflow.

An obvious downside to the use of targeted methods is the need to have a panel of compounds, which for this study was limited to only compounds within the particular drug classes. Adding additional, commonly co-observed compounds to the method is simple, though it does require some time. The targeted methods also highlighted how class-specific methods designed for enhancing separation can address limitations with general purpose methods. This was observed for multiple compounds (acetyl fentanyl, FIBF, dibutylone, and α-PVP) in the sample set. The use of different chromatographic thresholds for confirmation can also, obviously, lead to differences in the number of compounds that can be identified.

While implementation of targeted methods may be appealing, they do require the use of an information-rich screening tool. Success of the targeted methods was largely due to the fact that DART-MS provided sufficient results to enable accurate identification of nearly all controlled substances in the samples. This approach would not have been successful had color tests been used as the screening tool. Another possible use for targeted GC-MS could be to supplement existing general-purpose screening methods where sufficient separation of compounds cannot be achieved (such as acetyl fentanyl and FIBF). The use of targeted methods requires minimal additional cost and effort beyond the purchase of consumables and method validation. Depending on the compound classes of interest, however, systems with different stationary phases may be required, which could be problematic for laboratories with only one or a few instruments.

This study highlights some of the strengths and limitations of two specific analytical workflows. Though there are clear limitations in the experimental workflow, it does highlight some reasons why laboratories may want to consider changes to their protocols. An ideal workflow would certainly look different across laboratories and would be dependent on factors such as caseload, personnel, types of cases frequently

examined, jurisdictional requirements, and access to instrumentation. While it may not be practical to measure all gains and drawbacks prior to implementing changes to analytical protocols, the ability to test these changes, comparing potential workflows on a small scale can minimize the risk of new instrumentation being procured but never implemented into casework. Additional studies investigating different analytical workflows are still ongoing and are the focus of current research.

Artifacts

The following section provides a list of artifacts that were produced and/or disseminated as a result of this award.

List of Products

- Publication: Sisco, E.; Burns, A.; Moorthy, A. S. *A Framework for the Development of Targeted Gas Chromatography Mass Spectrometry (GC-MS) Methods: Synthetic Cannabinoids.* Journal of Forensic Sciences. 2021; 66: 1908-1918. DOI: https://doi.org/10.1111/1556-4029.14775.
- Publication: Sisco, E.; Burns, A.; Moorthy, A. S. *Development and Evaluation of a Synthetic Cathinone Targeted Gas Chromatography Mass Spectrometry (GC-MS) Method.* Journal of Forensic Sciences. 2021; 66: 1919-1928. DOI: https://doi.org/10.1111/1556-4029.14789.
- Publication: Sisco, E.; Burns, A.; Moorthy, A.S. *Development and Evaluation of a Synthetic Opioid Targeted Gas Chromatography Mass Spectrometry (GC-MS) Method.* Journal of Forensic Sciences. 2021; 66: 2369-2380. DOI: https://doi.org/10.1111/1556-4029.14877.
- Publication: Sisco, E.; Burns, A.; Schneider, E.; Ikpeama, I. *Evaluation of Internal Standard Inclusion for Qualitative Analysis of Seized Drugs using DART-MS.* Forensic Chemistry. 2022: 27; 100392. DOI: https://doi.org/10.1016/j.forc.2021.100392.
- Publication Under Review: Sisco, E.; Burns, A.; Schneider, E.; Hardy, L; Ikpeama, I. *A Template for the Validation of DART-MS for Qualitative Seized Drug Analysis.* Pre-print available at ChemRXiv. DOI: https://doi.org/10.26434/chemrxiv-2021-pc716.

- Publication: Sisco, E.; Burns, A.; Schneider, E.; Miller, C.R.; Hardy, L. *Comparing Two Analytical Workflows for Seized Drug Analysis of Synthetic Cannabinoids, Cathinones, and Opioids.* Journal of Forensic Sciences. 2021; 00: 1-12. DOI: https://doi.org/10.1111/1556-4029.14936.
- Published Dataset: Sisco, E. *Data Supporting the Development of Targeted GC-MS Methods for Seized Drug Analysis.* DOI: 10.18434/MDS2-2367. Available for download at https://data.nist.gov/od/id/mds2-2367.
- Document Repository: Sisco, E. *Templates for the Implementation of DART-MS for Seized Drug Analysis.* DOI: 10.18434/mds2-2424. Available for download at https://data.nist.gov/od/id/mds2-2424.

Datasets Generated

The following datasets were generated as a result of this award.

- Data supporting the development of targeted GC-MS methods. This dataset contains method files, retention time lists, and replicate mass spectra (in .csv and .pdf formats) that were used in the creation of targeted GC-MS methods. These datasets are freely available at https://data.nist.gov/od/id/mds2-2367.
- Data supporting validation of the DART-MS. This dataset contains all of the data generated for the validation of DART-MS for casework at MSP-FSD. This dataset contains both the raw and processed data as well as summaries of the different components of the data. The summary data will be included in the publication titled *A Template for the Validation of DART-MS for Qualitative Seized Drug Analysis*.
- Data supporting the comparison of analytical workflows. This dataset contains all of the data generated for the workflow comparison study. This includes DART-MS, GC-FID, and GC-MS data as well as summaries of this data and results of color tests. The summary data will be included in the publication titled *Comparing Two Analytical Workflows for Seized Drug Analysis*

of Synthetic Cannabinoids, Cathinones, and Opioids.

- Data and code supporting the *min-max test*. This dataset includes code to run the min-max test in R as well as representative data for its use. This dataset is freely available at: https://data.nist.gov/od/id/mds2-2418.

Dissemination Activities

- Sisco, E. *Analytical Strategies to Advance Forensic Operations*. Presented at the National Institute of Justice's Forensic Technology Center of Excellence 2019 National Opioid and Emerging Drug Threats Policy and Practice Forum, Washington DC, July 2019.
- Sisco, E. *Utilizing Trace Drug Residues in Forensic Analysis of Opioids.* Presented at the Washington Baltimore Mass Spec Discussion Group, Washington DC, January 2020.
- Burns, A. *Novel Workflows for Seized Drug Analysis*. Present at the ASCLD Lightning Talk Series, Virtual, October 2020.
- Sisco, E.; Burns, A. *Development of Novel Workflows for Seized Drug Analysis*. Presented at Forensics@NIST, Virtual, November 2020.
- Burns, A. *The Decision to Implement: Lessons Learned*. Presented as part of the *Applications, Implementation, and the Future of DART-MS in Forensic Laboratories* workshop at Forensics@NIST, Virtual, November 2020.
- Sisco, E. *Towards a Re-envisioned Workflow for Seized Drug Analysis*. Presented at 2021 Online Forensic Symposium: Current Trends in Seized Drug Analysis, Virtual, January 2021.
- Burns, A. *The Decision to Implement: Lessons Learned*. Presented as part of the *Applications, Implementation, and the Future of DART-MS in Forensic Laboratories* workshop at the American Academy of Forensic Sciences, Virtual, February 2021.
- Burns, A. *Increasing Safety, Speed, Sensitivity, and Selectivity or Controlled Substance Analysis*. Presented at the American Academy of Forensic Sciences, Virtual, February 2021.

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