FINAL REPORT

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Project Title: ***Chemometric Processing of DART-HRMS-derived Dark Matter for the Identification of New Psychoactive Substances***

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PURPOSE OF THE PROJECT

The central hypothesis of the project is that the capabilities of DART-HRMS can be applied to elucidate the structures of unknown novel variants of synthetic cathinones, cannabinoids, opioids, and tryptamines for the benefit of forensic science practitioners. The approach combines: (1) the information inherent in DART-HRMS collision induced dissociation (CID)-derived neutral loss spectra; and (2) chemometric processing of the data. Demonstration of the proof of this principle will allow the building of a database of known psychoactive substances that crime labs can use to determine the identity of emerging psychoactive substances found at crime scenes.

Project Goals: The hypothesis was investigated through pursuit of the following four Specific Aims:

Specific Aim I: Collection of soft ionization and CID spectra of the commercially available standards of synthetic cathinones, cannabinoids, opioids, and tryptamines by direct analysis in real time-high resolution mass spectrometry (DART-HRMS).

***Specific Aim II*:** Creation of DART-HRMS CID-derived neutral loss spectra to compile a database of neutral losses for each of the classes of molecules listed in Specific Aim I.

Specific Aim III: Application of multivariate statistical analysis of neutral loss spectra to categorize molecules based on shared structural features.

Specific Aim IV: Use soft ionization spectra (Specific Aim I), neutral loss spectra (Specific Aim II), and clustering information (Specific Aim III) to elucidate the structures of novel, emerging synthetic cathinones, cannabinoids, opioids, and tryptamines.

PROJECT DESIGN AND METHODS

Plan of Action—Sample types, description, rationale and analysis plan

Specific Aim I: Towards accomplishing Specific Aim I, standards of forty-seven cathinones, one hundred-twelve cannabinoids, fifty three tryptamines, and four opioids were purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA). All mass spectra were acquired in positive ion mode in the range of 40-800 *m/z* using sing a DART-SVP™ ion source (Ionsense, Saugus, MA, USA) coupled to a JEOL AccuTOF high-resolution mass spectrometer (JEOL USA, Inc., Peabody, MA, USA). For acquiring soft ionization and CID spectra, orifice 1 was cycled through 20, 30, 60, and 90 V. Other optimal instrument parameters were determined to be; orifice 2 and the ring lens voltages: 5 V, ion guide voltage: 400 V to allow for the detection of ions above *m/z* 40, helium flow rate: 2 L min^{-1} and gas heater temperature: 350 °C. The standards were analyzed in ten replicates by dipping the closed end of a melting point capillary (VWR, Radnor, PA, USA) into the powdered standards and waving it for ~5 s in open air gap between ion source and mass spectrometer inlet.

Specific Aim II: Towards accomplishing Specific Aim II, Mass Mountaineer software was used to determine the neutral masses lost during fragmentation caused by raising the orifice 1 voltage from 20 to 90 V. For this, each of the *m/z* values of the fragment peaks in the CID spectra were subtracted from the peak representing the protonated precursor of the psychoactive substances at 20 V. This resulted in an intensity vs *m/z* value plot which constituted the neutral loss or “loss spectrum” for each psychoactive compound. The peak intensities shown in the neutral loss spectra are equivalent to the intensities of the various fragments.

Specific Aim III: Chemometric processing of the neutral losses acquired in this study was conducted, and advanced workflows were developed by writing in-house codes in into MATLAB 9. 3. 0, R2019a software (The MathWorks, Inc., Natick, MA, USA).

Specific Aim IV: Toward Specific Aim IV, the analysis workflow was tested using both internal and external validation approaches.

DATA ANALYSIS

Over the course of the project, the majority of the chemical standards (forty-seven cathinones, one hundred-twelve cannabinoids, fifty three tryptamines, and four opioids) were successfully analyzed by DART-HRMS, and their neutral losses were compiled to populate a comprehensive database. These compounds are represented in Tables 1-4. As illustrated by the groups in the Tables (which emerged from the statistical analysis treatment of the data), the DART-HRMS derived-neutral loss spectra revealed the validity of the original hypothesis, which was that the neutral losses spectra of synthetic designer drugs exhibit striking similarities for those that share key structural features, and that that this can be used for structure elucidation purposes, particularly in those cases where EI-MS results are ambiguous. Replicates of each standard were acquired to generate a robust database of the neutral losses for each compound. The statistical analysis processing of this data involved the development of procedures that enabled implementation of the following workflow: background subtraction; binning (with defined bin widths and relative abundance threshold cut-offs) to align the spectra along common *m/z* values; normalization; data fusion of CID neutral loss spectra; application of unsupervised classification methods to reveal clusters of structures that share similar structural features; and application supervised machine learning methods to discriminate the detected clusters, with the goal of characterizing the distinct markers that characterized each cluster. Using this approach, the new designer drugs could be rapidly analyzed by DART-MS within ~5 s and identified

based on markers and their similarities to structures in the database. The approach provides the level of certainty associated with the prediction of unknowns, which was found to be 90-100% in examination of all the external validation samples. Furthermore, over course of the project, we developed an analysis protocol to integrate ATR-IR spectral data (acquired using a Perkin Elmer spectrum 2 in the wavenumber range of 4000 to 400 cm^{-1}, with a resolution of 1 cm^{-1}, and with each spectrum being an average of 16 scans) and DART-HRMS-derived neutral loss data. Both methods are simple and rapid, and combining the output of both methods resulted in more consistent and accurate identification results, and reduced the rate of false positives. For example, the application of this protocol for analysis of tryptamines revealed that both types of data have similar information content in terms of enabling the characterization of similar structures and therefore, their fusion has a synergetic effect on accurate identification of emerging psychoactive substances.

Table 1. Tryptamines analyzed and the groups in to which they were categorized following multivariate statistical analysis processing of DART-HRMS-derived neutral loss data.

• **Group 1** 4-Methyl-α-ethyltryptamine; 5-Methoxy-α-ethyltryptamine; *N*-Ethyl-*N*-methyltryptamine; α-Ethyltryptamine; • **Group 2** 5, 7-Dichloro tryptamine; 5-(2-Aminopropyl)indole; 5-methoxy-α-methyltryptamine; 6-(2-Aminopropyl)indole; 7-Fluoro tryptamine; 5-Nonyloxytryptamine; 5-Hydroxytryptamine; α-Methyltryptamine; • **Group 3** *N*,*N*-Dipropyltryptamine; *N*,*N*-Diisopropyltryptamine; *N*,*N*- Diethyltryptamine; *N*,*N*- Dimethyltryptamine; • **Group 4** *N*-Acetyl-5-hydroxytryptamine; *N*-Acetyltryptamine; • **Group 5**	• **Group 6** 4-Methoxy-*N*-methyl-*N* isopropyltryptamine; *N*,*N*-Diallyl-5-methoxy tryptamine; 5-Methoxy-*N*,*N*-dibutyltryptamine; 5-Methoxy-*N*,*N*-diethyltryptamine; 5-Methoxy-*N*,*N*-dimethyltryptamine; 5-Methoxy-*N*,*N*-dipropyltryptamine; 5-Methoxy-*N*,*N*-dibutyltryptamine; 5-Methoxy-*N*,*N*-diisopropyltryptamine; 5-Methoxy-*N*-ethyl-*N*-propyltryptamine; 5-Methoxy-*N*-ethyl-*N*-isopropyltryptamine; 5-Methoxy-*N*-methyl-*N*-isopropyltryptamine; 6- Methoxy-*N*-methyl-*N*-isopropyltryptamine; 7- Methoxy-*N*-methyl-*N*-isopropyltryptamine; • **Group 7** 5-Hydroxy-*N*-methyl tryptamine; *N*-methyl tryptamine; • **Group 8** Sumatriptan; • **Group 9**	4-Hydroxy-*N*,*N*-dimethyltryptamine; 4-Hydroxy-*N*,*N*-Dipropyltryptamine; 4-Hydroxy-*N*,*N*-Dipropyltryptamine; 4-Hydroxy-*N*-ethyl-*N*-methyltryptamine; 4-Hydroxy-*N*-methyl-*N*-propyltryptamine; 4-Hydroxy-*N*-methyl-*N*-isopropyltryptamine; 5-Hydroxy-*N*,*N*-dimethyltryptamine; 4-Hydroxy-*N*,*N*-dimethyltryptamine; 4-Hydroxy-*N*-methyl-*N*-allyltryptamine; • **Group 10** 4-Acetoxy-*N*,*N*-diethyltryptamine; 4-Acetoxy-*N*,*N*-dimethyltryptamine; 4-Acetoxy-*N*,*N*-dipropyltryptamine; 4-Acetoxy-*N*,*N*-diisopropyltryptamine; 5-Acetoxy-*N*-methyl-*N*-ethyltryptamine; 4-Acetoxy-*N*-methyl-*N*-isopropyltryptamine; 4-Acetoxy-*N*-methyl-*N*-allyltryptamine; 4-Acetoxy-*N*-methyl-*N*-propyltryptamine; 4-Propanoyloxy-*N*,*N*-dimethyltryptamine;

6-Fluoro-*N*,*N*-diethyltryptamine;	4-Hydroxy-*N*,*N*-diethyltryptamine;	

Table 2. The opioids analyzed during the project and derived group of structures that share similar structural features.

Acetyl fentanyl; α-Methyl acetyl fentanyl; α-Methyl fentanyl; Fentanyl.

Table 3. The cannabinoids analyzed during the project and derived group of structures that share similar structural features.

• **Group 1** 1-naphthoyl indole; AM679; JWH 016; JWH 018 N-(1-ethylpropyl) isomer; JWH 018 N-(2-methylbutyl) isomer; JWH 018 N-(3-methylbutyl) isomer; JWH 022; JWH 122 N-(4-pentenyl) analog; JWH 175; JWH 182; JWH 210; JWH 210 7-ethylnaphthyl isomer; RCS-4 2-methoxy isomer; RCS-4 3-methoxy isomer; XLR11 N-(4-pentenyl) analog; • **Group 2** JWH 018 6-methoxyindole analog; JWH 398 N-pentanoic acid metabolite; MAM2201 N-pentanoic acid metabolite; RCS-4 N-(5-carboxypentyl) metabolite;	• **Group 7** (S) AM1241; (S) JWH 073 N-(3-hydroxybutyl) metabolite; A-796260; AB-005; ADB-FUBICA; ADBICA; AKB 48-N-(4-hydroxypentyl); metabolite; AM 2232; AM1248; AM2201 2-hydroxyindole metabolite; JW 642; JWH 015; JWH 018 N-(5-bromopentyl) analog; JWH 018 adamantyl carboxamide; JWH 020; JWH 030; JWH 030 2-naphthoyl isomer; JWH 031; JWH 073 2-methylnaphthyl analog; JWH 073 N-(3-hydroxybutyl) metabolite; JWH 145;	• **Group 8** JWH 018 N-(2-hydroxypentyl) metabolite; JWH 018 N-(3-hydroxypentyl) metabolite; JWH 018 N-(4-hydroxypentyl) metabolite; JWH 018 N-(5-hydroxypentyl) B-D-glucuronide; JWH 122 N-(4-hydroxypentyl) metabolite; JWH 122 N-(5-hydroxypentyl) metabolite; JWH 210 N-(4-hydroxypentyl) metabolite; JWH 398 N-4-hydroxypentyl metabolite; RCS-4 N-(4-hydroxypentyl) metabolite; RCS-4 N-(5-hydroxypentyl) metabolite; UR-144 N-(4-hydroxypentyl) metabolite; UR-144 N-(5-hydroxypentyl) B-D-glucuronide; UR-144 N-(5-hydroxypentyl) metabolite; • **Group 9** A-834735; JWH 201; JWH 203 3-chlorophenyl isomer; JWH 203 4-chlorophenyl isomer; JWH 249; JWH 251; JWH 302; • **Group 10**

UR-144 N-pentanoic acid metabolite; • **Group 3** AM2233; AM2233 azepane isomer; JWH 073 2-hydroxyindole metabolite; • **Group 4** JWH 007; JWH 018 N-(4,5-epoxypentyl) analog; JWH 098; JWH 213**;** • **Group 5** AM2201 5-hydroxyindole metabolite; AM2201 7-hydroxyindole metabolite; AM2201 N-(4-hydroxypentyl) metabolite; JWH 018 N-(5-chloropentyl) analog; MAM2201 N-(4-hydroxypentyl) metabolite; • **Group 6** Hu-331 MDMB-FUBICA	JWH 147; JWH 198; JWH 250 N-(4-hydroxypentyl) metabolite; JWH 250 N-pentanoic acid metabolite; JWH 307; JWH 368; JWH 369; JWH 370; JWH 398 N-pentanoic acid metabolite; JWH 031 2-isomer; MDA 19; MDA 77; MDMB-FUBICA metabolite 3; MMB-FUBICA; RCS-4-C4 homolog; RCS-8 3-methoxy isomer; RCS-8 4-methoxy isomer; STS 135; Pravadoline; WIN 55, 212-2 (mesylate);	AM2201 2-naphthyl isomer; AM694 3-iodo isomer; AM694 4-iodo isomer; MAM2201 N-(2-fluoropentyl) isomer; MAM2201 N-(3-fluoropentyl) isomer; MAM2201 N-(4-fluoropentyl) isomer; XLR11 N-(2-fluoropentyl) isomer; XLR11 N-(3-fluoropentyl) isomer; XLR11 N-(4-fluoropentyl) isomer; • **Group 11** JWH 200 4-hydroxyindole Metabolite; JWH 200 7-hydroxyindole metabolite; JWH200 5-hydroxyindole metabolite; • **Group 12** AB-FUBICA JWH 176 HU-211 JWH 133

Table 4. The cathinones analyzed during the project and derived group of structures that share similar structural features.

• **Group 1** 2,3-Dimethylethcathinone; 3,4-Dimethylethcathinone; 2-Methylethcathinone; 3-Methylethcathinone; 2-Ethylethcathinone; 2-Fluoroethcathinone; 3-Fluoroethcathinone; 4-Fluoroethcathinone; • **Group 2** Diethylcathinone; 2,5-Dimethoxy-4-methyl-phenethylamine; α-Ethylaminopentiophenone; *N*-Ethyl-*N*-methylcathinone Isopentedrone; 4-Methyl-α-ethylaminobutiophenone Pentedrone;	• **Group 3** 3,4-Dimethoxy-α-pyrrolidinopentiophenone; 3-Fluoro-α-pyrrolidinopropiophenone; 4-Fluoro-α-pyrrolidinopropiophenone; 4-Methoxy-α-pyrrolidinopentiophenone; 2-Methyl-α-pyrrolidinopropiophenone; 3-Methyl-α-pyrrolidinopropiophenone; Naphyrone; Pyrovalerone; α-Pyrrolidinoheptiophenone; α-Pyrrolidinohexiophenone; α-Pyrrolidinopentiophenone; α-Pyrrolidinopropiophenone; • **Group 4** *N*-Ethylbuphedrone; 3-Methylbuphedrone;	4-Methylbuphedrone; 4-Methyl-*N*-methylbuphedrone; • **Group 5** Ethylone; Eutylone; 3,4-Methylenedioxy-*N*-benzylcathinone; 3,4-Methylenedioxy-5-methylethcathinone; 3,4-Methylenedioxypyrrolidinobutiophenone; 3,4-Methylenedioxypyrrolidinohexanophenone; 3,4-Methylenedioxypyrrolidinopropiophenone; 3,4-Methylenedioxypyrovalerone; 3,4-Methylenedioxypyrovalerone metabolite 2; Methylone; Pentylone; • **Group 6** 4-Bromomethcathinone; 3,4-Dimethylmethcathinone; 2-Fluoromethcathinone; 4-Fluoromethcathinone; Mephedrone

PROJECT FINDINGS AND DELIVERABLES

From the perspective of the Specific Aims, the major findings of the project were: (1) chemometric analysis of DART-HRMS mass spectrometry-derived neutral losses of the commercially available standards of synthetic cathinones, cannabinoids, opioids, and tryptamines can be used to identify new psychoactive compounds; (2) there are a reliable set of experimental parameters that can be used to reproducibly generate neutral loss mass spectra and ATR-IR spectra, and that these optimized parameters can be used to identify new psychoactive substances; (3) an optimized statistical analysis protocol can be applied to elucidate the structures of novel psychoactive substances using fused neutral loss spectra and fused DART-HRMS neutral loss/ATR-IR spectra; and (4) a highly robust and accurate database of DART-HRMS derived-neutral loss spectra, along with an accompanying statistical analysis processing workflow can be developed against which the neutral loss spectra of unknowns can be screened, and which enables identification of the class of structure to which the unknown belongs, with reporting of the statistical accuracy. The deliverables associated with these findings appear in the form of 4 journal articles (one published and 3 in preparation); 1 book chapter; 4 conference presentations; 1 university talk; a database of DART high resolution mass spectra for tryptamines, cannabinoids, cathinones, and opioids, along with their corresponding neutral loss spectra; a partially completed graphical user interface that can be used by practitioners to process DART-HRMS data acquired from unknowns, in order to make predictions; and the training of 1 postdoctoral associate, 6 graduate students and 2 undergraduate students.

PRODUCTS

1) Publications, conference papers, and presentations

**Graduate student; **Postdoctoral trainee; ***Undergraduate student*

Publications

1. Fowble, K. L.; Musah, R.A. *Utilizing direct analysis in real time-high resolution mass spectrometry-derived dark matter spectra to classify and identify unknown synthetic cathinones,* **Methods Mol Biol.** (2018), *1810*, 217-225.
2. Fowble, K.L.; Shepard, J.R.E.; Musah, R.A. *Identification and Classification of Cathinone Unknowns by Statistical Analysis Processing of Direct Analysis in Real Time-High Resolution Mass Spectrometry-Derived "Neutral Loss" Spectra*, **Talanta** (2018), *179*, 546-553.

Presentations

1. **Standardizing Future Smart and Connected Forensic Evidence Rooms Using Internet of Things (IoT) Based AutoID Enabled Technologies Conference**, Arlington TX; *Development of SPME Approaches to the Detection of Psychoactives*; Musah, R. A., June 11, 2018. Oral Presentation.
2. **PITTCON 2019**, Philadelphia, PA; "*Chemometric Processing of Direct Analysis in Real Time (DART) Mass Spectrometric Data for the Identification and Classification of New Psychoactive Substances*"; Musah, R. A., Beyramysoltan, S. Fogerty, M.G., Fowble, K.L., R. A. March 20, 2019. Oral Presentation.
3. **Northeastern Association of Forensic Scientists 2019 Annual Conference**, Lancaster, PA; "*Trippin' on Tryptamines: The Use of Chemometric Processing of DART-HRMS Data for Identification of This Subset of Psychoactive Substances.*" Ventura, M.I., Musah, R.A. November 14, 2019. Poster.
4. **PITTCON 2020**, Chicago, IL; "*Determination of The Structures of New Psychoactive Substances Using DART-MS-Derived Collision Induced Dissociation Data*"; Musah, R. A., Beyramysoltan, S., Ventura, M.I., Fowble, K.L., March 3, 2020. Oral Presentation.
5. **Northeastern Association of Forensic Scientists 2020 Annual Conference**, Mystic, CT; "*Trip or Treat: Data Fusion and Multivariate Statistical Analysis Treatment of DART-HRMS Collision-induced Dissociation Data to Enable the Rapid Detection and Identification of Tryptamines*." Ventura, M.I., Beyramysoltan, S., Musah, R.A. October 15, 2020. Oral Presentation.

IMPLICATIONS FOR CRIMINAL JUSTICE POLICY AND PRACTICE IN THE US

The project has resulted in the development to several sorely needed innovations that were heretofore lacking in forensic analysis of psychoactive substances. These are: (1) **Rapid Analysis and Straightforward Protocols** through the use of DART-MS and ATR-IR—The rapidity of the analyses can reduce the burdens of backlogs in forensics casework by cutting down on the sample preparation time and extraction steps used in conventional analyses; (2) **High Throughput Screening**—The methodology enables analysis of up to 250 samples per hour, vs. an average of one sample being analyzed every 2-12 hours (from start of finish) if conventional methods are used; and (3) **Psychoactive substances Database with Statistical Analysis Reporting**—This work furnished an psychoactive substances database analogous to controlled substance databases, against which unknowns can be screened and rapidly identified with a defined level of confidence.

Furthermore, this database serves as a “living” resource, in that it will be continuously and rapidly expanded with data on new emerging products, and become more robust with the addition of data from more and more samples of a given designer drug. This has immediate and long-range impacts on forensic science practice and criminal justice, and could facilitate the crafting of legislation for the control of emerging psychoactive substances. In summary, the developed techniques circumvent the challenges in identification of new psychoactive substances as identified by United States Office on Drugs and Crime. Among the expected advantage for forensic practice are: (1) reduction in crime lab sample testing backlogs; (2) streamlining of sample analysis protocols; (3) reduction of human resource and chemical reagent costs; (3) re-deployment of laboratory equipment such as GC- and LC-MS instruments for other necessary types of analyses; (4) more timely completion of sample analyses so that prosecutions can be expedited; and (5) statistical reporting of results.

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