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FINAL REPORT

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Project Title: ***Development of Ambient Ionization Mass Spectrometric and Multivariate Statistical Analysis Methods for Rapid High Throughput Forensic Analysis and Identification of Psychotropic Plants***

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PURPOSE OF THE PROJECT

The central hypothesis of the project is that the unique mass spectrometry-derived chemical fingerprints of abused psychoactive plants can be rapidly determined and subjected to multivariate statistical analysis, and that the results of such processing can be used for quick genus or species-level identification for the benefit of forensic science practitioners. Demonstration of the proof of this principle will allow: (1) the building of a database of abused psychotropic plants that crime labs can use to determine the identity of unknown plant materials found at crime scenes; and (2) statistical reporting of the level of certainty of the results.

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

Specific Aim I: Determination of whether chemometric analysis of mass spectrometry-derived chemical fingerprints of plants of abuse can be used to identify and discriminate one from another.

***Specific Aim II*:** Determination of the experimental parameters required to reproducibly generate data that can be used in Specific Aim I, and development and validation of experimental protocols for analysis of plant drugs of abuse.

Specific Aim III: Development of mass spectral quantitation methods that can be used in the analysis of forensically relevant small molecule biomarkers from plants of abuse.

Specific Aim IV: Design and demonstration of a classification system (database) for forensically relevant plants of abuse evidence that is based on chemometric analysis of mass spectral data.

PROJECT DESIGN AND METHODS

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

Specific Aim I: Towards accomplishing Specific Aim 1, model plants, plant material (e.g. seeds) and products (e.g. dried material or powders; tinctures; extracts etc.) were purchased from various and when possible, each species was acquired from several vendors in order to compare the fingerprints obtained from each and assess the degree of variability between cultivars and products, and ensure that the developed approach enabled all products representing a given species to be identified as having been derived from that species. Where relevant, live plants were also cultivated and analyzed as a function of time of day, plant age, growing season and plant parts (e.g. leaves, flowers, roots, bark) in order to acquire a comprehensive database of chemical signatures associated with a particular species.

Specific Aim II: Towards accomplishing Specific Aim II, optimal DART-MS instrument parameters were determined. Specifically, analysis of plant material and standards was performed using a JEOL AccuTOF© time-of-flight mass spectrometer (JEOL USA, Peabody, MA) coupled to a Direct Analysis in Real Time (DART)-SVP ion source (IonSense, Saugus, MA). Spectra were collected in both positive and negative ion modes and the merits of both were assessed. Since it was deemed essential that forensics labs to be able to integrate sample analysis automation protocols into their DART-MS analysis methods manual sampling methods alongside automated sampling methods utilizing such tools as the Linear Rail sample delivery system from IonSense, were also developed. Method validation was conducted using the strict and detailed guidelines provided by the United States Food and Drug Administration (US FDA) for Bioanalytical Method Validation on selectivity, accuracy, precision, recovery, sensitivity, stability, reproducibility, calibration curves, dilution, carry over, and matrix effects.

Specific Aim III: The presence of relevant small molecule biomarkers such as psychoactive compounds in the plant material was confirmed through collision induced dissociation (CID) of

authentic standards. Internal standards were developed to permit quantitation of these biomarkers. Corresponding calibration curves were then be constructed. Following determination of the optimal extraction solvent system for each plant and product type, the plant material was extracted. Quantification procedures were optimized using the Linear Rail system (IonSense, Saugus, MA) for semi-automation and enhanced reproducibility.

Specific Aim IV: Chemometric processing of the mass spectral profiles acquired in this study was conducted and advanced classification systems were developed by writing in-house codes in MATLAB 9.0. They were tested using both internal and external validation approaches.

DATA ANALYSIS

Over the course of the project, the majority of the plants designated by the United Nations Office on Drugs and Crime (UNODC) as "Plants of Concern" were successfully analyzed by DART-HRMS, and their chemical signatures were compiled to populate a comprehensive database. The species represented are: *Picralima nitida*, *Banisteriopsis caapi*, *Nymphea caerulea*, *Calea ternifolia*, *Psychotria viridis*, *Datura stramonium*, *Turnera diffusa*, *Argyreia nervosa*, *Sceletium tortuosum*, *Piper methysticum*, *Catha edulis*, *Mitragyna speciosa*, *Leonotis leonurus*, *Mimosa tenuiflora*, *Ipomea* spp., *Salvia divinorum*, *Peganum harmala*, *Voacanga africana* and *Lactuca virosa*. Over the course of the project, it was also learned that several species that are related to the aforementioned plants by having similar psychoactive compounds, are also enjoying increased recreational use and are of forensic interest. In order to ensure that they could also be identified and would not be confused with those members of the UNODC list, these additional species were also analyzed and included in the database. They are *Echinopsis* spp. that are used as legal alternatives to peyote; several Datura spp. that are used as alternatives to *D. stramonium* (i.e. *D. ceratocaula*, *D. discolor*, *D. ferox*, *D. innoxia*,

D. leichardtii, *D. metel*, *D. quercifolia*, and *D. wrightii*); and numerous additional species that contain scopolamine and atropine *Atropa baetica*, *A. belladonna*, *A. komarovii*, *Brugmansia arborea*, *B. aurea*, *B. sanguinea*, *B. suaveolens*, *B. versicolor*, *Hyoscyamus albus*, *H. aureus*, *H. muticus*, *H. niger*, *H. pusillus*, *Mandragora autumnalis*, and *M. officinarum*). The DART-HRMS results revealed the validity of the original hypothesis, which was that plant derived psychoactive materials exhibit diagnostic chemical signatures that have intraspecies similarities and show interspecies differences. In all, tens of thousands of spectral replicates were acquired to generate a robust and well-populated database of their representative chemical signatures. The sequence of steps associated with statistical analysis processing of this data involved the development of code that enabled implementation of the following workflow: background subtraction and binning (with defined bin widths and relative abundance threshold cutoffs; normalization; application of variable selection using tools; and application of machine learning. Using this approach, all the species could be rapidly identified simply based on their DART-MS chemical signature acquired within ~5 s. Furthermore, the approach provides the level of certainty associated with the identification, which is 98-100% in most cases! For all plants for which the psychoactive constituent is known, validated quantification protocols using the same analytical tool (DART-HRMS) were developed. These include methods for the quantification of: dimethyltryptamine (DMT); mescaline; salvinorin A; mitragynine; and atropine. Lastly, a number of psychoactive substances of forensic interest are comprised of mixtures of plant materials. The most notable of these is ayahuasca, and brew of South American origin that is comprised of a mixture of one DMT-containing species and a second harmala alkaloid containing species. Since abuse of this material is of forensic interest and it can be difficult to identify in a crime scene analysis context, we also included its chemical signature in the aforementioned database, along with the chemical signatures of the plant materials of which it is comprised. A validated protocol for the quantification of its psychoactive component (DMT) was also developed.

PROJECT FINDINGS AND DELIVERABLES

When couched from the perspective of the Specific Aims, the major findings of the project were: (1) chemometric analysis of mass spectrometry-derived chemical fingerprints of plants of abuse can be used to identify and discriminate one from another; (2) A reliable set of experimental parameters that could be used to reproducibly generate species-specific mass spectral fingerprints that can be used to identify legal high plants of abuse; (3) Validated protocols were developed that enabled quantification of the psychoactive components of plant-based legal highs; and (4) a highly robust and accurate database, along with an accompanying statistical analysis processing workflow has been developed against which the spectra of plant-based legal high unknowns can be screened, and which enables their species-level identification with reporting of the statistical accuracy. The deliverables associated with these findings appear in the form of 14 journal articles, 2 book chapters, 60 conference presentations; 1 Ph.D. thesis; 5 University talks; and the training of 2 postdoctoral associates, 8 graduate students and 2 undergraduate students.

PRODUCTS

1) Publications, conference papers, and presentations

Publications

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

1. Longo, C.M.* and Musah, R.A. *An Efficient Ambient Ionization Mass Spectrometric Approach to Detection and Quantification of the Mescaline Content of Commonly Abused Cacti from the Echinopsis Genus.* J Forensic Sci. (2020), 65, 61-66. doi: 10.1111/1556-4029.14134.
2. Appley, M.G.,* Beyramysoltan, S.,** and Musah, R.A. *Random Forest Processing of Direct Analysis in Real-Time Mass Spectrometric Data Enables Species Identification of Psychoactive*

Plants from Their Headspace Chemical Signatures. ACS Omega (2019), 4, 15636-15644, doi: 10.1021/acsomega.9b02145

3. Chambers, M.I.,* Osborne, A.M.,* Musah, R.A. *Rapid Detection and Quantification of Psychoactive Compounds in Complex Plant Matrices by Direct Analysis in Real Time-High Resolution Mass Spectrometry – Application to "Kava" Psychoactive Pepper Products.* Rapid Commun. Mass Spec. (2019), in press, doi:10.1002/rcm.8532.
4. Beyramysoltan, S.,** Abdul-Rahman, N.,*** and Musah, R.A. *Call it a "Nightshade" - A Hierarchical Classification Approach to Identification of Hallucinogenic Solanaceae spp. Using DART-HRMS-derived Chemical Signatures.* Talanta. (2019), 204, 739-746, doi: 10.1016/j.talanta.2019.06.010.
5. Fowble, K.L.* and Musah, R.A. *A validated method for the quantification of mitragynine in sixteen commercially available Kratom* (*Mitragyna speciosa*) *products.* Forensic Sci. Int. (2019), 299, 195-202.
6. *Analysis of Drugs of Abuse*, Methods in Molecular Biology, R.A. Musah, Ed.; Springer Nature; 2018.
7. Lesiak, A.D.;* Fowble, K.L.;* Musah, R.A. *A rapid, high-throughput validated method for the quantification of atropine in Datura stramonium seeds using direct analysis in real time-high resolution mass spectrometry (DART-HRMS)*. Methods Mol. Biol. (2018), 1810, 207-215.
8. Longo, C.M.;* Musah, R.A. *Detection of Diagnostic Plant-Derived Psychoactive Biomarkers in Fingerprints by MALDI-SpiralTOF-Mass Spectrometry Imaging*. Methods Mol Biol. (2018), 1810, 125-132.

9. Giffen, J.E.;* Lesiak, A.D.;* Cody, R.B.; Dane, A.J.; Musah, R.A. *Rapid Species-level Identification of Salvias by Chemometric Processing of Ambient Ionization Mass Spectrometry-derived Chemical Profiles*, Phytochem. Anal. (2017), 28, 16-26.

10. Lesiak, A.D.;* Musah, R.A., *Rapid High-throughput Identification of Botanical Material Using Direct Analysis in Real Time High Resolution Mass Spectrometry*. JoVE (2016) (doi: 10.3791/54197).

11. Lesiak, A.D.;* Musah, R.A. *Application of Ambient Ionization High Resolution Mass Spectrometry to Determination of the Botanical Provenance of the Constituents of Psychoactive Drug Mixtures.* Forensic Sci. Int. (2016), 266: 271-280.

12. Lesiak, A.D.;* Cody, R.B.; Dane, A.J.; Musah, R.A. *Direct Analysis in Real Time High Resolution Mass Spectrometry as a Tool for Rapid Characterization of Mind-altering Plant Materials and Revelation of Supplement Adulteration—the Case of Kanna.* Forensic Sci. Int. (2016), 260: 66-73.

13. Lesiak, A.D.;* Musah, R.A. *More than just heat: ambient ionization mass spectrometry for determination of the species of origin of processed commercial products—application to psychoactive pepper supplements*, Anal. Methods (2016), 8: 1646-1658.

14. Lesiak, A.D.;* Cody, R.B.; Dane, A.J.; Musah, R.A. *Plant Seed Species Identification from Chemical Fingerprints—A High-Throughput Application of Ambient Ionization of Mass Spectrometry,* Anal. Chem. (2015), 87: 8748-8757.

15. Musah, R.A.; Espinoza, E.O.; Cody, R.B.; Lesiak, A.D.;* Christensen, E.D.; Moore, H.E.; Maleknia, S.; Drijfhout, E.P. *A High Throughput Ambient Mass Spectrometric Approach to Species Identification and Classification from Chemical Fingerprint Signatures*. Sci. Rep. (2015), doi: 10.1038/srep11520.

IMPLICATIONS FOR CRIMINAL JUSTICE POLICY AND PRACTICE IN THE US

The project has resulted in the development to several sorely needed innovations (as identified by the National Academy of Sciences and the United Nations Office on Drugs and Crime) that were heretofore lacking in forensic analysis of mind-altering plants. These are: (1) **Rapid Analysis and Straightforward Protocols** through the use of DART-MS—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) **Genus and Species-level Identification of Plant-based legal highs**; (3) **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; (4) **Validated protocols for the quantification of the psychoactive components of legal high complex plant matrices**; (5) **Abused Plant Database with Statistical Analysis Reporting**—This work furnished an abused plant 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 species. 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 mind altering plant drugs. In summary, the developed techniques circumvent to varying extents many of the challenges associated with analysis of complex plant matrices, as well as allow consistent reporting of the statistics associated with the analyses themselves. Among the expected dividends are: (1) reduction in crime lab sample testing backlogs; (2) provision of novel validated methods for analysis of samples for which no SOPs exist; (3) streamlining of sample analysis protocols; (3) reduction of

human resource and chemical reagent costs; (4) re-deployment of laboratory equipment such as GC- and LC-MS instruments for other necessary types of analyses; (5) more timely completion of sample analyses so that prosecutions can be expedited; and (6) statistical reporting of results.