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Gabby DiEmma and Erica Fornaro, Editors

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Abstract

The 2023 National Institute of Justice (NIJ) Forensic Science Research and Development (R&D) Symposium is intended to promote collaboration and enhance knowledge transfer of NIJ-funded research. The NIJ Forensic Science R&D Program funds both basic or applied R&D projects that will (1) increase the body of knowledge to guide and inform forensic science policy and practice or (2) result in the production of useful materials, devices, systems, or methods that have the potential for forensic application. The intent of this program is to direct the findings of basic scientific research; research and development in broader scientific fields applicable to forensic science; and ongoing forensic science research toward the development of highly discriminating, accurate, reliable, cost-effective, and rapid methods for the identification, analysis, and interpretation of physical evidence for criminal justice purposes.

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Introduction

The National Institute of Justice (NIJ) is the federal government's lead agency for forensic science research and development as well as the administration of programs that facilitate training, improve laboratory efficiency, and reduce backlogs. The mission of NIJ's Office of Investigative and Forensic Sciences is to improve the quality and practice of forensic science through innovative solutions that support research and development, testing and evaluation, technology, information exchange, and the development of training resources for the criminal justice community.

Through the research, development, testing, and evaluation process, we provide direct support to crime laboratories and law enforcement agencies to increase their capacity to process high-volume cases and provide needed training in new technologies. With highly qualified personnel and strong ties to the community, NIJ's Office of Investigative and Forensic Sciences plays a leadership role in directing efforts to address the needs of our nation's forensic science community.

RTI International and its academic- and community-based consortium of partnerships work to meet all tasks and objectives for the Forensic Technology Center of Excellence (FTCOE), put forward under the National Institute of Justice (NIJ) Cooperative Agreement No. 15PNIJ-21-GK-02192-MUMU.

The FTCOE is led by RTI International, a global research institute dedicated to improving the human condition by turning knowledge into practice. With a staff of almost 6,000 providing research and technical services to governments and businesses in more than 75 countries, RTI brings a global perspective. The FTCOE builds on RTI's expertise in forensic science, innovation, technology application, economics, DNA analytics, statistics, program evaluation, public health, and information science.

On February 14, 2023, NIJ and the FTCOE held the 2023 NIJ Forensic Science Research and Development (R&D) Symposium. Hundreds of attendees joined us online and in person for this hybrid event to learn about NIJ research awards given to several talented researchers spanning the forensic disciplines.

For more than a decade, NIJ has hosted an annual R&D Symposium to showcase great scientific innovations and promote the transition of research into practice. NIJ supports research to advance efficiency, quality, reliability, and capacity in the criminal justice and forensic science communities; this research focuses on developing new technologies, providing proof for evidence-based practices, and evaluating findings for case investigations and legal proceedings.

This year, members of the NIJ Office of Investigative and Forensic Sciences R&D team—including program managers Gregory Dutton, Danielle McLeod-Henning, Frances Scott, and Tracey Johnson—worked to create a phenomenal research agenda. The full program included 16 presentations and 26 posters from principal investigators and their research partners; these presentations and posters represent accomplishments from NIJ R&D grants awarded during 2018–2021. Most presentations are archived on the FTCOE's website and available to view for free.

Dr. Dutton, Ms. McLeod-Henning, Dr. Scott, and Ms. Johnson were moderators. Dr. Dutton moderated Session I, Impression and Pattern Evidence/Trace Evidence; Ms. McLeod-Henning moderated Session II, Forensic Anthropology and Forensic Pathology; Dr. Scott moderated Session III, Seized Drugs and Toxicology; and Ms. Johnson moderated Session IV, Forensic Biology/DNA.

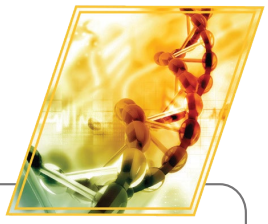
Summary of Oral Presentation and Poster Session Topics

NIJ Forensic Science Research and Development Symposium

February 14, 2023

Oral Presentation and Poster Session Topics



Sessions I & II

Impression and Pattern Evidence/ Trace Evidence | Forensic Anthropology and Forensic Pathology

Sessions III & IV

Seized Drugs and Toxicology | Forensic Biology/DNA

512 Total Attendees

191 In Person

319 Online

16 Podium Presentations

26 Posters

Over 100 Chat Messages

3,584 Total Learning Hours

46 Online Q&A Submissions

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Forensic Technology CENTER OF EXCELLENCE — A program of the National Institute of Justice

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SESSION ABSTRACTS

SESSION I

IMPRESSION AND PATTERN EVIDENCE/TRACE EVIDENCE

Moderated by NIJ Program Manager
Gregory Dutton

Applying the NIST Footwear Impression Comparison System to Comparisons Involving Realistic Crime Scene Impressions

NIJ AWARD #: DJO-NIJ-20-RO-0010

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Martin Herman
Hari Iyer
Adam Pintar
Gunay Dogan
National Institute of Standards and Technology (NIST)
*Presenting author

The National Institute of Standards and Technology (NIST) footwear research team will summarize progress in developing the NIST Footwear Impression Comparison System (FICS) and in applying FICS to comparisons from the recent black box study for footwear examiners conducted by the Federal Bureau of Investigation. The presentation will overview the end-to-end FICS comparison process, including manual markup, alignment, and evaluation of similarity metrics for comparing both general outsole patterns (e.g., design, size, and wear) and randomly acquired characteristics. Previously presented versions of FICS showed strong performance in picking the true shoe from a lineup of close nonmatches (i.e., same make, model, size) across a variety of questioned impressions intended to represent casework. This system used alignment and similarity metrics based on a user's interpretation of the outsole pattern found in a questioned impression, which was provided as a binary contact/noncontact mask and sometimes required hours of manual image annotation for a single impression. The FICS process has been revamped by developing similarity metrics that do not rely on binary contact/noncontact interpretations of questioned impressions to reduce this burden. The presentation will include a discussion of the results from the revised process and will explain how the relevance of validation comparisons to a given case comparison is assessed using image features designed to reflect questioned impression quality and outsole pattern complexity. Finally, the presentation will introduce an approach to strengthening comparison performance by using test impressions available from other shoes similar to the shoe of interest (e.g., same make, model, and same or similar size) when comparing to a given questioned impression.

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Novel Ambient Oxidation Trends in Fingerprint Aging Discovered by High-Resolution Mass Spectrometry

NIJ AWARD #: 2019-DU-BX-0134

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The time elapsed after a fingerprint has been deposited on a surface—fingerprint age—is critical temporal information that allows a forensic practitioner to determine the relevance of the evidence to the timeline in question. Our previous work demonstrates that ambient ozonolysis of unsaturated triacylglycerols (TGs) in fingerprints can be monitored with laser desorption/ionization–mass spectrometry techniques and that unsaturated TGs decrease as the fingerprint ages (Hinners et al., 2020). Though TGs are a promising class of molecules to model the fingerprint age, other chemical markers may aid in fingerprint age determination. However, a fingerprint's chemical complexity (i.e., wax esters, diacylglycerols, fatty acids, squalene, and their oxidation products) can make spectral interpretation challenging. This work uses high-resolution mass spectrometry and Kendrick mass defect (KMD) analysis to deconvolute spectral trends and identify features that can be used in fingerprint age modeling. Groomed sebaceous fingerprints were acquired from an individual onto glass slides. Fingerprints were aged in the ambient laboratory environment for 0, 3, and 7 days. Sodium acetate in methanol (10 mM) was sprayed onto the fingerprints with a HTX TM-Sprayer™ (HTX Technologies, LLC), followed by a 20-second Au sputter at 40 mA using a Cressington 108 Auto Sputter Coater (Ted Pella, Inc.). Samples were analyzed using a Q Exactive™ HF Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific™) with a matrix-assisted laser desorption ionization, or MALDI, source (Spectroglyph) in positive mode with a mass resolution of 240,000 at *m/z* 200. The feature clustering provided by KMD analysis is consistent with ozonolysis playing a central role in the degradation of unsaturated lipids in fingerprints, extending the process beyond unsaturated TGs. Thus, many new spectral features are available for subsequent modeling. However, this prompts us to consider possible competition between observed lipid substrates for the ozone, which may explain the increased error in TG degradation at longer time intervals (i.e., 7 days). Importantly, the analyzed mass range was extended below *m/z* 500, which was not previously investigated. Aided by the KMD plots, the researchers identified a reporter ion at *m/z* 217, decanoic acid, that increases over time. This is attributed to the ozonolysis of all omega-10 unsaturated lipid species in the fingerprint. Using this reporter ion would remove most issues regarding competition between lipid species and has the potential to improve estimations of late-stage aging (5–7 days) and possibly extend an ozonolysis-based model further. This work is published in a high-impact open-access journal, *ACS Central Science* (Paulson & Lee, 2022).

References

Hinners, P., Thomas, M., & Lee, Y. J. (2020). Determining fingerprint age with mass spectrometry imaging via ozonolysis of triacylglycerols. *Analytical Chemistry, 92*(4), 3125–3132. https://doi.org/10.1021/acs.analchem.9b04765

Paulson, A. E., & Lee, Y. J. (2022). Novel ambient oxidation trends in fingerprint aging discovered by Kendrick mass defect analysis. *ACS Central Science, 8*(9), 1328–1335. https://doi.org/10.1021/acscentsci.2c00408

Surface-Enhanced Raman Spectroscopy Enables Highly Accurate Identification of Different Brands, Types, and Colors of Hair Dyes

NIJ AWARD #: 15PNIJ-21-GG-04169-RESS

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Because hair is present at nearly all crime scenes, forensic analysis of hair can be used to establish a connection between a suspect and a crime scene or demonstrate the absence of such connection. Although hair coloring is popular worldwide, there is no robust and reliable forensic approach that can be used for a confirmatory analysis of artificial colorants present on hair. The presenter's research group pioneers the development of surface-enhanced Raman spectroscopy (SERS), a modern analytical technique, for the detection and identification of colorants present on hair. The presenter will discuss the potential use of SERS in the identification of more than 30 different colorants. The presenter's laboratory found that the accuracy of detection and identification of individual hair colorants is 98%, on average. The presenter's laboratory also investigated the extent to which SERS can be used to differentiate between different brands and types of colorants and to identify hair color regardless of the type and brand of the colorant used to dye hair. The results showed that individual colorants could be identified with on average 98% accuracy, whereas different brands can be predicted with nearly 100% accuracy. The presenter also found that SERS offered nearly 100% accurate identification of the type of the colorant and 97.6% accurate prediction of the hair color on average. These results demonstrate that SERS can facilitate the forensic analysis of hair, providing highly important information about the artificial colorants present on the analyzed specimens.

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Application of Particle Correlated Raman Spectroscopy (PCRS) for the Forensic Examination of Soils

NIJ AWARD #: 2019-DU-BX-0017

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Soil is a valuable trace evidence that, when properly recognized, analyzed, and interpreted, has the potential to provide investigative leads and to associate an unknown specimen with a collected known specimen. Although this is widely recognized by criminalists with a plethora of case examples, criticisms of forensic soil analysis as being subjective, too labor-intensive, and too time-consuming have resulted in a considerable decline in its use in forensic investigations. As a result, exemplary soil samples are not being collected in the field, which eliminates the possibility of later laboratory analysis. Furthermore, forensic laboratories are not equipped with criminalists who are currently capable of performing a comprehensive forensic soil examination. Thus, the potential of soil traces is not being realized. The purpose of this presentation is to share our research steps and progress in developing a statistically supported, automated, and objective analytical method for forensic soil analysis using particle correlated Raman spectroscopy (PCRS). PCRS, also known as particle-driven or morphologically directed Raman spectroscopy (MDRS), is a novel yet reliable analytical technique capable of delivering particle size distribution and microscopic morphological characteristics for the particles present within a sample (e.g., minerals) while providing secure chemical identification. The presentation will include a report on the results of the method optimization, which includes determining recommended analysis parameters for soil sample preparation, mineral dispersion, imaging, Raman spectroscopy, and data analysis. An important aspect of the data analysis has been the collection and testing of a comprehensive Raman spectral library of minerals. Although reference spectra of minerals have been published before and are available through both the Know-It-All (Wiley Science Solutions, 2022) and RRUFF databases (Lafuente et al., 2015), there are considerable limitations to each database. Thus, to ensure the correct identification of mineral grains in our unknown soil samples, a comprehensive searchable Raman library of >9000 mineral reference spectra was created by combining these spectral libraries with our own. This database was evaluated with Raman spectra collected from the 60 common rock minerals from Cargille's comminuted mineral set. This is important for determining and understanding the limitations of the analysis with regard to the ability to identify soil mineral grains with Raman spectroscopy. Last, the results from a study comparing the results of PCRS with traditional forensic soil analysis by experienced forensic microscopists will be presented.

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Wiley Science Solutions. (2022, July 7). *KnowItAll Raman spectral database collection*. Distributed by John Wiley & Sons, Inc. https://sciencesolutions.wiley.com/solutions/technique/raman/knowitall-raman-collection/

SESSION ABSTRACTS

SESSION II

FORENSIC ANTHROPOLOGY AND FORENSIC PATHOLOGY

Moderated by NIJ Program Manager
Danielle McLeod-Henning

GIS Application for Building a Nationally Representative Forensic Taphonomy Database

NIJ AWARD #: 2020-DQ-BX-0025

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Postmortem interval (PMI) determination is a critical piece of information to provide when human remains are discovered. A large and representative number of forensic cases are required to accurately model uncertainty and provide known error rates for PMI predictions. This project uses data from the geoFOR application, which is an ArcGIS-based survey using a mass collaboration model of data collection. Researchers, practitioners, and interns at several coroner and medical examiner offices around the country entered data about the characteristics of decomposition, general demographic characteristics, and location of death. geoFOR was designed to allow for quick and efficient data input while minimizing interobserver error. The application records the individual decomposition characteristics observed on the remains to facilitate more robust modeling of decomposition across a variety of different environments without presuming an artificial data structure related to stages of decomposition. The geocoded information collected from the scene allows for numerous variables from environmental databases, such as the National Oceanic and Atmospheric Administration (NOAA), to be included in the model. Although data collection through geoFOR is ongoing, 1,800 individuals are included in the current results. Our current best model, a tree-based ensemble machine learning model (XGBoost), achieves high performance ($R^2 \sim 0.855$) in predicting log-scaled PMI using the information submitted through the geoFOR application. Looking at the effect on R^2 of adding in covariates such as insect activity, scavenger activity, and weather variables, there is a small improvement compared with just including the decomposition characteristics. All covariates collected through geoFOR generate the best R^2 score for the current best model, although the improvement is only marginal compared with the model considering only the decomposition characteristics. This method provides a significant improvement on estimates of PMI compared with previous methods. Therefore, focusing on recording individual characteristics of decomposition observed on the remains instead of classifying decomposition into stages, as frequently used in previous studies, improves methods that can be broadly applicable and systematically evaluated.

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Germ-Line Transformation of Forensically Important Flies

NIJ AWARD #: 2019-DU-BX-0013

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The most common forensic entomological analysis is to estimate the age of a carrion insect, which usually equals a minimum time since death. Empirical validation of such age estimation methods under realistic conditions (i.e., a large corpse or carcass exposed to the elements and natural insect colonization) is technically daunting because it is extremely difficult to know the true age of the insect in that setting. This difficulty could be overcome if a carrion insect, typically a blow fly maggot, could be marked in a way that persisted and could be recognized throughout its life. Eggs of known age from a laboratory colony could be added to a corpse and would develop under natural conditions. When those individuals were later recognized and recovered, they would be of a known age. Because larvae increase tremendously in size and shed their skin, marking with pigment would not work. The research team developed a marked strain of *Phormia regina* (Calliphoridae), the most forensically useful insect in North America, by inserting a gene coding for a fluorescent protein into the insect's genome. The protein is expressed in all life stages and fluoresces under green or blue light. Preblastoderm embryos were microinjected with two types of DNA plasmid: a piggyBac® transposon donor plasmid with the DsRedExpress2 gene and a helper plasmid with the transposase gene. Both genes were driven by the *Lucilia cuprina* heat shock promoter Lchsp83. Of the approximately 3,500 injected individuals, about 33% survived and were screened for fluorescence as mature larvae using a fluorescent microscope to look for signs of mosaicism. All individuals were outcrossed with the wild type, the resulting larvae were screened for fluorescence, and the six positive individuals from a single injected male cross were outcrossed for two generations then incrossed to produce a single homozygous line. The proportion of heterozygous and homozygous individuals from a particular cross approximated Mendelian expectations. Homozygous larvae fluoresce more brightly than heterozygotes. Genomic analysis indicated a single transgene insertion, although we have not yet identified the location. Because insertion of a transgene can have deleterious effects, we are currently evaluating the suitability of the transgenic strain for future validation experiments (i.e., to be sure that development rate was not altered to a practical extent). The typical laboratory development experiments used to estimate age in casework were performed for wild type, heterozygotes, and homozygotes under the same conditions. The planned analysis is to use wild type development data to support a statistical method for predicting the age of transgenic individuals as a 95% confidence set. If >95% of such age predictions include the true value, we will conclude that transformation did not make these *P. regina* unsuitable for age estimation validation studies. Specimens representing 18 ages have been collected and preserved but not yet completely processed. In a preliminary analysis based on the first of two development experiment replicates from each genotype, >98% of age predictions included the true value.

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Bone Trauma and Thermal Alteration of Human Remains

NIJ AWARD #: 2019-75-CX-0019

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The overarching goal of the project is to provide the criminal justice system, forensic anthropologists, and other professionals involved in trauma analysis a standard protocol to confidently recognize blunt force fractures after a burning event and to distinguish perimortem blunt force trauma from thermal trauma. To achieve this goal, the objectives are to provide data on the rate of mis- or nonidentification of perimortem blunt force trauma fractures following a burning event and to provide error rates on thermal and blunt force trauma fractures misinterpretation. The project consists of two phases over the course of 3 years: data collection and analysis (Phase I) and protocol design and validation (Phase II). The goal of Phase I is to document and analyze blunt force trauma and thermal-induced fractures on human remains. To facilitate this, complete and intact human cadavers from the Forensic Anthropology Center (FAC) Body Donation Program (donors) are mechanically impacted and thermally altered. The specially designed impact gantry system allows researchers to control force (in psi), duration of impact, location of impact, and impact head shape (e.g., square, rectangular, or ball). Donors are impacted in three locations: the cranium above the ear (i.e., landmark pterion), one radius and ulna (the other side serves as a control), and one tibia and fibula (the other side serves as a control). Data that record the amount of force are captured. Once the donors are impacted, the fractured regions are X-rayed. We have completed blunt force impact on 16 donors resulting in 16 fractured radii and ulnae (16 as control), 16 tibiae and fibulae (and 16 as control), and 14 crania (two as control). Following blunt force impact, all donors were exposed to thermal trauma using an outdoor forensic pyre. Temperature data and timed photographic data points were collected for all burns. The time of burn is approximately 2 to 3 hours to reach Glassman-Crow Level 2–3 (i.e., charred, arms and legs possibly disarticulated, head still attached) (Glassman & Crow, 1996). Following the burn, the donors were placed at the FAC outdoor decomposition/anthropological research facility. To date, 11 donors have completely decomposed and have been recovered from the outdoor decomposition facility, and seven have been inventoried and fracture assessment has started. Fracture data are being collected by examining the remains directly and examining the X-rays. Thus far, 50% of lower limbs and 25% of upper limbs do not follow the expected pugilistic pattern indicating that the pugilistic pattern is not reliable for assessment of perimortem trauma. Phase II will rely on the continuing data analysis for protocol design and validation.

Reference

Glassman, D. M., & Crow, R. M. (1996). Standardization model for describing the extent of burn injury to human remains. *ASTM International, 41*(1), 152–154. https://doi.org/10.1520/JFS13915J

Development of the Forensic Anthropology Skeletal Trauma (FAST) Database

NIJ AWARD #: 2019-DU-BX-0040

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Skeletal trauma research is critical to improve the understanding of the response of human bones to loading and the interpretation of the resulting fractures. One of the greatest limitations researchers face is ensuring they can communicate their findings to practitioners and offer a means for their findings to be employed. The development of the Forensic Anthropology Skeletal Trauma (FAST) database is a critical component of the National Institute of Justice grant, Skeletal Trauma in Forensic Anthropology: Improving the Accuracy of Trauma Analysis and Expert Testimony, and was designed primarily to bridge the gap between researchers and applied professionals and students. The FAST database will be a searchable database comprising images and all test variables, with the intent of providing objective trauma interpretation for young scholars and professionals. To date, few researchers get quality hands-on training with trauma cases. Even fewer have experience with known cases. Therefore, the ability for students and professionals, at all stages in their career, to be exposed to skeletal trauma with known parameters has the potential to be transformative for the field. The FAST database will include specimen, experimental, fracture characteristic, and imaging data for each record (i.e., for each skeletal element). Individual-level variables (e.g., age) and element-level variables (e.g., total length) are included in the database for each specimen. The experimental data consist of the boundary conditions (e.g., loading direction) and test output variables (e.g., force). All specimens will have pre- and post-photographs, X-rays, and computerized tomography (CT) scans, which will be included in the FAST database. Additionally, the post-test CT scans are used to create a three-dimensional model, which the user will be able to manipulate to visualize all orientations of the resulting fracture. The inclusion of these data makes the FAST database unique both in the amount and quality of data within it, enabling the database to be extremely useful for both scholars and professionals in the field of forensic anthropology. This custom-built database will allow users to conduct queries ranging from basic to advanced searches, creating a distinctive skeletal trauma research tool that is valuable for both academics and practitioners. Previous and future results of controlled experimental bone trauma studies, conducted at the Injury Biomechanics Research Center, The Ohio State University, will also be added to the FAST database to begin the expansion to other skeletal elements and varied testing scenarios. A long-term goal of the FAST database is to include a section dedicated to statistical model creation. In essence, users will be able to input the element, fracture characteristics, and any other demographic variables of their current case work into the FAST database. Based on the combination of predictor variables, a probability statement will be provided that the practitioner can use to substantiate their interpretation of the injury. The FAST database will contain an extensive amount of data, which is uncommon for forensic anthropological trauma research and will provide considerable contributions to the current knowledge and methods of skeletal trauma analysis and interpretation.

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SESSION ABSTRACTS

SESSION III

SEIZED DRUGS AND TOXICOLOGY

Moderated by NIJ Program Manager
Frances Scott

Detectability of Δ10-THC's Chiral Analogs in Urine by Six Commercially Available Homogeneous Immunoassays

NIJ AWARD #: 15PNIJ-21-GG-04188-RESS

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Following the legalization of hemp (Agricultural Improvement Act of 2018), there has been an increased presence and use of cannabinoids in the United States. This growth is not only attributed to Δ9-tetrahydrocannabinol (Δ9-THC) and cannabidiol (CBD), the most abundant phytocannabinoids of cannabis and hemp, respectively, but also other THC analogs. Recently, Δ10-tetrahydrocannabinol (Δ10-THC) has become readily available for commercial use in various products, including e-cigarettes, edibles, and powders. Currently, Δ10-THC is considered legal; however, with similar potency to Δ9-THC, it has the potential for abuse. Δ10-THC is a positional isomer of Δ9-THC, which consequently presents the potential to interfere with current immunoassay screening methods used for the detection of 11-nor-9-Carboxy-Δ9-THC (Δ9-THC carboxylic acid) in urine and regularly employed in forensic, clinical, or pain management testing laboratories. The federally regulated screening cutoff for Δ9-THC carboxylic acid is 50 ng/mL, and in some scenarios (i.e., compliance testing), a lower 20 or 25 ng/mL cutoff is used to further extend the detection window. Δ9-THC or (−)-(6aR,10aR)-6,6,9-trimethyl-3-pentyl-6a,7,8,10a-tetrahydro-6H-benzo[c]chromen-1-ol has several stereochemical analogs. The most predominant is (−)-trans-Δ9-tetrahydrocannabinol, which is commonly referred to as Δ9-THC. Δ10-THC exists as four chiral analogs: 9(R)-Δ6a,10a-THC; 9(S)-Δ6a,10a-THC; (6aR,9R)-Δ10-THC; and (6aR,9S)-Δ10-THC. Currently, these are the only commercially available Δ10-THC analogs. The Δ10-THC analogs were analyzed using six commercial homogenous immunoassays at 50 ng/mL and lower cutoffs. The immunoassays used were as follows: Abbott Cannabinoids (enzyme immunoassay)–Abbott Diagnostics (Abbott); CEDIA™ Multi-Level THC (cloned enzyme donor immunoassay)–Thermo Scientific™ (CEDIA™); DRI® Cannabinoid Assay (enzyme immunoassay)–Thermo Scientific™ (DRI); ONLINE DAT Cannabinoid II (kinetic interaction of microparticles in a solution, KIMS)–Roche Diagnostics (Roche); LZI Cannabinoids (cTHC) Enzyme Immunoassay (enzyme immunoassay)–Lin-Zhi International, Inc. (LZI); and Syva® EMIT® II Plus (enzyme immunoassay)–Siemens Healthineers (Syva®). The analysis was performed on an Abbott ARCHITECT c4000 (Abbott Diagnostics). The limit of detection was evaluated by preparing each analog at 20, 50, 100, and 1000 ng/mL in urine. Samples were analyzed at both cutoff concentrations to determine if the analyte could be detected at one or both cutoffs. Analytes not detected at 1000 ng/mL for a cutoff were considered not detectable. If detected, the appropriate concentration was used as the decision point to determine the precision at the immunoassay's cutoff. Precision was assessed using three quality control pools of the analyte prepared at −50%(QCN), decision point, and +100%(QCP), which were analyzed in five different runs (n=3) along with the immunoassay's respective control materials. The total mean (n=15), standard deviation, and percent coefficient of variation were calculated for each quality control concentration. The four Δ10-THC analogs were detected at 100 ng/mL using the federal cutoff (50 ng/mL) by Abbott, CEDIA™, DRI, LZI, and Syva®. The four Δ10-THC analogs were detected at 50 ng/mL using the lower cutoff (20 or 25 ng/mL) by Abbott (20 ng/mL), CEDIA™ (25 ng/mL), DRI (20 ng/mL), LZI (25 ng/mL), and Syva® (20 ng/mL). None of the analogs were detected by Roche at 20 or 50 ng/mL.

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Assessment of the Contribution to Drug-Impaired Driving from Emerging and Undertested Drugs

NIJ AWARD #: 2020-DQ-BX-0009

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Impaired driving is an increasing problem in the United States and presents a public health and public safety concern. The extent, nature, concentrations, and prevalence with which impairing substances contribute most to drug-impaired driving cases are poorly understood because of a lack of standardization of methods. Since 2007, the National Safety Council Alcohol Drugs and Impairment Division (NSC-ADID) has promoted the standardization of testing scope and analytical cutoffs for investigations of impaired driving cases and traffic fatalities, contributing to more consistency in testing practices. Currently, only 35 drugs or metabolites are recommended for the primary scope of testing (Tier I). The objective of this research was to collect authentic specimens submitted for analysis in suspected impaired driving cases and test them using a comprehensive method to determine what drugs are identified with the greatest frequency, assess polydrug use, and evaluate positivity rates of drugs outside the Tier I recommendations. To date, 2,127 samples have been comprehensively tested. Positivity for Tier I drugs include methamphetamine (n=331; 15.5%), fentanyl (n=303; 14.2%), amphetamine (n=295; 13.8%), benzoylecgonine (n=146; 6.8%), and cocaine (n=79; 3.7%). Ethanol was found in 851 (40%) cases with a median concentration of 0.16 g/100 mL and range from the limit of detection (0.01 g/100 mL) to 0.61 g/100 mL. Tetrahydrocannabinol (THC) was found in 1,032 (48.5%) cases with a median concentration of 5.1 ng/mL and a range from the limit of detection (0.5 ng/mL) to 96 ng/mL. Positivity rates for drugs outside of Tier I drugs (Tier II) were 5.6% (diphenhydramine) or less. At a blood alcohol concentration (BAC) of 0.10 g/100 mL, a common threshold in which drug testing is not performed if a BAC exceeds it, 11.4% of cases were positive for Tier I drugs with 16.4% of samples being positive for a drug in Tier I, Tier II, or both. Data collected as part of this research support the Tier I recommendations for drug testing in impaired driving cases. Testing for the recommended Tier I scope and ethanol captures 64% of positive cases with an impairing substance, with 55% of those cases being positive for Tier I or Tier II drugs only. Drug positivity in cases with a BAC greater than 0.10 g/100 mL suggests that comprehensive testing even in high BAC cases provides insight into polysubstance impaired driving and is best practice.

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A Molecular Networking Approach to Processing Untargeted High-Resolution Mass Spectrometry Data in Forensic Toxicology

NIJ AWARD #: 15PNIJ-21-GG-04171-COAP

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Untargeted screening of psychoactive substances in biological matrices is one of the most difficult yet exciting goals of forensics. Admittedly, truly untargeted screening is still out of reach. Recent advances in liquid chromatography and high-resolution mass spectrometry (HRMS) have enabled sensitive full-spectrum high-resolution and accurate mass data acquisition. However, untargeted data acquisition generates files with highly multiplexed ion spectra with no direct relation between the precursor ion and its fragments. This makes unknown compound annotation incredibly time-consuming, costly, and necessitates analysis by highly trained and experienced scientists. Because of these limitations, purported untargeted screening methods available in the literature rely on targeted data processing and dereplication such as spectral library matching. Recently, other scientific disciplines such as metabolomics, proteomics, and natural products discovery have developed accessible tools to carry out systems-level analysis of HRMS data generated from diverse molecular environments. One promising approach is called molecular networking and uses a vector-based computational algorithm to organize tandem mass spectral data without any prior knowledge of the sample's chemical composition. Based on the assumption that structurally relevant compounds produce similar fragmentation patterns, molecular networking produces maps of structural similarity. Nearly since inception, tremendous effort has been focused on making the molecular networking algorithm accessible to the scientific community. This work has resulted in the Global Natural Products Social Molecular Networking (GNPS) interactive online data curation and analysis infrastructure. Herein, the researchers present on efforts to apply this web-based, crowd-sourced, and community-driven GNPS HRMS analysis infrastructure to forensic toxicology. The presenters have developed an untargeted sample preparation and data acquisition method using a Waters Xevo® G2-XS quadrupole time-of-flight mass spectrometer. Method validation efforts focused on the National Safety Council's Tier I and Tier II drugs, and results such as matrix effects and recovery will be presented. Next, a hybrid data processing and spectral annotation method was developed by using GNPS to create a molecular network of the entire sample and seeding it through targeted dereplication of compounds. To facilitate targeted dereplication, a drugs of abuse HRMS library was curated and is publicly available on the GNPS platform. Because this project aims to demonstrate the power of molecular networking for untargeted identification of drugs of abuse and their metabolites in forensic samples, molecular networks of several biological samples and seized drugs will be presented and discussed.

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High-Resolution Mass Spectrometry Screening in Forensic Toxicology: Cost Benefit Analysis

NIJ AWARD #: 2018-75-CX-0040

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The proliferation of new psychoactive substances (NPS) in the United States over the past two decades has significantly changed the landscape of forensic toxicology investigations. The criminal justice and medicolegal community relies upon effective and reliable toxicological drug screening to identify new threats, ensure the effective and impartial administration of justice, and protect public safety. The emergence of numerous designer drug classes, including synthetic cannabinoids, cathinones, psychedelics, benzodiazepines, novel opioids, and fentanyl analogs, presents a formidable challenge to operational forensic laboratories. Immunoassay (IA) is the most widely used technique for forensic toxicology screening. Despite the many advantages of this approach, its effectiveness has been called into question in light of the need to identify an ever-growing number of new and emerging drugs. Thus, it has been suggested that high-resolution mass spectrometry (HRMS) will replace IA-based screening because it offers flexibility, sensitivity, and improved selectivity. However, operational laboratories have been slow to adopt this new technology because of limited resources, complexity of operation, and training needs associated with its implementation. In this study, liquid chromatography-quadrupole-time-of-flight mass spectrometry (LC-QTOF-MS) was used to develop, optimize, and validate a comprehensive drug screening procedure that met published standards and recommendations for forensic toxicology investigations. The new method was used to re-analyze adjudicated blood samples from two populations of impaired drivers in Texas and California who were previously tested using IA. Following the direct comparison of IA- and HRMS-based screening results, a full cost benefit analysis was undertaken. The performance and effectiveness of each approach was evaluated in terms of fiscal impact, resource management, training, and technological challenges. This study not only provides new scientific methods for toxicological screening that meet the needs of the forensic community, but it also provides valuable insight regarding the costs and benefits for operational laboratories that may be considering this technological shift.

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SESSION ABSTRACTS

SESSION IV

FORENSIC BIOLOGY/DNA

Moderated by NIJ Program Manager
Tracey Johnson

Validation of a Confirmatory Proteomic Mass Spectrometry Body Fluid Assay for Use in Publicly Funded Forensic Laboratories

NIJ AWARD #: 15PNIJ-21-GG-02712-SLFO

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DNA short tandem repeat (STR) identity testing has become foundational to forensic investigations. It is scientifically sound, statistically rigorous, and above all confirmatory. With the ability to produce a full DNA STR profile from a small number of cells, a suspect may claim their DNA profile came from shed skin cells or DNA transfer. Demonstrating through a confirmatory test that the source of an STR profile is blood, saliva, or semen can prove vital to the defense or prosecution. However, current serology methods, except the microscopic identification of sperm, are presumptive and do not carry the scientific weight and therefore evidentiary significance of confirmatory tests. The confirmatory body fluid identification method presented here uses protein markers with tissue-specific functions (e.g., hemoglobin in blood to carry oxygen, amylase in saliva to digest carbohydrates, and semenogelin in semen for reproduction), which are therefore predominately and abundantly expressed in their respective body fluids, giving them both specificity and sensitivity as markers. With support from the National Institute of Justice, the New York City Office of the Chief Medical Examiner has completed all developmental aspects of our mass spectrometry body fluid assay from sample extraction, protein quantitation, and digestion to high-performance liquid chromatography and mass spectrometry methods, peptide marker selection for targeted Multiple Reaction Monitoring, and data analysis metrics and decision criteria (Butler et al., 2021; Yang et al., 2013). Here, we describe the rigorous validation and thorough statistical evaluation of the performance of this assay for implementation in routine casework and production of legally defensible results (Jarman & Merkley, 2019). Validation testing included large numbers of blood, semen, and saliva samples, including mock casework type samples, aged samples, and mixtures as well as vaginal fluid, menstrual blood, semen from vasectomized donors, and non-human blood, semen, and saliva. No false positives for identification of blood, semen, or saliva occurred, including in non-human samples. Overall sensitivity for each bodily fluid out of all samples known to contain that body fluid, including mixtures, was 98.3% for blood, 98.7% for saliva, and 95.2% for semen.

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Biological Sample Analysis Using Prediction Modeling for Early Mixture Detection

NIJ AWARD #: 2019-DU-BX-0003

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Analysis of evidentiary samples containing DNA from multiple contributors ("mixtures") is a time-intensive process for a forensic analyst and one where the contributor nature of a sample is not revealed until the end of the workflow. At this stage, retesting or additional testing of mixture samples is often not possible, particularly if only a trace amount of a contributor's DNA is present. Thus, a method that would allow for the quick and accurate identification of single-source (versus mixture) samples, prior to the endpoint of short tandem repeat (STR) analysis, would be beneficial. To meet this need, a high-resolution melt (HRM) curve screening assay has been developed and integrated into an existing step in the laboratory evidence workflow—the real-time PCR-based DNA quantification step. Using the developed assay, the resulting HRM data are coupled with prediction modeling approaches to allow for the contributor status of an evidence item to be identified and a genetic comparison to be made without additional steps or delays in processing. The developed HRM assay adds two new amplification targets (STR loci D5S818 and D18S51) and an intercalating dye into two existing commercial human DNA quantification chemistries, the Investigator® Quantiplex® kit and Quantifiler™ Trio DNA Quantification kit. These can then be used in combination with added transition and melt steps post-amplification on the QuantStudio™ 6 Flex. Approximately 170 single-source samples and 32 two-person mixture samples were tested using both novel integrated assays; the complete HRM datasets were exported for prediction modeling using linear discriminant analysis and support vector machine algorithms in RStudio software. For proof of concept, only eight different genotypes, including a genotype of "mixture," were represented (for each locus) in testing. When samples were tested in the integrated Quantiplex®-HRM assay, an overall accuracy of 87.88% was exhibited, correctly classifying 87.5% of single-source samples and 90% of mixture samples. Similarly, when samples were tested in the integrated Quantifiler™ Trio-HRM assay an overall accuracy of 79.2% was exhibited, accurately classifying 89.2% of single-source samples and 43.8% of mixtures. Additionally, quantification values obtained from the integrated assays and the standard curve quality metrics used to evaluate runs (slope, R^2, and y-intercept) were not significantly different than those obtained in the standard assays. These proof-of-concept results support further study of this assay as a new tool for forensic laboratories. However, for implementation of this assay into forensic laboratories, the dataset used for modeling must be expanded to encompass HRM data from all common genotypes for both STR loci. For this, synthetic melt curve data will be generated for each common genotype not represented in the aforementioned datasets. The synthetic data will then be incorporated into the final reference dataset used to train the prediction models, expanding the applicability of this assay. To date, synthetic data have been generated for the D18S51 locus and are being tested, increasing the training set used for prediction modeling from 7 genotypes to all 66 common genotypes. With key developmental validation experiments

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(e.g., limit of detection, reproducibility) and a user-friendly web interface (in development), the application will be ready to deploy to accredited practitioner laboratories for external testing. With successful validation and implementation, this tool could provide forensic examiners with a powerful way to screen and triage evidence items prior to the end point of analysis with the possibility of providing early exclusionary information about those potentially involved in a crime. This low-cost, low-effort assay will thus contribute to saving time and resources, which continue to be limiting factors in forensic DNA laboratories.

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DNA Analysis Findings from Male Sexual Assault Victims: Multidisciplinary Practice Implications

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Few studies have explored DNA analysis findings from male sexual assault victims. In our study, we found a significant difference between the development of short tandem repeat (STR) DNA CODIS-eligible profiles in female and male victims. In an analysis of 4,626 sexual assault kits, 34% of victims with female genitalia developed an STR DNA profile entered into CODIS compared with 19% of victims with male genitalia (chi-squared $p \approx 2.33 \times 10^5$ for female vs. male). We explored victim and assault features associated with the development of STR DNA CODIS-eligible profiles in male victims and found that the time between assault and examination, victim's age, and multiple suspect assaults were statistically significant. Other variables nearing statistical significance included penetration of victim's anus by assailant (e.g., penis, finger, hand), hit by assailant, weapon use, and non-anogenital physical injuries. DNA analysis findings between female and male victims will be shared. Additional descriptive data comparing female and male victims will be provided to improve knowledge regarding sexual assault of male victims. Multidisciplinary practice implications will be discussed to improve case outcomes for male sexual assault victims.

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Selectively Analyzing and Interpreting DNA from Multiple Donors with a Full Single-Cell Strategy

NIJ AWARD #: 2020-R2-CX-0032

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Interpreting mixture samples from a bulk pipeline is arduous because the signal is a cacophony of low-fidelity fluorescence from noise, artifact, and allele signal from an unknown number of contributors. The resultant electropherograms (EPGs) are sometimes so complex they require significant computational power to complete the interpretation. An alternative to the bulk processing pipeline is a single-cell pipeline, where the sample is collected and each is sequestered. The DNA is then extracted, amplified, and electrophoresed. The result is *n* single-source single-cell EPGs from n cells. Efficient direct-to-PCR treatments compatible with downstream processes are needed if single-cell forensics are to translate to operations. First, we improved single-cell EPG (scEPG) qualities—measured by peak height, allele drop-out, and scEPG sloping—by applying a direct-to-PCR laboratory pipeline that incorporated phosphate buffer saline, Proteinase K, and dithiothreitol at concentrations significantly less than those applied to bulk procedures. At optimal conditions, a significant 77% of the scEPGs exhibited at least a 50% allele detection rate and a median peak height of 550 relative fluorescence units. In addition to examining the effects of candidate laboratory treatments, we confirmed the signal quality is unchanged between manual and semi-automated processes while exploring signal quality with respect to cell type. Next, the researchers explored a two-stage interpretation strategy that clusters scEPGs into distinct groups and calculates a likelihood ratio (LR) for all scEPGs in that cluster. Because this LR assumes the group of scEPGs is from the same individual, effective clustering and good scEPG quality are paramount. Gaussian Mixture Models (GMMs), as implemented in mclust, were found to be an effective unsupervised learning approach because they simultaneously determined the number of clusters and assigned a group index to each scEPG. The researchers report on the efficacy of GMMs to accurately cluster with a set of blood or epithelial cell admixtures consisting of two to five persons. Once EPGs with poor total signal were excluded, GMM was deemed fit-for-purpose, offering high-fidelity clustering into groups, with early results showing that the propensity for GMMs to render clusters with scEPGs from more than one contributor is rare (<0.1% across all mixtures tested).

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POSTER ABSTRACTS

SESSION I

IMPRESSION AND PATTERN EVIDENCE/TRACE EVIDENCE

A Universal Method for the Detection of Organic and Inorganic Gunshot Residue Based on Fast Fluorescence Mapping and Raman Spectroscopic Identification

NIJ AWARD #: 15PNIJ-21-GG-04153-RESS

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Gunshot residue (GSR) is an important type of trace evidence, which is often associated with a violent crime. Traditionally, scanning electron microscopy coupled with energy dispersive X-ray spectroscopy, also known as SEM-EDS or SEM-EDX, is used for detection and identification of GSR particles. The application of this two-step method is limited to inorganic GSR (IGSR) because it relies solely on the detection of heavy metals (i.e., lead, barium, and antimony). This is problematic because environmental concerns have led to the increased popularity of heavy metal–free or "green" ammunition. It has been found that accurate identification of GSR samples using current elemental analysis techniques is severely hindered in the absence of heavy metals. Additionally, the probability of environmental and manufacturing particles being incorrectly assigned as GSR has increased with the onset of "green" ammunition. Recently, organic GSR (OGSR) has been the focus of many forensic researchers for several reasons. First, the total amount of OGSR generated because of the discharge of a firearm is much larger than the amount of IGSR. Second, OGSR particles are typically much larger than IGSR particles. In addition, the chemical composition of OGSR is quite complex and includes partially burned and unburned smokeless powder, stabilizers, and plasticizers. As a result, it is easier to detect and identify OGSR particles, although new methods are required. The research team's laboratory has developed a new two-step approach for fast detection of OGSR particles using fluorescence spectroscopy followed by a confirmatory identification by Raman microspectroscopy. The method uses a single instrument combining a confocal scanning Raman microscope and a fluorescence microscope working in reflection mode. In the first proof-of-concept study, the research team used adhesive tape to collect OGSR particles. Most recently, they significantly expanded this emerging methodology by demonstrating the possibility of detecting and identifying IGSR particles. In addition, they explored the ability of the method to detect GSR particles on original common substrates (e.g., cotton fabric), eliminating the initial GSR particle transfer stage. This presentation will demonstrate and discuss the results of these recent studies and the challenges and future steps for the proposed two-step method development for the detection and confirmatory identification of both OGSR and IGSR particles. In addition, results from the most recent proof-of-concept study involving the direct detection of GSR particles on a fabric substrate (without using an adhesive tape) will be reported.

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Development of an Empirical Fingerprint Aging Model Using Fingerprints Analyzed with Laser Desorption/Ionization Mass Spectrometry

NIJ AWARD #: 2019-DU-BX-0134

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Fingerprints are invaluable evidence in the criminal justice system because of their ability to identify individuals. The presenters introduced a simple kinetics model in the 2022 National Institute of Justice (NIJ) Forensic Science Research & Development Symposium using triacylglycerol (TG) standards based on the ozonolysis of TGs. Although this simple model revealed many important factors in TG aging, such as temperature and ozone concentration, there was some discrepancy with the aging of real fingerprint TGs. In the current study, an empirical model was developed based on aging real fingerprint samples in a controlled environment. The fingerprint samples include two sets: set #1 advanced aging (four individuals at 100 ppb ozone, 30°C, 0–8 hours) and set #2 ambient aging (one individual at 13 ppb, 20.6°C, 0–7 days). All samples were sprayed with 10 mM sodium acetate and sputtered with gold. Analysis was completed using a Q Exactive™ HF (Thermo Scientific™) equipped with a matrix-assisted laser desorption/ ionization (MALDI) source (Spectroglyph, LLC) set to positive ion mode. The data were then analyzed using Xcalibur™ Qual Browser (Thermo Scientific™) and Python. There are two most significant limitations in the previous simple kinetics model. First, the simple model ignores the difference in the ozonolysis decay rate resulting from varying levels of unsaturation in TGs. Second, it also ignores the difference between standard TGs and fingerprint TGs, such as the effect of other lipids, including wax esters and fatty acids. In the current study, the researchers empirically derive kinetics parameters to address the latter issue. Furthermore, they address the former issue by deriving kinetics parameters separately for mono-, di-, tri-, and tetra-unsaturated TGs. The advanced kinetics model derived from set #1 could predict the deposition time quite accurately for the midrange aging (4 hours, 30°C, 100 ppb) with 12% root mean square (RMS) error compared with 42% in the simple kinetics model. Though the empirical model best predicts midrange aging, early and late aging show higher relative errors of 45% and 38%, respectively, which are still much better than the 63% and 50% in the simple kinetics model. When the empirical model was applied to ambient aging set #2, it produced higher errors in the range of 29%–39%, probably because of insufficient temperature correction and ozone concentration measurement error. To conclude, the empirical model shows notable improvements to the time since deposition estimations and is applicable to ambient conditions found at crime scenes. Future studies will continue to further improve the kinetics model and test over wide environmental conditions.

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Revisiting the Set Value for the Minimum Drip Stain Size

NIJ AWARD #: 2020-DQ-BX-0006

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Bloodstain pattern analysis uses the location, size, shape, and distribution of stains to identify potential mechanisms of a bloodletting event from the bloodstains detected at crime scenes. For instance, whether a certain circular stain was generated from dripping or another mechanism, such as a satellite droplet, is determined based upon the size of the stain. The current cutoff value for the smallest stain size from dripping has been suggested to be 3.9 mm in diameter. However, more recently, it has been demonstrated that blood impacting the same surface can cause varied stain sizes because of surface residues, which may affect the advancing and receding contact angles. Additionally, other factors such as the impact condition and the hydrophobicity of the surface can alter the size of the resulting stain as well. This presentation will revisit the minimum size of drip stains using human blood impacting multiple surfaces along with the application of various coatings. The data indicate that the cutoff for the minimum drip stain size is not always obvious. This research examined the role of contact angles, impact speed, and initial droplet volume resulting in the final stain size. The results demonstrate that drip stains are generally larger than the previously reported value. Furthermore, the hydrophobicity and low contact angle hysteresis of the surface can lead to a drip stain as small as 2 mm, not 3.9 mm as originally proposed.

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Extraction and Quantification of Fentanyl and Metabolites from Complex Biological Matrices to Support Medicolegal Death Investigations

NIJ AWARD #: 2019-R2-CX-0037

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Beyond 72 hours, entomological evidence is considered the most reliable source to estimate the minimum postmortem interval (minPMI), the minimum amount of time that it would take for an insect to reach a physical characteristic such as length or life stage. The succession that occurs on a corpse after death is a relatively confined and closely packed ecosystem that is typically restricted to the remains and environment proximity. The incorporation of drugs into the insects is possible and opens an opportunity for assessing drug presence and developmental effects in insect tissue. If drugs are present when the insects are actively feeding, their size or stage of growth could be impacted and ultimately lead to an incorrect minPMI calculation. This study investigates the effects of fentanyl on the development of blow fly (*Lucilia sericata*) larvae and the ability to detect and quantify the drugs or metabolites present in each stage of the fly's life cycle. The researchers evaluated human tissue fortified with fentanyl at four treatment concentrations. Blow fly larvae were placed to feed on the drug-infused tissue then collected at seven time frames. A quick, easy, cheap, effective, rugged, and safe (QuEChERS) extraction method was developed, validated, and applied to extract fentanyl and metabolites from the larvae, pupae, pupae casings, and adult flies. This research represents an advancement for forensic toxicology laboratories and medical examiner's offices for complex biological matrices by providing (1) a validated protocol to extract and quantify fentanyl and metabolites from complex matrices using QuEChERS, (2) an evaluation of any growth effects fentanyl has on the development of the insects and the potential implications for minPMI estimations, and (3) conclusions on the ability to correlate the concentration of fentanyl and metabolites from the insect tissue to the initial concentration of the feeding media.

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Utilizing eDNA from Four Biological Taxa Associated with Geologic Evidence for Sample-to-Sample Comparisons and Study Site Separation

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Soil and dust are often submitted to crime laboratories as trace evidence and can be used to link an individual to a crime scene or to determine an evidentiary sample's origin. Methodologies that are routinely applied to analyze these geologic materials aim to characterize their physical properties (e.g., color, pH) and inorganic components (e.g., mineral content). However, sample size is often a limiting factor in these analyses; supplemental methods requiring a small amount of geologic material as input could provide additional evidentiary information from evidence. DNA metabarcoding is a commonly used approach to identify the biological taxa present in various environmental samples by amplifying and sequencing short, informative regions of the genome and is not restricted by sample amount. The goal of this research was to determine the utility and stability of environmental DNA (eDNA) from four biological taxa associated with soil and dust for sample-to-sample comparisons and sample origin. In this study, five mock geologic evidence items were collected monthly from agricultural and urban locations in North Carolina over a 1-year period. Mock items included (a) soil removed from t-shirts, boot soles, and trowels; (b) exposed dust collected from brick pavers using polyurethane swabs; and (c) dry dust from air filters (approximately 1" × 1" area used). DNA was isolated from mock geologic evidence using the QIAGEN DNeasy® PowerSoil® Pro Kit, and DNA metabarcoding was applied to characterize bacteria (16S), fungi (ITS1), arthropods (COI), and plants (ITS2, trnL) associated with each sample (n = 1026). Libraries were generated using custom indexed primers and were subsequently sequenced using the Illumina® MiSeq™. Raw sequencing reads were processed through a bioinformatic pipeline that removes primer sequences, identifies amplicon sequence variants (ASVs) via DADA2, and searches the ASVs against GenBank® for taxonomic identification. This presentation will focus on the experimental design and workflow and will include a preliminary assessment of temporal and spatial variables on the recovery of bacteria, fungi, arthropods, and plants from mock geologic evidence.

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Analysis of Less Volatile Components in Ignitable Liquids by Direct Analysis in Real Time Mass Spectrometry and Versatile Sampling Strategy

NIJ AWARD #: 2020-DQ-BX-0003

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The current protocols (e.g., ASTM E1618) for ignitable liquid (IL) residue detection target the volatile compounds more effectively as the gas chromatography–mass spectrometry (GC/MS) instrument is sensitive to these compounds. The less volatile compounds are more likely to be retained in burned or arson debris, yet they have not received as much attention in forensic investigation. For example, glycol ethers with relatively low vapor pressures are commonly mixed with other volatile organic solvents in paint thinner products. In gasoline and diesel fuels, detergents such as polyisobutylene succinimides and polyether amines are commonly added to control the deposits. These chemicals could be important markers for gasoline and diesel fuels. Nevertheless, these detergents cannot be readily analyzed by traditional GC/MS methods because of their high boiling points (>250°C), high molecular weights, and complex polymeric structures. The less volatile fractions of ILs have relatively less variable chemical profiles and therefore could be more desirable for yielding reliable evidence in arson investigation. In the present study, direct analysis in real time mass spectrometry (DART-MS) was applied to analyze various IL samples, including gasoline, paint thinner, lighter fluid, diesel, and kerosene, and various less volatile compounds were identified in the IL. The similarity and difference of the DART-MS spectra from these IL were compared by chemometric methods. In addition, a sorbent tube–based sampling device and sample introduction strategy for DART-MS were developed to enhance the applicability and data quality for forensic chemical analysis from complex matrices. The rationale for the design of this sampling method is based on the ASTM E1413-13 standard for the separation and concentration of IL residues from fire debris samples by dynamic headspace concentration. This method is designed to prepare extracts from fire debris in both positive and negative pressure systems. A positive system employs an inert gas, such as nitrogen, to purge the headspace vapors through an adsorbent tube, whereas a negative system uses a vacuum to evacuate the headspace vapors out of the container. The samples (i.e., substrates with IL residues) were placed in a one-quart paint can and sealed with a modified lid. The paint can was then placed into the heating mantle, which was heated to the desired temperature, forcing the volatile and less volatile compounds to vaporize into the headspace. For the positive system, the N_2 gas from the N_2 generator was connected to the copper tube on the lid of the paint can to force the headspace vapors through the sorbent tube attached to the lid. The negative system employed a portable air sampling pump connected to the sorbent tube using a Tygon® or polytetrafluoroethylene (PTFE) tube, which provides a negative pressure to evacuate the headspace vapors from the paint can into the sorbent tube. The effectiveness of the sampling strategy and DART-MS analysis for less volatile compounds in the IL will be discussed in this presentation.

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Development of an Interactive Database of Contemporary Material Properties for Computer Fire Modeling

NIJ AWARD #: 2019-DU-BX-0018

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Fire models are commonly used extensively by fire and arson investigators to test hypotheses and improve the understanding of the fire dynamics involved in an incident. Recently, the most sophisticated of these models has improved such that a wide range of material properties are required to completely define the materials in these models. Underwriters Laboratories Fire Safety Research Institute has undertaken a project to measure all the properties required to completely define a material in a computational fire model for over 70 materials that were selected by a technical panel comprising academics, consultants, fire investigators, and firefighters. The database includes pyrolysis kinetics and energetics data, temperature-dependent specific heat capacity and thermal conductivity, milligram-scale and bench-scale heat release rate and burning rate data, large-scale calorimetry data, and optical property data. The database is hosted on a dedicated website that includes background information on experimental and analytical techniques and guidance on the use of the properties in models of various levels of sophistication. With these data, fire investigators have all the information required to conduct detailed analyses and test hypotheses involving the most common materials in the built environment.

The presentation will cover conception of the experimental methods, population of the database, validation of the data sets, and navigation of the interactive site.

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POSTER ABSTRACTS

SESSION II
FORENSIC ANTHROPOLOGY AND FORENSIC PATHOLOGY

Rapid Association of Commingled Remains by Their Chemical Profile

NIJ AWARD #: 15PNIJ-21-GG-04151-SLFO

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The commingling of human remains poses an obstacle for death investigations in both modern and archaeological forensic contexts. After recovering a mixed assemblage, anthropologists face the challenge of sorting each skeletal element to its proper individual. Using physical features and osteometric methods, the reassociation process can be tedious, especially if bones have undergone fragmentation or taphonomic changes. However, in addition to specific physical traits, bones also have chemical profiles representative of the individual. This information provides useful discriminatory data for sorting. This study proposes that the elemental signatures obtained from bones in commingled assemblages can be used as a preliminary sorting technique. Laser-induced breakdown spectroscopy (LIBS) is an analytical technique well-suited for acquiring chemical information from bones. It requires no sample preparation and provides an emission spectrum within seconds that is representative of the sample surface composition. LIBS is also a quasi-nondestructive method, showing no noticeable indication that material has been removed from the bone during analysis. Furthermore, LIBS technology is available in portable, field-deployable instruments. Because much of the casework for forensic anthropology begins out in the field, handheld instrumentation conveniently aids in efficient analysis. To simulate data collection from a mass grave, the skeletal remains of 12 individuals were obtained from the Forensic Osteology Research Station (FOREST) decomposition facility at Western Carolina University. A dataset was created by acquiring LIBS spectra from multiple locations on 28 bones for each individual, providing more than 2,000 chemical signatures for classification. Following data reduction and optimization, supervised learning algorithms were used to build discriminant models for the classification of each individual. These models were able to correctly match unclassified bones to their corresponding individuals with greater than 90% accuracy. Further statistical analysis of the spectral dataset provided insight on the significance of some trace elements responsible for the variation between each set of skeletal remains as well as the minimum number of bones required to classify individuals. The results of this study illustrate how the chemical profiles of bones help expedite the sorting process for skeletal assemblages and demonstrate the usefulness of portable LIBS as a potential tool to help forensic anthropologists reassociate commingled remains directly in the field.

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Personal Identification Using Part-to-Part Comparison of 3D Lumbar Geometry Using Antemortem and Postmortem Computed Tomography

NIJ AWARD #: 2019-DU-BX-0031

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This presentation will discuss the most up-to-date results of our real-world application of using computed tomography (CT)–derived three-dimensional (3D) models of the L1–L5 vertebrae of antemortem CT (AMCT) scans of known individuals and comparing them with postmortem CT (PMCT) scans for personal identification. This presentation expands upon the preliminary results that were previously presented at the 2022 National Institute of Justice Forensic Science Research and Development Symposium. The confirmation of identification for an unknown individual is a critical part of forensic practice especially in disaster victim identification (DVI). The comparison of AM imaging for the purposes of personal identification is a common tool in pathology, odontology, and anthropology. The authors performed a simulated version of this study as a proof of concept of using 3D-rendered lumbar vertebrae comparisons for personal identification (Decker & Ford, 2019). The University of Leicester's East Midlands Forensic Pathology Unit uses PMCT as part of their daily practice and in DVI situations. To date, the University of Leicester has acquired 40 matching AM scans for individuals who passed through their facility for PMCT scanning. The University of Leicester anonymized the scans so researchers at the University of South Florida Health Department of Radiology were blinded to the identities of the AM and PM scans. Each scan was imported into the Mimics® Innovation Suite 24 (Materialise™). The L1–L5 vertebrae were modeled via segmentation and thresholding. Every vertebra was isolated and separated from the most proximal vertebral levels at the superior and inferior facet joints. Each series of 40 antemortem vertebrae were registered with a target unknown PMCT derived vertebra. A part-to-part comparison was conducted for each vertebra, and a match ratio was recorded. A threshold of ±1 mm was set for the part comparison. Every unknown PMCT L1–L5 was correctly matched to the corresponding AMCT L1–L5, signifying complete accuracy for this sample. A receiver operating characteristic curve was calculated to determine 100% sensitivity and specificity with a cut-off point of a 0.73 match ratio. The increased use of clinical CT scans and the growing use of PMCT have allowed for the expansion of means of personal identification in a forensic setting.

Reference

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You Are What You Eat! Identification of the Matrices on Which Necrophagous Insects Feed Using Direct Analysis in Real Time–High Resolution Mass Spectrometry (DART-HRMS) and Chemometrics

NIJ AWARD #: 2020-MU-MU-0016

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Necrophagous insects that have colonized decomposing remains can play a critical role in forensic investigations, because their species identity can be used to estimate postmortem interval. However, insect evidence has the potential to reveal much more about the circumstances associated with a death. In this regard, it would be useful to be able to determine whether retrieved insect evidence fed on human versus animal remains, because this may be relevant to a crime. Recently, the mass spectral chemical fingerprints of insect species at various life stages, acquired by direct analysis in real time–high resolution mass spectrometry (DART–HRMS), were shown to enable accurate determination of species identity. The presenters will report an investigation of whether these DART–HRMS chemical profiles can also reveal the food resource ingested by the flies. Eggs of three species (*C. vicina*, *L. sericata*, and *P. regina*) were reared on five resources: beef liver, pork chop, dog feces, chicken breast, and decaying tilapia. The emergent subsequent life stages were collected and stored in 70% aqueous ethanol until analysis. The DART–HRMS data of these samples were acquired from analysis of their aqueous ethanol suspensions. The data were binned and scaled, and the resulting matrix was explored by the multifactor Analysis of Variance (ANOVA)–Simultaneous Component Analysis technique to reveal variations in the chemical profiles that were a function of species and resource type. A fusion of partial least square-discriminant analysis and principal component analysis–discriminant analysis was performed to create a discriminative model for the reliable identification of not only species but also food resource using selected *m/z* values. The performance analysis of the method showed 95% and 52% accuracy by five-fold cross validation for larvae and adults, respectively. The results illustrated that the chemical profiles of adult samples were more influenced by external conditions than larva samples, which affected identification accuracy. Therefore, analysis of the larval life stage, which is the most commonly encountered insect form in forensic investigations, can be used not only for species determination but also for determination of resource substrate.

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Developing Subadult Sex Estimation Standards Using Adult Morphological Sex Traits and an Ontogenetic Approach

NIJ AWARD #: 2020-R2-CX-0024

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Forensic anthropologists routinely assist law enforcement agencies in the identification of unknown human skeletal remains by creating a biological profile, an estimate of a decedent's sex, population affinity, stature, and age at death. Currently, the only parameter of the biological profile that is routinely estimated for subadults is age at death. The primary reason for the exclusion of sex from the subadult biological profile is the ongoing belief that sex estimation can only be conducted on "adults," a term associated with the legally defined chronological age of 18 years and older. The arbitrary designation of 18 years as the age at which sex estimation becomes tenable is problematic, because it is based upon a culturally determined definition of adulthood that is not at all grounded in biological processes pertaining to skeletal development, growth, or maturation. Because many traits used in sex estimation, specifically those of the skull and pelvis, express dimorphism as a result of puberty, they should be useful in estimating sex prior to adulthood. A new two-step method for subadult sex estimation has been developed using an ontogenetic perspective. Various skeletal maturity indicators associated with puberty are first evaluated to estimate stage of maturation. If the maturity indicators suggest puberty has commenced, traditional adult sex traits of the skull and pelvis that express dimorphism are then evaluated using modified definitions specifically for use with immature remains, and a sex estimate is generated using robust statistical analyses. The method will be made freely available to forensic practitioners as a graphical user interface, the Ontogenetic Subadult Sex Estimation System (OnSEt), providing practitioners with a user-friendly interface to include sex as part of the subadult biological profile, ultimately increasing identifications involving children in forensic contexts and improving outcomes in medicolegal death investigations.

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An Osteometric Approach to Separating Commingled Pelvic and Foot Joints

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Commingled human remains can result in a significant loss of information regarding the individuals present and make the biological profile difficult to establish. One common technique for separating commingled remains at joint surfaces is osteometric sorting (Byrd & LeGarde, 2014). However, current models have only been applied to large joints of the body, for instance the hip or knee, while smaller joints like the sacroiliac (SI) or first tarsometatarsal (1st TMT) joint are unable to be sorted. Osteometric sorting separates commingled joints with a singular width measurement (Byrd & LeGarde, 2014). However, linear measurements may be limited in their ability to describe the entirety of joint surfaces (Rösing & Pischtschan, 1995). Therefore, this study's first hypothesis is that commingled individuals can be sorted at the SI and 1st TMT joints using osteometric sorting. The second hypothesis is that the joint surface area values will exclude more potential matches than the width measurements. The measurements and surface area values were calculated from virtual models created from the William M. Bass Donated Skeletal Collection. The left os coxae, sacra, medial cuneiforms, and first metatarsals of 56 individuals were scanned with a SHINING 3D® EinScan Pro 2X Plus handheld surface scanner. Each articular surface was cropped from the virtual model, and the widest portion of each facet was measured with the distance tool while the surface area was calculated using the compute area function in Geomagic Wrap 2017. The measurements were recorded in a Microsoft Excel Workbook, where the osteometric sorting models were also calculated (Byrd & LeGarde, 2014). Four reference samples were formed to generate the models: SI width (Model 1), SI surface area (Model 2), 1st TMT width (Model 3), and 1st TMT surface area (Model 4). Each sample was composed of 51 known individuals with varying demographics. Shapiro-Wilks tests were conducted to identify any outliers. These four models were then used to identify commingled individuals in the four test samples. Each test sample consisted of five known individuals and 20 artificially commingled pairs. Each model's efficiency was calculated as an indicator of overall success at determining the correct classification rate of true positives and negatives (Byrd & LeGarde, 2018). In all test samples, several potential pairs were excluded through the implementation of the new models. Model 1 was the least efficient (0.28) and only eliminated 10% of the commingled pairs. Model 2 was the most efficient (0.72) and correctly rejected 65% of the commingled pairs. Furthermore, two of the true SI joints were correctly reassociated using Model 2. Model 3 excluded 60% of the commingled pairs; however, it also eliminated one of the true matches (efficiency = 0.64). A similar result occurred for Model 4, where only 45% of commingled pairs were rejected as well as one true match (efficiency = 0.52). These results illustrate that osteometric sorting can help exclude potential matches at these particular joints. If future researchers are interested in osteometric sorting at these joints, then the reference sample sizes should be increased.

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POSTER ABSTRACTS

SESSION III
SEIZED DRUGS AND TOXICOLOGY

Combining Surface-Enhanced Raman Spectroscopy (SERS) and Mass Spectrometry Techniques to Increase Sensitivity and Specificity in Toxicological Drug Analysis in Blood Plasma

NIJ AWARD #: 2018-75-CX-0034

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Nationally, the Centers for Disease Control and Prevention (CDC) reports that from March 2020 through April 2021 nearly 101,000 people died from drug overdoses, the largest number of drug overdoses for a 12-month period ever recorded, including 1,150 adolescents (CDC, 2020b). The epidemic has accelerated during the COVID-19 pandemic. The CDC reports between 38%–50% increases in opioid overdoses in 28 states (CDC, 2020a). Such dismal statistics impose ever greater challenges for criminal justice and drug analysis in forensic toxicology. Liquid chromatography–mass spectrometry (LC–MS) is the gold standard for analysis, but this technique is labor- and time-intensive and has proven too complex to implement in local toxicology laboratories. Consequently, serious backlogs have emerged nationally across jurisdictions. Herein, we demonstrate uniquely shaped gold nanostructures in the fabrication of a new nanoparticle (NP)-decorated microneedle array, which functions as both a highly sensitive surface-enhanced Raman spectroscopy (SERS) substrate for Raman analysis and an efficient sample preparation and ionization device for substrate-supported electrospray ionization–mass spectrometry (ssESI-MS). SERS analysis provides a unique spectroscopic fingerprint for each drug, with signal strength correlated to concentration. Electrospray MS is faster and less labor-intensive than LC–MS, with near equivalent specificity and selectivity. Together, SERS and ssESI-MS are orthogonal and complementary techniques, the combination of which is highly specific and able to unambiguously identify a large majority of potent drugs. The array also enables high-throughput toxicology drug analysis by SERS and MS simultaneously without the need for offline sample preparation. Together, multimodal SERS–ssESI-MS analysis enhances the specificity and selectivity compared to LC–MS in forensic toxicology. Therefore, we expect that this method would be suitable for implementation for high-throughput drug analysis in local forensic laboratories nationwide to battle the drug overdose epidemic.

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Getting into the Weeds—A Combined Ambient Mass Spectrometric and Chemometric Approach for Differentiating Hemp and Marijuana Varieties of *Cannabis sativa*

NIJ AWARD #: 2019-DU-BX-0026

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Hemp (fiber-type) and marijuana (drug-type) are the two major varieties of *Cannabis sativa*. Although both varieties contain Δ9-tetrahydrocannabinol, or THC (i.e., the primary psychoactive component of this species), they differ in the amount of this active ingredient present. Currently, federal laws designate that *C. sativa* materials that contain greater than 0.3% THC are classified as marijuana (i.e., a Schedule I controlled substance) while *C. sativa* that has less than or equal to 0.3% THC is hemp (i.e., a legal agricultural commodity). In the context of analyzing *Cannabis* materials in a forensic laboratory, distinguishing between these two varieties can be difficult. Furthermore, the increased workload associated with differentiating between hemp and marijuana, which derives from the requirement to quantify the THC content in all *C. sativa* samples submitted as evidence, has imposed additional challenges. Therefore, one of the primary goals of this National Institute of Justice–funded project was to develop a method to rapidly distinguish between hemp and marijuana plant materials. To accomplish this, advanced chemometric techniques were applied to data obtained by direct analysis in real time–high resolution mass spectrometry (DART-HRMS). A diverse set of *C. sativa* plant materials was acquired, including commercial hemp flower from multiple vendors, marijuana samples from two Drug Enforcement Agency–registered suppliers (i.e., the National Institute on Drug Abuse and the National Institute of Standards and Technology), and recreational marijuana flower. All plant materials were analyzed by DART-HRMS in positive-ion mode under soft ionization conditions (i.e., orifice 1 voltage of 20 V). No sample pretreatment steps were required prior to interrogating the samples by DART-HRMS, which was accomplished by simply introducing the closed end of a glass melting point capillary tube into the sample and presenting the coated surface of the tube to the open-air gap between the ion source and mass spectrometer inlet (i.e., DART gas stream). After obtaining the mass spectral chemical profiles for all samples, chemometric techniques (i.e., principal component analysis and random forest) were applied to optimally differentiate between the hemp and marijuana samples with high accuracy. A set of external validation samples (i.e., samples that were not used to develop the model) were used to assess the model's prediction ability to classify "unknowns." The assessment resulted in 100% accuracy for determining the varieties of the *C. sativa* unknowns. During the development of the model, a set of *m/z* values was identified as diagnostic for differentiating between the two varieties. During investigations to determine the identities of all variables found to be crucial for this distinction, it was found that several markers had masses that were consistent with cannabinoid and terpene molecules known to be present in *Cannabis*. Furthermore, fragments of these major molecules, which are formed during the ambient ionization analysis by DART-HRMS, also appear to aid in the differentiation of these varieties. In summary, the developed mass spectrometric and chemometric approach for differentiating between hemp and marijuana would assist crime laboratories in the rapid identification of unknown *C. sativa* material prior to the launch of time-consuming and resource-intensive confirmatory methods.

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Tracking and Disseminating Data on Novel Psychoactive Substances (NPS) through NPS Discovery's Drug Early Warning System

NIJ AWARD #: 2020-DQ-BX-0007

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Novel psychoactive substances (NPS) continue to increase in prevalence in the United States. Constant turnover of these drugs is a concern because of their unknown effects and toxicity. Many laboratories may not have the resources to stay current with NPS trends, which means a central authority is needed to provide assistance and guidance. To that end, and with National Institute of Justice funding, the Center for Forensic Science Research and Education (CFSRE) developed NPS Discovery—a drug early warning system—in 2018 to streamline the identification of emerging NPS and to disseminate important information to stakeholders. The CFSRE's NPS Discovery program monitors NPS through sample-mining and data-mining analytical techniques. The sample populations that feed these workflows are facilitated through collaborations with medical examiner and coroner offices, crime laboratories, police departments, hospitals, and other public health and safety agencies and include raw drug materials and toxicology specimens. Additionally, our program monitors online resources (e.g., gray market sites, drug use forums) for newly emerging NPS. NPS benzodiazepines, although the smallest subclass in terms of newly discovered drugs, are the most prevalent subclass of NPS observed in toxicology samples, accounting for approximately 50% of positivity. Beginning around early 2022, a sharp increase in positivity for etizolam and flualprazolam was observed, often in combination with opioids. Since 2020, etizolam has remained the most encountered NPS benzodiazepine, while the positivity of flualprazolam has fluctuated. During this time, notable detection increases for clonazolam, flubromazepam, flubromazolam, and bromazolam have been observed, albeit with less frequency. NPS opioids are the second most prevalent subclass observed, accounting for approximately 20% positivity in toxicology samples. In early 2020, isotonitazene was the most prevalent synthetic opioid encountered but was quickly replaced by brorphine in mid-to-late 2020 and then by metonitazene in early 2021. This demonstrates the quick pace of the current NPS opioid market with drugs that have similar or higher potency than fentanyl. The only fentanyl analog observed during this time with noted prevalence was fluorofentanyl, although its characteristics are atypical for an NPS. Other NPS opioids to emerge more recently include N-pyrrolidino etonitazene, protonitazene, and etodesnitazene. NPS stimulant and hallucinogen positivity has remained static in the last several years with one drug dominating the market at a given time. Eutylone was the primary NPS stimulant between 2020 and 2022; however, it was recently replaced following international control and now the prevalence and positivity of N,N-dimethylpentylone are skyrocketing. The synthetic cannabinoid subclass of NPS has changed the most in recent years because of a class-wide ban implemented by China. This ban has caused a significant reduction in positivity in 2022. MDMB-4en-PINACA has been the most prevalent synthetic cannabinoid observed, followed by ADB-BINACA and others. Much uncertainty remains for this NPS subclass. NPS—the drugs themselves and their subclasses—are not created equal. Emergence, prevalence, and other characteristics vary greatly from drug to drug and among the subclasses themselves. Laboratories and scientists must remain abreast of ever-changing drug trends and impacts (e.g., chemistry, pharmacology). NPS Discovery strives to collect and share this data and information.

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Biomarkers of *Cannabis* Exposure in Exhaled Breath Condensate and Oral Fluid

NIJ AWARD #: 2020-DQ-BX-0011

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The continuous increase in *Cannabis* use for its perceived medical benefits and as a recreational drug in the United States has created a great demand in forensic toxicology for biomarkers of *Cannabis* exposure that can reliably indicate medicinal versus recreational *Cannabis* use, recent versus past exposure use, and occasional versus chronic use. In addition, markers that can be sampled non-invasively and with relative ease are needed. Current markers of *Cannabis* intake include Δ9-tetrahydrocannabinol (Δ9-THC) and its metabolites 11-hydroxy-Δ9-tetrahydrocannabinol (11-OH-THC) and 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH). However, these exhibit complex pharmacokinetics, making it difficult to interpret concentrations to determine the time and frequency of exposure. More importantly, blood or urine levels of THC and metabolites correlate poorly with degree of impairment in driving under the influence scenarios. *Cannabis* contains ~120 unique chemical entities considered to be "cannabinoids," some of which are psychoactive and some that can modify the overall activity of the combined product. These include major and minor components, few of which have been assessed as potential exposure biomarkers in conventional or alternative sample matrices. The possibility that profiles or patterns of multiple cannabinoids may provide more reliable correlations with *Cannabis* exposure or impairment than individual molecules has not been extensively explored. Oral fluid (OF) and exhaled breath condensate (EBC) are alternative, non-invasive sample matrices that hold promise for identification of *Cannabis* exposure biomarkers. OF is currently being explored as a matrix for *Cannabis* exposure analysis. EBC is an easily collected specimen derived from lung lining fluid that consists of condensed water vapor, dissolved volatiles, and water-soluble polar and non-volatile small molecules, including metabolites. Although licit drugs and metabolites have been measured in EBC, this matrix has not been explored as an alternative sample in forensic toxicology studies, despite its obvious advantages. This presentation describes the development of an analytical method to determine a battery of major and minor cannabinoids and metabolites as potential *Cannabis* exposure biomarkers in EBC and OF. Liquid chromatographic-triple quadrupole tandem mass spectrometry (LC-QqQ-MS/MS) can detect and quantify 25 cannabinoid analytes, including the difficult to separate analytes Δ8-THC, Δ9-THC, cannabichromene, and cannabinol, which have similar multiple reaction monitoring transitions. Limit of quantitation values for the great majority of target cannabinoids in OF and EBC are in the 1–10 ng/mL range. In addition, the recruitment of a cohort of 240 adult *Cannabis* users with well-characterized *Cannabis* use profiles is described. Participants provided samples of EBC, OF, and urine and completed an exposure questionnaire detailing characteristics of exposure and type of *Cannabis* used. OF was collected using Quantisal™ (Immunalysis™) devices. EBC was obtained using RTube™ devices (Respiratory Research, Inc), which yield ~1 mL of sample with 5–10 minutes of normal breathing. Urine was also

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collected for initial confirmation of recent or chronic use using dipstick analysis. Initial cannabinoid data analysis will use descriptive statistics, one-way analysis of variance, and correlation analysis to isolate possible trends and correlations. Then, data will be further investigated with multivariate analyses, including principal component analysis, to explore the relationships between individual or groupings of analytes and exposure parameters, including type of *Cannabis* used and the timing and frequency of use.

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Validation of a LC-DAD Method with Optional ESI/TOFMS Detection for the Accurate Measurement of Δ9-THC and Δ9-THCA Among Twenty Cannabinoids in *Cannabis*

NIJ AWARD #: 2020-DQ-BX-0021

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There are two primary species of *Cannabis*: marijuana is the cultivated plant used as a psychotropic drug because of high concentrations of Δ9-tetrahydrocannabinol (Δ9-THC), whereas hemp is characterized by a low concentration of Δ9-THC and high concentration of cannabidiol (CBD), which is antipsychoactive. The Federal Controlled Substance Act of 1970 defined *Cannabis* as a Schedule I substance, which made both marijuana and hemp illegal. The 2018 Farm Bill excluded hemp from the statutory definition of *Cannabis* with a total concentration of Δ9-THC and Δ9-tetrahydrocannabinolic acid (Δ9-THCA) not more than 0.3% (w/w) on a dry basis, which made hemp compliance testing mandatory. To meet this demand, a liquid chromatography diode array detector (LC-DAD) method has been developed and validated. So far, published LC-DAD methods have focused on the quantification of 17 or fewer cannabinoids. In addition, resolution of critical pairs of cannabinoids (e.g., cannabigerol [CBG]/CBD, Δ9-/Δ8-THC) often did not meet the required minimum resolution of 1.5 for appropriate method validation, which was especially true when LC separation was carried out at high concentration of cannabinoids or involved a large number of cannabinoids. In our study, a thorough separation optimization led to baseline separation of both critical pairs. Precision and accuracy were assessed using quality control samples at three concentration levels (0.02, 0.5, and 12.5 μg/mL) in triplicates with inter-day and intra-day precision of less than 15% relative standard deviation (RSD) and accuracy of less than ±15% relative error, therefore meeting the requirements by the ISO 17025 standards. Unlike most published methods that had to analyze the same sample at more than one concentration because of a narrow linear calibration range, samples were analyzed with this method at one concentration (50 μg/mL in methanol) because of a wide linear calibration range (i.e., 0.02–25 μg/mL or 0.04%–50% [w/w]). In the literature, published recovery experiments were limited by the unavailability of cannabinoid-free matrix and the high cost of cannabinoid standards. This problem was solved by spiking abnormal CBD, a cannabinoid not naturally present in *Cannabis* products and commercially available with a reasonable price, into the samples. The researcher's assessment in triplicate showed that the recovery ranged from 93.6% to 106.1%, while the RSD values ranged from 1.1% to 7.0%. The method had overall good specificity with only a few minor interferences from compounds in the samples, which was verified by electrospray ionization time-of-flight mass spectrometry (ESI/TOFMS). Additionally, ESI/TOFMS has discovered seven unknown cannabinoids, including one structural isomer of CBG, one structural isomer of cannabinolic acid, four structural isomers of Δ9-THC, and one structural isomer of Δ9-THC acetate. The method was applied in the analysis of cannabinoids in 23 samples of plant materials of *Cannabis*. Eighteen cannabinoids were found at concentrations higher than the limit of quantification and ranged from 0.04 to 26.97% (w/w) in the samples. The analysis also uncovered that one of the two samples of Δ8-THC fortified hemp flowers contained 5.16% (w/w) Δ9-THC, an alarm to the current Δ8-THC craze by the public.

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Sample Homogenization, Extraction, and Clean Up Procedures at NIST for the Determination of Total Δ9-THC in Hemp-Derived Finished Products

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Forensic laboratories have been forced to start differentiating seized *Cannabis* products as either legal hemp or illegal marijuana in many parts of the United States after the passage of the 2018 Farm Bill. In 2021, hemp production in the United States was worth an estimated $824M with approximately 76% focused on floral hemp that is used to produce hemp-derived finished products such as smokable plant products, vape cartridges, oils, or edibles (US Department of Agriculture, 2022). Historically, forensic laboratories have only performed a series of qualitative measurements: macro- and microscopic identification of plant features, colorimetric test for presence of tetrahydrocannabinol (THC), and confirmation of Δ9-THC by gas chromatography-mass spectrometry (GC/MS). With new legislation, forensic laboratories are now required to quantify the level of total Δ9-THC in seized evidence to distinguish them as either hemp or marijuana products. In response, the National Institute of Standards and Technology (NIST) started developing and evaluating analytical methods to provide forensic scientists with the tools necessary. This presentation will summarize sample homogenization, extraction, and clean-up procedures for the determination of Δ8-THC, Δ9-THC, tetrahydrocannabinolic acid (THC-A), and total Δ9-THC in smokable hemp plant samples, hemp oils, and vape liquids. Additionally, commercial hemp samples that were supposed to be at or below the federal limit of 0.3% were analyzed by liquid chromatography photodiode array detector (LC-PDA); however, in the case of smokable hemp plant samples, approximately 93% were measured by NIST to have a total Δ9-THC value above this limit. Similar studies will be highlighted for other *Cannabis* matrices and comparisons will be provided between NIST mass fractions and the manufacturers' fractions.

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POSTER ABSTRACTS

SESSION IV
FORENSIC BIOLOGY/DNA

Improved Nucleic Acid Recovery from Trace Samples Using Affinity Purification

NIJ AWARD #: 15PNIJ-21GG-04149-RESS

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An increasing proportion of DNA casework in the United States comprises trace levels of DNA. Traditional kits for nucleic acid recovery present potential for loss of DNA during extraction and purification caused by multiple wash steps and buffer exchanges during which the small amount of DNA can be lost. Although this loss may be insignificant on body fluids such as blood or semen where DNA content is high, when working with trace DNA, it could mean the difference between an informative profile and unusable data. In addition, commercial kits for DNA recovery disregard the potential value of RNA, proteins, and metabolites and focus exclusively on DNA isolation. RNA and metabolites are highly valuable because they can identify the type of biological sample within the evidentiary sample. There is an immediate need for an efficient workflow that effectively extracts and purifies DNA from challenging trace samples while simultaneously preserving RNA, proteins, and metabolites for further analysis. The researchers' new approach combines high-efficiency recovery of trace DNA with retention of proteins and metabolites in the unbound material. The significance of additional analytes has been demonstrated in recent literature; however, adaptation of these techniques has been delayed because of a lack of an efficient multi-analyte isolation kit. This research seeks to fill that gap by developing a novel workflow incorporating a highly efficient DNA binder imbedded into a paramagnetic bead in combination with buffers that are non-destructive to protein and other analytes. Preliminary data using known quantities of DNA applied to the DNA binder and from swabs of glass slides handled by volunteers (with/without spiked DNA) demonstrated near complete binding of nanogram quantities of sheared (size range 500 bp to 12 kb) human genomic DNA, while the remaining analytes are retained in the unbound material for further analysis. Current elution protocols achieve up to 90% recovery from the DNA binding vector, confirmed by the Quant-iT™ PicoGreen™ and Quantifiler™ Trio assays (Thermo Scientific™). With DNA of 25 bp to 500 bp, 95% elution efficiency was obtained, indicating usefulness of this method with severely degraded DNA that is often problematic with conventional kits using DNA binding columns. Experimentation is in progress to improve DNA elution efficiency through enzymatic release agents and buffer optimization and to develop optimal protocols for co-binding and differential elution of RNA from the binding vector. Results will be presented on DNA elution efficiency using enzymatic release agents and several buffer combinations and on different strategies for co-binding and differential elution of RNA. Side-by-side comparisons to currently available commercial kits will also be presented. In time, this innovative research will result in the development of a robust method of successfully isolating challenging trace DNA while retaining RNA, protein, and metabolites from the same sample, thus increasing the value of a single piece of biological evidence.

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Development of a Probe Capture Next-Generation Sequencing Assay for Analysis of the Mitochondrial Genome and Nuclear STR and SNP Markers

NIJ AWARD #: 2018-DU-BX-0183

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The clonal property of next generation sequencing (NGS), also known as massively parallel sequencing, facilitates the de-convolution of mixtures, a common and challenging category of crime scene evidence. Using a probe capture strategy for target enrichment enables the analysis of the short and degraded DNA fragments common in many forensic specimens because the alternative strategy of polymerase chain reaction (PCR) enrichment requires the presence of two intact priming sites. Moreover, a single shotgun library prepared from a forensic sample can, in principle, be analyzed with probe panels for mitochondrial DNA (mtDNA), single nucleotide polymorphisms (SNPs), and short tandem repeats (STRs), maximizing the genetic information that can be extracted from quality- and quantity-compromised forensic samples. The presenters have developed NimbleGen probe panels for the entire mtDNA genome (Shih et al., 2018), for 496 SNPs (Bose et al., 2018), and for 44 STR genetic markers. The captured libraries are sequenced on the Illumina® MiSeq™ platform. When sequencing the entire mitochondrial DNA genome, a haploid lineage marker allows de-convolution of mixtures by counting individual clonal sequence reads and assigning them to one of the contributors, aided by mixemt, a phylogenetic-based software (Vohr et al., 2017). This software can estimate the number of contributors and their proportions in the mixture; this information can be helpful in interpreting the SNP and STR analyses of the same mixtures. The presenters have recently reported the analysis of several contrived forensic type mixtures (e.g., hair, blood) using the mtDNA probe panel and mixemt (Wisner et al., 2021). Recently, the presenters have developed a probe panel for 44 different STR loci that includes 30 autosomal STRs, five X-STRs, eight Y-STRs, and the amelogenin marker. The probes target unique flanking sequences, and the sequence files are analyzed with the web-based software toaSTR (Ganschow et al., 2018), developed by LABCON-OWL, for genotyping NGS STRs. The toaSTR analysis of STR markers enables the identification of STR alleles that are identical by size but different by sequence and can distinguish biological (iso-)alleles from stutter and other artifacts, which is useful in mixture interpretation. The SNP analysis is valuable in highly degraded forensic samples where the STR analysis may be compromised. The custom SNP probe panel consists of a set of 496 nuclear SNPs (367 SNPs and 129 microhaplotype markers). The presence of tri-allelic, tetra-allelic, and microhaplotype markers in the SNP probe panel facilitates possible mixture detection and analysis. The presenter's experience with contrived mixtures indicates that counting sequence reads for mtDNA markers can be used to estimate proportions but estimating proportions for SNP and STR markers by counting reads is less reliable at DNA inputs <1 ng. This study presents the results obtained from a single shotgun DNA library made from contrived mixtures of hair and blood target enriched using the STR, SNP, and mtDNA probe capture panels, demonstrating proof of concept for application to forensically relevant degraded and mixed samples.

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Single-Cell Likelihood Ratios Are Highly Informative and Robust Across Multifarious Mixture Complexities

NIJ AWARD #: 2018-DU-BX-0185

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Traditional mixture interpretation works within a likelihood ratio (LR) paradigm and requires assumptions on relatedness and whether a known contributor's DNA is present in the data. In the bulk pipeline, DNA is extracted using laboratory treatments that force volume fractionation between the extraction and polymerase chain reaction (PCR) steps. This fractionation leads to variation in the number of copies of each allele before PCR cycling starts, which drives peak height ratio differences between alleles within a locus and drop-out. The resultant bulk electropherogram (EPG) is, therefore, a mix of short tandem repeat (STR) peaks from possibly related individuals who may not have their full profiles represented in the data. It is for these reasons that multifarious number of contributors assignments and contexts are required to evaluate the evidence. As the number of reasonable propositions increases so do the cost burdens associated with training, proficiency testing, and research. Though technologies, such as sequencing, might improve the ability to resolve more alleles, full resolution can only be acquired through a single-cell approach that invokes a direct-to-PCR methodology. Thus, the researchers endeavor to develop a single-cell pipeline, complete with interpretation and capable of reporting the strength of the consistency between group(s) of single-cell data and a person of interest (POI). Despite its potential, single-cell analysis has not received widespread consideration within the forensic domain and therefore requires studies that affirm its relevance. To do this, this study must demonstrate the flexibility of this strategy by showing that for a set of mixtures containing two to five persons, the LR for a cluster of single-cell electropherograms (scEPGs), grouped by similarity, approaches that of the known genotype regardless of the number of individuals comprising the mixture. This study shall also present a treatment that demonstrates how the strength of consistency between scEPGs in a cluster and the POI can be combined to determine the strength of evidence for the entire mixture. The presentation shall continue by demonstrating two boundary cases: (1) where the LRs for a mixture consisting of 15 persons is determined and (2) where each cluster contains only one scEPG. In the first case, the mixture is tested against all 15 true contributors and finds that all the LRs approach those of the ground truth genotype. From the case where each cluster contains only one scEPG, this study found that a total peak height of 5,000 RFU, representing ca. 10 allele peaks, renders LR ≈ 106 and that over 80% of the cells exhibit at least this allele detection rate. The scLRs from this software application, named EESCIt for Evidentiary Evaluation of Single Cells, were perfectly repeatable and were obtained in less than 1 minute for any admixture. These results demonstrate the relevance, salience, and legitimacy of this strategy to the forensic domain, justifying further development.

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Adaptation, Optimization, and Validation of a Semi-automated DNase I-Based Differential Extraction Procedure on the Beckman Coulter Biomek® NX^P Automated Workstation

NIJ AWARD #: 2019-NE-BX-0002

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Processing evidence in sexual assault cases is time-consuming, which has led to high backlogs in many localities. The key challenge has been to separate DNA in sperm cells from DNA in epithelial cells prior to purification to simplify the mixture before DNA profiling. The differential extraction (DE) method, developed by Gill et al. (1985) and still routinely used in forensic laboratories, is based on the higher density of sperm cells and their resistance to cell lysis in the absence of a reducing agent such as dithiothreitol (DTT) relative to epithelial cells. Following lysis of epithelial but not sperm cells in buffers containing proteinase K and detergent, samples are centrifuged to pellet sperm cells (sperm fraction, or SF) leaving lysed epithelial cells in the supernatant (non-sperm fraction, or NSF). Removal of the NSF and repeated washing of the SF prior to DNA extraction is used to develop separate DNA profiles for the SF and NSF. Although effective, the process is a challenge to fully automate and the repeated wash steps are relatively slow, labor-intensive, can lead to loss of sperm, and provide more opportunities for cross contamination. Multiple procedures to improve the differential lysis/centrifugation method have been developed, including laser-, acoustic-, pH-, pressure-, affinity-, and enzyme-based methods. Critical aspects of new methods are to demonstrate that they work as well or better than methods in use in the forensic laboratory and have an ability to seamlessly integrate into existing workflows. Virginia Department of Forensic Science (VADFS) optimized and fully integrated an automated DNase I-based DE method into the current automated DNA processing pipeline using Promega's DNA IQ™ DNA isolation system and Beckman Coulter's Biomek® NX^P automation workstation. DNase I is a nuclease that degrades DNA to oligonucleotides. Starting from reports by Garvin et al. (2009, 2012). and Wong and Mihalovich (2019) and the VADFS's current DE procedure, DNase I digestion of SFs was performed using a new method developed for the Biomek® NX^P following manual epithelial cell lysis. The presenters report the results of testing multiple buffers, enzyme concentrations, incubation times and temperatures, and semen dilutions using vaginal and buccal mock sexual assault samples from multiple donors. The effects of commonly encountered vaginal contaminants and aging on results and tests to measure cross-contamination rates to validate the optimized protocol on the Biomek® NX^P are also reported. This research found that multiple buffers and conditions worked as well as or better than the current VADFS standard DE protocol with respect to Y-DNA yields (measure of male DNA), ratios of autosomal-DNA to Y-DNA yields (A/Y ratios, which measure the relative quantity of female DNA), and the quality of sperm fraction DNA profiles. No evidence for increased rates of cross contamination was observed due to the automated DNase I treatment. The automated DNase I DE method is rapid and deposits the DNase I-treated SFs into the deep well sample plate, in which the SFs will be purified for DNA along with other casework samples using the existing automated extraction procedure.

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Paper-Based Chemiresistive Biosensor Array for Rapid, On-Site Identification of Multiple Body Fluids at a Crime Scene

NIJ AWARD #: 2019-NE-BX-006

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Identifying body fluids left at a crime scene is a fundamental piece of evidence in forensic science. Often, knowing what the body fluid is serves as the key in a criminal investigation. However, the identification of human body fluids has not always been an easy task because of their resemblance to other fluids or substances. Over the years, a variety of methods have been developed for the identification of commonly found body fluids at crime scenes, but most of these tests are only qualitative, suffer from low sensitivity and selectivity, and consume a significant quantity of valuable samples. To alleviate these drawbacks, this study developed a single-walled carbon nanotube (SWNT)–based chemiresistive nanobiosensor array for quantitative, selective, on-site identification of multiple body fluids, namely blood, semen, saliva, urine, and sweat, through detection of their protein biomarkers using corresponding antibodies. Chromatography paper was used as the substrate, and electrical leads were inkjet printed on its surface. The sensor array consisted of five individual sensing channels made by a simple wax-printing method, and they only required a microliter-sized sample, which was equally split into aliquots by the built-in paper microfluidics. For fabricating the chemiresistors, the researchers synthesized a dimethylformamide (DMF)-based PBASE (1-pyrenebutanoic acid succinimidyl ester)/SWNT ink where PBASE was attached to SWNTs through non-covalent π-π stacking interaction and the ink was deposited on the paper substrate by inkjet printing, keeping an optimal three-dimensional semiconductor density. The researchers identified two sets of unique biomarker proteins for each of the five body fluids from literature to be tested with this sensor. Individual antibodies were immobilized onto the SWNT surface, and any specific antigen-antibody interaction near the interface of the SWNTs and the environment caused a significant increase in the measured electrical responses because of surface charge–induced gating effect, which gave the sensor response values. Furthermore, to facilitate easy, multiplexed sensing and reduce the time of fabrication, the researchers developed a water-based all-in-one SWNT-Antibody Bioink, containing both transducer (SWNT) and biological elements (antibody) that could be drop-casted or printed on the paper substrate to form the chemiresistor arrays. The described sensing methods showed promising results, with specific interactions giving an average response of >70% and non-specific interactions giving an average response of <10% in each case, thereby proving the specificity of the sensor. Quantitative and selective detection of the tested biomarker proteins could be demonstrated with the very low limit of detection values obtained. The paper-based chemiresistive biosensor system reported here was easy to fabricate and handle and designed for rapid, sensitive, selective detection of biomarkers. It also followed the ASSURED criteria (i.e., Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free and Deliverable to end users). This biosensing platform exhibited promising performance and potential for unprecedented on-site use during crime scene investigations and is expected to promote the future development and adoption of chemiresistors on paper substrates in other sensing scenarios.

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Sex-Based Targeted Recovery of Cells in a Heterogeneous Mixture: Separating Male and Female Like-Cells

NIJ AWARD #: 2020-DQ-BX-0019

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Mixture interpretation remains a central challenge in the forensic DNA field. Much research and development has focused on methods to improve the interpretation of complex samples, including software solutions (probabilistic genotyping [Bleka et al., 2016; Brenner, n.d.; Perlin et al., 2011; Taylor et al., 2013] and statistical/machine learning-based methods [Alfonse et al., 2017; Marciano & Adelman, 2017]). These methods are used to improve mixture interpretation on the "back-end" rather than address the separation of the individual biological components (e.g., cells) to wholly avoid mixtures. This project will address this need in the forensic biology field, aiming to evaluate an emerging method of targeted male cell analysis in mixtures of like-cells (e.g., male and female epithelial cells collected on a vaginal swab). This method will either eliminate or significantly ease the complexity of mixture interpretation for specific case types where male and female like-cells are collected, improve laboratory efficiency, and lower the cost of processing such cases. The central aim of this project is to develop and optimize a method to identify and recover male cells from a mixture of male and female like-cells (e.g., epithelial cells); for example, the identification of a vasectomized male in a sexual assault of a female victim. The method will adapt two well-characterized methods into a single unified protocol for use in forensic DNA analyses—Y chromosome targeting via the Abbott Molecular Vysis™ CEP Y DYZ1 probe and male cell recovery using the Menarini-Silicon Biosystems DEPArray™ NxT or PLUS. Separating male from female cells in like-cell mixtures will result in single-source male autosomal profiles and provide unprecedented levels of resolution that will lead to stronger statistical support for the comparisons. It may also allow the profiles to be uploaded to CODIS in cases that yielded profiles that were not previously able to be uploaded. The proposed method would also simplify the data interpretation because probabilistic genotyping would not be required to deconvolute a mixture. The benefits extend to any sample with a male and female mixture of like-cells. This study is in progress.

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A Fully Integrated Microfluidic Tool for Forensic Epigenetic Sample Preparation

NIJ AWARD #: 2020-R2-CX-0030

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The toolkit for forensic human identification (HID) has expanded beyond comparative methods for individualization to include investigative tools based upon nucleic acid (NA) analysis. The determination of chronological age from forensically relevant stains has garnered considerable attention, with over 300 research articles published and countless predictive models developed from the multiplexed analysis of few age-associated, epigenetic loci (Maulani & Auerkari, 2020). Unfortunately, analysis of epigenetic "marks" requires both DNA extraction and chemical conversion of the DNA to conserve NA modifications for downstream analysis. The gold-standard techniques for both DNA extraction and chemical conversion by sodium bisulfite are time-consuming, labor-intensive, prone to analyst contamination with multiple tube transfers, and result in extensive DNA loss. In particular, sodium bisulfite conversion (BSC) is associated with extensive DNA damage, with even the most well-established methods resulting in more than 50% template loss (Leontiou et al., 2015). Despite the development of more sensitive detection and analysis strategies downstream, including massively parallel sequencing (MPS), studies have demonstrated that the interrogation of epigenetic targets for forensic purposes requires increased sensitivity for reproducible and accurate predictions (Aliferi et al., 2018). To increase the analytical range associated with forensic epigenetic analysis, the presenter has developed a microfluidic system for the automated sample preparation that includes both DNA extraction and chemical conversion. The extraction step employs a thermophilic enzyme for lysis, producing an eluate in PCR-compatible buffer; this step omits the use of any solid phase materials to immobilize and wash the extract to conserve template (Turiello et al., 2022). Released DNA proceeds through a miniaturized, dynamic solid phase BSC (dSP-BSC) process that has been previously optimized to conserve NA templates and maintain high conversion efficiency. All sequential unit operations are performed via a rotationally driven disc, roughly the size of a CD (Thompson et al., 2015), in concert with a mechatronic system to drive fluid toward the periphery of the disc through centrifugal force, induce magnetic mixing (Dignan et al., 2021), perform sacrificial valving (Woolf et al., 2021), and complete targeted incubation steps through contact heating. The system was tested with epigenetic standards of known methylation status, human K-562 erythroleukemia cell lines, and human blood via multiple downstream strategies, including real-time polymerase chain reaction (RT-PCR), high-resolution melting (HRM), and pyrosequencing using age-associated targets FHL2 and ELOVL2 for proof of concept (Hamano et al., 2017). Preliminary results confirm the extraction strategy is compatible with the dSP-BSC chemistry sans nucleic acid purification. Future work is focused on the complete automation of the protocol with the in-house engineered systems and optimization of the tool with forensically relevant samples.

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Quality Control Procedures Required for the Generation of Forensic Quality Mitogenome Reference Data

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Mitochondrial DNA (mtDNA) analysis plays a specialized role in forensic applications, overcoming certain limitations of autosomal DNA markers. The high copy number and uniparental inheritance pattern are advantageous characteristics of mtDNA in cases involving shed hairs and aged skeletal elements, such as decades-old missing persons cases. Though the discriminatory power of mtDNA is limited by common haplotypes, next generation sequencing (NGS) offers feasible access to entire mitochondrial genome (mitogenome) data that can provide increased resolution of common haplotypes to unique sequences. The primary implementation challenge of mitogenome analysis is a lack of forensic-quality reference data, which are required to determine the evidentiary weight of a match. To meet this need, the Armed Forces Medical Examiner System–Armed Forces DNA Identification Laboratory (AFMES-AFDIL) proposed to generate 5,000 mitogenomes as part of a National Institute of Justice (NIJ)–funded project. Mitogenome data were produced using robust laboratory procedures and automated processing, followed by independent data reviews by two experienced analysts and a multi-step quality control (QC) process. During review of the data, analysts assessed haplotypes for misalignment of homopolymer regions, nuclear mtDNA segment (NUMT) interference, sequencing errors, and other artifacts. The mtDNA haplogroup, which was predicted as part of the NGS analysis workflow, provided the analyst with phylogenetic nomenclature guidance and an invaluable QC check of the haplotype during data review. Replicate processing of samples with questionable variants, often with an alternate enrichment method, was performed to confirm the authenticity of the mtDNA haplotype. Once data review was completed for a population, samples with shared mitogenome haplotypes were subjected to nuclear DNA testing to assess their relatedness (i.e., nuclear family members and potential second-degree relatives). If maternal relatives were identified, only one sample from the lineage was included to ensure that the mitogenome data represented a random sampling of the population. Lastly, a series of QC checks was performed during submission to the EMPOP database. In addition to the confirmation of the haplogroup prediction, haplotypes were compared against a curated mitogenome dataset to identify any "abnormal" variants or combination of variants (e.g., variants never observed before, known phantom mutations, or other haplotype irregularities). Haplotypes that did not pass the EMPOP QC checks were flagged for further review, resulting in the reanalysis of the existing NGS data or replicate processing to confirm the reported variants. Haplotypes that ultimately could not meet the QC criteria were excluded from reference dataset. In the end, the AFMES-AFDIL produced over 6,000 forensic-quality mitogenome haplotypes for use by the forensic community.

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