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The Future of Forensic Analysis

October 2024

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Cover Art:
Expert Using Professional Tools and Equipment at Crime Scene: Robbery victim's wallet containing DNA of the criminal. Detective working in the background.

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WHITE PAPER

White Powder Incidents: Identifying Narcotics in Complex Samples

Mira DS handheld Raman System

A person suspected of possessing a narcotic can be charged with a crime only after the identity of the illicit substance is confirmed. This confirmation is typically provided by analytical chemists in forensic laboratories and requires highly technical separation and detection methods. Unfortunately, such labs often have deep caseloads that lead to delays in testing. Handheld Raman analyzers bring the reliability and accuracy of lab analysis to first responders in the field, allowing for rapid and accurate identification of street drugs with a white powder appearance. With such tools, demand for forensic analysis can be reduced and enforcement agencies can enforce drug policies with greater safety, speed, and precision.

Metrohm

INTRODUCTION

Presumptive tests, including colorimetric tests that detect classes of drugs, establish suspicion and reduce demands on labs. Such tests are designed for officers in the field, but often lack precision and robustness to field conditions in addition to user-error, subjectivity, and exposure. Moreover, presumptive testing by field-based professionals must be suited to analysis of complex drug street samples. Raman spectroscopy brings the precision of laboratory analysis to presumptive testing. The handheld Metrohm Instant Raman Analyzer for Defense and Security (Mira DS) equipped with Orbital Raster Scanning (ORS) technology provides any user, anywhere, the ability to accurately identify white powder street drugs through library comparison and mixture matching.

INTRODUCTION

«White powder incidents» might involve methamphetamine, cocaine, or designer drugs contaminated with excipients. This application describes analysis of real street drug samples using proprietary Library and Mixture Matching routines included with Mira DS. Much research has been dedicated toward identifying related substances and establishing tagged and treed libraries of known combinations of illicit materials. In other words, detection of certain substances activate algorithms that search for specific correlated materials. These libraries inform Mixture Matching routines, which can be described as deconvolution of multiple spectra from components in a mixture.

For example, excipients may comprise 70–80% of street drug samples. These are cutting agents that bulk up the sample, residual chemicals from production, or other drugs. For example, caffeine is used as a cutting agent for cocaine – its powdered form has a similar appearance to cocaine, upon inhalation it gives a user a similar euphoria as pure cocaine, and its stimulant properties complement those of cocaine. When caffeine is detected in a complex sample, established library correlations cause the software to analyze the same sample for associated materials, leading to identification of cocaine.

Figure 1. Mira DS with Right Angle Attachment.

EXPERIMENT

A local Drug Task Force used Mira DS to scan seized street drug samples with crystalline white powder appearance. Notably, Mira DS uses embedded algorithms that automatically optimize acquisition parameters in order to acquire highest quality spectra: an analytical chemist is available at the touch of a screen.

The resulting spectra were imported into the MiraCal DS software and compared within on-board libraries of illicit materials: the results follow. Each search resulted in preliminary identification with a match score indicating how well the sample spectrum correlates to a library spectrum. Mixture matching routines determine if a spectrum is possibly the result of a combination of substances. Up to 3 components in a mixture are identified and reported with accompanying information that includes a qualifier and a color-coded hazard warning.

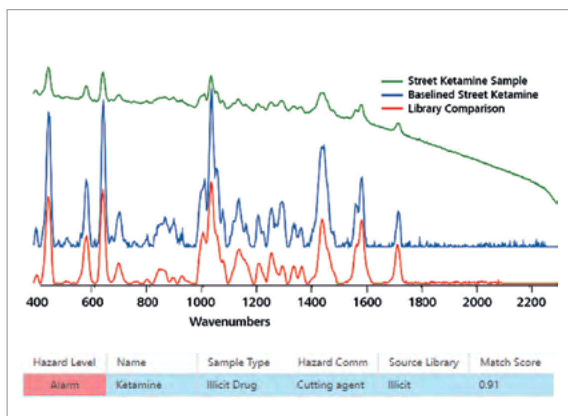

Figure 2. Ketamine Spectra with Match Score.

Street ketamine exhibits fluorescence with Raman excitation at 785 nm, as the green spectrum above shows. Baselining emphasizes peaks of interest, but may result in a spectrum with poor signal-to noise-ratio (blue spectrum above.) Even with this complication, MiraCal DS successfully identifies the sample as ketamine with an HQI= 0.91.

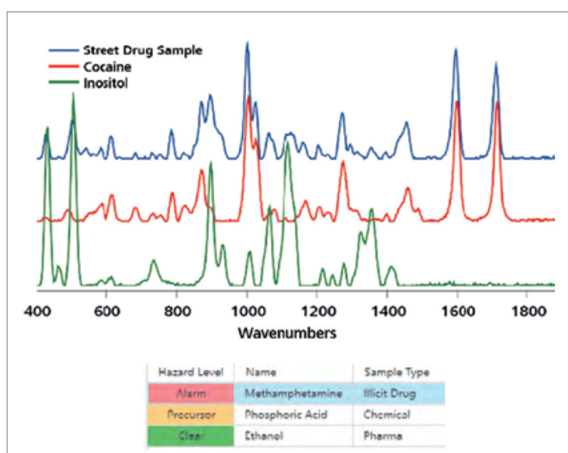

Figure 3. Street Cocaine cut with Inositol.

Inositol is a sugar alcohol commonly used to cut cocaine: it has a similar appearance and less sweetness than many other sugars used as excipients. Similarly, sodium bicarbonate is a white powder used frequently to add extra bulk to illicit drugs. Figures 3 and 5 illustrate that the complex spectrum from the street sample is consistent with both the active substance and the cutting agent. These spectra also provide an excellent example of the colorcoded warnings provided by Mira DS.

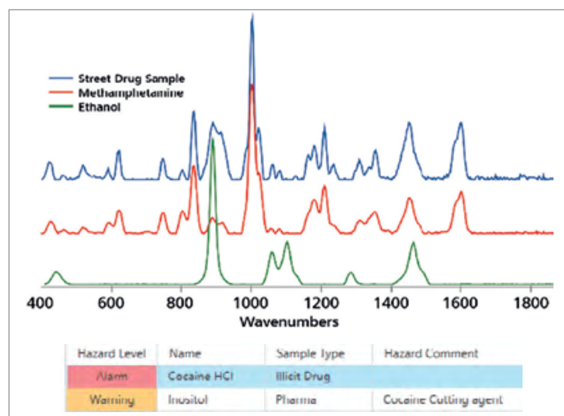

Figure 4. Methamphetamine with residual Ethanol.

Ethanol is used in the production of methamphetamine. Figure 4 is an example of successful identification of an excipient through mixture matching, despite its spectral similarity to the compound of interest.

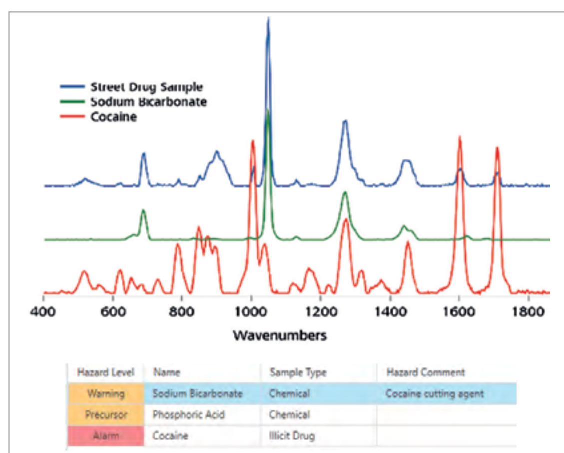

Figure 5. Sodium Bicarbonate is a Common Cutting Agent.

CONCLUSION

Mira DS is a powerful tool for Defense and Security professionals who encounter white powder street drugs. Metrohm Raman delivers fast evaluation of potentially hazardous substances in the field. With Mira DS, first responders can safely get immediate information about unknowns without concerns about contact or sample consumption. Metrohm Raman is excited to offer a rugged little handheld Raman analyzer that brings sophisticated chemical analysis out of the lab and onto the streets.

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FEATURED ARTICLE

Mass Spectrometry for Forensic Analysis: An Interview with Glen Jackson

Will Wetzel

As part of "The Future of Forensic Analysis" content series, *Spectroscopy* sat down with Glen P. Jackson of West Virginia University to talk about the historical development of mass spectrometry in forensic analysis.

Dr. Glen P. Jackson of West Virginia University.

Mass spectrometry (MS) is an analytical technique used in analytical chemistry and spectroscopy. It measures the mass-to-charge (*m/z*) ratio of ions to identify and quantify molecules in a sample (1). This method involves ionizing chemical compounds to generate charged molecules or molecule fragments, then measuring their *m/z* ratios. The resulting data, typically presented as a spectrum, provides detailed information about the molecular weight and structure of the analytes, making it a powerful tool for chemical analysis.

MS has been used in forensic and criminal investigations to analyze many substances. In particular, MS analysis has been used to study biological samples such as urine, hair, and blood to identify drugs and other toxic samples (1). MS can help uncover evidence in cases of poisoning or drug abuse.

MS is also employed in the examination of environmental samples, such as soil and water, to detect pollutants and residues related to criminal activities. Furthermore, MS analysis contributes to uncovering biological evidence, including DNA and proteins, aiding in the identification of individuals and establishing connections between suspects, victims, and crime scenes (1).

Glen Jackson, a Ming Hsieh Distinguished Professor of Forensic and Investigative Science at West Virginia University, has been conducting extensive research in forensic analysis applications. Jackson earned his BS in Chemical and Analytical Science from the University of Wales Swansea, an MS degree in Analytical Chemistry from Ohio University, and a PhD in Analytical Chemistry from West Virginia University. After receiving his PhD, Jackson conducted his postdoc at Oak Ridge National Laboratory before starting his career as a member of the chemistry faculty at Ohio University. Following his tenure at Ohio University, he returned to West Virginia University, where he is currently exploring MS instrumentation development in forensic and biological applications.

As part of "The Future of Forensic Analysis" content series, we invited Jackson to talk about his laboratory group's current research in forensic analysis. Our interview with Jackson covers a breadth of topics, including the historical developments of MS in forensic analysis, current research efforts in this field, and how the role of MS is currently evolving.

Photo Credit: Glen P. Jackson.

Can you elaborate on the major historical developments in MS that have significantly impacted its application in providing evidence in the criminal justice system?

The three most important developments have been: 1) the electron ionization (EI) source, which was driven by Bleakney and others in 1937 (2); 2) the coupling of MS with gas chromatography (GC–MS), which was demonstrated by Gohlke in 1959 (3); and 3) the invention of the quadrupole mass analyzer by Paul and Steinwedel in 1953 (4). The hyphenated GC-EI-MS instrument, which was first commercialized by Finnigan in 1968, became the technology of choice for many areas of chemical analysis, and it is still by far the most trusted and commonly used instrumental method of analysis for seized drugs and ignitable liquid residues.

How has MS been utilized in casework involving trace metal impurities in hair and glass? Could you provide specific examples?

The oldest case that I know of involving MS analysis of trace metals in hair involved ion microprobe mass spectrometry (IMSS), now called secondary ion mass spectrometry (SIMS). The technique was first presented by R. Castaing and G. Slodzian in 1962 (5,6). In its first forensic application in 1977, Walter McCrone used IMSS in *United States v. Brown* to link a suspect's hair with hair strands found at a Planned Parenthood clinic that had been bombed (7). The court struggled with the validity and application of IMSS for human hair because it had never been applied for this purpose before. Although IMSS was found to reliable as a scientific technique, its application to human hair did not meet admissibility standards because it had not gained the level of general acceptance in the scientific community for this purpose (7).

The first casework applications to glass that I know of occurred after Houck and others successfully coupled MS to an inductively coupled plasma (ICP-MS) instrument in 1980 (8). Following commercialization of ICP-MS instruments a few years later, the forensic community demonstrated that the intra- and inter-variability of different elements in glass were sufficient to distinguish glasses from different sources because of their impurity differences. Today, both ICP-MS and laser-ablation (LA)-ICP-MS are widely used to compare glass samples in forensic contexts (9–11).

What applications of mass spectrometry are most common and successful for criminal investigations?

By far, the most common application of MS is the use of GC-EI-MS to confirm the identity of seized drugs. A recent white box study involving 71 laboratories showed that they all used GC–MS to help with the identification of a controlled substance (12). GC–MS is also the method of choice for ignitable liquid residues to support suspected arson cases (13). Finally, although GC–MS was also the method of choice to detect drugs and drug metabolites in human body fluids for several decades, liquid chromatography–MS (LC–MS) now tends to dominate the market in toxicology laboratories.

What are some notable cases where MS has been used to identify drugs, explosives, polymers, and ignitable liquids?

Gosh! There are so many. I recently wrote a review article on this topic in which I attempted to include all the notable early cases (14). One of the earliest examples for seized drugs was by Martin and Alexander at the U.S. Food and Drug Administration (FDA) in 1968. They published a report that explained how they used cracking patterns and high-resolution mass spectrometry (HRMS) to identify the hallucinogen dimethyltryptamine (DMT) in a casework sample (15). They noted that the use of mass spectrometry changed the project from a major research project, which would have taken weeks or months using conventional techniques, to "an exercise problem in spectroscopic identification" (15).

For polymers, the earliest application of MS to casework I could find was the use of pyrolysis-GC–MS (Pyr-GC–MS) by Zoro and Hadley in 1976. They described a case where they linked an antioxidant in the trace fragments of a polymer in blades of a hacksaw to those of a stolen cable that was coated with a polymer (16).

> Ion mobility spectrometry has some untapped potential in forensic science, especially if differences in drift times enable the resolution of isomers that are difficult to resolve by GC–MS or LC–MS.

How do the scientific foundations of MS compare to other forensic techniques like pattern-based methods and physical matching?

The theory and practice of MS is based on physics and the fundamental properties of matter. In an EI source, the branching patterns of a molecule can be tied to statistical mechanics through the Rice–Ramsperger–Kassel–Marcus (RRKM) theory. In these ways, our measurements have scientifically rigorous foundations. However, the result of our measurements is usually a mass spectrum of a substance, which can be thought of as a pattern. Although the spectral patterns can be linked to chemical structures through spectral interpretation, our comparisons of one spectrum to another—such as in a database search—are hardly distinguishable or superior to

pattern-based disciplines. Sure, we have a plethora of algorithms for comparing and interpreting spectra, but even the most advanced algorithms struggle to provide meaningful statistics, probabilities, or likelihood ratios for specific compound identifications. Quite simply, the spectra of some drugs are more unique than others. For these reasons, we should not rest on our laurels, and we should continue to improve the evidential power of our MS results.

Can you discuss any landmark legal precedents where mass spectrometric evidence played a critical role in the outcome of the court's decision-making process?

Again, I would refer you to my recent review that specifically covers influential legal precedents in a wide variety of forensic MS applications (14). If I had to pick two favorites, one would be GC–MS identification of cocaine. For most of the 1970s, GC–MS was basically useless for the identification of *l*-cocaine—the controlled isomer—because it could not distinguish *l*-cocaine from the uncontrolled *d*-cocaine. For most of the 1970s, analysts often struggled on cross-examination about how many isomers of cocaine exist and which ones they had identified—there are four chiral centers in cocaine, so eight theoretical isomers in total, but only two are commonly considered (17). In 1981, a Federal Bureau of Investigation (FBI) analyst testified that because plants make exclusively *l*-cocaine and humans make racemic mixtures of *d*- and *l*-cocaine via laboratory-based synthetic methods, *d*-cocaine had never been observed independently from *l*-cocaine (18). Therefore, if any cocaine is identified, at least 50% will be *l*-cocaine. Since then, analysts have never really had to identify the isomeric form of cocaine, so GC–MS regained its use.

One case in which MS proved essential was in 1991, when a mother was found guilty of poisoning her 5-month-old child with ethylene glycol. Ethylene glycol had been identified based on GC retention time data using a flame ionization detector (GC-FID) (19). She gave birth to a second child in prison, and after that child also became ill, doctors were able to diagnose a genetic disorder in the newborn called methylmalonic acidemia. Scientists then reanalyzed the serum from the first child using the same GC-FID method, and of course, they still found ethylene glycol. Methylmalonic acidemia does not cause a buildup in ethylene glycol, so there was no evidence to overturn the conviction for poisoning her first child. Finally, a toxicologist named Jim Shoemaker whose laboratory had worked on the original case developed a more selective GC–MS approach that proved that the toxin was in fact propionic acid. Propionic acid has the same GC retention time as ethylene glycol, but it has a different fragmentation pattern. Unlike ethylene glycol, propionic acid could be linked to the genetic disorder, so, thankfully, the mother was ultimately exonerated (19,20).

The theory and practice of MS is based on physics and the fundamental properties of matter.

Despite its reliability, MS evidence has been challenged in court. What are some reasons for these challenges, and how have courts typically responded?

In 1981, mass spectrometric evidence was discussed at length in a case against 2,116 boxes of boned beef (21). The U.S. District Court of Kansas was appalled at the GC–MS expert witnesses in the case. The case concerned the alleged adulteration of beef with the hormone diethylstilbestrol. Apparently, the court witnessed mass spectrometrists from each side arguing for opposite conclusions about the exact same data. The Court bemoaned the GC–MS experts and reported that "they are disregarded as being of any scientific assistance to the Court. Simply stated, a review of these exhibits suggests that the experts can read into them about what they want to read, the Court perceiving nothing and is totally helpless" (21). The example above from the McCrone institute in 1977 regarding the analysis of trace elements in human hair showed that although MS techniques can be perfectly valid for the scientific community, their application to a particular case needs to be fit for purpose to be accepted by the court. This criterion is an important part of Daubert standards and the Federal Rules of Evidence that help judges assess the validity of forensic evidence.

What future developments or improvements do you foresee in the application of MS within the criminal justice system?

I'm quite sure that vendors will continue to deliver instruments with lower limits of detection, greater signal-to-noise (S/N) ratios, and shorter analysis times than we have today. These instruments will have trivial effects on most applications, since in most applications, we already have the required sensitivity to identify substances at biologically relevant levels. However, portable MS instruments have long been proposed to speed up the analysis of seized drugs and enable on-site detection; in so doing, they could save a fortune for the criminal justice system. One might wonder why they have struggled to fulfill

their potential. I suspect that one problem could be that cost saving is most beneficial for the criminal justice system and not directly for the crime laboratory. For example, on-site testing could help reach plea deals with suspected drug dealers/possessors in a matter of days, thereby avoiding the average wait of approximately 100 days in jail at an average cost of ~$150 per day. That is a total of ~$15,000 per case in jail costs alone, not including legal fees and laboratory expenses. If a portable GC–MS instrument could be used to conduct on-site confirmatory testing, thusly circumventing jail time, legal proceedings, and legal fees, the criminal justice system could save a fortune. The eventual savings would more than compensate for the upfront and maintenance costs.

I believe that we have the most work to do with data analysis. We have barely scratched the surface in terms of maximizing the evidential value of mass spectrometric evidence. We need to help the forensic community by providing more meaningful probabilities or error rates with our mass spectral identifications.

Can you talk about some of the current projects your laboratory group is working on?

The forensic applications in our laboratory have two main themes: spectral interpretation and spectral identification. For spectral interpretation, we help analysts understand the mechanisms of fragmentation that lead to the observed product ion spectra of certain seized drugs. We typically use a variety of age-old techniques, including isotope labeling, MS^n, and HRMS, to help identify the observed fragmentation pathways of various synthetic drugs, like cannabinoids, cathinones, and fentanyl analogs. We've identified novel structures and pathways for cathinone fragmentation (22), and we've identified some unprecedented skeletal re-arrangements for fentanyl analogs (23).

Regarding spectral identification, we're developing a new algorithm that can help overcome the problems caused by spectral variance when substances are analyzed on different days or on different instruments (24,25). The algorithm will allow analysts to identify substances in the absence of a standard analyzed contemporaneously with the casework sample. It's still a work in progress, but we've made some promising developments.

Can you explain the importance of your research within the broader field of spectroscopy or in a specific industry or application?

Our new algorithm for spectral identification has the most potential outside of forensic science. We are currently demonstrating that the algorithm is applicable to protonated precursors from electrospray ionization (ESI) and direct analysis in real time (DART) ion sources in addition to EI sources. Therefore, the algorithm should be applicable to identifying peptides, lipids, metabolites, and almost any substances for which replicate spectra can be (or have been) acquired.

How do you stay updated with advancements in mass spectrometry and spectroscopy techniques and technologies?

I read the table of contents for most MS journals when they arrive in my inbox. I find most articles that way. My students occasionally share new and interesting articles with me, too. Sometimes, I will come across other articles when I perform targeted literature searches during a literature review for a manuscript or grant proposal. I also keep up to date with developments by paying attention to the work of my colleagues at conferences, meetings, and college visits.

Can you discuss a recent innovation or development that you find particularly impactful or exciting in mass spectrometry and forensic analysis?

I think ion mobility spectrometry has some untapped potential in forensic science, especially if differences in drift times enable the resolution of isomers that are difficult to resolve by GC–MS or LC–MS. For example, ortho-, meta-, and para-isomers of fentanyl analogs are often difficult to resolve with tandem mass spectrometry, so it would be helpful—and apparently no slower—if IMS-MS could resolve such isomers as they eluted from a chromatographic column. A couple of groups have recently shown that the site of protonation within a fentanyl analog—such as on the amide or amine group—can significantly impact the drift time of the protomer (26–28). Therefore, in contrast to most drugs, many fentanyl analogs show a bimodal distribution of arrival times in IMS-MS. However, the work is strictly academic now and has yet to be generalized or made practicable. So, although IMS-MS is popular in areas like structural biology, there are very few researchers exploring and maximizing opportunities in forensic science.

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The Future of Forensic Analysis: An Interview with Brooke Kammrath

Will Wetzel

As part of "The Future of Forensic Analysis" content series, *Spectroscopy* sat down with Brooke Kammrath of the University of New Haven to talk about the significance of spectroscopy in forensic analysis.

Dr. Brooke Kammrath of the University of New Haven.

Photo Credit: Lindsey Weinger

Brooke Kammrath of the University of New Haven forged a unique path to becoming an internationally recognized forensic science researcher.

As a chemistry student at Northwestern University in Chicago, Illinois, Kammrath initially planned on becoming a chemistry and physics teacher, which led to her to pursue a master's degree in chemistry education from New York University (NYU) back in 2003.

However, after a few years of teaching, she opted for a career change and took steps to become a criminalist specializing in forensic chemistry. Kammrath received an MS in forensic science from John Jay College of Criminal Justice before pursuing a PhD in Criminal Justice at the CUNY Graduate Center.

After obtaining her PhD in 2012, Kammrath joined the faculty at the University of New Haven, pursuing research endeavors that included combining microscopy with spectroscopy, identifying and characterizing microscopic forensic samples, statistical analysis of trace, pattern, and impression evidence, developing portable instrumentation, and investigating the significance and impact of physical evidence. She has served as the president of the New York Microscopical Society (NYMS) and was on the Governing Boards of NYMS, the Society for Applied Spectroscopy (SAS), and is currently on the Eastern Analytical Symposium (EAS) board. Additionally, she is a certified criminalist by the American Board of Criminalistics (ABC).

Recently, she has collaborated with other researchers in the field on several published books, including *Solving Problems with Microscopy: Real-life Examples in Forensic, Life and Chemical Sciences, Blood Traces: Interpretation of Deposition and Distribution, Portable Spectroscopy and Spectrometry 1: Technologies and Instrumentation,* and *Portable Spectroscopy and Spectrometry 2: Applications.*

Kammrath currently serves as a Professor in the Henry C. Lee College of Criminal Justice and Forensic Sciences at the University of New Haven, as well as the Executive Director of the Henry C. Lee Institute of Forensic Science.

As part of "The Future of Forensic Analysis" content series, the editors of *Spectroscopy* spoke to Kammrath about her career in forensic science and her ongoing research in this field. Our interview with Kammrath covers a breadth of topics, including her collab-

orations with some of the most well-known spectroscopists and how recent developments in spectroscopic instrumentation will impact forensic analysis moving forward. Our conversation reveals her unique path to becoming one of the leading forensic analysts of our time.

I would like to start this interview by offering our readers a glimpse into your background. How did you become interested in forensic science, and how has your academic career shaped your research in this field?

I first learned about forensic science when I was a college chemistry major at Northwestern University and *CSI* premiered on television. I thought it featured the most outstanding application of natural sciences (chemistry, physics, and biology). But I continued on the path I had planned for myself for the next several years, as a high school chemistry and physics teacher, even getting a master's in chemistry education from New York University (NYU). After a few years of wishing I could have a different career, I took the steps needed to become a forensic scientist. Forensic science is more than just applying chemistry to a crime scene problem, because there are nuances and questions in forensic science that are unique to this discipline that require specialized knowledge. I attended John Jay College of Criminal Justice in NYC for my master's in forensic science, which is where I was fortunate to learn from extraordinary professors about all aspects of forensic science. It was at John Jay College that I was inspired to pursue a career in academia, which would allow me to teach and mentor students, do meaningful research with brilliant collaborators, and consult on interesting and complex forensic cases. To achieve this, I continued at the CUNY Graduate Center for my Ph.D. in Criminal Justice with a concentration in Forensic Science.

I have been incredibly lucky to have some phenomenal mentors, most notably Professors John A. Reffner, Peter De Forest, Nicholas D.K. Petraco, and Nicholas Petraco, Sr., who have shaped all aspects of my research and approach to forensic science problem solving. Regarding my research, my academic journey has taught me to rely on the fundamentals of good science, to take chances because you never know where opportunities will be uncovered, to ask relevant questions (which is critical for meaningful forensic science research), to seek out diverse collaborations because alternative perspectives are always valuable, and to believe in the power of kindness.

> My academic journey has taught me to rely on the fundamentals of good science, to take chances because you never know where opportunities will be uncovered.

You are also a certified criminalist by the American Board of Criminalistics (ABC). Can you talk about what the process was for certification, and how this has aided your research and career?

As an academic and private consulting criminalist, I recognized early in my career the importance of certification. For me, certification by the ABC is a credential that verifies my qualifications (skills, knowledge, experiences, and abilities) as a forensic professional. I find external validation particularly valuable in my role as an expert for the court and as a professor in an applied field, but I have not found it to aid my research. At the time of my initial certification in the mid-2000s, I obtained the General Knowledge certification from the ABC, which was like the current Comprehensive Criminalistics one, which covers a variety of forensic disciplines rather than one specific specialty. The initial process involved passing a notoriously challenging exam, but maintaining certification requires demonstrating continued competency yearly. This can include casework, teaching, publishing research, attending conferences or workshops, and more.

Over the past few years, you've published several books in this space, collaborating with other well-known spectroscopists, such as Richard Crocombe and John A. Reffner (1–4). How did you get involved in these projects?

Reffner is my mentor, having advised both my master's and PhD theses in which the use of infrared (IR) microscopy was investigated for the identification of soil minerals and the forensic discrimination of glass fragments. He is an incredibly brilliant, thoughtful, generous, and accomplished man who has been a source of wisdom, teaching, and support for me since the day I met him in 2006, when I attended a New York Microscopical Society (NYMS) professional microscopy workshop he was teaching. In 2018, John and I were talking, and he expressed that he had always wanted to write a book. He asked if I would help him realize this last professional bucket list item, which was a true honor. When discussing possible topics, it became obvious that we didn't want to write a typical "how-to" book that instructs people how to perform a specific microscopical technique; instead, we wanted to capture the more elusive "why-to" that can help people understand the powerful capabilities of microscopy and microspectroscopy for solving problems. I was able to take a semester sabbatical from my university work to spend every day brainstorming, writing, and doing microscopy

and spectroscopy with John, which was one of the most incredible professional experiences of my career.

While a master's student, I completed a summer internship at Smiths Detection under the supervision of Reffner and Pauline E. Leary. Leary is an incredible scientist, collaborator, and friend. For over 15 years, Leary and I have worked together on numerous research projects on a range of topics of forensic interest, from investigating the dispersive effects of barium fluoride cover slips on the quality of IR spectra for potentially hazardous samples in a sealed cell (5) to the field analysis of explosives and illicit and counterfeit drugs (6–8). Richard Crocombe and Leary teach an informative workshop on portable spectroscopy and spectrometry, which is still offered at select conferences. Crocombe recognized the need for a comprehensive resource on portable spectroscopy and spectrometry, and when the project expanded to a two-volume book with 45 chapters, I was invited to join as a co-editor. It was an incredible opportunity to work with Richard and Pauline on this project, and even more wonderful is that I am able to expose my students to their brilliance when they come to the University of New Haven for continued collaborations.

Your most recent publication, *Solving Problems with Microscopy*, presents examples and lessons regarding the value the microscope brings to problem solving by experienced scientists in various industries, including in criminal and civil forensic science (1). Can you provide our readers with a couple of highlights from the book that you found particularly interesting when working on it?

That is an incredibly difficult question because each case example included in the book is fascinating to me and highlights a different aspect of scientific problem-solving using microscopy, microspectroscopy, or both. As a forensic scientist, I am partial to murder investigations, which include the cases of the Green River Killer, the Buttonier Case, A Connecticut Murder Case, the Red Hooded Sweatshirt, the Atlanta Child Murders Investigation, the Hog Trail Murders, the Hoeplinger Murder (co-authored with Dr. Henry Lee), and the Preppy Murder (co-authored with Peter R. De Forest). A highlight, in my opinion, is the Green River Killer case, where the microscopical and Fourier-transform infrared (FT-IR) microspectroscopical analysis of vacuum sweepings from the victims' and suspect's clothing was shown to contain microscopic spray paint spheres. This work was completed by Skip Palenik and other scientists at Microtrace, who, with this work, elegantly demonstrated the value and potential of the interrogation of microscopic traces.

Additionally, two cases from scientists at the US FDA Forensic Chemistry Center, "A Mouse, a Soft Drink Can… and a Felony" by S. Frank Platek and Nicola Ranieri, and "Optical Microscopy Takes Center Stage: Melamine in Pet Food," which is authored by Mark Witkowski and John Crowe, are fascinating, brilliant, and have the most elegant scientific problem-solving.

Thorough testing of new methods needs to be done not only on mock evidence, but in real world cases as well, which is why collaboration with practitioners is essential for impactful forensic research and development.

What are the key spectroscopic techniques you use in your forensic research (for example, IR, UV-vis, Raman, NMR)?

I have used a variety of spectroscopic techniques in my research because there isn't just one instrument used to interrogate forensic traces. My research involving portable instruments uses a variety of spectroscopic techniques, including FT-IR, Raman, near-infrared (NIR), gas chromatography–mass spectrometry (GC–MS), high-pressure mass spectrometry (HPMS), and ion mobility spectrometry (IMS). My research into soil mineral identification has focused on IR and Raman microspectroscopy, specifically particle-correlated Raman spectroscopy and morphologically-directed Raman spectroscopy. I've also used laser-induced breakdown spectroscopy (LIBS), scanning electron microscope–energy dispersive X-ray spectroscopy (SEM-EDX), atomic absorption (AA), and X-ray fluorescence (XRF) for elemental analysis of a variety of traces, including gunshot residue (GSR) and copper metal.

How do you determine which spectroscopic method to use for a particular forensic analysis? Is there a specific spectroscopic technique that you've often used in your research?

The problem needing to be solved will direct the scientific investigation. For me, it's about understanding the fundamental question (or questions) being asked, balancing that with the limitations and capabilities of the available tools and circumstances, then coming up with a logical plan. For example, if sample size is limited, non-destructive microspectroscopical methods (for example, FT-IR or UV-vis microspectrophotometry [MSP]) will be used and explored first (after macroscopic and microscopic examinations, both with proper documentation) prior to destructive methods like pyrolysis-GC–MS (Py-GC–MS). Forensic paint analysis is

an excellent example which shows the integration of spectroscopic methods within a forensic analytical scheme and is brilliantly detailed by Scott Ryland and Ed Suzuki in their book chapter, "Analysis of Paint Evidence" (9).

When evaluating new spectroscopic tools, the question often asked is how they compare to existing methods, and if they provide added value to that which is already being done. Spectroscopic techniques must demonstrate that they are fit-for-purpose, and in the forensic landscape, that can be very difficult and take a long time.

How does one ensure the reliability and accuracy of spectroscopic data in forensic investigations?

There are well-known good laboratory practices and methods that are used in all scientific pursuits to ensure reliability and accuracy that must be completed in all forensic scientific investigations. These include, but are not limited to, adherence to the scientific method (and its iterative process of critically testing hypotheses), the maintenance of standards and controls, and transparent documentation of everything (from sample selection through analysis and including interpretation). Thorough testing of new methods needs to be done not only on mock evidence, but in real-world cases as well, which is why collaboration with practitioners is essential for impactful forensic research and development.

What recent advancements in spectroscopic techniques do you find most exciting or promising for forensic applications?

There are quite a few exciting new advancements in spectroscopy that I think may have meaningful applications for interrogating forensic traces. I'll discuss three.

Of particular interest to me are methods that can correlate physical and optical properties analysis with chemical analysis, such as IR or Raman spectroscopy. These methods include FT-IR and Raman mapping as well as automated imaging paired with Raman spectroscopy methods, such as PCRS and MDRS.

Optical photothermal IR spectroscopy (O-PTIR) is an incredibly powerful technology that has great potential for discriminating material traces at a level not yet explored. I have been fortunate enough to work with this technology, albeit in a limited capacity, and I saw how O-PTIR can add value to molecular spectroscopic methods currently used in forensic laboratories, such as with the discrimination of automotive paint samples. O-PTIR is able to provide comprehensive non-destructive analysis of automotive paint layers without contact, including those previously too thin to analyze (< 10 µm). The spatial resolution afforded by O-PTIR, combined with the potential for pairing it with Raman and fluorescence spectroscopy, makes it, for me, a technology to watch.

Lastly, portable spectroscopic technologies have the exciting potential to bring powerful science to the scene. Some portable spectrometers are incredibly advanced with capabilities on par with their benchtop counterparts (for example, FT-IR spectrometers), whereas others are relatively new technologies that need additional research and real-world validations to understand their capabilities and limitations. Ultimately, Star Trek's tricorder is still far from reality, and there is no single portable spectrometer able to analyze all samples. Thus, a "toolkit" approach is recommended, and research into the tools in this kit is much needed and very exciting.

In addition to the different portable spectrometer tools available, I find it exciting that there is a plethora of forensic traces that may be analyzed at the scene with portable spectrometers to provide investigative information. Suspected illicit drugs and explosives are the most researched and used items of forensic interest with regard to portable spectroscopy, but there is also great potential with regard to the analysis of paint, GSR, bullet impact or ricochet marks, body fluids, comingled remains, and much more.

How do you stay updated with the latest developments and research in the field of forensic spectroscopy?

In my opinion, there are three essential ways to stay abreast of the latest developments and research in the field of forensic spectroscopy: (1) conferences, (2) professional organizations, and (3) journals. Attendance at professional conferences, both chemistry-focused ones like EAS, SciX, and Pittcon, and forensic-centered ones, such as AAFS and NEAFS, is incredibly rewarding on numerous levels, including learning about the next new tools for forensic problem solving. Just as important for continual growth is membership in professional organizations, both chemistry-focused ones, like the Society for Applied Spectroscopy (SAS), the Coblentz Society, the New York Microscopial Society (NYMS), and forensic-centered ones, such as the American Academy of Forensic Sciences (AAFS), the Northeastern Association of Forensic Scientists (NEAFS), and the American Society of Trace Evidence Examiners (ASTEE). Finally, I also read and critically evaluate published research and reports from a variety of forensic, chemistry, and spectroscopy journals.

What advice would you give to someone looking to pursue a career in forensic science with a focus on spectroscopy?

The best advice I have is borrowed from Louis Pasteur: "Fortune favors the prepared mind," so prepare yourself. Preparation is first done through education, and you will need quality chemistry (especially analytical chemistry) and forensic science courses to achieve this goal. An aspiring forensic scientist can also get fantastic opportunities through membership in professional organizations and by attending in-person conferences, both for forensic science (for example, AAFS, NEAFS, and CAC) and chemistry (for example, EAS, SciX, and Pittcon). These experiences will not only add to your resume, but they will also add to your knowledge, thus making you a more valuable forensic scientist.

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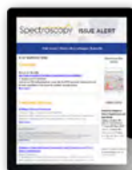

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The Role of Forensic Analysis at the Scene of the Crime: An Inside Look

The Editors of *Spectroscopy*

Forensic analysis plays a critical role in investigating, solving, and prosecuting criminal activity. To assist law enforcement professionals in conducting quick and accurate investigations, forensic analysts use spectroscopic techniques to help investigators uncover the truth. In this feature from "The Future of Forensic Analysis," we spotlight several recent studies that show how several spectroscopic techniques, including attenuated total reflectance Fourier transform infrared (ATR FT-IR) spectroscopy, near-infrared (NIR) spectroscopy, laser-induced breakdown spectroscopy (LIBS), and others, are contributing to solving criminal cases at the scene of the crime.

Determining the Age of Bloodstains at Crime Scenes Using ATR FT-IR Spectroscopy and Chemometrics

By Will Wetzel

This article offers some insight into using attenuated total reflectance Fourier transform infrared (ATR FT-IR) spectroscopy at crime scenes.

According to a recent study, attenuated total reflectance Fourier transform infrared (ATR FT-IR) spectroscopy combined with chemometrics can effectively estimate the time since deposition of bloodstains at crime scenes. This study was led by Antonio Ortiz and María Dolores Pérez-Cárceles of the University of Murcia and published in the journal *Chemometrics and Intelligent Laboratory Systems* (1).

Bloodstains are among the most common pieces of evidence found at crime scenes. Determining how long a bloodstain has been present can provide crucial information about the sequence of events in a crime (1). Traditionally, this estimation relies on assessing the physical and chemical changes that blood undergoes over time (1). However, these methods have often been limited by their reliance on specific environmental conditions and the surfaces on which the blood is deposited (1).

Numerous studies in the past have explored analyzing bloodstains using other spectroscopic techniques, such as Raman spectroscopy (2). In their study, the researchers at the University of Murcia analyzed 960 bloodstains on various surfaces, both indoors and outdoors, to develop predictive models for time since deposition (1). This process was done over 212 days. The study focused on four different surfaces: white cotton woven fabric; regular cellulose paper; filter paper; and glass (1). These surfaces were chosen because these surfaces represented a range of materials commonly found at crime scenes.

The research team used ATR FT-IR spectroscopy because it provides a detailed molecular fingerprint that can be analyzed to determine how the blood's composition changes over time (1). By using this method, the researchers were able to create a series of partial least squares regression (PLSR) models that predict the time since deposition (TSD) of bloodstains with high accuracy (1).

The PLSR models exhibited good predictive capabilities. The residual predictive deviation (RPD) values ex-

ceeded 3, and the R^2 values were greater than 0.90 (1). Interestingly, the models performed better on non-rigid surfaces, such as fabric and paper, compared to rigid surfaces like glass (1).

One of the most promising aspects of the study is the development of a global model for non-rigid surfaces. This model accounts for the various conditions under which bloodstains might be found, making it highly versatile for real-world applications (1). Whether a bloodstain is found on fabric in a dark indoor environment or on paper exposed to outdoor conditions, this model can provide a reliable estimate of how long the stain has been there (1).

To ensure the accuracy of their models, the researchers meticulously controlled the experimental conditions. Blood samples were collected from four healthy donors and deposited on the various surfaces under both indoor and outdoor conditions. For each surface and condition, 24-time measurements were taken, ranging from the day of deposition to 212 days later (1). The bloodstains were then analyzed using a FT-IR spectrometer, with spectra processed using specialized software (1).

This research represents a significant advancement in the field of forensic science and the impact spectroscopic techniques can make in this space. Raman spectroscopy was not susceptible to false positive assignments for bloodstains (2). ATR FT-IR spectroscopy was proven to be an effective tool for in situ determination of the total determination of time since deposition of bloodstains (1). Despite the promising results, the researchers acknowledge that further research is needed. Future studies will likely focus on expanding the range of surfaces and environmental conditions tested to create even more comprehensive models that can be applied in diverse forensic scenarios.

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Compact LIBS Sensor Crime Scene Forensics

By Jerome Workman, Jr.

Researchers have developed a cutting-edge, portable LIBS sensor designed for crime scene investigations, offering both handheld and tabletop modes. This device enables on-the-spot analysis of forensic samples with unprecedented sensitivity and depth, potentially transforming forensic science.

A team of researchers from the Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA) and the Fraunhofer Institute for Chemical Technology (ICT) has unveiled a novel laser-induced breakdown spectroscopy (LIBS) sensor. This groundbreaking tool, designed to meet the stringent demands of law enforcement, promises to revolutionize how forensic evidence is analyzed at crime scenes. The sensor, which can operate in both handheld and tabletop modes, is set to offer law enforcement agencies a versatile, portable, and highly sensitive tool for on-the-spot forensic analysis (1).

Read More: LIBS in Forensic Analysis

Details of the Development

The LIBS sensor was meticulously designed to address the technical gaps identified by law enforcement agencies participating in the Real-time on-site forenSic tracE qualificatioN (RISEN) project. This project aims to enhance crime scene investigations by integrating advanced on-site analysis tools with interactive 3D models of crime scenes. The LIBS sensor plays a crucial role in this initiative, providing a fast, reliable means of detecting trace elements on a variety of surfaces without the need for extensive sample preparation (1,2).

Key features of the sensor include its ability to operate in diverse environments, both indoors and outdoors, and its capacity for precise analysis of trace materials. The device's compact design includes a detachable, lightweight sensor head connected to an instrument box via a 2-m umbilical. This configuration allows for flexibility in operation, enabling the sensor to be used directly at the crime scene or in a laboratory setting for more detailed analysis (1).

Performance and Findings

The research team, comprising Violeta Lazic, Fabrizio Andreoli, Salvatore Almaviva, Marco Pistilli, Ivano Menicucci, Christian Ulrich, Frank Schnürer, and Roberto Chirico, conducted extensive testing of the LIBS sensor to optimize its performance. The results were impressive, demonstrating the sensor's ability to detect trace elements with a sensitivity below 10 picograms on silica wafers. The device also proved effective in analyzing a range of common forensic samples, including fingerprints, soil, gunshot residue, and varnished surfaces (1).

One of the most notable capabilities of the LIBS sensor is its depth profiling function. During tests on car paint samples, the sensor successfully identified all four layers of paint, highlighting its potential for detailed forensic investigations. This capability is particularly useful in cases involving automotive paint fragments, where determining the make, model, and year of a vehicle can be critical to an investigation (1).

Implications for Forensic Science

The introduction of this LIBS sensor marks a significant advancement in forensic technology. Its portability and versatility make it an invaluable tool for crime scene investigators, offering the ability to conduct detailed chemical analyses on-site. This reduces the need for sample transportation and minimizes the risk of contamination, ultimately leading to more accurate and reliable forensic evidence (1).

The sensor's design also prioritizes user-friendliness, with a graphical user interface (GUI) that simplifies operation and ensures that even those without specialized knowledge of LIBS technology can use it effectively. Additionally, the system's ability to transmit data to the central RISEN 3DA-CSI system enhances the overall efficiency of crime scene investigations by allowing for real-time data integration and analysis (1).

Future Developments

While the current LIBS sensor prototype has shown remarkable potential, the research team has already identified areas for further improvement. Plans are underway to reduce the size of the instrument box to fit into a backpack, making the device even more portable. Other proposed upgrades include motorized slits for sample positioning, enhanced spatial resolution for the viewing camera, and improved software for automatic data analysis (1).

The potential applications of this LIBS sensor extend beyond forensic science. The researchers envision its use in various fields, including cultural heritage preservation, environmental monitoring, and other areas where in situ analysis of materials is required (1).

Conclusion

The development of this LIBS sensor represents a significant leap forward in forensic technology, providing law enforcement agencies with a powerful new tool for crime scene investigations (1,2). As the research team continues to refine and enhance the device, it is likely to become an essential component of forensic workflows, offering unmatched precision and efficiency in the analysis of forensic evidence (1,2).

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SEM/EDX Analysis on Suspected Cigarette Burns in a Forensic Autopsy Case of Child Abuse

By John Chasse

Scanning electron microscopy/energy-dispersive x-ray (SEM/EDX) spectrometry analysis of cigarette burns on the corpse of a child led to adding child abuse to the charges against the alleged perpetrator.

A recent investigation by the Laboratory of Forensic Histopathology and Forensic Microbiology at the Department of Biomedical Sciences for Health of the University of Milan in Italy has dealt with a possible child abuse case resulting in the death of the child where three suspicious cigarette burn lesions were found on the body. The laboratory decided to perform scanning electron microscopy–energy-dispersive X-ray (SEM–EDX) spectrometry analysis on these lesions as well as the cigarette butt found at the crime scene. At the same time, SEM–EDX was applied to the analysis of an unlit cigarette in its entirety (obtained from the same source package as the cigarette butt), a positive control skin sample with an iatrogenic cigarette burn injury, and a negative control skin sample. Test results indicated a highly significant distribution pattern by being found in the autopsy samples, the cigarette butt, the tobacco of the unlit cigarette, and the positive skin control (1).

According to the article resulting from this investigation (1), cigarette burn lesions present forensic scenarios that are often difficult to investigate, both from a morphological diagnostic point of view as well as the mode of infliction, especially if the victim is unable to speak or has died (2). Although there may be the suspicion for a lesion to be produced by a lit cigarette, to date one can only rely on the morphological aspects that characterize it, and there is a lack of tools to reach the most evidence-based diagnosis possible.

In the case investigated, a man alerted emergency services one morning as his two-year-old son was not breathing. Upon arrival, emergency services found the child dead, without clothes, and characterized by multiple bruises over his entire body. The child's mother accused the man of violently beating the child in a fit of rage and while under the influence of hashish. The man claimed instead to have only pushed the child, as he was disturbed during his sleep. The man was arrested, and the victim's body was transported to the Institute of Legal Medicine, where a judicial autopsy was carried out two days later (1).

At the autopsy's conclusion, while the cause of death appeared clear, the skin lesions observed in the left zygomatic region, left clavicular region, and right hand third finger still left some questions unanswered; histological examination had shown clear signs of heat damage occurring a few hours before death. Also, the morphological characteristic of the lesions (rounded shape and reddish background) suggested cigarette burns; however, this

could not be proven with certainty, although the presence of a cigarette butt in the bedroom pointed in that direction. However, it was necessary to pursue a diagnosis that was as evidence-based as possible for legal purposes. Authorities believed that it was even more necessary in this case, as there could be a charge of child abuse preceding the homicide (1).

Given the great versatility of SEM–EDX in forensic investigations, the authors believed it appropriate to attempt analysis of the skin lesions in question and the cigarette butt seized from the scene with an electron microscope equipped with EDX detectors. In detail, it was used as a "standardless" EDX system, in which no standards are needed, as the system reads and measures the unknown sample, comparing it with internal references, created specifically using a wavelength dispersive spectroscopy (WDS) microprobe. Analysis of the morphology and severity of the burns resulted in the analysts believing that the injuries were the result of intentional harm, constituting a crime of child abuse, which, due to the histological findings, was chronologically placed before the murder (1).

The authors believe that their pilot approach to a suspected cigarette burn with SEM–EDX highlights promising results that seem to make this technique valid for the study of these lesions and, therefore, worthy of further investigation. They also believe that advances in the study of cigarette burns can only be effectively made by raising the bar, resorting to more sophisticated investigative techniques (1,2).

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The Scene of the Crime: Using NIR and UV-Vis Spectroscopy in Bloodstain Dating

By Jerome Workman, Jr.

A recent study explores the effectiveness of Near-infrared (NIR) and ultraviolet-visible (UV-vis) spectroscopy in determining the time since deposition (TSD) of bloodstains, a critical aspect of forensic investigations. By comparing these two methods, researchers aim to improve the accuracy and reliability of bloodstain dating, with potential implications for real-world forensic applications.

In the field of forensic science, accurately determining the time since bloodstains were deposited at a crime scene is vital for reconstructing events and establishing timelines. Traditional methods, which relied on visual changes in blood color, have evolved into sophisticated analytical techniques, including spectroscopy. However, while UV-vis spectroscopy has been widely studied, there has been less focus on near-infrared (NIR) spectroscopy, despite its potential to overcome certain limitations of UV-vis methods. A recent study published in *Talanta* by researchers from the University of Genova seeks to address this gap by critically comparing the effectiveness of NIR and UV-vis spectroscopy in forensic bloodstain dating (1,2).

Read More: NIR in Bloodstain Analysis

The Study

The research, led by Sara Gariglio, Cristina Malegori, Alicja Menżyk, Grzegorz Zadora, Marco Vincenti, Monica Casale, and Paolo Oliveri from the Department of Pharmacy (DIFAR) at the University of Genova, aimed to evaluate the applicability of NIR spectroscopy for estimating the age of forensic bloodstains. The study involved collecting capillary blood samples from volunteers, which were then aged over 16 days and repeatedly analyzed using both NIR and UV-vis spectroscopic methods. The study utilized classical preprocessing techniques like Savitzky-Golay derivatives and standard normal variate (SNV) transform, along with targeted strategies such as class centering, which was shown to be beneficial through principal component analysis (PCA). Finally, partial least squares (PLS) regression models were applied to assess the accuracy of both NIR and UV-vis spectroscopy in estimating the time since bloodstain deposition (1).

Findings and Implications

The study's findings revealed that both NIR and UV-vis spectroscopy demonstrated comparable performance in estimating the TSD of bloodstains, with root mean square errors of prediction (RMSEP) of approximately 40 hours for UV-vis and 55 hours for NIR spectroscopy. These results indicate an improvement in NIR spectroscopy compared to existing literature, suggesting that NIR could be a valuable tool in forensic bloodstain dating. Moreover, the study explored data fusion strategies, combining both spectroscopic methods to enhance the accuracy of TSD estimation. Mid-level data fusion showed promise, reducing the RMSEP to 35 hours under optimal conditions (1).

Despite the positive results, the study acknowledges the challenges of applying these methods in real-world forensic scenarios. NIR spectroscopy's relative independence from preprocessing steps and its robustness make it more suitable for practical forensic applications, where environmental factors and other variables can

complicate analysis. However, the research also highlights the potential of combining multiple spectroscopic techniques to achieve more accurate and reliable bloodstain dating (1).

Conclusions and Future Directions

The study concludes that NIR spectroscopy, when integrated with appropriate chemometric strategies, deserves increased recognition in the field of forensic bloodstain dating. While both NIR and UV-vis spectroscopy offer valuable insights into blood degradation processes, their combined use could lead to more accurate TSD estimation. However, further research is needed to refine these methods and develop standardized procedures that can be reliably applied in real forensic cases (1).

Exploring the synergies between different spectroscopic methods and advanced chemometric approaches, such as data fusion, holds promise for refining forensic practices and strengthening the reliability of age estimation in criminal investigations. The study sets the stage for future research aimed at bridging the gap between laboratory findings and real-world forensic challenges, ultimately enhancing the accuracy and effectiveness of forensic investigations.

This groundbreaking work by the University of Genova team not only advances the scientific understanding of bloodstain dating but also paves the way for more reliable forensic practices, contributing to the broader field of criminal justice (1,2).

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Illicit Drug Analysis in Blood Samples with Multivariate Analysis Using Surface-Enhanced Raman Spectroscopy

Güneş Açıkgöz and Abdullah Çolak

This study aims to discriminate different types of illicit drugs (MDMA and THC) in blood samples using surface-enhanced Raman spectroscopy (SERS) combined with chemometric techniques such as principal components analysis (PCA) and partial least squares discriminant analysis (PLS-DA). A PLS-DA classification model was built using a training data set containing Raman spectra from control and experimental groups (drug-detected blood). PLS-DA was performed for discrimination and classification among blood samples. The scores obtained in the PLS-DA model were used to evaluate the performance of the created model. The leave one out cross-validation (LOOCV) method was used for calibration and validation of the PLS-DA model. In the study, it was observed that the SERS method and chemometric techniques could be used together in drug analysis, even at low concentrations in complex body fluids such as blood. As a result, Raman spectroscopy with PCA and PLS-DA methods of data analysis could be used extensively to build similar or different classification models.

Illicit drug use or addiction has become an important problem all over the world, as it causes many serious problems (1). The World Drug Report in 2020 stated that the estimated number of past-year users of any drug utilization globally increased from 210 million to 269 million from 2009–2018. Around 20.5 million people globally were estimated to have used "ecstasy" in the past year, corresponding to 0.4% of the global population aged 15 and older in 2018 (2).

Since 1971, amphetamine, methamphetamine, methcathinone, and "ecstasy"-group substances (3,4-methylenedioxymethamphetamine [MDMA] and its analogues) have been called amphetamine-type substances (3). Ecstasy and cannabis (tetrahydrocannabinol [THC]) are two of the most frequently encountered drugs in blood analyses for the detection of drug use. THC is the most important psychoactive ingredient in cannabis (4).

Biological samples, such as blood, urine, hair, nails, and saliva, are used to determine whether a person uses drugs or not (5). Today, different analysis methods are used for body fluid identification. These analysis methods can be classified as chemical tests, immunological tests, protein catalytic activity tests, or spectroscopic and microscopic methods. The methods are applied for the harmless examination of obtained evidences, such as blood, urine, and saliva, as they continue to develop. Although the results of both screening and validation tests are reliable, the methods take time and may delay treatment (6,7).

Raman spectroscopy is one of the most recommended methods, since it is a method that does not damage the sample and provides rapid results, with even a small amount of sample being sufficient for examination (7–11). Raman spectroscopy has been increasingly used in all scientific areas in recent years in different analyses, and has enabled the determination of illicit drugs without using additional chemicals (8). However, although Raman spectroscopy has many advantages, it is sometimes insufficient by itself to analyze a small amount of a component in blood. This is because the components in biological samples may generate very weak Raman peaks. For this reason, the use of the surface-enhanced Raman spectroscopy (SERS) technique is more advantageous in Raman examinations. The SERS method significantly increases the applicability of Raman spectroscopy (12). The SERS method is a spectroscopic technique used to

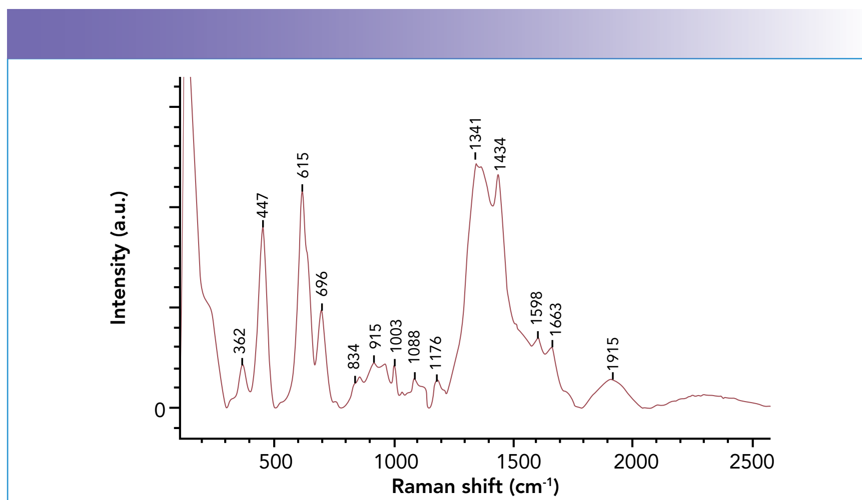

FIGURE 1: SERS spectrum of the blood sample.

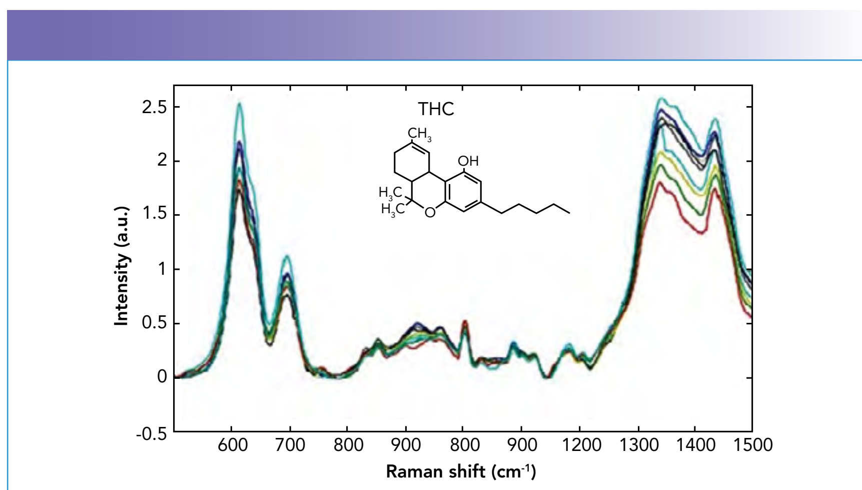

FIGURE 2: SERS spectra of the blood samples from patients with THC content.

induce vibrational transmission of adsorbed molecules on a rough metallic surface. Thanks to their electromagnetic and charge transfer mechanisms, metallic nanoparticles (Ag, Au, and others) are widely used in SERS applications. In addition, being able to control properties such as the size and the shape of nanoparticles facilitated the widespread application of the SERS technique in biomedical analytics and clinical diagnostics (13–16).

It is difficult to obtain an exact result, due to many different reasons (such as the classification of the spectra obtained by Raman spectroscopy examination of biological samples, as well as the limited number of samples). For this reason, chemometric methods are needed to classify many variables (17) and are used for both qualitative and quantitative analysis of the data while being categorized as supervised or unsupervised methods. Principal component analysis (PCA) is an unsupervised method, and is convenient for analyzing unknown spectra. A small amount of unknown data can be obtained, such as principal components (PC) and loadings (eigenvectors). Another preferred reason for applying PCA to the data obtained from the spectra in the studies is that only some components in a mixture are known (18). Partial least square (PLS) regression is a supervised method, and generally applied in cases where there is a limited number of samples. Calibration and validation are a two-stage process for the supervised methods (19–21). Conditions, such as the fact that the PCA method is an unsupervised method, do not necessarily reveal the relevant patterns, and the performance of PLS largely depends on the number of components to be used in discriminant analysis (DA) (17). PLS-DA is a linear classification method based on PLS regression, and it is the most popular chemometric tool used for qualitative and quantitative modeling of multidimensional data. The large data in human blood samples can give rise to problems in the analysis. Thus, the study aims to discriminate different types of illicit drugs (MDMA and THC) in blood samples using the SERS method combined with multivariate analyses. For this purpose, SERS spectra of the blood samples were analyzed. PLS-DA and PCA were performed for discrimination and classification among blood samples.

Materials and Methods

Preparation of Samples

This study was approved by the Health Sciences Central Ethical Committee (14.10.2021/01). Consent forms were signed and a total of 22 blood samples were taken from two groups: an experimental group (drug users) who were brought to the health service for different reasons, and the control group (non-drug users) in whose blood drugs were not contained. Blood samples were taken into ethylenediaminetetraacetic acid (EDTA) tubes and stored at -20 °C in a freezer until the examination was made. In all examinations, slides that were compatible with Raman spectroscopy and did not change the peak of the sample were used. Silver nanoparticles (AgNPs) were synthesized by reduction of $AgNO_3$ using trisodium citrate according to the Lee and Meisel method (22). Chemicals were supplied to synthesized AgNPs from Sigma Aldrich. An ultraviolet-visible (UV-Vis) spectrophotometer and Scanning Electron Microscopy (SEM-EDS) analysis unit (JEOL 5500-OXFORD Inca-

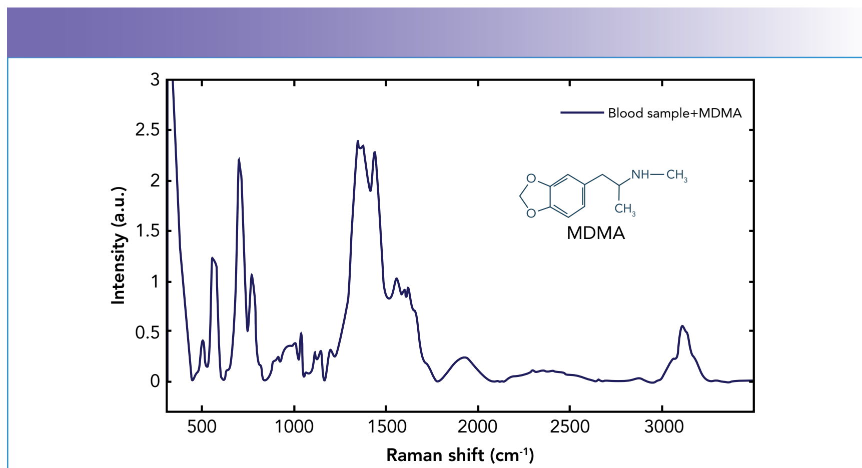

FIGURE 3: SERS spectrum of the blood serum samples from a patient with MDMA content.

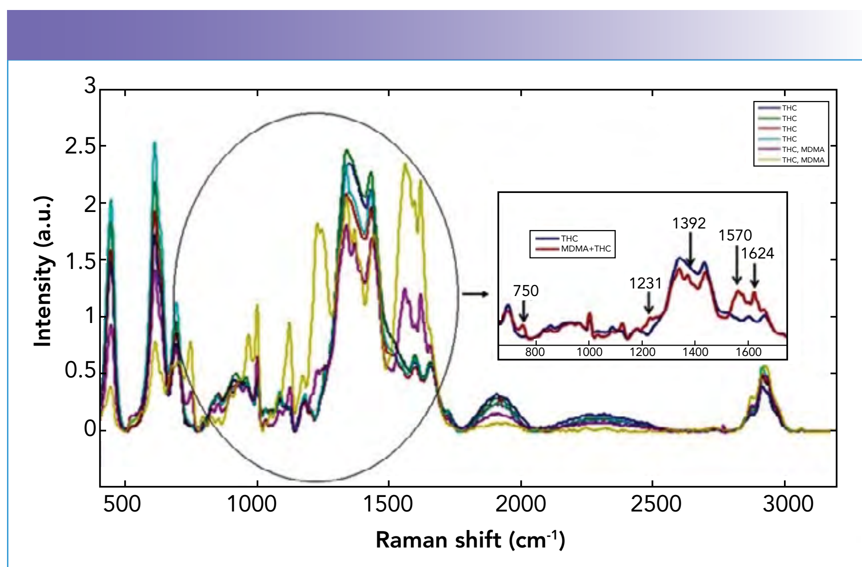

FIGURE 4: Raman spectra of the blood samples with THC and both THC and MDMA (different peaks observed in blood with MDMA are indicated by black arrows).

X) were used to characterize AgNPs. Before SERS measurements, 15 µl of AgNPs and 15 µl of blood samples were separately dropped on the slides and allowed to dry at room temperature and in the dark. After drying, Raman spectra of blood samples were obtained by the SERS method.

Equipment

All samples were measured with Renishaw's Via Raman spectroscopy instrument equipped with a charge-coupled device (CCD) and the same parameters. The parameters were taken as 5 mW laser power, 50x objective, 785 nm diode laser for excitation, and 40 s exposure time. Raman bandwidth was chosen as 100 to 3200 cm^{-1}.

Data Analysis

Spectral collection and data pre-processing (such as smoothing, fourth polynomial baseline correction, and vector normalization) from Raman spectroscopy were acquired using the WiRE 3.2 software (Renishaw plc). In the study, each sample was analyzed five times, then averaged to form a spectrum representing one sample. A total of 889 spectral data were created in the region between 500–1600 cm^{-1}, which forms the fingerprint region and has different peaks, from the Raman spectra of 22 (11 positive, 11 negative) blood samples. The data were transferred to the MATLAB (Matlab 7.13, The Mathworks) program and analyzed using PLS Toolbox (Eigenvector Research) for Matlab users, accounting for both the PCA and PLS-DA methods.

The PCA method was used for the analysis of 11 blood samples (2 MDMA, 1 THC + MDMA, and 8 THC), which were determined to be different drugs. Second, the PLS-DA method was used to optimize the separation between drug-containing and drug-free blood.

Results and Discussion

SERS Analysis

In the study, a SERS spectrum of the blood sample was obtained, and Raman bands were analyzed (Figure 1). The Raman band at 495 cm^{-1} involved vibrational modes of amino acids (23). The peaks at 1003, 1341, 1434, 1598, and 1663 cm^{-1} were assigned to hemoglobin and its derivatives (10). Raman bands at 1341 and 1434 cm^{-1} corresponded to deformations of the carbon rings and the characteristic bending motions of the CH, CH_2, and CH_3 group amino acid deformation modes. The bands at 1003 cm^{-1} and 1663 cm^{-1} involved stretching motions of the phenylalanine, C-C skeletal and amide, C=C groups in unsaturated fatty acids correspondingly (24,25). The bands at 1176 cm^{-1} and 1598 cm^{-1} correspond to the vibrations of C-C and C=C stretching.

The SERS spectra obtained in blood samples containing THC and MDMA are shown in Figure 2 and Figure 3, respectively. It was observed that the strong SERS bands obtained in blood samples containing THC were the same in the fingerprint range of 500–1600 cm^{-1} (Figure 2). However, it was observed that there were changes at the 615, 696, 1341, and 1434 cm^{-1} bands of blood samples containing different amounts of THC. The weak Raman bands at 830–860 cm^{-1} were assigned to stretching of the middle tetrahydro-

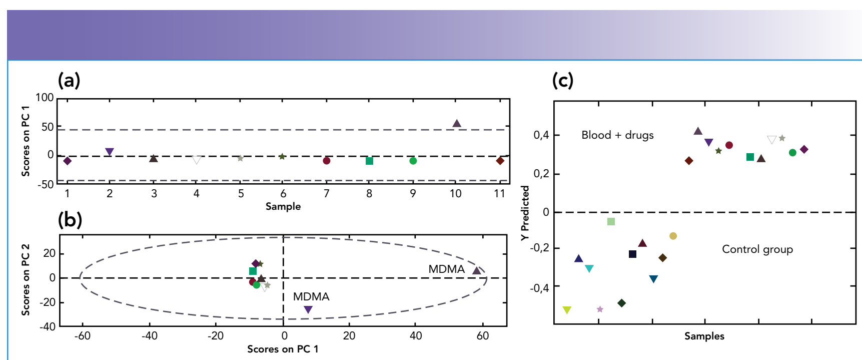

FIGURE 5: PCA scores plot for the first two components of the spectral variance of eleven blood samples: (a) PC1 was the most discriminatory where PCs indicated the importance of drugs in a given blood sample. PC1, which explains most of the variance, was located on the top as positively and strongly correlated with MDMA; (b) The figure of PC2 versus PC1; (c) Prediction scores for blood samples using PLS-DA model with control group and different drugs loaded as predictions. Dashed line represents the default classification threshold.

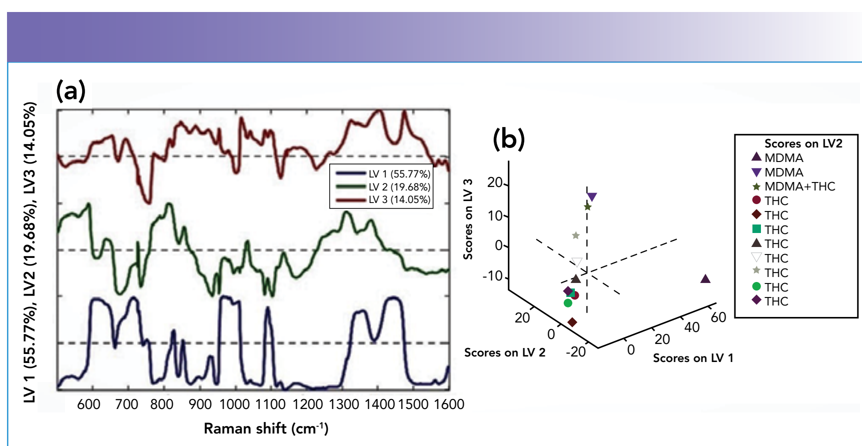

FIGURE 6: Loading plots; (a) Latent variables representation with different percentages obtained by the PLS-DA method; (b) Scores on LV2.

pyran ring, the weak Raman bands at 1003 and 1088 cm^{-1} corresponded to phenyl ring peak intensities, and the weak Raman bands at 1341 cm^{-1} were assigned to CH deformation modes (26). The characteristic Raman bands of blood samples containing different amounts of THC were detected at 712, 1006, 1310, 1390, 1450, and 1590 cm^{-1}. These bands corresponded to vibrations of, respectively, (C-H) deformation, (C-C) stretching, (C-H) deformation, (=C-H) deformation, (C-H) deformation, and C=C stretching (27).

In a study by Barnett and Rathmell, the Raman peaks of THC were examined by changing the THC concentrations and it was observed that these peaks were observed at 710, 999, 1130, 1232, 1352, and 1390 cm^{-1}. It was also shown that stronger peaks could be obtained at increasing concentrations (28).

The MDMA peaks differed in a blood sample taken from a person using both MDMA and THC (Figure 4). The most noticeable Raman peaks that distinguished MDMA from THC were found at 750, 1231, 1392, 1570, and 1624 cm^{-1}. These bands can be used to confirm the presence of MDMA. The Raman band at 1624 cm^{-1} corresponded to vibrations of NH deformation.

Sagmuller and associates stated that there are peaks of C-H and C-C vibrations belonging to methyl and ethyl groups in the region between 900 and 1650 cm^{-1} for MDMA analysis (1). Westa and coauthors (9) stated that the most prominent Raman peaks for MDMA were at 716 cm^{-1} and 810 cm^{-1}. Ryder and associates applied different chemometric analyses by dividing into regions between 450 and 1100 cm^{-1} by micro-Raman spectroscopy. Four different Raman peaks were observed between 700 cm^{-1} and 900 cm^{-1} as MDMA peaks in solid mixtures (29). It was observed that the results obtained from the studies were compatible with our study.

Inscore and coauthors performed a drug substance analysis in saliva with the SERS method in which it was shown that the obtained Raman peaks according to the type of drug used and the varying amount of the same substance were also different in their study (30). In the study, SERS of MDMA was found to be at 530–535, 715–720, 1250, 1365–1370, 1430–1435, 1470–1480, and 1620 cm^{-1}, largely due to the dioxole ring alone or coupled with the phenyl ring (26).

PCA Analysis

PCA, which is used as the most preferred method for qualitative classification, has proven very useful in different studies (21,31,32), as well as in our study, to visualize spectral data and examine possible sample groupings according to spectral characteristics.

When the SERS spectra of the blood samples containing THC and MDMA were examined, distinctive peaks were obtained at 500–1600 cm^{-1}. The PCA method was performed for pattern identification of different types of objects in cluster form among illicit drugs of blood samples. In the PCA model, three different principal components (PCs) with different percentages were obtained. It was observed that the percentages of these variables were 55.77% and 19.68%, respectively, and the model was classified depending on two PCs, which would make up 75.45% of the total variance. When PCA was applied to the data sets obtained for the analysis of blood samples containing dif-

ferent drugs, it was observed that the data were clustered differently. The PC1 and PC2 scores interpreted the highest variance, and the plot and loadings of two factors are shown in Figure 5a and Figure 5b. The figure of PC2 versus PC1 was illustrated in Figure 5b. PC1 was the most discriminatory, as illustrated in Figure 5a. The PCs indicated the importance of drugs in a given blood sample. In Figure 5a, PC1, which explains most of the variance, was located on the top as positively and strongly correlated with MDMA.

Using a small number of PCs is sufficient, as it uniquely captures the effect of a certain combination of relevant identifiers. For this reason, two PCs were sufficient for the visualization of the investigated blood samples (33). Another preferred reason for the application of PCA to the obtained data from the spectra in the study was that only some components in a mixture were known (18).

PLS-DA Analysis

In this study, since the variability of the data obtained by Raman spectroscopy was high, PLS-DA analysis was applied to determine the variable to be examined. Blood taken from the experimental group (drug users) and the control group (non-drug users) were divided into two groups for PLS-DA analysis. SERS spectra obtained from these two separated groups were transferred into the PLS toolbox as a training and test set. A PLS-DA classification model was built using a training data set containing SERS spectra from the control and experimental groups. The scores obtained in the PLS-DA model were used to evaluate the performance of the model. The leave one out cross-validation (LOOCV) method was used for calibration and validation of the PLS-DA model. Sensitivity, specificity, class error, the root mean square error of calibration (RMSEC), the root mean square error of cross-validation (RMSECV), and R^2 values were calculated to measure the difference. These values were used to describe the performance of the classification model.

RMSEC values were used to differentiate between the reference and the test set. CV values were used for the low number of samples for validation of a classification model, and RMSEC values were also used to evaluate the compatibility of the calibration model with the calibration set (31,34).

The PLS-DA models gave sensitivity and specificity values on the test set in the range of 88–100%. The sensitivity values were found to be on the test set in 100%, and specificity values were found to be on the test set in the range of 81.8–90.9% for the PLS-DA models. RMSEC values were found to be 2% and 3%. RMSECV values were found to be 0.37% and 0.58%. Class error values were found to be between 0 and 9%. The obtained values were found separately, calculated, and cross-validated. Obtained scores using the PLS-DA model with the control and experimental groups were loaded as predictions (Figure 5c, and the model was built with three latent variables (LVs). When the loading plots were examined, it was observed that there were significant differences in blood samples, blood samples with THC, and blood samples with MDMA in the region between 500 and 1600 cm^{-1} (Figure 6a). When the wavelengths of LVs were examined, it was observed that they resembled the spectra obtained from blood samples, blood samples with THC, and blood samples with MDMA (LV1, LV2, and LV3, respectively) and were compatible with the stated respective Raman bands. Scores on LV2 were used to create three-dimensional plots of blood using latent variables (Figure 6b). The LV values obtained in this method allowed for the visualization of different variables. Over-fitting in PLS models reduced the probability of estimation of the results. Therefore, the selection of latent variables was important. Too many latent variables can cause high bias and low variance (35).

Conclusion

The combination of Raman spectroscopy and chemometric techniques allows for the analysis of even the smallest analyte that can be found in biological samples. We have demonstrated an analysis of Raman spectroscopy that is a viable tool for the rapid, non-invasive detection of different illicit drugs in blood samples. It has been observed that the SERS method and chemometric techniques together can be used in drug analysis, even at low concentrations in complex body fluids. RS with PCA and PLS-DA methods of data analysis can be used extensively to build similar or different classification models.

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Physicochemical Analysis and Detection of Rice Syrup Adulteration in Kelulut Honey Using Portable Near-Infrared Spectroscopy

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Kelulut honey is prominent and valuable in the honey market because of its abundant health benefits. However, many adulteration cases of Kelulut honey have been reported. Rice syrup is one of the emerging adulterants used in Kelulut honey. This study aimed to assess and detect adulteration of Kelulut honey with different percentages of rice syrup using near-infrared (NIR) spectroscopy. All honey samples and rice syrup adulterants were stored at low temperatures. In this research, a NIR spectrometer from Texas Instruments was used to detect moisture content, electrical conductivity, water activity, hydroxy methyl furfural content, and honey color. The moisture content, electrical conductivity, water activity, hydroxy methyl furfural (HMF) content, and the color of the samples decreased when the adulterated percentage of honey increased except pH. The HMF content and pH did not comply with Malaysian standards. The physicochemical tests showed a significant positive correlation with each other, excluding the pH (p < 0.01). Later, spectra were collected for NIR spectroscopy and were pre-processed using cutting, extended multiplicative signal correction (EMSC), and Savitzky-Golay filter, followed by prediction of regression models. For NIR spectroscopy detection, the principal component regression (PCR) model showed higher accuracy (R^2 = 0.914) than the partial least squares (PLS) model. This result suggests that the PCR model was the better prediction model. Therefore, this study facilitated the development of a rapid and non-destructive method to detect Kelulut honey adulteration using handheld NIR spectroscopy technology.

Honey is a natural sweetener made by bees from nectar. It has been utilized for centuries for its nutritional and therapeutic benefits (1). Kelulut honey is produced by *Heterotrigona itama* bees, whereas Melaleuca flowers provide the majority of the nectar. Honey is often used as a sweetener or an ingredient in confectionery and pastry products. It is also used in the pharmaceutical and medical sectors (2). Natural honey imports climbed from 6454 to 6695 tons between 2017 and 2019, according to International Trade Statistics, owing to a growth in the population as well as a rise in the inclination for natural foods (3).

According to BERNAMA Malaysia (4), stingless bee farming, also known as Kelulut, is gaining attraction in the honey business, bringing in RM 33.6 million in Malaysia in 2020. Kelulut honey is often found in tropical and subtropical regions of the world. When compared to honeybees, Kelulut bees are more disease- and pest-resistant, non-toxic, and superb pollinators, according to several studies, resulting in their honey having antibacterial, antiulcer, and other health advantages (5,6). However, adulteration of honey has becoming more common over time (7). Adulteration is the process of adding or removing components from food, resulting in a decrease of quality (8). An adulterant is a material introduced to food that could be harmful or harmless to human health (9). According to the Deputy Director of MARDI, 15 out of 270 samples of Kelulut honey at the local market were fake honey (10). Since 2016, seven samples out of 77 examined samples from various honey brands have been found to be in violation of the Food Regulations 1985, according to the Director-General of Health Malaysia (11).

Adulteration is commonly accomplished by mixing sucrose syrup from sugar beets, maltose syrup, high-fructose syrup, or

FIGURE 1: DLP NIRscan Nano EVM (NIR spectroscopy equipment).

TABLE I: Preparing adulterated samples for physicochemical tests (*n* = 1)

Adulteration Percentage (%)	Amount of Kelulut Honey (g)	Amount of Rice Syrup (g)
0	26	0
10	23.4	2.6
20	20.8	5.2
30	18.2	7.8
40	15.6	10.4
50	13	13
60	10.4	15.6
70	7.8	18.2
80	5.2	20.8
90	2.6	23.4
100	0	26

TABLE II: Preparation of adulterated samples for NIR spectroscopy (*n* = 1)

Adulteration Percentage (%)	Amount of Kelulut Honey (g)	Amount of Rice Syrup (g)
0	70	0
10	63	7
20	56	14
30	49	21
40	42	28
50	35	35
60	28	42
70	21	49
80	14	56
90	7	63
100	0	70

industrial syrups like glucose and fructose straight into honey (12). Rice syrup is now one of the most often used adulterants (9). It is made from C3 plants, which are similar to honey (13). Calvin and Benson photosynthesis cycles are followed by C3 plants. This made conventional detection procedures like carbon isotope ratio analysis and thin-layer chromatography (TLC) ineffective in detecting honey adulteration with rice syrup (14). High performance liquid chromatography (HPLC), electronic nose, Fourier transform infrared spectroscopy (FT-IR), TLC, mid-infrared spectroscopy (MIR), carbon isotope ratio analysis test, and other detection techniques have been used in the honey business to detect adulteration (15–19). Detection techniques, such as HPLC, TLC, and MIR, take longer and may damage the sample. Because the carbon isotope ratio profiles of rice syrup and honey are similar, ^{13}C:^{12}C isotope ratio analysis is useless for detecting rice syrup in adulteration.

Furthermore, rice syrup is made using hydrolysis of polysaccharides and oligosaccharides, making it difficult to identify adulteration using TLC and HPLC (20). As a result, simple, reliable, and nondestructive techniques for determining the chemical composition of honey are urgently needed (21). Recent research has looked at near-infrared (NIR) spectroscopy, which is a fast and sensitive detection tool. It requires only a tiny sample amount, and it can be operated on-site, making it ideal for bulk monitoring (22). As a result, the purpose of this research was to improve the quality assessment and detection of rice syrup adulteration in Kelulut honey. To do so, the impact of various percentages of rice syrup in Kelulut honey was evaluated by examining the physicochemical properties and comparing the results to the Malaysian standard (23). Aside from that, the goal of this research was to see if utilizing NIR spectroscopy and regression models can identify the addition of varying amounts of rice syrup in Kelulut honey.

Materials and Methods

All the samples of H&B honey (Bee farm Malacca) were kept in a clean, dry container with labeling in the storage room between 0 °C and 4 °C. The rice syrup (Radiant Whole Foods Organic) adulterant was kept cold as well. Other materials used were the Carrez solution I, Carrez solution II, filter paper, volumetric flask, test tubes, sodium bisulphite, and the spectrophotometer. In this research, the NIR spectroscopy equipment used was the DLP NIR scan Nano EVM by Texas Instruments (Figure 1).

Sample Preparation

The Kelulut honey was mixed with rice syrup in various quantities to create adulterated samples. The proportion

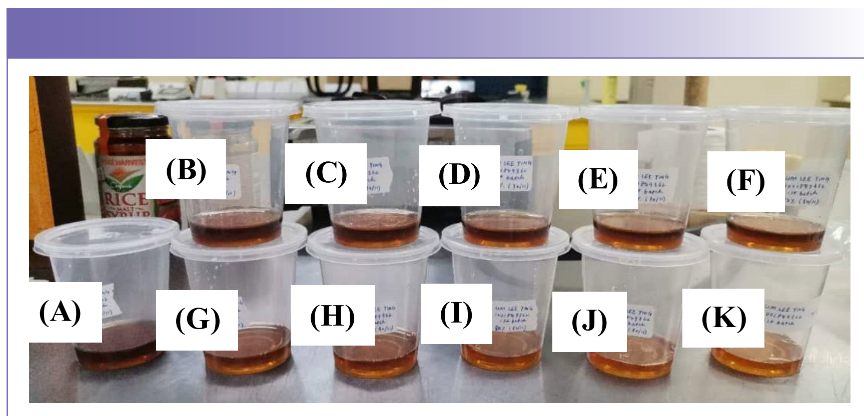

FIGURE 2: Adulterated Kelulut honey samples with 0% (A), 10% (B), 20% (C), 30% (D), 40% (E), 50% (F), 60% (G), 70% (H), 80% (I), 90% (J) and 100% (K) rice syrup added.

of adulteration for the study was 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% (w/w) (Figure 2, Tables I and II).

Physicochemical Analysis

The moisture content, HMF, and pH were determined using a Digital Brix refractometer, spectrophotometer, and pH meter, respectively (23). The HMF content was then calculated using Equation 1. The electrical conductivity was measured with an electrical conductivity meter, according to Fatima and others (24). The sample's water activity (25) and color (26) were also determined.

$$((A284 - A336) \times 149.7 \times 5 \times D)/W \quad [1]$$

A_{284} refers to the absorbance at 284 nm; A_{336} refers to the absorbance at 336 nm; D is the dilution factor; and W is the weight of honey taken.

NIR Spectroscopy Detection

Samples were scanned in triplicate, placed in a cuvette, and scanned for 14 d at wavelengths ranging from 900 to 1700 nm, both inside and outside. Each spectrum was collected in an average of 6 scans in 30 s. For more accurate results, aluminum foil was wrapped around the samples. To increase the accuracy, a total of 1848 spectra were obtained using the same cuvette. The spectra were then submitted to a regression model after pre-processing. To maximize the chemical composition contribution, pre-processing was required (27) and Orange Data Mining software was utilized (Bioinformatics Lab, University of Ljubljana, Slovenia). Cutting, extended multiplicative signal correction (EMSC), and the Savitzky-Golay filter were all included. Following the cutting, the spectra from 950 nm to 1650 nm were saved. The EMSC was carried out in the polynomial order of two. After that, a Savitzky-Golay filter was used with a window of 5 and a polynomial order of 2. Principal component regression (PCR) and partial least square (PLS) regression were used as regression training models in this investigation. The pre-processed spectra data was split into two groups, with 80% of the data serving as training data and 20% of the data serving as testing data. After that, the training and testing data sets were given to the PCR and PLS for prediction, with the correla-

TABLE III: Physicochemical properties of Kelulut honey, adulterated honey samples with rice syrup and rice syrup

Percentage of Rice Syrup Added (%)	Moisture Content (%)	Electrical Conductivity (mS/cm)	pH	Water Activity	HMF (mg/kg)	Color (Abs)
0	28.58 ± 2.06[a]	0.441 ± 0.005[a]	3.11 ± 0.00[b]	0.77 ± 0.01[a]	446.74 ± 4.56[a]	0.160 ± 0.025[a]
10	25.64 ± 1.00[ab]	0.413 ± 0.005[b]	3.12 ± 0.02[b]	0.77 ± 0.01[ab]	435.25 ± 2.12[a]	0.128 ± 0.015[ab]
20	24.95 ± 0.02[b]	0.390 ± 0.006[c]	3.16 ± 0.01[b]	0.76 ± 0.00[ab]	408.8 ± 16.90[b]	0.137 ± 0.006[ab]
30	23.87 ± 0.12[b]	0.366 ± 0.015[d]	3.17 ± 0.05[b]	0.76 ± 0.01[ab]	397.08 ± 0.74[bc]	0.125 ± 0.010[b]
40	23.19 ± 0.32[b]	0.326 ± 0.004[e]	3.22 ± 0.04[b]	0.75 ± 0.01[b]	375.26 ± 0.69[c]	0.116 ± 0.002[bc]
50	22.45 ± 1.01[bc]	0.295 ± 0.012[f]	3.27 ± 0.07[b]	0.74 ± 0.02[bc]	342.55 ± 1.11[d]	0.114 ± 0.009[bc]
60	21.91 ± 2.73[bc]	0.253 ± 0.003[g]	3.34 ± 0.06[b]	0.74 ± 0.00[bc]	316.65 ± 2.38[de]	0.108 ± 0.010[bc]
70	19.48 ± 0.69[c]	0.212 ± 0.003[h]	3.43 ± 0.07[b]	0.72 ± 0.00[c]	288.92 ± 3.07[e]	0.097 ± 0.012[bc]
80	20.24 ± 2.97[c]	0.191 ± 0.007[i]	3.60 ± 0.10[b]	0.71 ± 0.00[cd]	268.87 ± 0.90[e]	0.087 ± 0.013[c]
90	17.41 ± 1.19[c]	0.122 ± 0.002[j]	3.87 ± 0.16[b]	0.70 ± 0.00[cd]	223.50 ± 17.80[f]	0.075 ± 0.014[c]
100	16.89 ± 0.46[c]	0.029 ± 0.002[k]	5.84 ± 1.02[a]	0.70 ± 0.01[d]	167.03 ± 10.43[g]	0.070 ± 0.007[c]

***The same letter in the same column indicates no significant differences from each other ($p > 0.05$)**

tion coefficient (R^2) and root mean square error (RMSE) being evaluated as prediction performance. The greater the R^2 and the lower the RMSE, the better the models' performance (28).

For multivariate data analysis, PCR is a linear regression model derived from principal component analysis (29). According to Salleh and others (30), PCR first uses PCA to deecompose the data matrix X into a new latent variable. The score gained is then used to do multiple linear regression between it and the selected property of interest, Y. Equation 2 represents the linear model of PCR. PCR was used to extract feature representations from collinear and high-dimension data (31):

$$Y = Ab + e \quad [2]$$

with A = weighted normalized matrix of order (n x p), which the new coordinates for the n objects in the new system; p = loading - PCs, which refers to the loading matrix and the column vectors; b = coefficients; and e = error vector.

On the other hand, because of its simplicity and modest volume of computations, PLS is one of the most often used chemometric algorithms in quantitative NIR spectroscopy analysis to create the calibration model (32,33). When a large number of dependent variables (Y) must be estimated from a large number of independent variables (X), this method is quite beneficial. The best number of latent variables is determined using cross-validation based on the minimum prediction error (30). In both domains, it is a latent method to modeling covariance structures (34).

Statistical Analysis

Three triplicates of each experimental treatment (n = 3) were taken. The diversity between the physicochemical characteristics of different percentages of rice syrup-adulterated

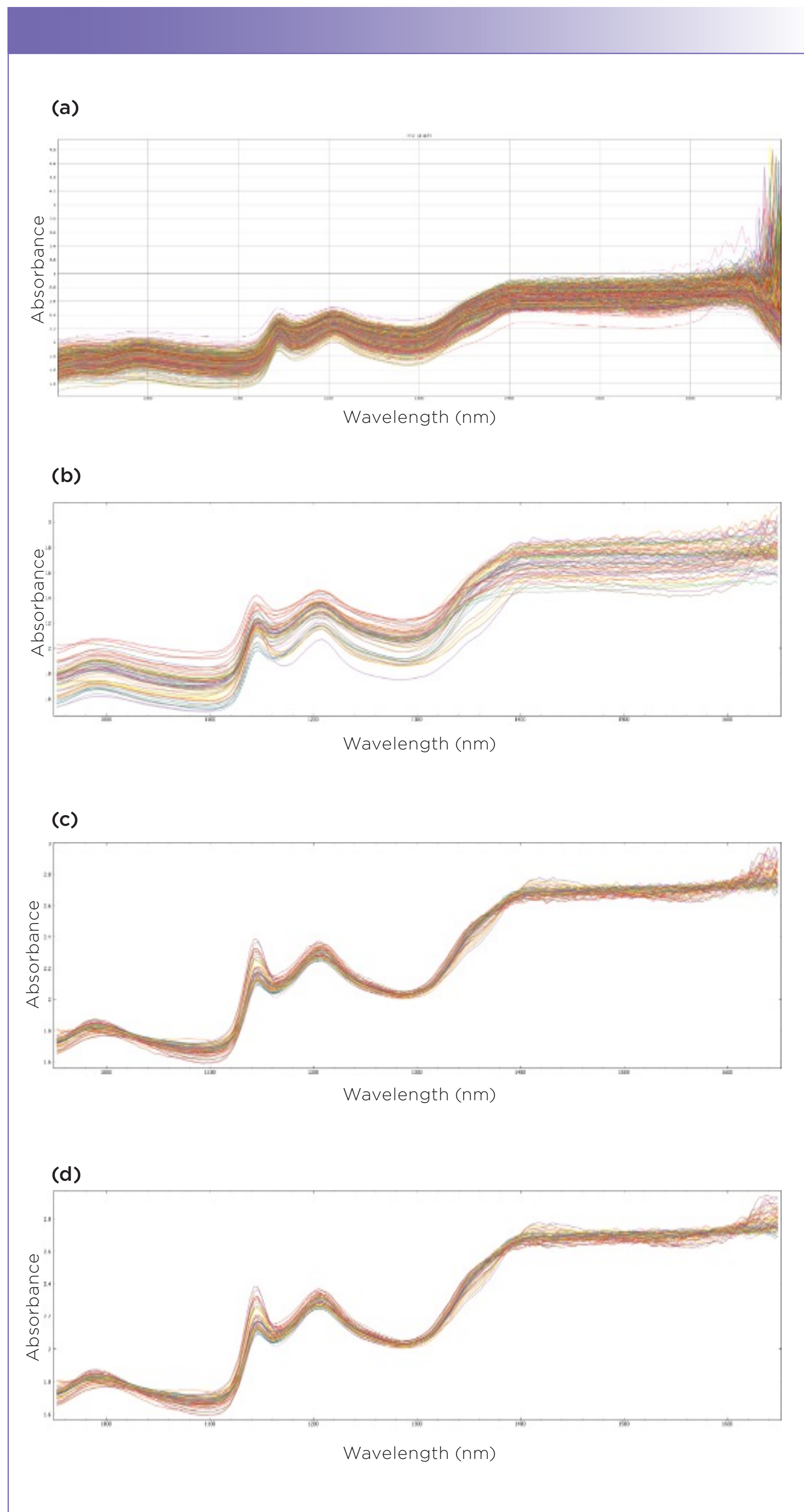

FIGURE 3: (a) Spectra before pre-processing; (b) spectra after cutting; (c) spectra after cutting and EMSC; (d) and final spectra after cutting, EMSC, and Savitzky-Golay filter.

TABLE IV: Pearson correlation coefficients between physicochemical properties of different adulterated honey samples

	Moisture Content	HMF	pH	Water Activity	Color
HMF	0.967				
pH	-0.703	-0.806			
Water activity	0.957	0.977	-0.699		
Colour	0.983	0.958	-0.704	0.951	
Electrical conductivity	0.968	0.999	-0.821	0.97	0.961

Kelulut honey was determined using an ANOVA test and Pearson correlation with a significance of $p \leq 0.05$. Minitab 17 (Minitab, LLC) was used to analyze the data gathered in this study.

Results and Discussion

Physicochemical Properties

Table III lists the physicochemical characteristics that were determined in this investigation. The moisture content was significant because it affects the honey's resistance to fermentation and granulation (35). When 0–70% rice syrup is applied, the moisture content drops from 28.58% to 19.48%, as shown in Table III. However, increasing the amount of rice syrup from 70% to 100% did not result in a significant reduction in moisture content ($p > 0.05$). This is in line with El-Bialee and Sorour's (36) findings that the moisture level of several contaminated samples was likewise lower than the pure honey sample. As a consequence, the findings are satisfactory, and the lower the moisture level of the honey samples, the greater the proportion of rice syrup applied. According to Ribeiro and others (17), the moisture content of honey increases progressively with the addition of adulterants, from 17.60% in pure honey to 22.80% in 100% adulterated honey. The moisture level of Kelulut honey shall not exceed 35%, according to Malaysian standards (23). Pure honey had a moisture content of 28.58%, whereas the contaminated honey samples had a moisture content of less than 35%, suggesting that the moisture content criterion could not be utilized to identify whether the honey was adulterated or not.

Honey's electrical conductivity is linked to its mineral content, organic acids, and protein content (25). When 0–100% rice syrup was applied, the electrical conductivity drops from 0.441 mS/cm to 0.029 mS/cm, as indicated in Table III. The results showed a decrease in the mineral level when the amount of rice syrup added increases. Geographical origin, botanical origins, and entomological origins were all plausible influences on the honey's electrical conductivity (25). According to Fatima and others (24), the electrical conductivity of the pure honey sample was 0.47 and 0.55 mS/cm, which was virtually the same to 0.441 mS/cm in the study.

When 0–90% rice syrup was applied, there was no significant difference ($p > 0.05$) in the pH change, which ranged from 3.11 to 3.87. However, increasing the amount of rice syrup from 90 to 100% results in a pH rise from 3.87 to 5.84. The pH of the honey increased as the amount of corn syrup added to the honey increased, according to Ribeiro and others (17). The pH of pure Kelulut honey was 3.11, which was within the Malaysian standard range of 2.5 to 3.8 (23). The pH, according to Malaysian standards, is comparable to 10–80% rice syrup adulteration. Hence, it is unable to determine whether the honey is adulterated or not because the pH of adulterated honey samples (10–80%) are also within this range, designed for the pH of pure Kelulut honey as per the Malaysian standards. The extraction, storage condition, and temperature were all factors in the increased pH of adulterated honey samples, according to Wan and others (37).

According to this study, the water activity in adulterated honey samples decreases from 0.77 at 0% to 0.70 at 100%, indicating that the food products are microbiologically stable (38). According to Chirife and others (39), there is a linear relationship between moisture content and water activity, as seen in Table III. Honey's water activity is usually between 0.60 and 0.65 (25). The water activity of the pure Kelulut honey sample in this investigation was 0.77, which is similar to the results that Shamsudin and others (40) and Yap and others achieved (41).

HMF is a highly reactive compound that can go through the Maillard process (38). It was influenced by the sugar content and the pH of honey, according to Singh and Bath (42). According to the study, HMF concentration drops from 435.25 mg/kg at 10% to 167.03 mg/kg at 100% with significant differences at 10–70% and 80–100%. However, there is no significant change ($p > 0.05$) in HMF content between 0–10% and 70–80% adulteration. According to the Malaysian standard (23), the HMF concentration of pure honey shall not exceed 30 mg/kg, but pure Kelulut honey in this study exceeded it. Nonetheless, Wan and others (37) reported that it was between 35.4 and 461.7. Variable storage conditions, processing, preparation, and ageing are all probable causes of increased HMF concentration in pure honey (37). Because of the heating used during adulteration, the HMF concentration of adulterated honey samples was substantially greater than that of pure honey, according to Samat and others (43).

For the color analysis, the absorbance of honey was reduced by adding rice syrup, which decreases from 0.160 at 0% to 0.087 at 80%. However, at 80–100% rice syrup adulteration,

there is no significant variation in absorbance ($p > 0.05$). According to Fatima and others (24), the absorbance of Kelulut honey ranged from 0.239 to 1.056, which is greater than the current study. This might be attributed to a variety of variables, including mineral content, geographical and botanical origin, storage time, and light duration of honey (24).

The Pearson correlation coefficients between the physicochemical parameters investigated in this study were shown in Table IV. The pH parameter was the only one that exhibited a significant negative association ($p < 0.01$) with moisture content, HMF concentration, water activity, color, and electrical conductivity. Except for pH, all other variables exhibited substantial positive correlations ($p < 0.01$), indicating that the results are significantly linked.

NIR Spectroscopy

The wavelength varied from 900 nm to 1700 nm, and a total of 1848 spectral data were obtained. Figure 3a shows the NIR reflectance spectra of Kelulut honey samples that were adulterated with 0 to 100% rice syrup. Because of the background data and noise generated by light scattering, it is impossible to differentiate between the honey samples (44). As a result, pre-processing is necessary to reduce noise and create highly twisted spectra. Figure 3b depicted the spectra after cutting; the wavelengths of the spectra varied from 950 nm to 1650 nm. The non-informative spectral data was removed, leaving the relevant spectral data. Then, the spectra were improved further using EMSC (Figure 3c). The slope and intercept of this linear fit are used to regress each spectrum to a model spectrum (45). Finally, the Savitzky-Golay filter was employed to minimize background data noise. Figure 3d shows the final condensed and uncluttered spectra after all of the pre-processing procedures.

To detect the adulteration level of the honey samples from the spectra of NIR spectroscopy, regression model needs to be developed and validated (21). In this study, PCR and PLS were used to be the predictive models and were regressed with actual recorded value in *x*-axis and predicted value by the models in *Y*-axis. PCR and PLS regression prediction models were built using the training data set while the predictions were made on the testing data set. Figure 4 shows the graph of predicted and actual adulterated percentages of the adulterated Kelulut honey samples from the training data and testing data using the PCR model. On the other hand, Figure 5 shows the predicted and actual adulterated percentages of the adulterated Kelulut honey samples from the training data and testing data using the PLS model. The majority of the prediction data falls into the same adulteration percentage group as the training data set, implying that these two models may be utilized to identify and forecast adulterated rice syrup percentages in Kelulut honey. When these two graphs were compared,

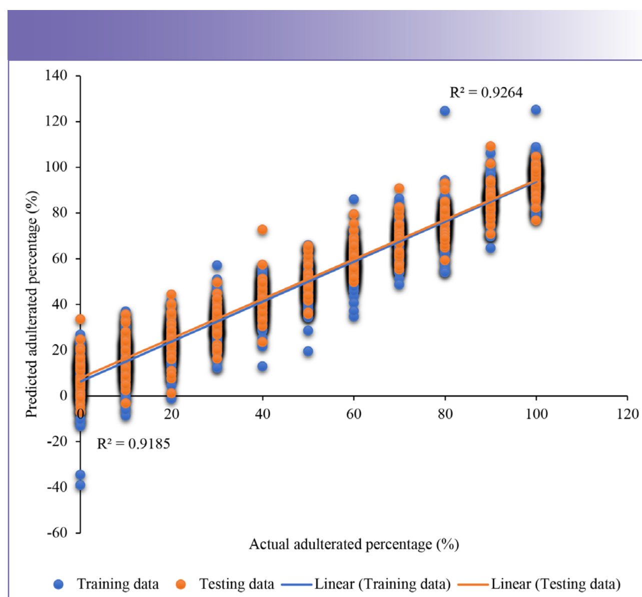

FIGURE 4: Plot of regression results using PCR for training (R^2 = 0.924) and testing (R^2 = 0.914). The blue dots represent the training data set, whereas the orange dots represent the testing data set.

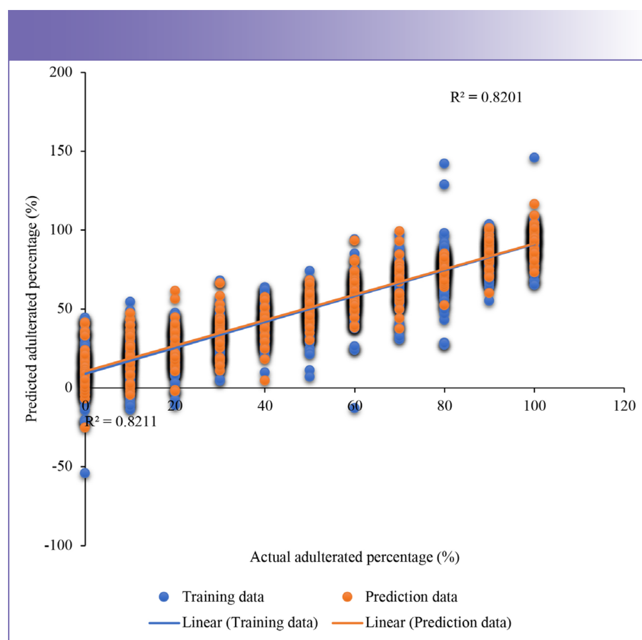

FIGURE 5: Plot of regression results using PLS for training (R^2 = 0.821) and prediction (R^2 = 0.818). The blue dots represent the training data set, whereas the orange dots represent the testing data set.

the PCR model had a better prediction performance since its R^2 in the training and testing data (0.924 and 0.914) was greater than the PLS model (0.821 and 0.818).

Increased noise levels in the NIR spectra above 1400 nm may have an effect on the precision and dependability of calibration models. High noise can increase uncertainty

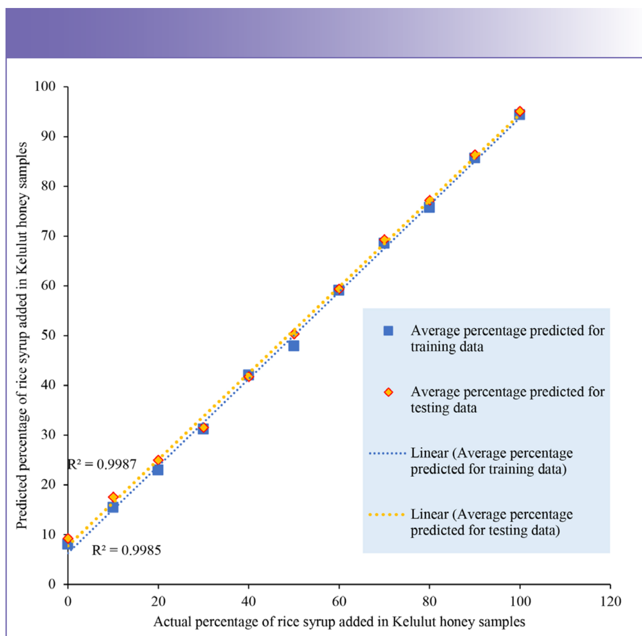

FIGURE 6: Scatter plot of rice syrup adulteration percentage in the Kelulut honey samples predicted by the PCR model for the training data and testing data.

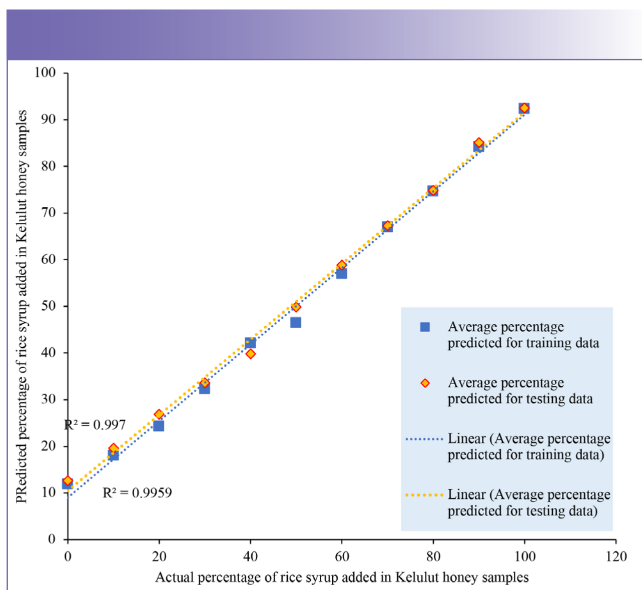

FIGURE 7: Scatter plot of rice syrup adulteration percentage in the Kelulut honey samples predicted by the PLS model for the training data and testing data.

TABLE V: Regression results using both PCR and PLS

Method	R^2 (training and prediction)	RMSE (training and prediction)
PCR model	0.924 & 0.914	8.724 & 9.366
PLS model	0.821 & 0.818	13.347 & 13.593

and make it more difficult for the model to accurately collect valuable spectral properties linked to the target variable. The target variable's important information may be obscured or distorted by the strong noise in this spectral area. In conclusion, it is important to draw attention to the high noise levels in the NIR spectra above 1400 nm since they may have an impact on the calibration task. Investigating the effects of leaving this region out of the calibration data set can help shed light on the difficulties brought on by noise and possibly enhance calibration performance. The precision and stability of NIR spectra measurements are also maintained by accounting for temperature sensitivity in DLP devices.

The scatter plots (**Figure 6 and 7**) were used to further explore the prediction performance of PCR and PLS on adulterated Kelulut honey samples from the training data and testing data. The average adulterated percentage of rice syrup was predicted similarly in both training and testing data, indicating that the PCR and PLS models have the ability to be employed in the prediction of Kelulut honey adulteration. As shown in **Figures 6 and 7**, PCR and PLS both performed well in terms of prediction, with the projected average percentage of rice syrup added in the samples nearly falling on the trendlines and the R^2 of all trendlines above 99%. The R^2 of the PCR model's trendlines was between 0.9987 and 0.9985, indicating that it performed better than the PLS model in terms of prediction. R^2 and RMSE were used to assess the performance of these two prediction models. The evaluation findings were presented in **Table V** using orange data mining software. RMSE stands for root mean square error, and the lower the RMSE, the better the predictive performance (46). In our analysis, the PCR model had the lowest RMSE.

However, the calibrations are frequently not applicable for "real world" sample prediction when using sophisticated regression methods on low-ranked data, such as intentionally created samples. Addressing the restrictions is essential to reducing these restrictions. obtaining a training data set that is diverse and indicative of the variety seen in real-world samples. To avoid overfitting and improve generalization, the regression model might be regularized or made simpler. By making sure the distribution of the training data closely resembles the distribution of the real-world data, one can account for covariate shift, including pertinent extra elements or variables that are known to or anticipated to have an impact on the samples taken from the real world.

Conclusion

The physicochemical properties moisture content, electrical conductivity, water activity, HMF content, color of the samples decreased when the adulterated percentage of rice syrup in Kelulut honey increased, with the exception of pH. However, it was observed that the values are in compliance

with the Malaysian standard, even in some percentage of adulteration with rice syrup. Hence, it is not applicable to detect the Kelulut honey adulteration by comparison of the physicochemical properties with the Malaysian standard. As a result, using training and testing data, regression models such as PCR and PLS were constructed for the NIR spectroscopy to estimate the adulterated percentage of rice syrup in Kelulut honey samples following pre-processing procedures. When compared to the PLS model, the PCR model exhibits higher R^2 values in both training and testing data, 0.924 and 0.914, respectively. In both the training and testing data, the RMSE of the PCR model was lower in the PLS model. The PCR model has a lower RMSE and a higher R^2 than the PLS model, indicating that it has a superior predictive performance in detecting adulterated amounts of rice syrup added honey samples. NIR spectroscopy in the field can offer a practical and effective way for analyzing tainted honey. It is significant to emphasize that accurate instrument calibration, representative reference samples, and reliable chemometric modeling are necessary for NIR spectroscopy to be successful in analyzing contaminated honey. For trustworthy and accurate analysis results, regular instrument maintenance and calibration, adherence to standard operating procedures (SOPs), and validation of the calibration model are essential.

In conclusion, this study investigated the physicochemical properties of Kelulut honey that were adulterated with different percentages of rice syrup. The study also examined the feasibility of using handheld NIR spectroscopy to detect the adulteration of Kelulut honey with different percentages of rice syrup. This may provide useful information for future research on identification of Kelulut honey adulteration with various types of syrup, allowing rapid and easy quality control in the honey market.

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Conflicts of Interest

The authors declare there are no conflicts of interest.

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