Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

# Preserving Evidence: Spectroscopic Fluid Identification

**Pushkar Joon**

operationbrightstar5@gmail.com

sgt university https://orcid.org/0009-0002-1115-3734

**Systematic Review**

**Keywords:** Forensic Serology, Raman Spectroscopy, ATR-FTIR, Non-Destructive Analysis, Bodily Fluid Identification, Chemometrics, Validation Metrics, Evanescent Wave, Likelihood Ratios, Chain of Custody.

**Posted Date:** November 5th, 2026

**DOI:** https://doi.org/10.21203/rs.3.rs-11615982/v1

**License:** This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

**Additional Declarations:** The authors declare no competing interests.

RESEARCH SQUARE PREPRINT MANUSCRIPT • SYSTEMATIC REVIEW • CC-BY 4.0 LICENSE

# Preserving Evidence: Spectroscopic Fluid Identification

*Advancements in Raman Spectroscopy and ATR-FTIR for Non-Destructive Bodily Fluid Identification*

**Pushkar Joon (Roll No.: 251728139)**

Department of Forensic Science, Faculty of Applied and Basic Sciences, Shree Guru Gobind Singh Tricentenary (SGT) University, Gurugram, Delhi-NCR, India • Subject: Forensic Serology and Biology (Sem III)

**ABSTRACT:** The definitive identification of biological fluids recovered at crime scenes represents a critical pillar of modern forensic serology and reconstruction. Conventional biochemical screening and immunochromatographic confirmatory assays—such as the Kastle-Meyer, Phadebas, and RSID platforms—remain inherently destructive, analyte-specific, and prone to false-positive or false-negative outcomes when confronted with trace or environmentally compromised stains. Over the past two decades, vibrational spectroscopy, specifically Raman spectroscopy and Attenuated Total Reflection Fourier-Transform Infrared (ATR-FTIR) spectroscopy, has emerged as a transformative, non-destructive analytical paradigm. By interrogating intrinsic molecular vibrations without chemical reagent consumption, both techniques preserve the structural integrity of genomic DNA and trace cellular architecture for downstream Short Tandem Repeat (STR) profiling. This systematic review examines the historical emergence, physical principles, instrumentation, characteristic spectral biomarkers, pre-analytical variables, substrate interference correction algorithms, degradation kinetics, and chemometric deconvolution strategies governing Raman and ATR-FTIR bio-spectroscopy. Furthermore, to bridge the gap between proof-of-concept spectroscopy and routine forensic deployment, this review synthesizes quantitative study characteristics, donor cohort sizes, validation metrics (sensitivity, specificity, AUC, $R^2$, RSD, and LOD/LOQ), inter-laboratory model transferability barriers, and courtroom admissibility standards.

**KEYWORDS:** Forensic Serology; Raman Spectroscopy; ATR-FTIR; Non-Destructive Analysis; Bodily Fluid Identification; Chemometrics; Validation Metrics; Evanescent Wave; Likelihood Ratios; Chain of Custody.

## 1. INTRODUCTION

Biological stains encountered during crime scene investigations—principally peripheral blood, menstrual blood, seminal fluid, saliva, vaginal secretions, sweat, and urine—serve as crucial physical links between perpetrators, victims, and physical scenes (**Virkler & Lednev, 2009**). While modern forensic genetics relies heavily on polymerase chain reaction (PCR) amplification of Short Tandem Repeat (STR) loci to establish individualization, DNA profiling alone does not disclose the tissue or cellular origin of the biological material. Determining whether a recovered DNA profile originated from innocuous salivary transfer or seminal deposition frequently dictates whether criminal charges of sexual assault can be sustained in a court of law (**Zapata et al., 2015**).

Historically, forensic laboratories have executed a sequential two-tier testing cascade: an initial catalytic or enzymatic presumptive test (e.g., benzidine, phenolphthalein, luminol, acid phosphatase, or starch-iodine assays) followed by a microscopic, immunological, or crystal-based confirmatory test (e.g., Takayama hemochromogen crystals, Christmas Tree staining for spermatozoa, or lateral flow immunochromatography) (**Butler, 2015**). Despite their operational familiarity, these wet-chemical protocols suffer from three major operational vulnerabilities. First, they are fundamentally destructive, solubilizing or chemically altering the scarce biological matrix and diminishing the yield of recoverable nuclear DNA. Second, they are single-analyte assays; if an investigator misjudges the suspected fluid type and executes an inappropriate test, precious micro-traces may be exhausted without yielding an identification. Third, catalytic tests exhibit well-documented cross-reactivity with plant peroxidases, oxidizing salts, and microbial enzymes (**Muro et al., 2015**).

Consequently, the forensic science community has prioritized the development of a universal, reagent-free, non-destructive confirmatory methodology capable of identifying any biological fluid through a single optical measurement. Vibrational spectroscopy—encompassing Raman scattering and Attenuated Total Reflection Fourier-Transform Infrared (ATR-FTIR) absorption—fulfills these stringent operational demands by recording comprehensive biochemical fingerprints composed of proteins, lipids, carbohydrates, nucleic acids, and low-molecular-weight metabolites (**Baker et al., 2014**).

## 2. METHODS: SYSTEMATIC REVIEW METHODOLOGY & EVIDENCE FRAMEWORK

### 2.1 Search Strategy, Inclusion/Exclusion Criteria, and Metrological Pillars

To move beyond purely narrative technical descriptions and evaluate whether vibrational bio-spectroscopy is ready for routine forensic casework, this systematic review synthesizes peer-reviewed experimental and validation studies indexed across forensic and analytical chemistry databases (Scopus, Web of Science, and PubMed) spanning 2008–2025. Studies were evaluated across four metrological pillars required by international forensic quality frameworks (ISO/IEC 17025 and SWGDAM/OSAC guidelines): (1) **Study Scale and Cohort Representativeness** (number of unique human donors *n*, biological fluid types, substrate diversity, and post-depositional aging intervals); (2) **Quantitative Analytical Validation Metrics** (diagnostic sensitivity, specificity, overall classification accuracy, Area Under the Receiver Operating Characteristic Curve [AUC-ROC], coefficient of determination [$R^2$], root-mean-square error of prediction [RMSEP], relative standard deviation [CV/RSD], and limits of detection/quantitation [LOD/LOQ]); (3) **Pre-Analytical and Matrix Robustness** (hydration state, laser power density, contact anvil pressure, coffee-ring heterogeneity, and background substrate interference); and (4) **Validation Architecture** (internal cross-validation versus blind external test sets and inter-laboratory model transferability) (**Muro et al., 2015**; **Takamura et al., 2018**).

**Inclusion Criteria:** (1) Peer-reviewed original experimental studies and validation protocols examining human peripheral blood, menstrual blood, semen, saliva, vaginal fluid, sweat, or urine using spontaneous/confocal Raman, SERS, SORS, or ATR-FTIR; (2) explicit reporting of donor cohort size ($n$), spectral band assignments, and multivariate chemometric classification/regression strategies; and (3) reporting of quantitative diagnostic validation metrics (sensitivity, specificity, accuracy, AUC, $R^2$, RSD, or LOD). **Exclusion Criteria:** (1) Non-forensic clinical liquid-biopsy diagnostics lacking dried stain or substrate evaluation; (2) qualitative reports lacking chemometric validation or multi-donor cohorts ($n < 3$); and (3) duplicate records, commentaries, or non-peer-reviewed abstracts.

**Systematic Review Literature Identification, Screening, and Eligibility Workflow (2008–2025)**

| Review Stage | Records & Screening Actions | Exclusion Criteria Applied |
|---|---|---|
| **1. Identification** | 792 total records identified across Scopus ($n$ = 312), Web of Science ($n$ = 284), and PubMed ($n$ = 196) covering 2008–2025. | 348 duplicate records removed across databases prior to title screening. |
| **2. Screening** | 444 unique records screened by title, abstract, and spectroscopic modality. | 319 records excluded (clinical serum oncology, non-forensic diagnostics, or non-vibrational assays). |
| **3. Eligibility** | 125 full-text forensic vibrational spectroscopy articles assessed against ISO/IEC 17025 and OSAC metrological criteria. | 108 articles excluded (54 lacked donor-stratified/external validation; 31 had $n < 3$ donors; 23 used destructive extraction). |
| **4. Inclusion** | 17 foundational and validation references synthesized; 6 primary multi-donor benchmark cohorts entered into quantitative meta-synthesis (Table 3). | Full compliance with donor-grouped cross-validation or blind external test cohort design. |

## 2.2 Documentation of Large Language Model (LLM) Usage

In compliance with Research Square authorship and accountability guidelines (Section 1.3), Google Gemini was utilized strictly as a formatting and editorial utility to convert the author's original research manuscript into a structured preprint layout and verify mathematical notation. No LLM satisfies authorship criteria, and full accountability for all literature synthesis, metrological evaluations, data verification, and scientific conclusions rests solely with the author.

## 3. EMERGENCE AND SCIENTIFIC PRINCIPLES

### 3.1 Historical Development of Vibrational Spectroscopy in Forensic Science

The integration of vibrational spectroscopy into forensic laboratories began in the mid-twentieth century with dispersive infrared spectrophotometers utilized for the bulk characterization of synthetic polymer fibers, automotive paint chips, illicit narcotics, and explosive residues (**Chalmers et al., 2012**). Early forensic practitioners valued infrared absorption because constitutional isomers and polymorphic crystalline solids yielded unambiguous fingerprint spectra in the mid-infrared region (4000–400 $cm^{-1}$). However, early dispersive instruments suffered from slow scan speeds, low optical throughput, and extreme sensitivity to ambient moisture, restricting their application strictly to dehydrated, purified chemical extracts rather than heterogeneous biological matrices.

### 3.2 Emergence of Raman Spectroscopy

Although the inelastic scattering of light was experimentally demonstrated by Sir C.V. Raman and K.S. Krishnan in 1928, Raman spectroscopy remained largely confined to specialized physical chemistry laboratories for over six decades due to the exceptionally low probability of the Raman scattering cross-section (approximately 1 in $10^6$ to $10^8$ incident photons) (**Smith & Dent, 2019**). The forensic emergence of Raman spectroscopy in the late 1990s was catalyzed by three technological breakthroughs: the invention of stable, monochromatic near-infrared (NIR) diode and solid-state lasers (e.g., 785 nm and 1064 nm) that mitigated biological autofluorescence; the development of holographic notch filters for Rayleigh line rejection; and the introduction of thermoelectrically cooled Charge-Coupled Device (CCD) arrays capable of quantum efficiencies exceeding 80% (**Khandasammy et al., 2018**).

### 3.3 Evolution of FTIR & ATR-FTIR

Simultaneously, infrared spectroscopy underwent a paradigm shift with the replacement of mechanical monochromators by Michelson interferometers and fast Fourier-transform digital computing in the 1970s. While transmission FTIR still required tedious sample preparation—such as grinding dried stains into potassium bromide (KBr) pellets or casting thin films onto infrared-transparent salt plates (NaCl or $BaF_2$)—the refinement of Attenuated Total Reflection (ATR) accessories by Fahrenfort and Harrick eliminated sample preparation entirely (**Griffiths & De Haseth, 2007**). By pressing a biological stain directly against a high-refractive-index crystal (such as diamond, zinc selenide, or germanium), forensic analysts could acquire high-signal-to-noise surface spectra within seconds without altering the physical evidence.

### 3.4 Transition to Forensic Bio-Spectroscopy

Between 2008 and 2015, pioneering research led by Igor K. Lednev at the University at Albany (SUNY) and Kazarian and colleagues in the United Kingdom systematically bridged vibrational spectroscopy and forensic serology (**Virkler & Lednev, 2009**). Recognizing that dried bodily fluids are intrinsically heterogeneous—comprising cellular particulates (erythrocytes, leukocytes, buccal epithelial cells, spermatozoa) suspended within dried extracellular protein matrices—researchers combined micro-spectroscopic mapping with multivariate statistical modeling (chemometrics). This transition established "forensic bio-spectroscopy" as a validated discipline capable of handling inter-donor biological variability while achieving near-perfect classification accuracy across diverse crime scene conditions (**Takamura et al., 2018**).

## 4. FUNDAMENTALS OF VIBRATIONAL SPECTROSCOPY

At temperatures above absolute zero, covalent bonds within polyatomic biological molecules undergo continuous quantized periodic motions, including symmetric and asymmetric bond stretching (ν) as well as in-plane scissoring (δ), rocking (ρ), out-of-plane wagging (ω), and twisting (τ) deformations. For a nonlinear biomolecule containing $N$ atoms, there exist $3N - 6$ fundamental normal modes of vibration (**Smith & Dent, 2019**). To first approximation, the vibrational frequency of a diatomic bond is governed by Hooke's Law:

$$\bar{\nu} = (1 / 2\pi c) \times \sqrt{(k / \mu)} \quad \text{where} \quad \mu = (m_1 \times m_2) / (m_1 + m_2)$$

Here, $\bar{\nu}$ represents the vibrational wavenumber ($cm^{-1}$), $c$ is the velocity of light in vacuum, $k$ is the bond force constant (reflecting bond order and electronic density), and μ is the reduced mass of the bonded nuclei $m_1$ and $m_2$. Consequently, light hydrogen-containing stretches (O–H, N–H, C–H) resonate at high wavenumbers (3600–2800 $cm^{-1}$), double and triple bonds resonate in the intermediate region (2300–1500 $cm^{-1}$), and heavy skeletal vibrations or porphyrin ring deformations populate the lower fingerprint region (1500–400 $cm^{-1}$).

### 4.1 Spectral Units and Interpretation

Both infrared and Raman spectra are plotted along an abscissa scaled in wavenumbers ($\bar{\nu} = 1/\lambda$ expressed in reciprocal centimeters, $cm^{-1}$), which is directly proportional to photon energy ($E = hc\bar{\nu}$). However, their ordinate axes differ fundamentally:

- **FTIR / ATR-FTIR Ordinate:** Plotted as Percentage Transmittance ($\%T = (I / I_0) \times 100$) or Absorbance ($A = -\log_{10}(I / I_0)$). According to the Beer-Lambert Law ($A = \varepsilon bc$), absorbance is linearly proportional to molar absorptivity (ε), optical pathlength ($b$), and analyte concentration ($c$), making absorbance units mandatory for quantitative chemometric preprocessing (**Baker et al., 2014**).

- **Raman Ordinate:** Plotted as Scattered Photon Intensity (arbitrary CCD counts per second) versus Raman Shift ($\Delta\bar{\nu}$ in $cm^{-1}$), defined as the energy difference between the incident laser excitation wavelength ($\lambda_0$) and the inelastically scattered wavelength ($\lambda_s$): $\Delta\bar{\nu} = (1 / \lambda_0) - (1 / \lambda_s)$.

## 5. RAMAN SPECTROSCOPY

### 5.1 Physical Principles

When monochromatic laser radiation of frequency $\nu_0$ interacts with the electron cloud of a biological molecule, it induces an oscillating electric dipole moment $P = \alpha E$, where $\alpha$ is the molecular polarizability tensor and $E$ is the electric field strength of the incident photon. The vast majority of incident photons undergo elastic Rayleigh scattering, emerging with unchanged frequency ($\nu_0$). However, if a molecular vibration causes a periodic deformation of the electron cloud polarizability with respect to the normal vibrational coordinate $Q$—satisfying the gross selection rule $(\partial\alpha / \partial Q)_0 \neq 0$—energy is exchanged between the photon and the molecule (**Smith & Dent, 2019**).

If the molecule transitions from the ground vibrational state ($v = 0$) to a virtual state and relaxes to the first excited vibrational state ($v = 1$), the scattered photon loses energy $h(\nu_0 - \nu_v)$, generating **Stokes Raman scattering**. Conversely, if the molecule already resides in an excited vibrational state ($v = 1$) due to thermal energy and relaxes to $v = 0$, the scattered photon gains energy $h(\nu_0 + \nu_v)$, producing **Anti-Stokes Raman scattering**. Because Boltzmann statistics dictate that most molecules occupy $v = 0$ at ambient temperatures, forensic Raman spectroscopy exclusively records the more intense Stokes-shifted spectrum. Furthermore, when the laser wavelength approaches an electronic absorption band of a chromophore (such as the Soret or Q-bands of hemoglobin), **Resonance Raman enhancement** selectively amplifies chromophoric vibrations by factors of $10^3$ to $10^6$ (**Virkler & Lednev, 2009**).

### 5.2 Instrumentation

A forensic dispersive confocal Raman micro-spectrometer integrates five precision optical subsystems: (1) continuous-wave excitation lasers spanning ultraviolet/visible to near-infrared wavelengths (typically 406.7 nm, 532 nm, 633 nm, 785 nm, and 1064 nm); (2) laser line-pass cleanup filters and beam-steering optics; (3) an epi-illumination research-grade optical microscope (10× to 100× objectives) that focuses the laser to a diffraction-limited spot ($d \approx 1.22\lambda$ / NA, typically 1–2 µm) and collects 180° backscattered light; (4) edge or holographic notch filters that reject the intense Rayleigh line while passing Stokes-shifted photons through a confocal pinhole aperture into an aberration-corrected Czerny-Turner spectrograph fitted with holographic diffraction gratings (600–1800 grooves/mm); and (5) a Peltier-cooled (−70 °C) back-illuminated CCD detector (**Khandasammy et al., 2018**).

### 5.3 Raman Spectral Signatures & Spatial Microscopy

Each human biological fluid exhibits a multi-component Raman profile dominated by its major functional proteins, porphyrins, or nitrogenous metabolites (**Muro et al., 2015**). Because dried biological stains undergo phase separation during desiccation (the "coffee-ring effect"), Lednev and coworkers demonstrated that a single Raman spectrum cannot fully represent a stain; instead, multi-component spectroscopic signatures combining fluorescence backgrounds and major Raman scattering peaks accurately capture fluid identity across multiple human donors (**Sikirzhytski et al., 2012**). Key vibrational assignments are summarized in Table 1.

Confocal Raman microscopy provides micrometer-scale spatial selectivity ($x$, $y$ lateral resolution ~1 μm; $z$ axial depth resolution ~2–3 μm). By mounting the sample on a motorized piezoelectric stage, automated 2D hyperspectral mapping collects hundreds of spatially resolved spectra across a dried biological stain (**Zapata et al., 2015**). This spatial capability is indispensable in forensic casework for three reasons: (1) it allows the analyst to focus directly onto an intact single erythrocyte (~7 μm diameter) or spermatozoon head (~4–5 μm) wedged between strongly fluorescing textile fibers; (2) it resolves heterogeneous co-precipitates in dried saliva and vaginal fluid; and (3) it enables non-destructive physical separation of overlapping mixed stains prior to statistical deconvolution (**Sikirzhytski et al., 2012**).

## 6. FTIR & ATR-FTIR SPECTROSCOPY

### 6.1 Fundamental Principles of FTIR

Infrared absorption spectroscopy probes direct transitions between quantized vibrational energy levels driven by resonant absorption of mid-infrared photons (4000–400 $cm^{-1}$). The quantum mechanical gross selection rule for infrared activity requires that a molecular vibration produce a periodic change in the net permanent electric dipole moment (μ) of the molecule with respect to the normal coordinate $Q$: $(\partial \mu / \partial Q)_0 \neq 0$ (**Griffiths & De Haseth, 2007**). Consequently, asymmetric and polar functional groups—such as carbonyls (C=O), peptide bonds (O=C–NH), phosphodiester linkages ($PO_2^-$), and hydroxyl groups (O–H)—exhibit intense infrared absorption coefficients, whereas homonuclear or centrosymmetric vibrations remain weakly infrared-active or silent. This physical complementarity ensures that FTIR and Raman spectroscopy interrogate distinct structural motifs within the same biological fluid.

### 6.2 Fourier-Transform Processing

Unlike dispersive instruments that scan individual wavelengths sequentially through narrow slits, an FTIR spectrometer routes polychromatic infrared radiation from a silicon carbide (Globar) source through a Michelson interferometer consisting of a KBr beamsplitter, a stationary mirror, and a moving mirror controlled via helium-neon (HeNe) laser fringe referencing. Constructive and destructive interference across optical retardation (δ) generates a time-domain interferogram $I(\delta)$, which is mathematically converted into a frequency-domain spectrum ($\tilde{\nu}$) via the Cooley-Tukey Fast Fourier Transform (FFT) algorithm (). Fourier-transform processing confers three classic instrumental advantages: (1) , whereby all wavenumbers reach the deuterated triglycine sulfate (DTGS) or liquid-nitrogen-cooled mercury cadmium telluride (MCT) detector simultaneously, drastically improving signal-to-noise ratio (SNR $\propto \sqrt{}$); (2) , resulting from circular optical apertures free of narrow dispersive slits; and (3) , where internal HeNe laser calibration locks wavenumber precision to within ±0.01 cm.$B$**Baker et al., 2014****Fellgett's (Multiplex) Advantage**$N_{\text{scans}}$**Jacquinot's (Throughput) AdvantageConnes' Advantage**$^{-1}$

### 6.3 Principles of Attenuated Total Reflection

In Attenuated Total Reflection (ATR-FTIR), the infrared beam is directed into an optically dense Internal Reflection Element (IRE) crystal of high refractive index $n_1$ (e.g., Type IIa Diamond, $n_1$ = 2.42; Zinc Selenide, $n_1$ = 2.40; or Germanium, $n_1$ = 4.00) at an angle of incidence θ exceeding the critical angle $\theta_c = \arcsin(n_2 / n_1)$, where $n_2$ is the refractive index of the biological stain ($n_2 \approx 1.50$) in intimate contact with the crystal surface (**Orphanou, 2015**). Total internal reflection at the crystal-sample boundary generates a standing electromagnetic wave—the **evanescent wave**—that penetrates exponentially into the biological sample to a characteristic penetration depth ($d_p$) governed by the Harrick equation:

$$\boldsymbol{d_p = \lambda / [2\pi n_1 \sqrt{(\sin^2\theta - (n_2 / n_1)^2)}]}$$

Because $d_p$ is directly proportional to wavelength λ, the evanescent wave penetrates deeper into the stain at lower wavenumbers (~2.0 μm at 1000 cm$^{-1}$) than at higher wavenumbers (~0.6 μm at 3300 cm$^{-1}$) using a 45° diamond crystal. Most modern forensic spectroscopy software applies an Advanced ATR Correction algorithm to normalize relative band intensities and correct anomalous dispersion (band shifts) prior to spectral library matching (**Takamura et al., 2018**).

### 6.4 ATR-FTIR Spectral Signatures of Bodily Fluids

The mid-infrared ATR-FTIR spectrum of dried human biological fluids is logically partitioned into five diagnostic biochemical spectral windows (**Orphanou, 2015**; **Sharma et al., 2020**):

- **Amide A & B Region (3500–3100 $cm^{-1}$):** Broad intense band centered near 3280–3300 $cm^{-1}$ arising from N–H stretching of proteins coupled with residual hydrogen-bonded O–H stretching of bound water.
- **Lipid C–H Stretching Region (3000–2800 $cm^{-1}$):** Sharp doublets at ~2958 $cm^{-1}$ ($\nu_{as}CH_3$), ~2924 $cm^{-1}$ ($\nu_{as}CH_2$), ~2872 $cm^{-1}$ ($\nu_sCH_3$), and ~2852 $cm^{-1}$ ($\nu_sCH_2$), reflecting cellular membrane phospholipids, triglycerides, and sebaceous esters.
- **Protein Amide I & II Region (1700–1500 $cm^{-1}$):** The Amide I band (1650–1635 $cm^{-1}$) originates primarily from peptide carbonyl (C=O) stretching (80%) and is exquisitely sensitive to protein secondary conformation (α-helices at ~1652 $cm^{-1}$ vs. β-sheets at ~1630 $cm^{-1}$). The Amide II band (1545–1535 $cm^{-1}$) stems from out-of-phase N–H in-plane bending (60%) coupled with C–N stretching (40%).
- **Amino Acid Side-Chain & Lipid Deformation Region (1480–1200 $cm^{-1}$):** Includes $CH_2/CH_3$ scissoring (~1450 $cm^{-1}$), symmetric carboxylate ($COO^-$) stretching of fibrinogen and amino acids (~1395 $cm^{-1}$), and the Amide III band (1310–1240 $cm^{-1}$).
- **Carbohydrate, Glycoprotein & Phosphodiester Region (1200–900 $cm^{-1}$):** Encompasses asymmetric ($\nu_{as}PO_2^-$ at ~1240 $cm^{-1}$) and symmetric ($\nu_sPO_2^-$ at ~1080 $cm^{-1}$) phosphate stretches of nucleic acids and phospholipids, alongside C–O and C–O–C glycosylation stretches of mucins, glycogen, and citric acid (1075–960 $cm^{-1}$).

### 6.5 Practical Advantages & Operational Constraints

ATR-FTIR offers exceptional spectral reproducibility, complete immunity to laser-induced fluorescence, rapid data collection (<30 seconds per sample), and ruggedness suitable for field-deployable handheld units. Furthermore, because the evanescent wave decays within ~1–2 μm of the stain surface, thick biological crusts deposited on substrates often yield pure fluid spectra without underlying fabric interference (**Zapata et al., 2015**). Conversely, its primary operational constraint is the requirement for firm physical contact between the sample and the diamond IRE crystal, which creates a risk of cross-contamination if the crystal is not meticulously sanitized with 10% sodium hypochlorite and 70% isopropanol between measurements. Additionally, water exhibits massive infrared absorption bands at ~3400 $cm^{-1}$ and ~1640 $cm^{-1}$ (H–O–H bending), requiring aqueous biological fluids to be air-dried prior to ATR-FTIR interrogation lest the water bending mode mask the diagnostic Amide I protein contour (**Baker et al., 2014**).

**Table 1: Comprehensive Raman (785 nm) and ATR-FTIR Diagnostic Spectral Signatures and Best-Performing Modalities by Bodily Fluid**

| Bodily Fluid | Key Biochemical Markers | Diagnostic Raman Shifts ($cm^{-1}$) & Vibrational Assignments | Diagnostic ATR-FTIR Peaks ($cm^{-1}$) & Vibrational Assignments | Best-Performing Modality & Casework Use-Case |
|---|---|---|---|---|
| **Peripheral Blood** | Hemoglobin (HbA), Oxyhemoglobin, Fibrin, Albumin, Glucose | 754 (pyrrole breathing $\nu_{15}$), 1003 (Phe ring breathing), 1224 (methine C–H deform), 1370 ($\nu_4$ pyrrole half-ring, $Fe^{3+}$ oxidation marker), 1547/1565 (C=C porphyrin), 1620 (vinyl C=C) | 3290 (Amide A), 2958/2925 (lipid C–H), 1651 (Amide I α-helix rich Hb), 1541 (Amide II), 1452 ($CH_2$ bend), 1395 ($COO^-$ sym stretch of amino acids), 1302 (Amide III) | **Confocal Raman (785 nm):** Resonance heme enhancement isolates single RBCs (~1 pg) and tracks TSD aging; ATR-FTIR preferred on strongly fluorescent dark denim. |
| **Seminal Fluid** | Spermine, Spermidine, Citrate, Acid Phosphatase, PSA, Fructose, Tyrosine | 715 (C–S stretch), 829/848 (Tyr Fermi doublet of SAP), 958/985 ($PO_4^{3-}$ & spermine), 1003 (Phe), 1327/1448 ($CH_2$ deform of spermine), 1668 (Amide I β-sheet/random coil) | 3282 (Amide A), 1638–1642 (Amide I shifted lower by high β-sheet content), 1545 (Amide II), 1396 (citrate $COO^-$ stretch), 1062 & 976 (fructose C–O & phosphate stretches) | **Complementary:** Confocal Raman maps individual sperm heads; ATR-FTIR rapidly screens azoospermic seminal plasma crusts via fructose/citrate bands. |
| **Saliva** | α-Amylase, Mucins (MUC5B/MUC7), Lysozyme, Potassium Thiocyanate ($SCN^-$) | 752 (Trp ring breathing in amylase), 852/938 (C–C skeletal & glycosidic linkages), 1003 (Phe), 1449 ($CH_2$ bend), 1655 (Amide I), 2065 (sharp C≡N stretch of thiocyanate $SCN^-$) | 3285 (Amide A), 2058 (C≡N stretch of $SCN^-$, pathognomonic when present), 1648 (Amide I), 1544 (Amide II), 1076–1035 (intense mucin oligosaccharide C–O stretching) | **ATR-FTIR / SERS:** Dried saliva films are thin and weakly scattering in spontaneous Raman; ATR-FTIR strongly captures mucin C–O stretches (1076–1035 $cm^{-1}$). |
| **Vaginal Fluid** | Glycogen, Lactic Acid, Mucins, Squamous Epithelial Cells, Urea | 853 (lactic acid C–COOH & glycogen α-glycosidic ring), 938 (C–O–C skeletal), 1004 (Phe), 1045/1083 (glycogen C–O), 1452 ($CH_2$ scissoring), 1658 (Amide I) | 1725–1740 (lactic acid & lipid ester C=O), 1645 (Amide I), 1542 (Amide II), 1152, 1078 & 1026 (prominent glycogen & mucin C–O/C–O–C carbohydrate triad) | **ATR-FTIR:** High dipole sensitivity to glycogen and mucin C–O–C triads (1152/1078/1026 $cm^{-1}$) and lactate esters provides superior SNR over spontaneous Raman. |

| Bodily Fluid | Key Biochemical Markers | Diagnostic Raman Shifts ($cm^{-1}$) & Vibrational Assignments | Diagnostic ATR-FTIR Peaks ($cm^{-1}$) & Vibrational Assignments | Best-Performing Modality & Casework Use-Case |
|---|---|---|---|---|
| **Menstrual Blood** | Hemoglobin + Lactic Acid, Glycogen, Endometrial Matrix Metalloproteinases | Combines Hb resonance peaks (754, 1224, 1370, 1565) with vaginal markers: 853 (lactate/glycogen) and elevated 1080 (phospholipids/exfoliated cells) | Hb Amide I/II profile (1650/1541) plus elevated phosphoric/carbohydrate envelope at 1240, 1080, and 1030 (endometrial glycogen & lactate C–O stretches) | **ATR-FTIR + PLS-DA:** Outperforms Raman in bulk stains because resonance heme scattering in Raman can overshadow weak lactate/glycogen co-markers. |
| **Sweat & Urine** | Urea, Creatinine, Uric Acid, Lactic Acid, Sodium Lactate, Amino Acids | **Urine:** 1003 (intense sharp symmetric C–N stretch of urea), 680 & 840 (creatinine). **Sweat:** 855 (lactate C–C), 1003 (urea), 1450 ($CH_2$), 1625 (urea $NH_2$ bend) | **Urine:** 3435/3340 (urea $NH_2$ doublet), 1660 (urea C=O), 1618 ($NH_2$ bend), 1458 (C–N stretch). **Sweat:** 1738 (sebum ester), 1585 & 1122 (lactate $COO^-$ & C–O) | **Confocal Raman / SERS:** Crystalline urea (1003 $cm^{-1}$) and lactate micro-crystals on fibers are sharply resolved by micro-Raman; diluted urine requires SERS or LASSO-FTIR. |
| **Mixed Stains** | Superposed Proteomic, Glycosidic & Metabolite Signatures (e.g., Semen + Vaginal) | Spatially segregated micro-domains: 829/848 & 1448 (semen pixels) vs. 853 & 1045 (vaginal epithelial pixels) or 1370/1565 (erythrocyte pixels) | Composite bulk envelope requiring MCR-ALS or SVM unmixing of 1638/1396/1062 (seminal citrate/fructose) from 1152/1078/1026 (vaginal glycogen) | **Confocal Raman 2D Mapping + MCR-ALS:** Superior spatial resolution (1–2 μm) physically separates unmixed cellular domains before chemometric unmixing. |

# 7. COMPARATIVE ANALYTICAL EVALUATION

## 7.1 Raman Spectroscopy vs. ATR-FTIR

While Raman and ATR-FTIR both probe fundamental vibrational eigenstates inside the 4000–400 $cm^{-1}$ range, their underlying quantum mechanical operators (polarizability tensor α vs. dipole moment μ) yield mutually complementary forensic capabilities (**Zapata et al., 2015**). A rigorous side-by-side comparison across operational and metrological parameters is detailed in Table 2.

**Table 2: Analytical and Forensic Operational Comparison between Confocal Raman and ATR-FTIR Spectroscopy**

| Analytical Parameter | Confocal Raman Micro-Spectroscopy | ATR-FTIR Spectroscopy |
|---|---|---|
| **Physical Mechanism** | Inelastic photon scattering (Stokes shift); requires change in molecular polarizability $(\partial\alpha / \partial Q)_0 \neq 0$. | Resonant mid-IR photon absorption via evanescent wave; requires dipole moment change $(\partial\mu / \partial Q)_0 \neq 0$. |
| **Sample Contact Mode** | Strictly Non-Contact; optical working distance of millimeters through glass evidence bags or slides. | Direct Physical Contact required against the IRE crystal under mechanical anvil pressure. |
| **Spatial Resolution** | Diffraction-limited (~1–2 µm with 50×/100× objective); resolves single red blood cells or sperm heads. | Macro-sampling (~1–2 mm crystal spot) or ~10–20 µm with specialized micro-ATR germanium objectives. |
| **Water Interference** | Minimal (water is a weak Raman scatterer); permits direct examination of fresh wet biological fluids. | Severe (O–H stretch and 1640 $cm^{-1}$ bend obscure Amide I); requires desiccation of wet stains. |
| **Fluorescence Sensitivity** | High vulnerability from dyed fabrics, biological degradation products, or humic soil contaminants. | Completely immune to electronic fluorescence; mid-IR energies do not excite electronic transitions. |
| **Substrate Penetration** | Confocal *z*-focus (~2 µm) isolates surface particles, though deeply absorbed stains co-scatter with fibers. | Shallow evanescent wave ($d_p \approx$ 1–2 µm) probes only the top crust, minimizing deep fabric interference. |
| **Sample Preservation & Cross-Contamination** | 100% Non-Invasive at ≤2–5 mW laser power; zero physical contact eliminates trace transfer between exhibits. | Non-Destructive to DNA, but anvil pressure can flatten fragile crusts and requires strict bleach/alcohol IRE sanitization. |
| **Field Suitability & Deployment** | Handheld 785/1064 nm units deployable for through-bag screening, though sensitive to ambient light and vibration. | Highly rugged monolithic diamond ATR portables; ideal for rapid on-scene triage of dried flakes and crusts. |

## 7.2 Criteria for Selecting a Technique & Decision-Tree Workflow

Selecting the optimal spectroscopic modality in a forensic workflow depends on three primary evidentiary variables: sample hydration state, substrate fluorescence, and trace particulate distribution (**Muro et al., 2015**). If an item of evidence consists of microscopic particulate droplets or sparse cellular deposits trapped within deep textile weaves—or if the sample is sealed inside an optically transparent glass container that cannot be opened due to contamination risks—confocal Raman microscopy is the mandatory choice. Conversely, if a biological stain is deposited on a heavily dyed, strongly fluorescing textile (such as dark indigo denim or neon synthetic polyester) where laser excitation induces overwhelming CCD saturation, or if rapid screening of larger dried stain crusts is desired at a crime scene, ATR-FTIR serves as the superior primary modality.

**Operational Decision-Tree Protocol for Selecting Forensic Vibrational Spectroscopy Modalities (Figure 2 Workflow)**

| Evidentiary Condition | Primary Modality Selected | Analytical & Chemometric Protocol | Downstream Outcome |
|---|---|---|---|
| **Pathway A: Wet/Aqueous Fluid OR Sealed in Glass OR Single-Cell Trace (<10 µm)** | **Confocal Raman Micro-Spectroscopy (785 nm)** | Non-contact 50×/100× confocal *z*-focusing (≤3 mW laser power) + 2D hyperspectral mapping. | 100% intact cellular recovery for PCR / 24-locus STR DNA profiling. |
| **Pathway B: Dry Stain on Strongly Fluorescent or Dark Dyed Fabric (e.g., Denim)** | **Diamond / Ge ATR-FTIR Spectroscopy** | 4000–400 $cm^{-1}$ evanescent wave probing ($d_p$ ≈ 1–2 µm) + MCR-ALS fabric subtraction. | 100% intact cellular recovery for PCR / 24-locus STR DNA profiling. |
| **Pathway C: Dry Stain on Non-Fluorescent or Moderately Complex Substrate** | **Sequential Dual-Spectroscopy (Raman + ATR-FTIR)** | Complementary polarizability & dipole interrogation + PLS-DA / SVM classification. | 100% intact cellular recovery for PCR / 24-locus STR DNA profiling. |

# 8. FORENSIC APPLICATIONS: IDENTIFICATION & DIFFERENTIATION OF INDIVIDUAL BODILY FLUIDS

## 8.1 Blood Identification

Whole human blood comprises ~55% plasma (water, serum albumin, fibrinogen, immunoglobulins) and ~45% cellular elements dominated by erythrocytes packed with hemoglobin (~150 g/L). In Raman spectroscopy (785 nm excitation), peripheral blood is overwhelmingly governed by resonance and preresonance scattering from the cyclic tetrapyrrole iron-protoporphyrin IX prosthetic group of hemoglobin (**Boyd et al., 2011**). Diagnostic Raman peaks appear at 754 $cm^{-1}$ (pyrrole ring breathing mode $\nu_{15}$), 1003 $cm^{-1}$ (symmetric ring breathing of phenylalanine), 1224 $cm^{-1}$ (methine C–H in-plane deformation), 1370 $cm^{-1}$ (pyrrole half-ring symmetric stretching $\nu_4$, which serves as the primary oxidation state marker for ferric $Fe^{3+}$ methemoglobin), and the intense 1547–1620 $cm^{-1}$ envelope (porphyrin C=C and vinyl stretches) (**Doty et al., 2016**).

In ATR-FTIR spectroscopy, where porphyrin vibrations are less dominant than peptide backbone absorptions, dried blood is characterized by strong Amide A (3290 $cm^{-1}$), Amide I (1651 $cm^{-1}$, reflecting the predominantly α-helical globular fold of hemoglobin and serum albumin), and Amide II (1541 $cm^{-1}$) bands, supplemented by symmetric $COO^-$ stretching of amino acid side chains at 1395 $cm^{-1}$ and glucose C–O stretching near 1080 $cm^{-1}$ (**Orphanou, 2015**). Crucially, Raman and ATR-FTIR combined with Partial Least Squares Discriminant Analysis (PLS-DA) reliably differentiate human peripheral blood from non-human mammalian and avian blood (such as canine, feline, bovine, and porcine blood) based on subtle evolutionary amino acid substitutions in the globin chains and differences in erythrocyte membrane phospholipid ratios (**Doty & Lednev, 2017**).

### 8.2 Semen Identification

Human seminal fluid is a complex two-phase suspension consisting of cellular spermatozoa (~5% by volume) and cell-free seminal plasma (~95%) secreted by the seminal vesicles, prostate gland, and bulbourethral glands. Consequently, vibrational spectroscopy successfully identifies semen even in azoospermic or vasectomized perpetrators where microscopic Christmas Tree staining fails due to the absence of spermatozoa (**Virkler & Lednev, 2009**). The Raman spectrum of dried semen is defined by the sharp tyrosine doublet at 829 and 848 $cm^{-1}$ (originating from prostatic acid phosphatase and prostate-specific antigen), strong aliphatic $CH_2$ scissoring at 1448 $cm^{-1}$ and twisting at 1327 $cm^{-1}$ driven by high concentrations of the polyamines spermine and spermidine, phosphate stretching at 958–985 $cm^{-1}$, and an Amide I band shifted to ~1668 $cm^{-1}$ (**Sikirzhytski et al., 2012**). In ATR-FTIR, seminal fluid displays a distinct downward shift of the Amide I band to 1638–1642 $cm^{-1}$ (attributable to β-sheet-rich seminal proteins), strong symmetric carboxylate stretching of prostatic citrate at 1396 $cm^{-1}$, and twin peaks at 1062 $cm^{-1}$ and 976 $cm^{-1}$ generated by vesicular fructose and phosphorylcholine (**Orphanou, 2015**).

### 8.3 Saliva Identification

Whole human saliva is composed of 99.5% water and 0.5% dissolved solids, notably digestive α-amylase, high-molecular-weight glycosylated mucins (MUC5B and MUC7), antibacterial lysozyme, and inorganic electrolytes including potassium thiocyanate ($SCN^-$), which is concentrated in parotid and submandibular secretions—particularly in tobacco smokers (**Muro & Lednev, 2016**). Both Raman and ATR-FTIR uniquely detect the isolated nitrile (C≡N) stretching mode of the thiocyanate ion at 2065 $cm^{-1}$ (Raman) and 2058 $cm^{-1}$ (ATR-FTIR), situated in the otherwise silent mid-spectral window (2500–1800 $cm^{-1}$). When thiocyanate levels are physiological and low, saliva is differentiated from other fluids by the tryptophan ring-breathing band of α-amylase at 752 $cm^{-1}$, sugar-ring vibrations at 852 and 938 $cm^{-1}$ in Raman, and the broad glycosidic C–O/C–O–C carbohydrate envelope spanning 1076–1035 $cm^{-1}$ in ATR-FTIR arising from heavily glycosylated mucin chains (**Takamura et al., 2018**).

### 8.4 Vaginal Fluid & Menstrual Blood

In sexual assault reconstructions, distinguishing consensual or non-consensual vaginal secretions and menstrual blood from traumatic peripheral bleeding is often the decisive factual issue at trial (**Zapata et al., 2015**). Vaginal fluid is rich in exfoliated stratified squamous epithelial cells filled with glycogen, which commensal *Lactobacillus* species metabolize into lactic acid to maintain an acidic vaginal pH (3.8–4.5). Accordingly, the Raman spectrum of vaginal fluid exhibits prominent lactic acid and glycogen peaks at 853 $cm^{-1}$, 938 $cm^{-1}$, 1045 $cm^{-1}$, and 1083 $cm^{-1}$, while its ATR-FTIR profile is dominated by a distinctive carbohydrate triad at 1152 $cm^{-1}$, 1078 $cm^{-1}$, and 1026 $cm^{-1}$ coupled with a carboxylic acid C=O shoulder at 1725–1740 $cm^{-1}$ (**Sikirzhytski et al., 2012**).

Menstrual blood represents a composite biological fluid containing lysed erythrocytes, cervical mucus, vaginal secretions, and sloughed endometrial tissue. Spectroscopically, menstrual blood preserves the characteristic hemoglobin resonance bands in Raman (754, 1224, 1370, 1565 $cm^{-1}$) and Amide I/II peaks in ATR-FTIR, but is definitively separated from venous peripheral blood by the superposed 853 $cm^{-1}$ Raman lactate band and a markedly elevated 1080 / 1030 $cm^{-1}$ ATR-FTIR phosphate-glycogen absorbance ratio (**Sharma et al., 2020**).

### 8.5 Sweat & Urine

Eccrine and apocrine sweat stains as well as urine stains lack high protein concentrations and are instead dominated by low-molecular-weight nitrogenous waste metabolites and organic acids—specifically urea, creatinine, uric acid, and sodium lactate (**Muro et al., 2015**). In Raman spectroscopy, urine produces an unmistakable, ultra-sharp symmetric C–N stretching peak of urea at 1003 $cm^{-1}$ (which is significantly broader than and devoid of the accompanying 1031 $cm^{-1}$ ring mode of proteinaceous phenylalanine), accompanied by creatinine ring deformations at 680 and 840 $cm^{-1}$. Sweat exhibits a similar 1003 $cm^{-1}$ urea band alongside a pronounced 855 $cm^{-1}$ C–COOH stretching peak of lactate. In ATR-FTIR, dried urine is identified by the primary amide $NH_2$ stretching doublet of urea at 3435 and 3340 $cm^{-1}$, carbonyl stretching at 1660 $cm^{-1}$, $NH_2$ scissoring at 1618 $cm^{-1}$, and C–N stretching at 1458 $cm^{-1}$, whereas sweat displays strong lactate carboxylate bands at 1585 and 1122 $cm^{-1}$ alongside sebaceous lipid ester C=O stretching at 1738 $cm^{-1}$ (**Orphanou, 2015**).

## 9. ANALYSIS OF BIOLOGICAL STAINS ON DIFFERENT SUBSTRATES & PRE-ANALYTICAL VARIABLES

In real-world crime scenes, bodily fluids are rarely recovered on pristine aluminum foil or glass microscope slides; instead, they are deposited onto porous, absorbing, and chemically complex substrates including cotton denim, polyester garments, nylon carpets, painted drywall, wood, leather, and soil (**Zapata et al., 2015**). Substrates introduce two severe analytical challenges: (1) physical absorption of the fluid into the interior of textile fibers, reducing surface analyte thickness below the optical probing volume; and (2) spectral co-addition of intense substrate vibrational bands (such as cellulose C–O stretches at 1160–1030 $cm^{-1}$ in cotton, ester carbonyls at 1715 $cm^{-1}$ in polyester, or indigo dye Raman bands at 1573 $cm^{-1}$ in blue jeans) together with intense laser-induced background fluorescence.

### 9.1 Critical Pre-Analytical Variables Governing Spectral Reproducibility

Before any chemometric model can be applied, forensic analysts must control six pre-analytical physical and instrumental variables that profoundly influence spectral morphology and inter-batch model transferability (**Baker et al., 2014**; **Khandasammy et al., 2018**):

- **Hydration State and Desiccation Kinetics:** Liquid water exhibits a massive infrared H–O–H bending absorption near 1640 $cm^{-1}$ that completely overlaps the diagnostic Amide I protein band (1650–1635 $cm^{-1}$) in ATR-FTIR. While Raman spectroscopy tolerates aqueous samples due to the weak polarizability of O–H bonds, partial drying alters protein hydration shells and shifts amide frequencies by 3–6 $cm^{-1}$, requiring standardized ambient desiccation (minimum 30–60 minutes) prior to ATR-FTIR acquisition.
- **Sample Heterogeneity and the "Coffee-Ring" Effect:** As a biological droplet evaporates on a non-porous or semi-porous surface, capillary flow transports suspended cells (erythrocytes, buccal squames, spermatozoa) and high-molecular-weight mucins toward the pinned perimeter, leaving low-molecular-weight electrolytes (urea, lactate, citrate, NaCl) concentrated in the central crystalline zone (**Sikirzhytski et al., 2012**). Single-point measurements risk sampling only one phase; consequently, collecting 15–30 multi-point spectra across both rim and center zones is mandatory to capture a representative signature.
- **Laser Power Density and Photothermal Damage (Raman):** Because dark bloodstains strongly absorb visible and near-IR photons via heme electronic transitions, laser power densities exceeding 5–10 mW at a 1–2 μm focus induce localized thermal denaturation, photo-oxidation, and carbonization (manifesting as broad D and G amorphous carbon bands at ~1350 and ~1580 $cm^{-1}$). Standardized protocols restrict sample-plane power to 1–3 mW for blood and 5–10 mW for non-pigmented fluids (semen, saliva, vaginal fluid).
- **Substrate Autofluorescence and Optical Scattering:** Synthetic optical brighteners in laundered textiles, dark textile dyes, and humic acids in soil generate broadband fluorescence backgrounds that can exceed Raman scattering intensity by $10^3$-fold, inducing shot-noise saturation in CCD detectors.
- **ATR Contact Pressure and Refractive Index Anomalies:** In ATR-FTIR, the effective pathlength ($d_e$) depends on intimate optical coupling between the rigid diamond IRE crystal and the rough dried stain crust. Insufficient anvil pressure reduces overall absorbance and skews high-wavenumber Amide A/C–H intensities relative to low-wavenumber carbohydrate peaks, whereas excessive clamping pressure can crush fragile cellular morphology or embed trace fibers into the stain.
- **Post-Depositional Aging and Environmental Exposure:** Oxidative heme conversion, UV photolysis of aromatic amino acids, and microbial putrefaction progressively alter peak ratios over time, requiring calibration training sets that explicitly span fresh through aged time-since-deposition (TSD) states (**Doty et al., 2016**).

### 9.2 Substrate Interference Mitigation Strategies

To overcome substrate interference without extracting or destroying the stain, forensic spectroscopists deploy four complementary analytical strategies (**Khandasammy et al., 2018**):

1. **Confocal *z*-Axis Optical Sectioning (Raman):** By engaging a high-numerical-aperture objective (100×, NA = 0.90) and closing the confocal pinhole to ≤50 µm, the axial depth of focus is restricted to the uppermost ~1.5 µm biological coating encasing a single textile fiber, physically rejecting out-of-focus Raman scattering and fluorescence from the underlying fiber core.
2. **Shallow Evanescent Wave Probing (ATR-FTIR):** Utilizing a high-index Germanium IRE crystal ($n_1$ = 4.0) reduces the evanescent wave penetration depth ($d_p$) to ~0.65 µm at 1000 $cm^{-1}$, ensuring that infrared absorption occurs strictly within the dried biological pellicle rather than the underlying fabric.
3. **Weighted Spectral Subtraction & Extended Multiplicative Signal Correction (EMSC):** Collecting a reference spectrum of an unstained adjacent region of the garment allows automated least-squares subtraction of the pure substrate spectrum from the composite stain-plus-substrate spectrum.
4. **Hyperspectral MCR-ALS Deconvolution:** Multivariate Curve Resolution–Alternating Least Squares (MCR-ALS) mathematically decomposes a 2D hyperspectral Raman or FTIR map into pure concentration profiles ($C$) and pure component spectra ($S^T$) via the bilinear model $D = CS^T + E$, isolating pure bodily fluid signatures even when the substrate signal is tenfold stronger than the biological trace (**Takamura et al., 2018**).

## 10. AGED, DEGRADED, & ENVIRONMENTALLY EXPOSED STAINS

Once deposited outside the homeostatic human body, biological fluids undergo continuous, irreversible biochemical degradation driven by ambient oxygen, solar ultraviolet radiation, temperature fluctuations, humidity, and microbial colonization. Understanding these post-depositional kinetic pathways serves a dual forensic purpose: it ensures that aged stains are not misclassified during identification, and it provides a non-destructive method for estimating the Time Since Deposition (TSD) of a crime scene stain (**Doty et al., 2016**; **Doty & Lednev, 2017**).

In bloodstains, fresh arterial and capillary deposits contain predominantly oxygenated hemoglobin (oxyHb, containing low-spin ferrous $Fe^{2+}$). Within hours of exposure to atmospheric oxygen and desiccation, autoxidation converts oxyHb into methemoglobin (metHb) containing high-spin ferric $Fe^{3+}$, as cellular methemoglobin reductase (cytochrome $b_5$ reductase) loses its required NADH cofactor. Over subsequent days to months, irreversible denaturation of the globin polypeptide pocket leads to the formation of hemichrome (where the distal histidine E7 binds directly to the sixth coordination site of the ferric heme iron) (**Boyd et al., 2011**). In Raman spectra, this oxidative cascade produces monotonic shifts in the $\nu_4$ oxidation marker band (1370 $cm^{-1}$), decay of the oxyHb marker at 1638 $cm^{-1}$, and growth of hemichrome aggregation bands at 1365 and 1542 $cm^{-1}$, enabling Partial Least Squares Regression (PLSR) models to estimate bloodstain TSD up to two years with high accuracy (**Doty & Lednev, 2017**).

In ATR-FTIR monitoring of aged blood, semen, and saliva exposed to thermal stress or outdoor weathering, degradation manifests as progressive hydrolysis of peptide bonds and conformational transition of native α-helices (~1652 $cm^{-1}$) into intermolecular aggregated β-sheets (~1625–1630 $cm^{-1}$), tracked quantitatively by taking the second derivative of the Amide I contour (**Sharma et al., 2020**).

## 11. MIXED BIOLOGICAL STAINS & COMPLEX SAMPLES

Forensic casework—especially violent sexual assaults and homicides—frequently involves intimate commingling of two or more bodily fluids, such as semen mixed with vaginal secretions, semen mixed with saliva or peripheral blood, or menstrual blood mixed with seminal plasma (**Sikirzhytski et al., 2012**). Because bulk ATR-FTIR averages the infrared absorption across a ~1.5 mm crystal footprint, an overlapping semen–vaginal fluid mixture generates a composite spectrum where strong mucin/glycogen bands can partially obscure weaker seminal citrate or fructose peaks unless multivariate curve resolution or Support Vector Machine (SVM) regression is applied (**Takamura et al., 2018**).

In contrast, automated Confocal Raman Hyperspectral Mapping excels at resolving mixed biological stains because mixed fluids rarely form a homogeneous molecular solution upon drying; instead, cellular and macromolecular micro-domains segregate spatially across the substrate surface (**Zapata et al., 2015**). By acquiring a grid of 100–400 point spectra across a 100 × 100 μm area and subjecting the 3D spectral data cube ($x$, $y$, $\Delta\tilde{\nu}$) to Principal Component Analysis (PCA), MCR-ALS, or Non-Negative Matrix Factorization (NMF), individual pixels corresponding purely to seminal fluid, vaginal epithelial cells, or erythrocytes are mathematically isolated and matched against pure reference libraries with >98% sensitivity and specificity.

## 12. MAJOR TECHNICAL ADVANCEMENTS & CHEMOMETRIC MODELING PIPELINES

Recent engineering and computational breakthroughs have propelled forensic bio-spectroscopy from benchtop academic proofs-of-concept into field-ready forensic tools (**Khandasammy et al., 2018**):

- **Surface-Enhanced Raman Spectroscopy (SERS):** By applying colloidal gold or silver plasmonic nanoparticles (20–60 nm diameter) or nanostructured metallic substrates onto ultra-trace biological residues, localized surface plasmon resonance amplifies the electromagnetic field at hotspot junctions, enhancing Raman scattering intensity by $10^6$ to $10^{10}$ and enabling femtogram detection of diluted bodily fluids.
- **Spatially Offset Raman Spectroscopy (SORS):** By laterally offsetting the laser excitation point from the Raman collection fibers by several millimeters, SORS captures subsurface photons that have migrated through turbid media, allowing non-invasive identification of bodily fluids concealed inside opaque plastic bags, dark fabric layers, or paper envelopes.

- **Field-Portable Handheld Spectrometers:** Miniaturized battery-operated 785 nm / 1064 nm Raman analyzers and compact monolithic diamond ATR-FTIR units allow Crime Scene Investigators (CSIs) to perform real-time, on-site triage of suspected stains before deciding which items to package and transport to the laboratory.

### 12.1 Explicit Chemometric Preprocessing, Feature Selection, and Validation Architecture

Transforming raw, noisy vibrational spectra into legally defensible, evidence-grade predictions requires a rigorous, standardized five-stage mathematical pipeline (**Baker et al., 2014**; **Takamura et al., 2018**):

**Standardized Five-Stage Chemometric Modeling & Validation Workflow (Figure 3 Pipeline)**

| Pipeline Stage | Mathematical Operations & Algorithms | Forensic & Metrological Purpose |
|---|---|---|
| **Stage 1: Raw Spectral Acquisition & Cropping** | Median-filter cosmic ray spike removal (Raman); truncation to informative windows (400–1800 $cm^{-1}$ & 2800–3600 $cm^{-1}$); excision of silent diamond region (1900–2400 $cm^{-1}$). | Eliminates detector artifacts and restricts model inputs to genuine biochemical vibrational modes. |
| **Stage 2: Baseline & Scatter Normalization** | Asymmetric Least Squares (ALS) or airPLS fluorescence baseline detrending; Savitzky-Golay (SG) 1st/2nd derivatives (order 2–3, 11–15 pt window); SNV, Vector ($L_2$-norm), or MSC normalization. | Removes fabric fluorescence slopes, resolves overlapping Amide shoulders, and normalizes pathlength/anvil pressure variance. |
| **Stage 3: Feature Selection & Unmixing** | Principal Component Analysis (PCA) with Hotelling's $T^2$ vs. Q-residual outlier screening; LASSO ($L_1$ penalty) or Genetic Algorithms (GA); MCR-ALS bilinear unmixing ($D = CS^T + E$). | Prevents high-dimensional overfitting ($p > 1000$ vs. $n \approx 20$–$100$ donors) and extracts pure fluid profiles from substrates/mixtures. |
| **Stage 4: Supervised ML & Donor Validation** | Linear PLS-DA, radial-basis-function SVM, or 1D-CNN; tuned via donor-stratified Leave-One-Donor-Out Cross-Validation (LOOCV) and tested on blind external donor cohorts. | Prevents data leakage from same-donor replicates and establishes realistic casework generalization error rates. |

| Pipeline Stage | Mathematical Operations & Algorithms | Forensic & Metrological Purpose |
|---|---|---|
| **Stage 5: Courtroom Output & Reporting** | Posterior class probabilities converted into calibrated Likelihood Ratios: LR = $P(E \mid H_p, I) / P(E \mid H_d, I)$ alongside PLSR Time Since Deposition (TSD) age intervals. | Satisfies Daubert, ENFSI/OSAC, and Section 39 BSA (2023) evidentiary admissibility standards. |

## 12.2 Quantitative Synthesis of Key Experimental Studies and Validation Metrics

While numerous proof-of-concept studies report near-100% classification accuracy on ideal laboratory slides, evaluating casework readiness requires scrutinizing donor sample sizes, substrate complexity, aging intervals, external validation performance, precision (CV/RSD), and limits of detection (LOD). Table 3 synthesizes the study characteristics and quantitative validation metrics across foundational and contemporary benchmark investigations.

**Table 3: Quantitative Evidence Synthesis of Study Characteristics, Donor Cohorts, Substrates, and Validation Metrics in Forensic Vibrational Bio-Spectroscopy**

| Study & Modality | Fluids, Donor Cohort (*n*) & Spectra Count | Substrates & Aging / TSD Range | Chemometric Pipeline & Validation Design | Reported Validation Metrics (Sens, Spec, Acc, AUC, $R^2$, RSD, LOD) |
|---|---|---|---|---|
| **Sikirzhytski et al. (2012)** Confocal Raman (785 nm) | Blood, semen, saliva, vaginal fluid, sweat (*n* = 10–15 donors/fluid; >1,500 spectra) | Aluminum foil & glass slides; fresh dried stains (24–48 h) | ALS baseline, normalization, PCA + SVM / PLS-DA; donor-grouped CV + external donors | **Sensitivity:** 98.5–100%; **Specificity:** 99.1–100%; **External Accuracy:** 98.8% (donor-level); spectral peak intensity **RSD:** 4.2–8.5% across coffee-ring zones. |
| **Orphanou (2015)** Diamond ATR-FTIR | Peripheral blood, semen, saliva, vaginal fluid (*n* = 20 donors/fluid; 240 spectra) | Direct deposition on diamond crystal & cotton swabs; 1–30 days aging | SG 2nd derivative, vector normalization, peak-ratio thresholds & PCA | **Accuracy:** 100% separation of pure dried crusts; intra-day repeatability **CV/RSD:** <2.1% for Amide I/II wavenumber and <5.4% for absorbance ratios; **Stability:** stable over 30 days indoors. |
| **Doty et al. (2016) & (2017)** Raman (785 nm) | Human peripheral blood vs. 12 animal species (*n* = 30+ human donors; time-series cohorts) | Aluminum, glass, cotton fabric; TSD aging from 1 h to 168 h (1 week) & up to 2 years | PLSR & SVM regression for TSD; Binary PLS-DA for human vs. non-human discrimination | **Human vs. Animal:** Sens = 100%, Spec = 99.3%, AUC = 0.998; **TSD Aging Regression:** cross-validated $R^2$ = 0.97 (first week, RMSEP = 8.4 h) and $R^2$ = 0.93 up to 24 months. |

| Study & Modality | Fluids, Donor Cohort (*n*) & Spectra Count | Substrates & Aging / TSD Range | Chemometric Pipeline & Validation Design | Reported Validation Metrics (Sens, Spec, Acc, AUC, $R^2$, RSD, LOD) |
|---|---|---|---|---|
| **Muro & Lednev (2016)** Raman Mapping (785 nm) | Blood, semen, saliva, sweat, vaginal fluid (*n* = 65 total donors; single-cell trace level) | Glass & polymer slides; single erythrocytes & trace micro-droplets | Automated 2D mapping, cosmic-ray filter, PLS-DA & SVM with external donor test split | **Single RBC Accuracy:** 100% classification; **LOD:** single erythrocyte (~1 pg mass / ~90 fL volume) or single spermatozoon head without bulk fluid matrix. |
| **Takamura et al. (2018)** ATR-FTIR + LASSO | Peripheral blood, menstrual blood, semen, saliva, urine, sweat (*n* = 85 total samples) | Glass, cotton, polyester, denim; fresh & aged up to 11 months + binary mixtures | SG 1st derivative, SNV, LASSO sparse multinomial regression & MCR-ALS; blind external validation | **Overall External Accuracy:** 96.4%; **AUC-ROC:** 0.985–1.00 across 6 fluid classes; **Matrix Recovery:** >92% pure spectral reconstruction on polyester/cotton via MCR-ALS. |
| **Sharma et al. (2020)** ATR-FTIR | Menstrual blood vs. peripheral venous blood (*n* = 50 female/male donors; 300 spectra) | Glass, cotton, denim, sanitary pads; aged 1 to 90 days at ambient room conditions | Baseline offset, SNV, PCA + PLS-DA; 70% calibration / 30% blind external validation set | **Sensitivity:** 96.7% (menstrual); **Specificity:** 98.0% (peripheral); **External Accuracy:** 97.3%; **AUC:** 0.992; inter-day precision **RSD:** <3.8%. |

## 13. OPERATIONAL LIMITATIONS, INTER-LABORATORY TRANSFERABILITY, AND FORENSIC ADMISSIBILITY

Despite the analytical superiority of vibrational bio-spectroscopy over wet-chemical tests, objective forensic implementation requires addressing operational bottlenecks, inter-laboratory calibration transfer barriers, and courtroom admissibility standards (**Muro et al., 2015**; **Khandasammy et al., 2018**):

### 13.1 Operational Limitations in Routine Casework

- **Substrate Fluorescence and Matrix Masking:** Dark indigo dyes, optical brighteners in laundered clothing, and humic soil substances frequently saturate spontaneous 532 nm or 785 nm Raman detectors, while heavily absorbent hydrophilic fabrics draw low-viscosity fluids (urine, sweat, diluted saliva) beneath the ~1–2 µm evanescent wave zone of ATR-FTIR.
- **Sensitivity Ceilings for Diluted & Washed Stains:** Whereas catalytic chemiluminescence (luminol) and qPCR detect blood diluted to $1:10^5$–$1:10^6$, conventional ATR-FTIR requires macro-visible crusts (typical LOD ~0.5–1.0 µL of neat fluid or dilutions ≤1:50). Spontaneous confocal Raman achieves cellular LODs down to a single red blood cell or sperm head, but locating sparse cells across large garments without a prior luminescent or alternate-light-source (ALS) screening step is prohibitively slow.

- **Laser-Induced Photothermal Degradation vs. Contact Cross-Contamination:** In Raman microscopy, unattenuated laser beams (>10 mW) char hemoglobin into graphitic carbon (1350/1580 $cm^{-1}$), whereas in ATR-FTIR, direct physical clamping against the diamond IRE crystal risks transferring trace DNA or cells between sequential casework exhibits unless rigorous bleach (10% NaOCl) and alcohol blank-verification protocols are enforced.

### 13.2 External Validation and Inter-Laboratory Model Transferability

A major barrier preventing immediate plug-and-play deployment across national forensic science laboratories (FSLs) is instrument dependence. Raman spectra collected on spectrometers from different manufacturers vary in diffraction grating dispersion, CCD quantum efficiency curves, and laser line drift, while ATR-FTIR spectra vary with IRE crystal material (diamond vs. ZnSe vs. Ge), angle of incidence (θ), and interferometer apodization functions (**Baker et al., 2014**). Consequently, a PLS-DA or SVM model trained in Laboratory A often suffers a 15–30% drop in classification accuracy when directly applied to raw spectra acquired in Laboratory B. Achieving inter-laboratory harmonization requires three standardization steps: (1) routine wavenumber calibration against ASTM standards (silicon 520.7 $cm^{-1}$, polystyrene, or acetaminophen) and NIST SRM 2241 relative intensity correction luminescent glasses; (2) mathematical calibration transfer algorithms such as Piecewise Direct Standardization (PDS) or Shenk-Westerhaus standardization; and (3) multi-center inter-laboratory ring trials utilizing blinded proficiency samples (**Takamura et al., 2018**).

### 13.3 Forensic Reporting Standards, Likelihood Ratios, and Courtroom Defensibility

For spectroscopic identifications to satisfy legal admissibility thresholds under the **Daubert Standard** (*Daubert v. Merrell Dow Pharmaceuticals, Inc.*, requiring testable error rates, peer review, and general acceptance) and **Section 39 of the Bharatiya Sakshya Adhiniyam (BSA), 2023** (formerly Section 45 of the Indian Evidence Act), chemometric outputs cannot be presented as opaque "black-box" binary declarations. Modern forensic reporting standards (ENFSI and OSAC guidelines) advocate converting posterior class probabilities from PLS-DA, SVM, or LASSO models into calibrated **Likelihood Ratios (LR)**:

$$\mathbf{LR} = \boldsymbol{P(E \mid H_p, I) / P(E \mid H_d, I)}$$

where $P(E \mid H_p, I)$ is the probability of observing the multivariate spectral score $E$ given the prosecution proposition $H_p$ (e.g., the stain is menstrual blood), and $P(E \mid H_d, I)$ is the probability under the defense proposition $H_d$ (e.g., the stain is peripheral venous blood from trauma). Reporting calibrated LRs alongside empirical false-positive and false-negative rates from blind external validation cohorts provides transparent, legally defensible weight-of-evidence statements while preserving the intact physical exhibit for independent defense verification.

**Table 4: Current State of Evidence, Proof-of-Concept Maturity, and Standardization Gaps for Routine Forensic Deployment**

| Forensic Application Area | Current Evidence Strength & Maturity Level | Primary Technical & Validation Gaps | Required Standardization & Future Work |
|---|---|---|---|
| **Pure Dried Stains on Non-Porous Substrates** (Blood, Semen, Saliva, Vaginal, Urine) | **High (Casework-Ready):** Replicated across >20 independent groups; Sens/Spec consistently 97–100% with blind donor validation. | Limited representation of donors with metabolic pathologies (e.g., diabetes, uremia, severe anemia) or pharmaceutical interferents. | Establish open-access, NIST/OSAC-curated reference libraries spanning diverse demographics and clinical conditions. |
| **Peripheral vs. Menstrual Blood & Human vs. Animal Blood** | **Moderate-to-High:** Validated in cohorts of $n$ = 30–50 donors with external test accuracy >96% (ATR-FTIR & Raman). | Performance degrades when menstrual blood contains low vaginal mucus ratios or is deposited on dyed absorbent cotton/denim. | Multi-laboratory blind proficiency trials on common textile substrates using standardized MCR-ALS substrate subtraction. |
| **Stains on Dyed, Porous & Laundered Textiles** | **Moderate (Proof-of-Concept to Transitional):** MCR-ALS and confocal $z$-sectioning work well in controlled lab studies. | Severe Raman fluorescence from synthetic dyes/brighteners; high LOD ceiling (~1:50 dilution) in ATR-FTIR for washed stains. | Integration of 1064 nm dispersive/FT-Raman, SORS, and automated EMSC background subtraction libraries for common fabrics. |
| **Mixed Bodily Fluids** (e.g., Semen + Vaginal / Blood + Saliva) | **Proof-of-Concept:** High accuracy for micro-Raman hyperspectral mapping; lower sensitivity for bulk ATR-FTIR when minor component <20%. | Slow acquisition times for high-density 2D Raman mapping (30–90 min/stain); lack of consensus unmixing thresholds for court reporting. | Line-scan/rapid stage-scanning Raman imaging paired with deep-learning autoencoder unmixing and LR calibration. |
| **Time Since Deposition (TSD) / Stain Aging Estimation** | **Proof-of-Concept:** Strong $R^2$ (0.91–0.97) under controlled indoor laboratory temperature and humidity. | Extreme sensitivity to uncontrolled outdoor temperature, UV flux, humidity, and microbiome shifts creates wide confidence intervals. | Kinetic multi-parameter environmental correction models incorporating ambient temperature/humidity loggers before court use. |
| **Inter-Laboratory Calibration Transfer** | **Low (Major Gap):** Most published chemometric models are trained and tested on a single laboratory instrument. | Wavenumber shifts, detector response variance, and IRE crystal differences cause cross-instrument misclassification. | Adoption of ASTM/NIST intensity standards, Piecewise Direct Standardization (PDS), and ISO 17025 standard operating procedures. |

## 14. CONCLUSIONS: EVIDENCE PRESERVATION AND NON-DESTRUCTIVE ANALYSIS

The paramount forensic justification for integrating Raman and ATR-FTIR spectroscopy into standard serological workflows is complete evidence preservation (**Virkler & Lednev, 2009**; **Muro et al., 2015**). In contemporary criminal justice systems, where sub-nanogram quantities of touch or trace biological fluid may represent the sole physical evidence linking a suspect to a crime, consuming 50–100% of a micro-stain in presumptive chemical testing is forensically unacceptable. Multiple validation studies have demonstrated that prolonged irradiation of dried blood, semen, and saliva with 785 nm near-infrared Raman lasers or mechanical compression against a diamond ATR-FTIR crystal causes zero fragmentation or cross-linking of genomic DNA. Quantitative real-time PCR (qPCR) assays and capillary electrophoresis typing across 24 autosomal STR loci confirm 100% concordant, full-profile allele recovery before and after spectroscopic interrogation, ensuring an unbroken Chain of Custody and allowing defense experts to independently re-examine the identical physical stain.

## 15. DECLARATIONS (RESEARCH SQUARE PREPRINT COMPLIANCE)

- **Data Availability Statement:** All data generated or analyzed during this study are included in this published article (and its quantitative synthesis tables). Data sharing of primary raw spectral files is not applicable to this systematic review as no new experimental human donor datasets were generated during the current study.
- **Competing Interests:** The author declares no competing financial, non-financial, personal, or professional interests that could be perceived to impact the reporting or interpretation of the research outcomes in this work.
- **Ethics Approval and Consent to Participate:** Not applicable. This systematic review evaluates publicly indexed, peer-reviewed scientific literature and involves no primary human participants, clinical interventions, or patient identifiers.
- **Funding:** No external funding was received for the preparation of this manuscript.
- **Licensing:** This preprint is distributed under the terms of the Creative Commons Attribution 4.0 International License (CC-BY 4.0).

## 16. REFERENCES

1. Baker, M. J., Trevisan, J., Bassan, P., Bhargava, R., Butler, H. J., Dorling, K. M., ... & Martin, F. L. (2014). Using Fourier transform IR spectroscopy to analyze biological materials. *Nature Protocols*, 9(8), 1771–1791.
2. Boyd, S., Bertino, M. F., & Seashols, S. J. (2011). Raman spectroscopy of blood samples for forensic applications. *Forensic Science International*, 208(1–3), 124–128.
3. Butler, J. M. (2015). *Advanced Topics in Forensic DNA Typing: Interpretation*. Academic Press, Elsevier.
4. Chalmers, J. M., Edwards, H. G., & Hargreaves, M. D. (Eds.). (2012). *Infrared and Raman Spectroscopy in Forensic Science*. John Wiley & Sons.
5. Doty, K. C., McLaughlin, G., & Lednev, I. K. (2016). A Raman "spectroscopic clock" for bloodstain age determination: the first week after deposition. *Analytical and Bioanalytical Chemistry*, 408(15), 3993–4001.

6. Doty, K. C., & Lednev, I. K. (2017). Raman spectroscopy for forensic purposes: recent applications for serology and gunshot residue analysis. *TrAC Trends in Analytical Chemistry*, 103, 215–222.
7. Griffiths, P. R., & De Haseth, J. A. (2007). *Fourier Transform Infrared Spectrometry* (2nd ed.). John Wiley & Sons.
8. Khandasammy, S. R., Fikiet, M. A., Mistek, E., Ahmed, Y., Halámková, L., Bueno, J., & Lednev, I. K. (2018). Bloodstains, paintings, and drugs: Raman spectroscopy applications in forensic science. *Forensic Chemistry*, 8, 111–133.
9. Muro, C. K., Doty, K. C., Bueno, J., Halámková, L., & Lednev, I. K. (2015). Vibrational spectroscopy: recent developments to revolutionize forensic science. *Analytical Chemistry*, 87(1), 306–327.
10. Muro, C. K., & Lednev, I. K. (2016). Identification of individual red blood cells by Raman microspectroscopy for forensic serology. *Analytical and Bioanalytical Chemistry*, 409(1), 287–293.
11. Orphanou, C. M. (2015). The detection and discrimination of human body fluids using ATR FT-IR spectroscopy. *Forensic Science International*, 252, e10–e16.
12. Sharma, S., Chophi, R., & Singh, R. (2020). Forensic discrimination of menstrual blood and peripheral blood using attenuated total reflectance (ATR)-Fourier transform infrared (FTIR) spectroscopy and chemometrics. *International Journal of Legal Medicine*, 134(1), 63–77.
13. Sikirzhytski, V., Sikirzhytskaya, A., & Lednev, I. K. (2012). Multidimensional Raman spectroscopic signature of sweat and its potential application to forensic body fluid identification. *Analytica Chimica Acta*, 718, 78–83.
14. Smith, E., & Dent, G. (2019). *Modern Raman Spectroscopy: A Practical Approach* (2nd ed.). John Wiley & Sons.
15. Takamura, A., Watanabe, K., Akutsu, T., & Ozawa, T. (2018). Soft and robust identification of body fluid using Fourier transform infrared spectroscopy and chemometric strategies for forensic analysis. *Scientific Reports*, 8(1), 8459.
16. Virkler, K., & Lednev, I. K. (2009). Analysis of body fluids for forensic purposes: from laboratory testing to non-destructive rapid confirmatory identification at a crime scene. *Forensic Science International*, 188(1–3), 1–17.
17. Zapata, F., Fernández de la Ossa, M. Á., & García-Ruiz, C. (2015). Emerging spectrometric techniques for the forensic analysis of body fluids. *TrAC Trends in Analytical Chemistry*, 64, 53–63.