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Detection of Oral Fluid Stains on Common Substrates Using SEM and ATR−FTIR Spectroscopy for Forensic Purposes

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Cite This: *ACS Omega* 2024, 9, 30142−30150

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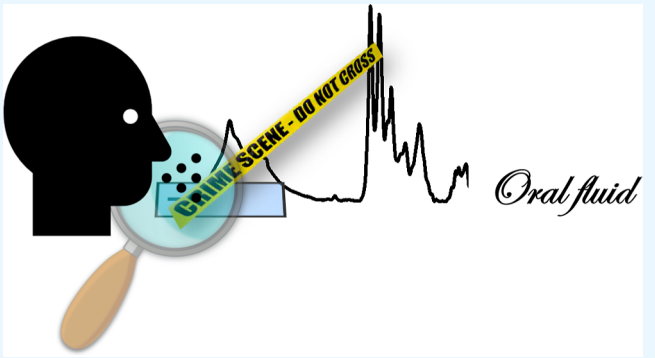
ABSTRACT: Attenuated total reflectance (ATR) Fourier-transform infrared (FTIR) spectroscopy has been pursued as a novel approach to detect and differentiate biological materials with high specificity owing to its ability to record unique spectral patterns corresponding to the biochemical composition of a specimen. This study expands the application of ATR−FTIR for detecting oral fluid (OF) stains on various common substrates, including four porous and six nonporous substrates. For nonporous substrates, the spectral contribution from the substrate was minimal, and no background subtraction from the substrate bands was required (except for mirrors). For porous substrates, the contribution from the surface was pronounced and was addressed via background subtraction. The results indicated that major OF bands were detected on all the surfaces, even six months after OF deposition. Furthermore, scanning electron microscopy (SEM) was used to probe the morphologies of OF stains on various substrates. SEM micrographs revealed characteristic salt crystals and protein aggregates formed by the dried OF, which were observed for fresh samples and samples after six months post-deposition. Overall, this study demonstrated the great potential of SEM and ATR−FTIR spectroscopy for detecting OF traces on porous and nonporous substrates for up to six months for forensic purposes.



1. INTRODUCTION

Human oral fluid (OF) has received considerable attention in recent years as valuable biological evidence in various forensic scenarios as a source of genetic information. For many years, it has been used as a rich and reliable source of nucleic acids, e.g., deoxyribonucleic acid—which is used for human identification and sex determination—and messenger ribonucleic acid, which enables OF sample age estimation.[1] OF is the specimen of choice for important forensic analysis, such as testing for the presence of illicit drugs and alcohol in cases involving driving under the influence, doping in sport, testing in the workplace, and others.[2] Compared to other biological specimens, OF undeniably requires limited personnel training, can be easily and noninvasively collected, and has minimum chances of adulteration.[3] In addition, its collection does not need special and expensive equipment; it can simply be drained, spat in a tube, or swabbed. In forensics, OF can be used to investigate several criminal cases, such as homicides and sexual and physical assaults that leave OF deposits on the skin or clothes. Moreover, it can be found on objects that contact the lips, such as glass, cigarettes, and spoons, or left on other surfaces through drooling or wiping the mouth with a tissue.[2]

Owing to its transparent nature, specifically in its dried state, detecting OF can be challenging; thus, there is a demand to develop analytical tools that can be routinely and rapidly employed for OF identification in forensic laboratories. Biochemical detection methods of OF are mainly based on targeting specific components, such as α-amylase, using the Phadebas paper method. Although α-amylase activity detection is an important way of detecting OF stains, it is inconclusive due to the presence of α-amylase in other body fluids, such as the pancreas, semen, and vaginal secretions.[4] Another test used for OF identification is the lateral-flow immune-chromatographic strip test to detect the presence of human salivary α-amylase (HAS);[5] however, this test can also produce positive results with human pancreatic amylase.[6] Owing to the lack of OF confirmatory tests, a demand exists to develop confirmatory and nondestructive tests for OF detection for forensic applications.

As emerging methods, several techniques have been employed for OF analysis by exploiting organic and inorganic constituents, including mass spectrometry[7] and X-ray spectrometry.[8] For example, proteomic mass spectrometry has shown considerable progress, where multiple endogenous

Received: November 23, 2023
Revised: February 11, 2024
Accepted: June 10, 2024
Published: June 18, 2024

https://doi.org/10.1021/acsomega.3c09358
ACS Omega 2024, 9, 30142−30150

proteins are directly linked to body fluid identification. As such, α-amylase, cystatin, histatin, and uncharacterized protein (c6orf58) are linked to OF identification.[9] Despite the success of these studies for qualitative and quantitative detection, these techniques remain destructive to the sample and require lengthy preparation, limiting their use for forensic applications. Recently, vibrational spectroscopic approaches, including attenuated total reflectance-Fourier-transform infrared (ATR-FTIR) spectroscopy and Raman spectroscopy, have proven to be suitable due to their nondestructive nature, minimum sample volume and preparation, and safety in handling procedures.[10] Vibrational spectroscopy methods enable the detection of several molecular components by providing a characteristic spectral signature of different types of evidence.[11] They have been employed to procure spectroscopic signatures of heterogeneous mixtures, such as biological samples (OF, blood, urine, sweat, and fingermarks)[12−15] and identify nonbiological samples, such as cosmetic products[16] and drugs of abuse.[17]

Over the years, various IR and Raman spectroscopies have been used to identify bodily fluids for forensic applications. These body fluids were detected and distinguished based on their unique spectral patterns that are specific to macromolecule groups within the body fluids, including proteins, sugars, and phosphates.[12] Takamura et al.[18] developed a modeling method based on ATR−FTIR and partial least-squares (PLS) linear-discriminate analysis (LDA) Q test (PLS-LDA-Q) for discriminating body fluids from nonbodily fluids and body fluids. In 2009, Raman spectroscopy was employed to differentiate between human, canine, and feline blood.[19] Subsequently, Raman spectroscopy was used to describe three components of OF: protein, saccharide, and amino acids.[20] They also indicated that the Raman spectral signature can be used to identify and differentiate OF stains from other unknown substances. Fujihara et al.[21] used a portable Raman spectrometer to analyze different blood species, including humans, cows, horses, sheep, pigs, rabbits, chickens, rats, mice, cats, and dogs. Mistek-Morabito and Lednev[22] employed ATR−FTIR spectroscopy and PLS discriminant analysis (DA) for discrimination between human and nonhuman blood.

Casework on body fluids collected from crime scenes involves the analysis of such evidence under unpredictable and nonideal situations. For example, body fluids can be present in trace amounts in a dried condition on different substrates with different properties (porous, nonporous, hydrophilic, rough, etc.); thus, examining biological evidence in situations stimulating a real forensic case is intriguing. Some articles have reported that the detection of body fluids on different substrates using Raman spectroscopy may be hindered by the interferences/fluorescence from the substrates.[23,24] Therefore, ATR−FTIR spectroscopy is practically better suited for detecting body fluids on different substrates. Gregório and co-workers[13] used ATR−FTIR spectroscopy to detect semen, vaginal fluid, and urine stains on superabsorbent pads. The results indicated that the method enabled the detection of these three fluids on the upper layer of all of the pads. In a preliminary study by Cano-Trujillo et al.,[25] ATR−FTIR spectroscopy and chemometric approach were used to detect and differentiate human and canine OFs deposited on different porous substrates. The results allowed the classification of human and canine OF stains regardless of the substrates on which they were placed.

To the best of our knowledge, only two studies have focused on the ATR−FTIR analysis of OF stains on various substrates.[25,26] The first study by Quinn and Elkins focused on ATR−FTIR spectra of venous and menstrual blood, semen, saliva, and breastmilk deposited on cotton, nylon, wood, paper, and glass substrates. Their findings indicated that the porosity and weave of the substrate affected the detection of these body fluids. The second study focused on differentiating human and canine OF samples deposited on various porous substrates. Therefore, this study aims to expand the use of ATR−FTIR spectroscopy for analyzing OF stains placed on common porous and nonporous substrates. We removed the substrate contribution from the ATR−FTIR spectrum of the OF to improve the identification. This approach enabled the detection of OF stains on all tested substrates soon after deposition and six months after the deposition. In addition, scanning electron microscopy (SEM) was used to probe the morphology of OF stains on various substrates, revealing characteristic features of salt crystals and protein aggregates in fresh OF stains and those after six months post-deposition.

2. EXPERIMENTAL SECTION

2.1. Oral Fluid Sample Collection and Preparation.

OF sample was collected from a healthy female donor with no history of illness. The donor was requested to rinse her mouth with water to remove any food particles and wait 15 min to avoid sample dilution. OF samples were collected in sterile, screw-top tubes via "spitting," after which they were refrigerated at 4 °C. This research was approved by the Health Sciences Centre Ethical Committee of Kuwait University and is in accordance with the ethical standards of the institutional and/or national research committee and the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Furthermore, informed consent was obtained from the donor.

This study used 10 substrates that were classified as porous and nonporous. The porous substrate group included cigarettes, printing and tissue papers, and wooden spoons, whereas the nonporous group involved aluminum foil, glass, mirror, and paper, plastic, and Styrofoam cups. It is worth mentioning that the internal surface of the paper cup was coated with a polymer, which is likely responsible for the nonporous characteristics of the cup. All items were acquired from local stores and were cut into approximately 1 cm × 1 cm pieces using sterilized scissors or a scalpel. The nonporous and hard substrates were thoroughly cleaned and sterilized using acetone, methanol, and Alconox detergent (Sigma-Aldrich) before OF sample deposition. Gloves were worn all the time and replaced between each substrate to avoid substrate contamination, and disposable forceps were used to handle each substrate. The OF sample was brought to room temperature and vortexed for 1 min before applying onto different substrates. 20 μL of OF was directly deposited on each substrate using a pipette and left to dry in a clean Petri dish before analysis. The drying time varied for each sample depending on the nature of the substrate; however, the analysis was only performed when the samples were completely dry. All the samples were stored at ambient conditions, not exposed to direct sun light, and left to dry overnight. Sample collection and handling were maintained consistent throughout the experiment and no inconsistency or irreproducibility were noticed when spectra were acquired.

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ACS Omega 2024, 9, 30142−30150

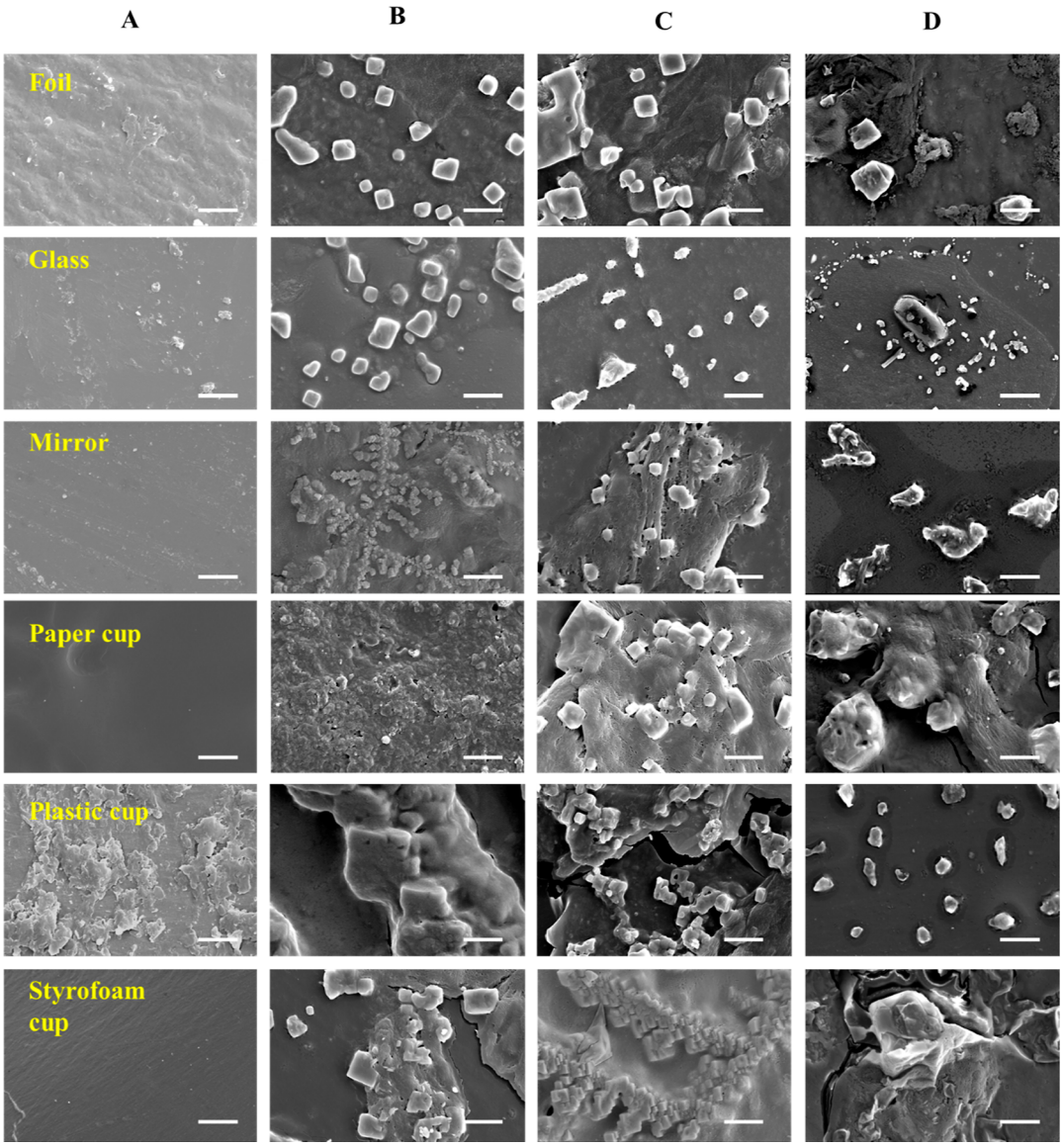

Figure 1. SEM micrographs of an OF on nonporous substrates: (A) substrate without OF, (B) substrate with fresh OF, (C) substrate with OF after 4 weeks, and (D) substrate with OF after six months. The magnification is ×2000, and the scale bar is 10 μm.

For aging experiments, the OF samples on the porous and nonporous substrates were left on the laboratory bench under ambient conditions, and the same samples were analyzed throughout the course of this study. Similarly, the samples were left on the stub (SEM sample holder) under ambient conditions and were analyzed after 4 weeks and six months using SEM.

2.2. Sample Analysis and Data Processing. The samples were subjected to SEM using a JSM-IT100 InTouch Scope scanning electron microscope JEOL operated at an accelerating voltage of 15 kV, a working distance of 10 mm, and a probe current of 60 to acquire the OF images on each substrate. Before analysis, the samples were coated with gold by using a spattering machine (LEICA EM ACE 200). Initial attempts involved optimizing the magnification employed to obtain clear and well-defined images. For this, magnifications of 100×, 1000×, 2000×, and 10,000× were used; however, the higher magnification 10,000× provided poor image quality, and components in the sample were not distinguished. The second attempt involved comparing low- and high-magnification images of OF on both porous and nonporous substrates. In this respect, 100× and 2000× magnifications were applied; nonetheless, the lower magnification did not produce a satisfactory resolution, particularly for absorbing substrates; therefore, ×2000 magnification was used for all experiments.

The ATR−FTIR spectra for each sample deposited on the 10 substrates were collected using a PerkinElmer Spectrum 3 instrument equipped with a 1.8 mm diamond crystal at a 45° incidence angle and a fixed spectral resolution of 4 cm^{-1} and a scan speed of 0.2 cm/s. At these parameters, good quality spectra and less spectral noise were obtained. The spectrum of air was utilized as a background upon the spectrum recording. All spectra were acquired at a room temperature between 21 and 25 °C. Spectra were collected from 10 locations on the periphery and central regions of the sample, and multiple scans of each spectrum were collected to account for heterogeneity. No inconsistency or irreproducibility was noticed when spectra were acquired. A moisture trap connected to the ATR−FTIR spectrometer was used to reduce the contribution of water from the atmosphere. Additionally, a CO_2/H_2O correction in

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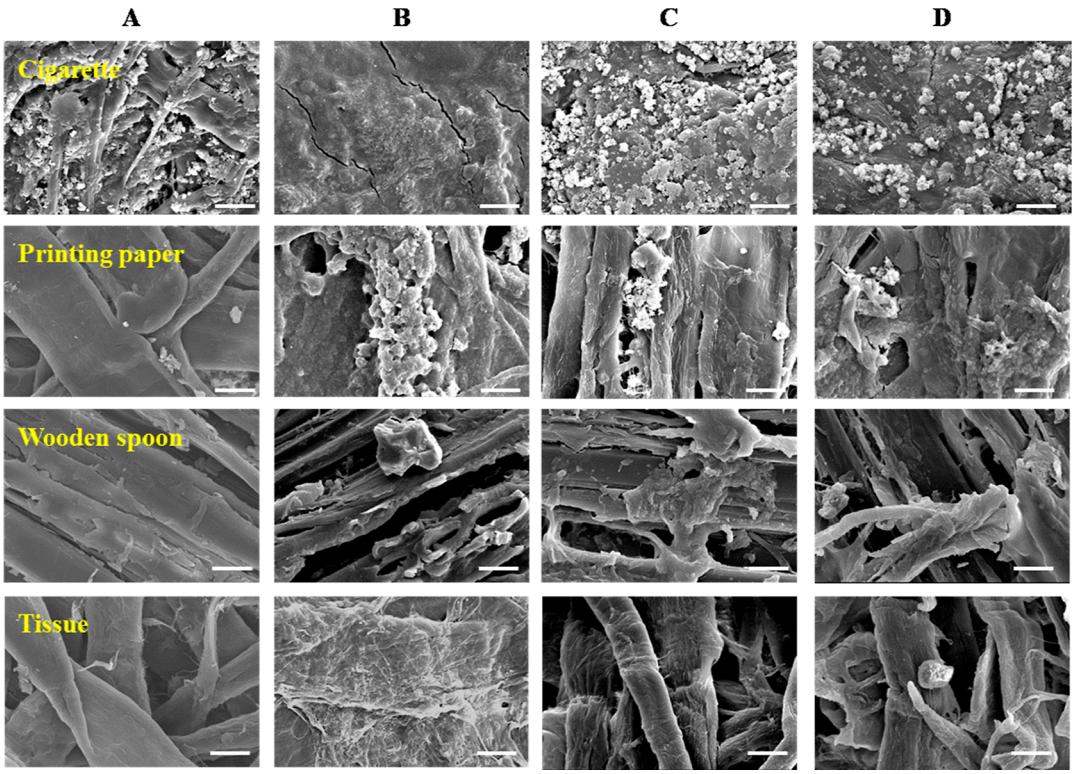

Figure 2. SEM micrographs of the OF on porous substrates: (A) substrate without OF, (B) substrate with fresh OF, (C) substrate with OF after 4 weeks, and (D) substrate with OF after six months. The magnification is ×2000, and the scale bar is 10 μm.

Spectrum IR software was employed upon data collection to remove the contribution of CO_2 and H_2O from the atmosphere. The ATR crystal was thoroughly cleaned with methanol after each analysis.

The ATR−FTIR spectra were imported into the PLS Toolbox 8.9.1 (eigenvector Research, Inc., Wenatchee, WA) operating in MATLAB R2020b (MathWorks, Inc., Natick, MA) software for preprocessing. The absorption spectra were obtained by applying the $\log(1/T)$ function. Then, the spectra were baseline corrected by automatic weighted squares and normalized by the total area. Additionally, Savitzky−Golay smoothing algorithm with a 15 nm filter width was applied.[27] Spectral subtraction was applied to minimize the substrate interference using eq 1

$$S_{\text{subtracted}} = S_{\text{sample}} - (S_{\text{substrate}} \times \text{SF}) \quad (1)$$

where $S_{\text{subtracted}}$ is the targeted OF spectrum with a minimum substrate contribution, S_{sample} is the acquired sample spectrum, $S_{\text{substrate}}$ is the substrate spectrum, and SF is the subtraction factor.[28] The SF was adjusted manually to resolve the OF bands and avoid oversubtraction by not allowing negative and derivative-like spectral features. Readers are encouraged to explore the following review article to have insights into limitations and potential of spectral subtractions in FTIR spectroscopy.[28]

3. RESULTS AND DISCUSSION

3.1. Morphology of Oral Fluid Stains on Porous and Nonporous Substrates. Forensic applications of SEM include the analysis of trace evidence such as gunshot residue, fibers, and glass.[29] However, investigating the morphology of dried stains of biofluids, such as OF, remains largely unexplored; thus, we studied the morphology of dried OF stains on 10 different substrates.

Initially, SEM was used to examine the morphology of nonporous and porous substrates in the absence and presence of OF. SEM images of the 10 substrates without OF were obtained, and the surfaces appeared clean without any noticeable particulate or dirt on them (Figures 1A and 2A). Subsequently, images of a fresh OF (Figures 1B and 2B) and OFs aged for 4 weeks (Figures 1C and 2C) and six months were acquired (Figures 1D and 2D).

OFs displayed different morphologies when deposited on nonporous substrates (foil; glass; mirror; and paper, plastic, and Styrofoam cups). Specifically, when deposited on glass and foil, the OF appeared as separated salt crystals of different sizes randomly spread over the substrates. However, when deposited on mirrors and paper, plastic, and Styrofoam cups, the OF appeared as highly fused heterogeneous salt crystals (Figure 1B). These findings correlate with those reported by Hughes et al.[30] regarding the morphology of OF deposits on nonporous substrates, except that they reported dendrite-shaped crystals on thermolaminated, polypropylene, silicon, and aluminum substrates, which were observed in our case with mirror and Styrofoam cup substrates. The amorphous crystals of the OF on nonporous substrates showed excellent stability with aging for 4 weeks and six months (Figure 1C,D).

However, fresh OF samples were visible on all four porous substrates (the neat substrates are presented in Figure 2A) and displayed some differences in their morphology. For example, the fresh OF on a tissue paper appeared as network-shaped meshes, whereas the OF on printing paper and a wooden spoon exhibited highly fused microglobule morphology, and the microglobules were thoroughly attached to the substrate fibers (Figure 2B). Finally, for the cigarette, the tipping paper

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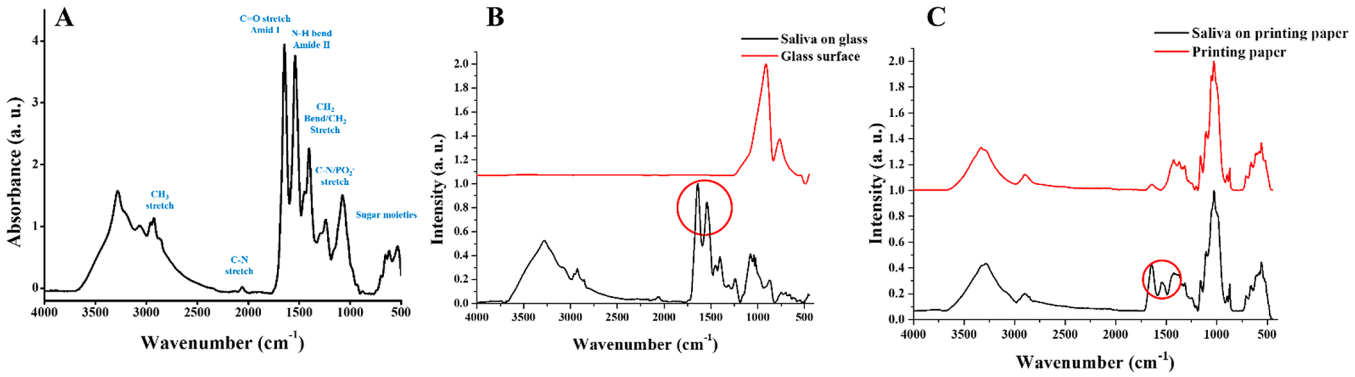

Figure 3. (A) ATR−FTIR spectrum of OF stains measured directly on diamond crystals. The most informative spectral region for identifying OF is 1500−1800 cm^{-1}. (B,C) Representative ATR−FTIR spectra of (B) nonporous and (C) porous substrates; the spectrum of the substrate is shown in red. The experimental spectra were baseline corrected.

portion of the cigarette filter was studied (which contacts the smoker's lips), which displayed a unique morphology without the OF. Figure 2A represents the tipping paper containing thick fibers with randomly distributed carbon particulates on its surface. After the addition of OF, a thick sample layer completely masked the fibers and particulates. The aging of the OF samples on porous substrates showed differences in morphology where the sample appeared dry and cracked when deposited on tissue and printing papers, with no considerable change in morphology even after six months post-deposition. However, the cigarette surface appeared pronounced in the images obtained for the samples after 4 weeks and six months of OF deposition, with the presence of some flakes that could be from the OF sample.

OF is a water-based liquid containing ions, such as calcium (0.88−1.51 mmol/L), magnesium (0.192−0.338 mmol/L), chloride (15.70−24.42 mmol/L), and phosphorus (2.39−3.61 mmol/L). It also contains proteins (0.35−0.65 mmol/L), such as albumin (0.169−0.394 mmol/L), and other constituents as reported previously.[31,32] When a drop of sample containing proteins and salts is allowed to dry (such as a drop of OF), the salt concentration increases and protein phase-separation occurs. This gradual increase produces protein ash (the dark part in the SEM image represents the region where salts are depleted), which is dominant in porous substrates, whereas in nonporous substrates, protein ash and salt crystals are formed.[33] Therefore, salt and protein structures are clearly distinguished in dried OF stains, depending on the type of surface on which the OF is deposited. These results indicate that the nature (porous and nonporous), roughness, and texture of the substrate rather than its hydrophobicity/hydrophilicity affect the final morphology of the OF stains.

3.2. ATR−FTIR Spectral Analysis of Oral Fluid. First, ATR−FTIR spectra were measured from OF stains deposited directly on the diamond crystals. The spectra were measured after deposition and drying for approximately 60 min. Figure 3A shows a characteristic spectrum of OF, and Table 1 summarizes the characteristic spectral bands. A 950−1080 cm^{-1} region is assigned to bands from carbohydrates and sugar moieties. The amide bands were also assigned: amide III at 1239 cm^{-1}, amide II at 1544 cm^{-1}, amide I at 1639 cm^{-1}, and amide A at 3279 cm^{-1}. Methylene groups of amino acids were observed at 1396 and 1452 cm^{-1}, and the bands at 2854 and 2958 cm^{-1} are attributed to the C−H stretching vibration of lipid ester groups. Finally, the band at 2054 cm^{-1} is assigned explicitly to the thiocyanate anions (SCN^-), a unique marker for OF.[12,18] Distinctly, the primary bodily fluid bands are between 1500 and 1800 cm^{-1};[34] therefore, we examined this region to determine the presence of OF stains on different porous and nonporous substrates.

Table 1. Major OF Absorption Bands (cm^{-1}) in the IR Spectra[12,18]

ATR−FTIR band (cm^{-1})	source	vibrational mode
3279	amide A	symmetric N−H stretching
2958, 2854	lipids in oral mucosa	CH_3 stretching
2054	thiocyanates	C−N stretching
1639	amide I (α-helix)	C═O stretching
1544	amide II	N−H bending coupled to C−N stretching
1452	methylene groups of amino acid side chains in proteins and lipids	in-plane bending
1396	amino acid side chains	CH_2^- stretching
1239	amide III/phospholipids	antisymmetric C−N stretching and PO_2^- stretching
1080−950	sugar moieties (glycosylated proteins)	CH_2OH groups, C−O stretching and COH groups bending, symmetric PO_2^- stretching

Initially, control experiments were performed using ATR−FTIR analysis of nonporous and porous substrates in the absence of OF (Figures S1 and S2). The major bands of the porous substrates are located at 600−1600 cm^{-1} (Figure S2), whereas the intensities of these bands were lower in the case of nonporous substrates (Figure S1). The effect of substrates on the ATR−FTIR pattern is less at >1500 cm^{-1}; a substantial contribution from the substrates is noticeable at <1500 cm^{-1}.

Similar ATR−FTIR bands were observed for some absorbing substrates (cigarettes, printing and tissue papers, and wooden spoons); an intense band located at ∼1000 cm^{-1}, ascribed to the presence of a large amount of cellulose on these surfaces.[35,36] Additional bands in the 1300−1700 cm^{-1} region were observed in the spectrum of a wooden spoon due to the food-safe finishing layer present on it (a common manufacturing addition). However, for the nonporous surfaces, each surface shows its unique spectrum, resembling the spectra reported in the previous study.[37] These control experiments were performed to understand any spectral contribution or overlap that might result from analyzing OF stains on these surfaces. OF stains have unique spectroscopic signatures, which are different from these surfaces. Therefore, ATR−FTIR

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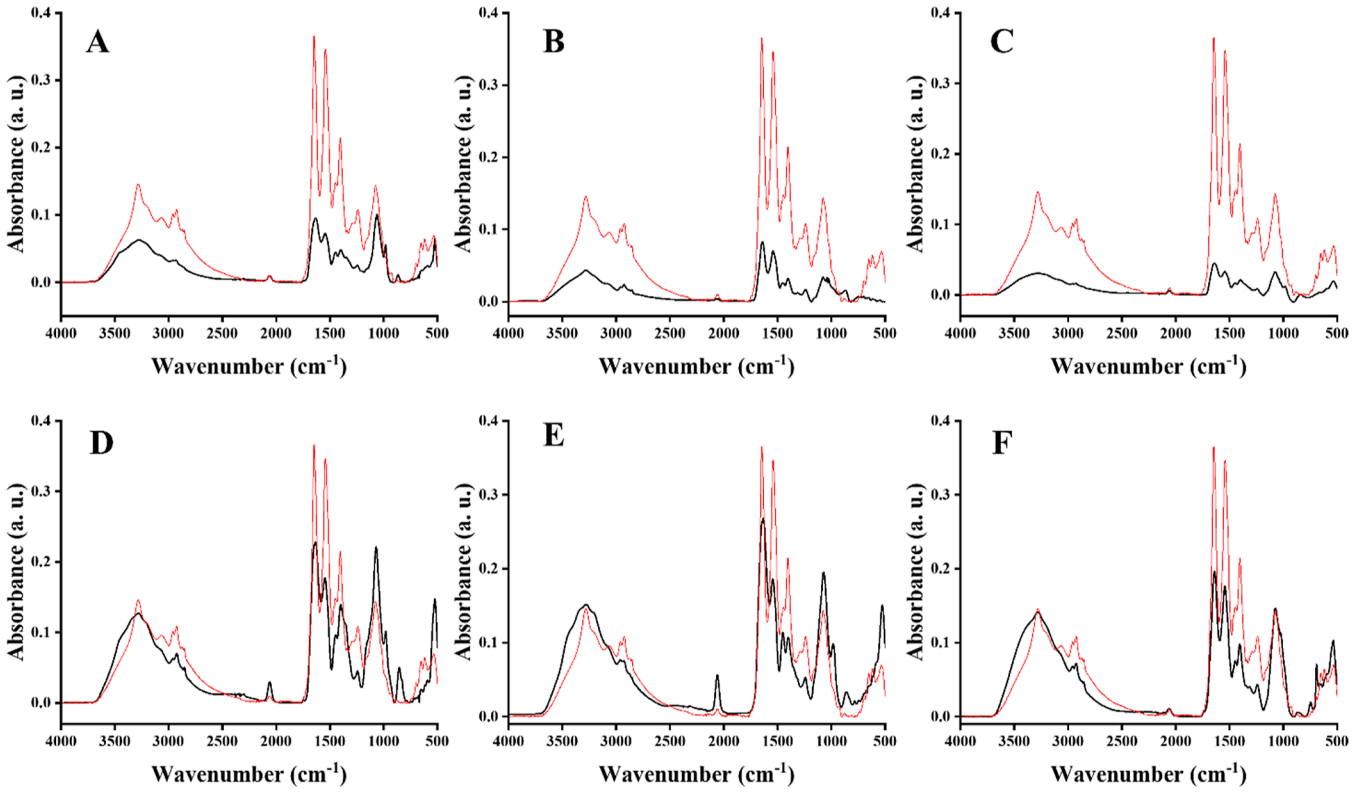

Figure 4. (A–F) ATR–FTIR spectra of OF stains on (A) aluminum foil, (B) glass, (C) mirror, (D) paper cup, (E) plastic cup, and (F) Styrofoam cup. The experimental spectra were baseline corrected; the red spectrum is the OF spectrum applied directly to the diamond crystal.

analysis can be used to probe the spectral features of OF stains found on these substrates.

Figure 3B,C shows the representative ATR–FTIR spectra of OF stains deposited on nonporous (glass) and porous (printing paper) substrates. It is clear that OF bands were clearly observed on nonporous substrate, while noticeable contribution from the substrate bands was observed when the OF was placed on the porous substrate. This result is not surprising because the OF placed on a nonporous substrate will form a droplet and remain on top of the substrate, minimizing the spectral contribution of the substrate bands. However, when OF is deposited on porous substrates, the fluid penetrates the substrate and becomes diluted, resulting in less intense OF bands, and the spectral contribution of the substrate becomes prominent. This result agrees with observations reported by Gregório and co-workers on semen, vaginal fluid, and urine stains placed on superabsorbent pads.[13] Another key point is the sample thickness; the exact knowledge thereof is important, especially when studying body fluids on porous substrates. This is governed by two factors: (1) absorption of fluids by the substrates to varying degrees and (2) the exponential decay of the amplitude of the ATR (E) when it penetrates the substrate layer, as expressed in the following equation

$$E = E_0 \exp(-z/d_p) \quad (2)$$

where E_0 is the amplitude of E at the interface between the internal reflection element and the sample, z is the depth of the interface, and d_p is the penetration depth.[38]

Controlling a liquid sample thickness is challenging, and at crime scenes, body fluids can be located on different substrates of varying nature, thickness, and roughness.[26] In this study, we used a simple approach to detect OF stains on different substrates by analyzing traces of OF stains on nonporous and porous substrates. Spectral subtraction was used to eliminate the spectral contribution of the substrate, particularly on the porous substrates using eq 1.

3.3. Analysis of Oral Fluid Stains on Nonporous Substrates. Figure 4A–F shows the OF stains placed on six nonporous substrates. The shape and intensity of the amide I and amide II vibration bands in the OF stains varied for each nonporous substrate, which depended on the nature of the substrate and the final form of the OF (drop or spread) when deposited on the substrate.[13] Additionally, vibrational modes of the protein and lipid methylene groups at ≈1452 cm^{-1} and the CH_2 band of amino acid side chains at ≈1396 cm^{-1} were detectable. Furthermore, the thiocyanate ion band at ≈2050 cm^{-1} was observed for OF stains on all nonporous substrates; however, this band appeared weaker in glass and mirror substrates. A slight spectral contribution from substrate bands was observed when OF stains were placed on the mirror, which was removed by using spectral subtraction (eq 1).

Figure S4A–F shows the ATR–FTIR spectra of OF stains on different nonporous substrates aged for six months; most OF bands were evidently observed in all spectra. In this case, a reduction in the intensity of amide I and amide II bands at ≈1639 and 1544 cm^{-1}, respectively, was noticeable, especially when the stains were placed on aluminum foil, mirror, and Styrofoam cup, indicating protein and macromolecule degradation, which is common in biological fluids during aging.[26] Furthermore, a decline in the intensity at 950–1080 cm^{-1} was noticeable, which could be attributed to the degradation of sugars and carbohydrate moieties.[18] Considering the 950–1080 cm^{-1} region, the bands originating from the

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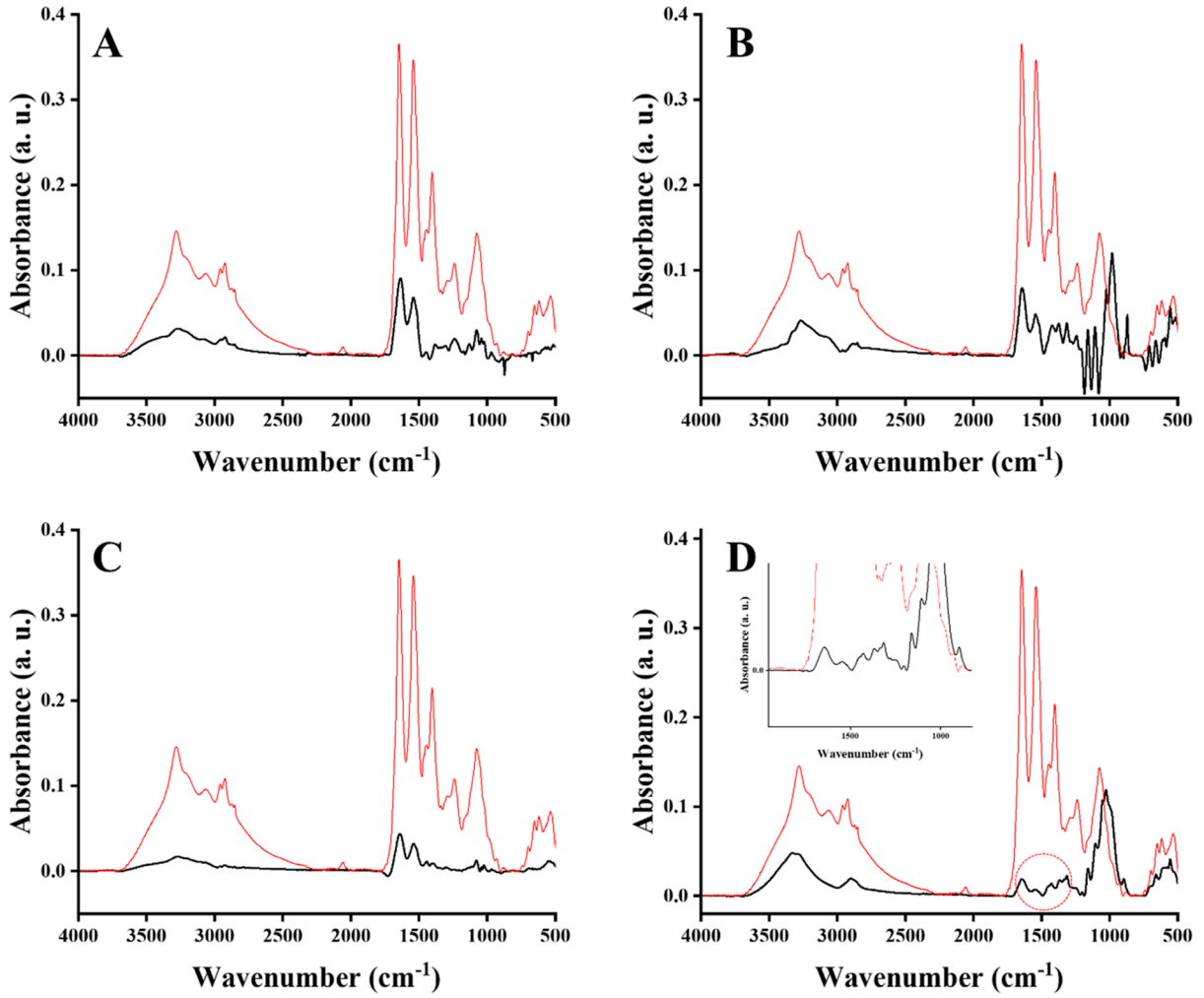

Figure 5. (A–D) ATR–FTIR spectra of OF stains on a (A) cigarette, (B) printing paper, (C) wooden spoon, and (D) tissue paper. The experimental spectra were baseline corrected; the red spectrum is the OF spectrum applied directly on a diamond crystal.

aluminum foil, glass, mirror, and Styrofoam cup were detectable and spectral subtraction was successfully applied. Therefore, ATR–FTIR spectroscopy can detect traces of OF stains on nonporous substrates even after six months since deposition.

3.4. Analysis of Oral Fluid Stains on Porous Substrates. OF stains were placed on four porous substrates: cigarettes, printing and tissue paper, and wooden spoon. In a recent study by Cano-Trujillo et al.,[25] ATR–FTIR spectroscopy and orthogonal PLS-DA enabled the identification of human and animal OF on porous substrates. This study applied spectral subtraction to remove the spectral interference from the substrate. The SF was adjusted manually to resolve the OF bands to avoid oversubtraction that could result in a derivative-like spectrum. The suitable SFs were 1.00, 0.60, 0.65, and 0.40 for the spectra of OF deposited on a cigarette, printing paper, wooden spoon, and tissue paper, respectively. After removing the substrate contribution, the characteristic bands of amide I and amide II at ≈1639 and 1544 cm^{-1}, respectively, were observed in all spectra; however, they were less intense on the tissue paper (Figure 5A–D).

Quinn and Elkins[26] reported similar observations for the bands observed for venous and menstrual blood, semen, OF, and breastmilk placed on porous substrates, and a decline in their intensity was observed compared with their intensity when placed directly on the ATR crystal. Additionally, Sharma and co-workers[39] reported that the amide I and amide II bands of menstrual blood placed on cotton, denim, paper, polyester, and sanitary napkins appeared less intense, and bands from the substrates dominated the spectra. The two main reasons behind these observations are (1) the high porous nature of the substrate that can cause OF to penetrate the substrate to a varying degree, resulting in sample dilution on the substrate surface, and (2) the relation of the absorbance of the OF bands is governed by the ability of the evanescent wave to penetrate the substrate layers and the thickness of the sample. To put this hypothesis to the test, a control experiment was performed by placing an excess amount of OF on two porous substrates (printing and tissue paper) and subjecting the samples to ATR–FTIR spectroscopy (Figure S3A,B). The recorded ATR–FTIR spectra illustrate an increase in the intensity of the amide I and amide II bands, and the contribution from the substrates was smaller when an excess amount of OF was applied. Although the spectral contribution of the surfaces can hinder the OF bands, these results demonstrate that ATR–FTIR spectroscopy can probe OF stains, even in case of highly interfering surfaces.

The findings reported here were further extended to detect OF stains on porous substrates aged up to six months since deposition (Figure S5A–D). Similar to nonporous substrates,

https://doi.org/10.1021/acsomega.3c09358
ACS Omega 2024, 9, 30142–30150

the results for porous substrates indicate that the OF bands become less intense with time, and a substantial spectral contribution from the substrates starts to emerge. However, the major bands of OF were detectable, even six months after deposition.

This proof-of-concept study involves only one donor (a healthy female) because the main aim was to investigate the effect of common porous and nonporous substrates on the analysis of OF stains. Collectively ten spectra were obtained for each OF sample on different substrates from different regions, and no inconsistency or irreproducibility was noticed when spectra were acquired. Despite the encouraging results that have been obtained in this study, additional work is required to understand the role of inter- and intradonor variabilities using a larger number of donors, both males and females. This, however, may increase the variation in the composition or characteristic of the biological fluids, and hence, statistical analysis will be useful to understand the information related to the stain source. Furthermore, we plan to perform a fundamental study on the effect of environmental conditions such as temperature, humidity, time, and others on OF samples deposited on different substrates.

4. CONCLUSIONS AND OUTLOOK

ATR−FTIR spectroscopy is a rapid, nondestructive, and universal tool commonly used for body fluid analysis. Owing to the high porosity and complexity of some common substrates, the ATR−FTIR analysis of biological fluids, such as OF, on these surfaces can be challenging, and substrate interference can limit the practical application of this technology. Thus, this study advances the application of ATR−FTIR spectroscopy as a promising tool for identifying OF stains on highly porous and interfering surfaces. We propose a simple approach based on substrate spectral subtraction to minimize the substrate interference and enable reliable identification of the OF bands. OF bands were observed on all nonporous substrates, particularly the band corresponding to the thiocyanate ion (specific to OF). A small spectral contribution from substrate bands was observed in the case of the mirror substrate, which was removed using spectral subtraction. Conversely, background correction based on the respective spectral signature of the substrate was applied for all porous substrates, and the OF bands were detected, except for the thiocyanate ion due to sample dilution. This approach expands the use of ATR−FTIR spectroscopy for detecting OF on various substrates, even after six months of deposition, with little to no contribution from interfering surfaces. Furthermore, SEM was used as a complementary technique to probe the morphology of the OF stains on various substrates. The SEM images showed characteristic structures of salt crystals (or particles) and protein aggregates formed by the dried OF, which were observed for fresh samples and the samples after six months post-deposition.

Therefore, this study offers a simple and reliable approach for solving one of the most challenging issues for the identification of bodily fluids, particularly OF, on different substrates. The stage of this project is on the technology readiness level (TRLs) TRL 3, which is an experimental proof-of-concept. Furthering this research study is needed before this method can be applied for field work. Other future plans include testing samples from multiple donors and investigating the effect of environmental conditions such as temperature, humidity, and time for OF placed on various common substrates. Additionally, the availability of portable devices will allow in situ analysis of OF on substrates at a crime scene; however, it is necessary to conduct studies to guarantee the suitability of our method for portable devices.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c09358.

ATR−FTIR spectra for nonporous and porous substrates, spectra of OF stain on printing paper and tissue when applied in excess amount, and spectra of aged OFs on different substrates (PDF)

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Author Contributions

Authorship contribution statement: Dalal Al-Sharji and Mohamed O. Amin performed the experiments, analyzed the spectral data, and drafted the manuscript. Entesar Al-Hetlani and Igor K. Lednev conceived the project and were responsible for project administration and supervision, as well as reviewing and editing the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors gratefully acknowledge the Kuwait Foundation for the Advancement of Science (KFAS-grant number CR19-12SC-01). The authors thank Mrs. Shobha Varghese for performing the ATR−FTIR analysis and Mr. Hamidu Ben Youngo for conducting the SEM analysis. The authors gratefully acknowledge support from the Kuwait University Research Administration (KURA) and the College of Graduate Studies (CGS) of Kuwait University.

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https://doi.org/10.1021/acsomega.3c09358
ACS Omega 2024, 9, 30142−30150