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OPTIMIZING DRYING TIMES AND SAMPLE VOLUMES OF PLASMA AND URINE FOR MID-INFRARED SPECTROSCOPY ANALYSIS

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ABSTRACT: **Purpose:** The attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy is an analytical technique capable of discerning alterations in the molecular composition of plasma and urine derived from both healthy and non-healthy individuals. However, the lack of standardized protocols for drying time and sample volume hinders reproducibility and comparability of results. This study assesses the optimal drying duration and volume of plasma and urine for ATR-FTIR analytical procedures. **Methods:** Plasma and urine samples were evaluated at volumes of 5, 10, and 20 μL, deposited on the equipment crystal or in aluminum plates. Spectral data were recorded for 0 to 120 min on the equipment and 0 to 8 hours for the plates. The data were pre-processed with vector normalization and Rubberband baseline correction, and the area under the curve was calculated using means and standard deviations. Statistical analysis was performed using one-way and two-way ANOVA. **Results:** Plasma samples were dry after 15, 12, and 18 min for the 5, 10, and 20 μL respectively, whereas urine samples were dry after 12 min for the 5 μL and 10 μL, and after 40 min for the 20 μL. No significant differences were found between the drying processes on the equipment and aluminum plates. **Conclusion:** The results indicate that a volume of 10 μl is the most appropriate for analyzing plasma and urine, as samples achieved dryness within 12 min at this specific volume. We hope these results will contribute to improving clinical findings using ATR-FTIR for screening various diseases.

Keywords: ATR-FTIR. Plasma. Urine. Drying time. Sample volume.

1. INTRODUCTION

Attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy is a sophisticated analytical method for identifying molecular modifications within biofluids and differentiating among various molecular subtypes via liquid biopsies, a non-invasive diagnostic technique that involves the analysis of biomarkers in body fluids [1-3]. It is a non-destructive technique, characterized by its relatively uncomplicated and expeditious methodology, which does not necessitate sample preparation, including reagents [4,5]. The ATR-FTIR analytical

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process relies on identifying chemical bonds within molecular structures through the absorption of infrared radiation, which induces distinctive molecular vibrations of functional groups, thereby generating a spectrum that conveys molecular-level insights into the analyzed sample [6]. Nonetheless, the presence of water within biofluids significantly compromises the quality of the spectra and complicates subsequent data interpretation and analysis; however, by employing drying techniques on the samples, it is feasible to diminish water-related interference in the spectral data [7].

Implementing ATR-FTIR within clinical frameworks involves validation and standardization protocols to ensure the reliability and reproducibility of outcomes. Each procedural phase must be meticulously regulated, from sample collection and preparation to spectral data analysis and interpretation. Recent discussions [8,9], have highlighted vital challenges, including why spectroscopy has not yet been widely adopted in clinical practice.

During the phase of spectral data interpretation, researchers in data science have been examining the ramifications of various methods, which range from spectral pre-processing techniques (including normalization, derivatives, and baseline correction) to the application of diverse artificial intelligence approaches for exploratory analysis or data classification, such as supervised and unsupervised techniques, machine learning, and deep learning [10,11]. An existing gap that warrants further investigation is the sample handling procedures before acquiring spectral data. This study aims to assess the optimal drying duration and volume of plasma and urine for ATR-FTIR analytical procedures, aiming to establish standardized protocols to enhance the reproducibility and comparability of analytical outcomes. Specifically, we tested (a) the optimal sample volume to use, (b) the ideal sample drying time, and (c) the collection method, either pipetting the sample directly onto the equipment's crystal or a separate aluminum plate.

2. Methods

2.1. Design and Ethics aspects

This experimental study was conducted following the Declaration of Helsinki and approved by the Research Ethics Committee of the Federal University of Espírito Santo, number CAAE #36894020.4.0000.5526.

2.2. Blood and Urine Samples Collection

Blood samples were collected after 8 hours of fasting and centrifuged at 1000g for 10 min at 10°C to separate the plasma. Greiner bio-one® 21 G needles and 5 mL capacity collection tubes were used. First-morning urine samples were collected, and the initial stream was discarded. Plasma and urine samples were transferred to 2.0 mL Criogenic microtubes with caps and stored in a -80°C freezer for later analysis.

2.3. ATR-FTIR spectroscopy analysis

The samples were thawed at room temperature (22.2°C ± 0.2 and 33.5% ± 2.1 relative humidity) for 30 min and then homogenized using a vortex mixer for spectral analysis. Spectra were acquired using an Agilent Cary 630 spectrometer, equipped with a diamond crystal, Attenuated Total Reflection (ATR) system and operated by Microlab software. The spectra were obtained in triplicate within the 4000-650 cm^{-1} absorbance range, with a resolution of 4 cm^{-1} and 32 scans. After each analysis, the crystal was cleaned with ultrapure water (Milli-Q®) and 70% (v/v) alcohol to prevent cross-contamination between samples. Before each reading, a background spectrum was obtained to normalize ambient conditions.

2.4. Experimental protocol

2.4.1 Biofluid volume and drying time

To investigate the optimal volume of biofluids for spectral analysis, initial volumes of 5, 10, and 20 µL were pipetted directly onto the equipment's crystal. Spectra were obtained at every 3-min interval (from 0 to 21 min), at every 10-min interval (from 21 to 61 min), and at every 20-min interval (between 61 and 120 min).

2.4.2. Drying method comparison

To evaluate the effectiveness of pipetting directly onto the equipment's crystal versus using an aluminum plate, 10 µL of biofluid was pipetted onto the crystal of the equipment (Fig. 1A), and the spectrum was obtained at 0, 2, 4, 6, and 8 hours. Additionally, 10 µL were pipetted onto a 3x3 mm aluminum plate (Fig. 1B), with the biofluids dried for 2, 4, 6, and 8 hours at room temperature (Fig. 1C). After the drying period, the aluminum plates were transferred to the equipment, and spectra were obtained in triplicate.

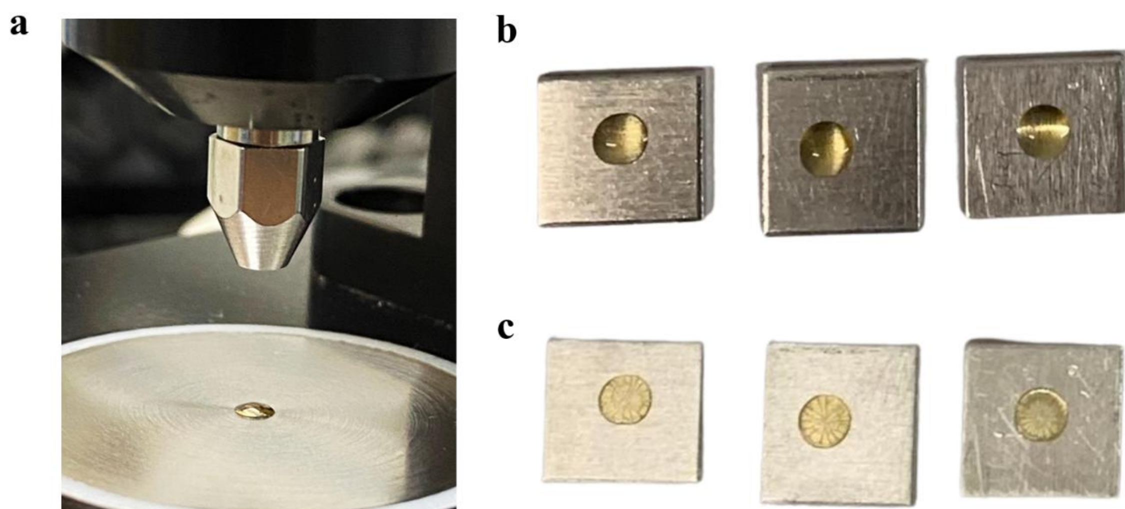

Fig. 1 Sample drying process: drying on the equipment (A), freshly deposited sample on the aluminum plate (B), and after 2 hours of drying (C)

2.5. Statistical analysis

Raw spectra were pre-processed using Orange software with vector normalization and Rubberband baseline correction. The mean spectra and the area under the curve (AUC) of the graphs were calculated. Data were analyzed using GraphPad Prism 8 with one-way and two-way ANOVA tests to assess drying time and compare drying methods respectively. The significance level adopted was 5%.

Bland-Altman analysis was performed to evaluate the agreement between the two drying methods. This method involves visualizing a scatter plot of the difference between two values versus their mean. In such a plot, it is possible to observe bias (how much differences deviate from zero), error (the dispersion of the difference points around the mean), outliers, and trends.

3. RESULTS AND DISCUSSION

The drying conditions of three volumes (5, 10, and 20 μL) of plasma and urine were analyzed with samples pipetted directly onto the equipment's crystal for up to 120 min. Samples were considered dry when no further variation in the AUC of the spectrum was observed over time.

Fig. 2 is divided into three sections (A, B, and C), each corresponding to one of the tested

volumes. The analysis of AUC variation for plasma suggests that the sample is dry after 15 min for the 5 μL volume (Fig. 2A), 12 min for the 10 μL volume (Fig. 2B), and 18 min for the 20 μL volume (Fig. 2C).

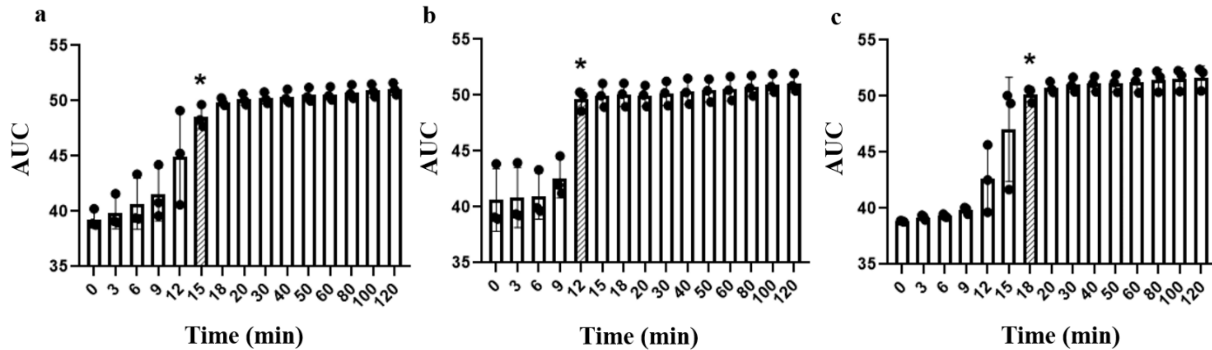

Fig. 2 Drying time of plasma for volumes 5 μL (A), 10 μL (B), and 20 μL (C). AUC: Area Under the Curve. One-way ANOVA tests. *Minimum drying time for volume stabilization (≤ 0.05)

The drying time of urine samples was analyzed for different volumes, as shown in Fig. 3. The AUC remained stable after 12 min for the 5 μL (Fig. 3A) and 10 μL (Fig. 3B), and after 40 min for the 20 μL volume (Fig. 3C), suggesting these times as optimal for spectral analysis for the respective volumes.

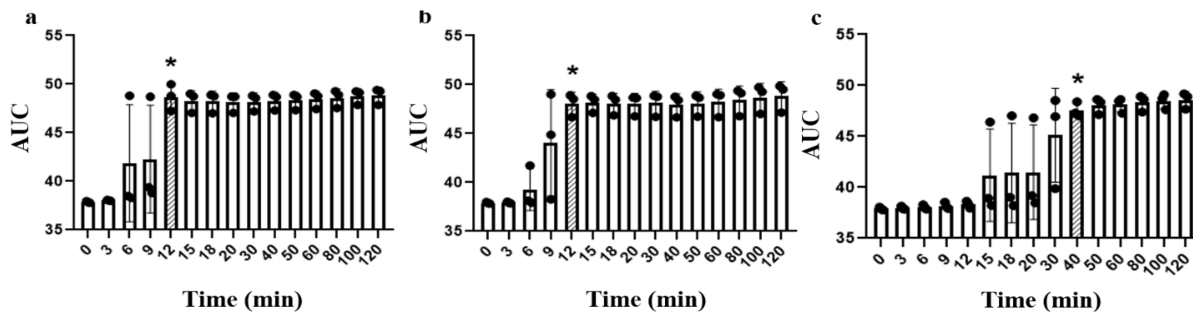

Fig. 3 Drying time of urine for volumes 5 μL (A), 10 μL (B), and 20 μL (C). AUC: Area Under the Curve. One-way ANOVA tests. *Minimum drying time for volume stabilization (≤ 0.05)

Based on the abovementioned data, we have determined to utilize a volume of 10 μL for the research study. This specific volume demonstrated a drying duration comparable to 5 μL and faster than 20 μL. Moreover, samples of 10 μL displayed a reduced variability in drying durations. Fig. 4 illustrates the spectral data acquired at intervals ranging from 0 to 120 min for the 10 μL volume concerning both plasma (Fig. 4A) and urine (Fig. 4B). No additional visual alterations in the spectrum were observed after the designated drying periods as indicated by the spectra depicted in red.

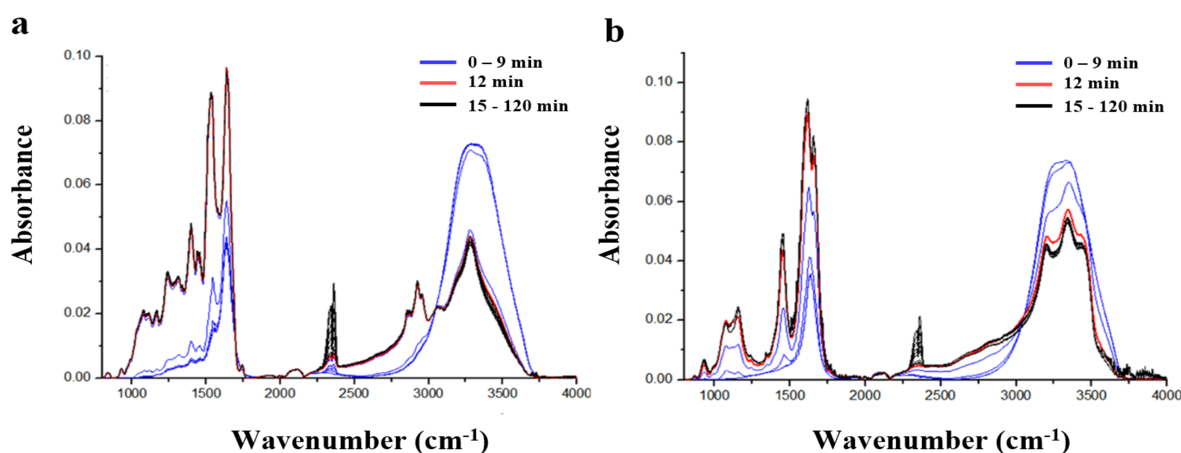

Fig. 4 ATR-FTIR spectra for 10 µL of plasma (A) and urine (B)

Determining 10 µL volume as ideal for both biofluids suggests the method's applicability in clinical settings, considering that reproducibility with smaller volumes could be affected by pipetting difficulties, requiring trained personnel. Larger volumes, on the other hand, may lead to greater sample consumption, which is particularly important in large-scale analyses or in clinical environments where samples may be limited. The ease of handling and minimization of sample consumption is essential for reproducing studies and clinical analyses.

In the present investigation, no significant alterations were detected in the spectra after 12 min for both plasma and urine with the 10 µL volume. It was possible to see that at these times, water bands (a strong absorber of infrared light) no longer influenced the quality of the spectrum. The presence of water in the spectra is evidenced by broadband related to water absorption in approximately 3200 to 3600 cm^{-1}, attributed to the stretching vibrations of water's hydroxyl group (OH group). These bands can overlap with bands of interest, which appear prominently only when the sample is dry, such as amide I and II peaks and the fingerprint region (800 to 1800 cm^{-1}) [7,8,12]. Achieving the ideal drying point eliminates the presence of water, which can interfere with spectral results, thereby increasing the quality and reliability of the obtained data [7].

The ability of ATR-FTIR to provide precise and relevant information about the molecular composition of biofluids [13], including plasma and urine, enables the detection of subtle changes associated with health conditions or diseases [14-16]. Moreover, ATR-FTIR can also be used quickly and cost-effectively in the long term to discriminate between different molecular subtypes from liquid biopsies [3].

Despite ATR-FTIR's advantages and broad applicability in clinical analyses and research, variability in sample preparation and analysis procedures remains challenging. A significant amount of variability in clinical laboratories occurs during the pre-analytical phase, before data collection and analysis [17], which can significantly affect the quality of results and data interpretation across laboratory settings. This study helps to address this limitation by establishing sample volumes and drying times, providing a more reliable framework for future research and clinical applications.

Given that each sample requires a minimum drying time of 12 min when directly applied to the equipment's crystal and acknowledging that only a singular sample can be deposited onto the crystal at a time, a triplicate examination would require no less than 40 min, including the background process and cleaning for sample exchange. Consequently, we tested the hypothesis of drying all samples together at room temperature outside the equipment before analyzing them. The selected drying timeframe ranged from 2 to 8 hours, during which the biofluids were applied onto a 3x3 mm aluminum substrate. For comparison, samples were also dried directly on the crystal for the same period, from 2 to 8 hours.

Fig. 5 compares drying on equipment versus on aluminum plates for plasma (Fig. 5A) and urine samples **(Fig. 5B)**. The analysis of the AUC suggests no significant differences in the spectra between the two drying methods at the evaluated times.

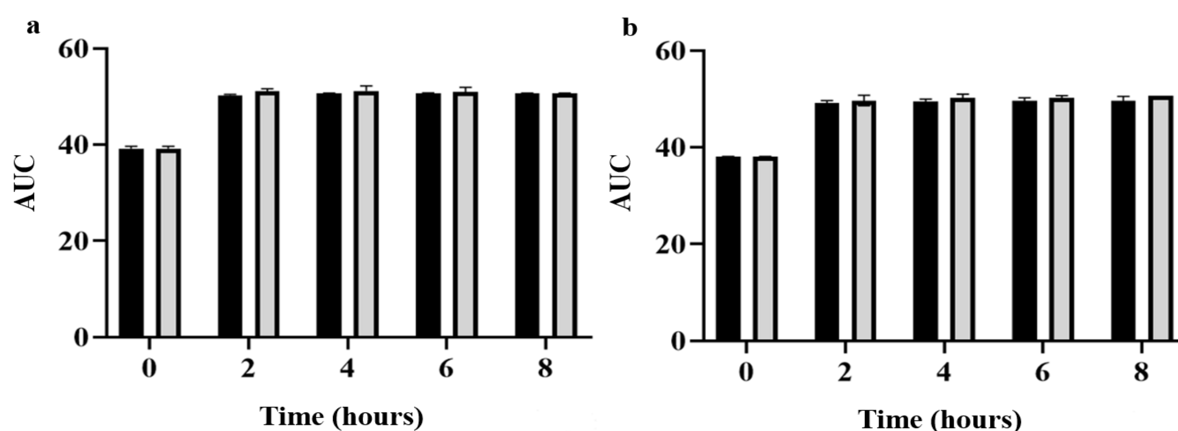

Fig. 5 Comparison of the Area Under the Curve (AUC) variation for plasma (A) and urine (B) with 10 μL volume on the equipment (black) and the aluminum plate (gray). Two-way ANOVA tests

Fig. 6 shows the spectra obtained from drying on the equipment and in aluminum plates. We observe no variations for plasma (Fig. 6A) and urine (Fig. 6B), suggesting that both methods can be used for sample drying.

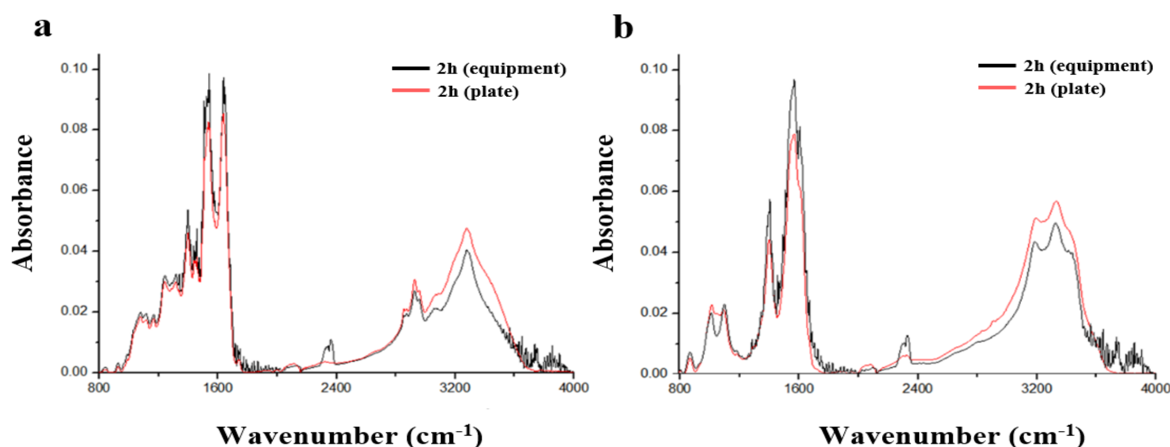

Fig. 6 Comparison of ATR-FTIR spectra for plasma (A) and urine (B) with 10 μL volume after two hours of drying on the equipment and aluminum plat

Statistical confirmation was conducted through Bland-Altman analysis (Fig. 7). This analysis demonstrates agreement between the spectra dried on the equipment (standard method) and on the aluminum plate for both plasma (Fig. 7A) and urine (**Fig. 7B**).

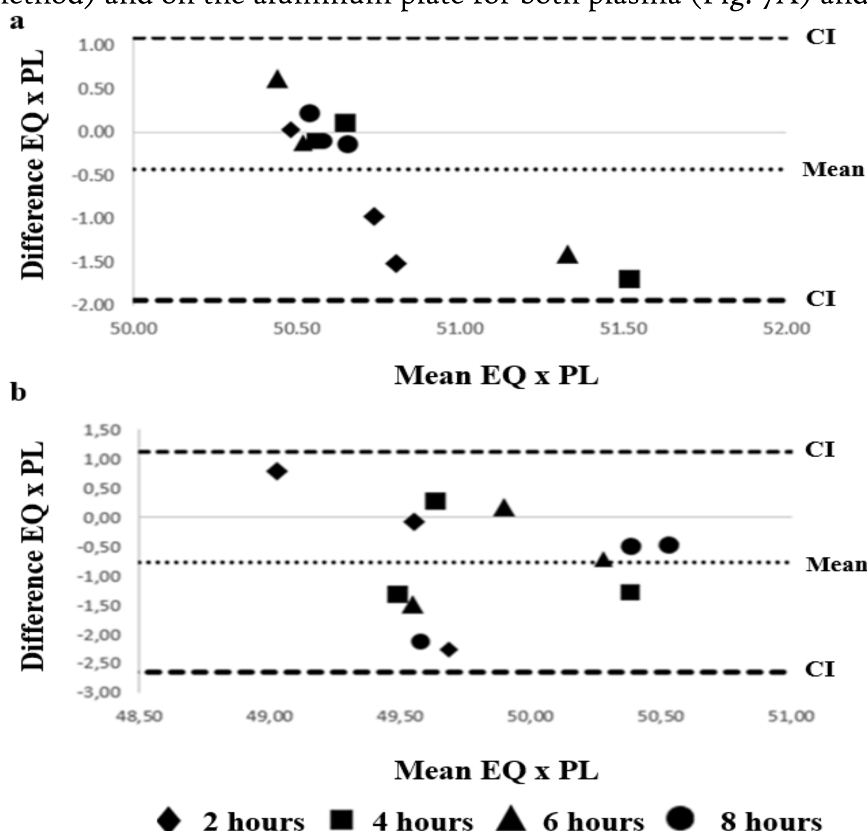

Fig. 7 Bland-Altman plot of the difference vs. mean for equipment (EQ) and aluminum plate (PL) for plasma (A) and urine (B). CI: confidence interval

The comparison between the drying methods demonstrated that drying on aluminum plates was comparable to drying on the equipment, offering a viable alternative, especially for

laboratories handling multiple samples [18]. Thus, implementing the aluminum plate drying method contributes to greater efficiency and productivity in the analytical process.

In this context, our findings significantly contribute to the standardization of experimental methods and protocols for analyzing plasma and urine by ATR-FTIR, essential for ensuring the reproducibility and reliability of results [19]. Standardization is crucial as ATR-FTIR has shown great potential for diagnosing and differentiating normal and pathological molecular profiles by detecting biochemical changes in biofluids based on their vibrational signatures [20]. By enabling more precise and consistent analysis, these improvements enhance the applicability of ATR-FTIR in clinical practice, making it a promising tool for identifying health conditions with greater accuracy. Therefore, our findings advance technical knowledge and expand the use of ATR-FTIR in biomedicine, opening new possibilities for early diagnosis, effective monitoring, and a deeper understanding of various diseases.

While FTIR provides a rapid and efficient method for biofluid analysis, it has intrinsic limitations that should be considered, such as potential interference from overlapping absorbance bands, variations in sample types [21], and the need for calibration to ensure accurate results. Nevertheless, the laboratory where the analysis was conducted adhered strictly to the equipment manufacturer's guidelines and regular equipment calibration and validation to ensure data accuracy and reliability.

4. CONCLUSION

The data from this study indicate that 10 μL is the optimal volume, with a drying duration of 12 min, for both plasma and urine for infrared spectral acquisition conditions. Furthermore, drying on aluminum plates proved a viable alternative to the traditional method (using drying equipment), maintaining both efficiency and reproducibility. This standardization process enhances the reliability of results and broadens the applicability of ATR-FTIR in clinical practice and research.

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