# ATR-FTIR spectroscopy and multivariate analysis for thermal burned skin classification

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**Abstract:** The treatment choice of skin burns depends on the determinations of the depth of injury. We demonstrated the feasibility of Fourier transform infrared spectroscopy (FTIR) with Attenuated Total Reflection (ATR) to characterize burned skin.

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## 1. Introduction

The burn depth has been a clinical diagnostic problem [1] because it is not quick to recognize the deep-degree damage. The treatment of burns considerably differ depending upon the results of the initial assessment, then characterization of deep-degree burns is a critical decision point [2]. Histological skin biopsy is the gold standard for deep-degree burn evaluation [3]. Nevertheless, the early surgical skin removal for histopathology analysis is an invasive, expensive clinical procedure and a critical time consuming. Thus, it is important to develop innovative techniques for early diagnosis.

Vibration spectroscopic methods, especially the Attenuated Total Reflection Fourier Transform Infrared Spectroscopy (ATR-FTIR), have been proven its efficient when applied to study biological samples [4-6]. In addition, the association of ATR-FTIR with multivariate analysis improves its discriminant capability. In case of this study, we aimed to evaluate the capability of ATR-FTIR associated to Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA) to discriminate health and burned skin [7].

## 2. Material and Methods

*Animal Experiment and Sample preparation*

The protocols used in this work have the approval of the institutional Animal Research Ethical Committee. The study was conducted on 5 male Wistar rats weighing 200 – 250 g, using intramuscular anesthesia with combination: 0.32 ml/ kg of ketamine and 0.2 ml/ kg of xylazine. To induce burn, one region (8 mm diameter) of each rat dorsum was exposed during 20 seconds to a water vapor source, prepared with a rubber tube attached to the outlet of steam.

The entire wound and another healthy skin sample were excised from the animals, resulting in 6 samples of healthy skin and 4 samples of burned skin. Sequentially, sample tissues were fixed in formaldehyde. Hence, the tissues were diaphanized in two baths of pure xylol for 30 min and dehydrated with ethanol baths of increasing concentrations (50%, 70% and 100%). When dehydration was concluded, the samples were mounted in wax blocks and slices of 5 µm were placed in MirrIR low-E-coated slides (Kevley Technologies, Chesterland, OH, USA). The paraffin provides spectral signatures in the same wavenumber range of interest. Thus, the dewaxing protocol was appied to the skin samples [8]. In this way, tissue slices were immersed in a series of baths consisting of two baths of xylene during 10 min and one bath of absolute ethanol during 5 min. Finally, it was kept in a desiccator for 24h prior to the spectroscopic measurements.

*ATR-FTIR Spectroscopy*

The spectra were measured in a FTIR system (model 6700, Nicolet Instruments, USA), using an ATR accessory (Smart Orbit, Thermo, EUA), in which samples were pressed onto a diamond crystal with an area of 2.25 mm² used as the internal reflection element. For the acquisition of each spectrum, 100 scans were averaged with a sampling interval of 4 $cm^{-1}$ wavenumbers, ranging from 4000 to 400 $cm^{-1}$. Then, for each sample (approximately 0.4 $cm^2$ of area) a visual observation led to the selection of about 10 different areas of interests for spectral measurement, that were averaged to obtain one representative spectrum per sample.

The data was vector normalized by the software MATLAB® R2015a (MathWorks®, EUA) and the second derivatives of absorbance were calculated to reduce baseline offset and assess the overlapping sub-bands in raw

spectrum. For signal-smoothing, spectra were submitted to Savitzky–Golay filter with a polynomial of second order in an eleven-points window. The data was then evaluated by PCA and the 20 first PCs were used as input data for LDA, which was subsequently validated by leave-one-out cross-validation (LOOCV), due to its ability to decrease the data overfitting and provide more robust predictors.

## 3. Results and Discussion

Figure 1 show the spectral range corresponding to the fingerprint region (900-1800 $cm^{-1}$) to ensure that the lipid removal caused by the dewaxing protocol has no influence on data interpretation. This region depicts vibrational modes often used in spectroscopic studies of biological tissues.

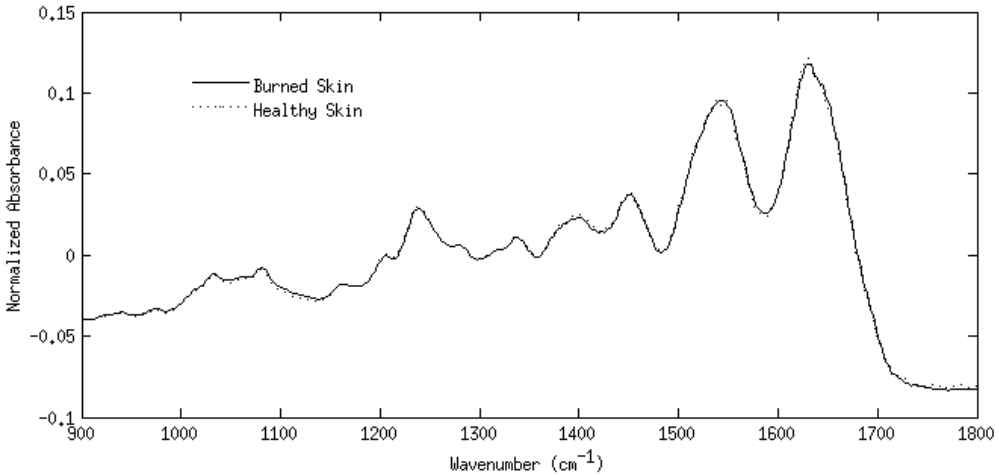


Fig. 1. Fingerprint region (900 – 1800 cm-1) of the normalized averaged spectra of healthy skin (dot line) and burned skin (continuous line)

The amplitude of second derivative of the bands related to protein content (Amide I and II) increased their intensity in the burned skin in comparison with a normal tissue. Sub-band of Amide I related to the β-sheet secondary structure of proteins shifted its position by 2 $cm^{-1}$ in the direction of higher wavenumbers, suggesting alterations in the molecular geometry of proteins in the burned skin. This finding indicates changes in hydrogen bonding between peptide groups of proteins, and may be related to the alterations promoted by healing process in the structural proteins that compose extracellular matrix. This result is in agreement with a previous study, in which we characterized biological alterations promoted by healing process in burned skin via nonlinear microscopy (second-harmonic generation, SHG and two-photon emission fluorescence microscopy, TPEFM) [9]. The second derivatives was obtained to evaluate the overlapped bands in the raw spectrum, as shown in Figure 2.

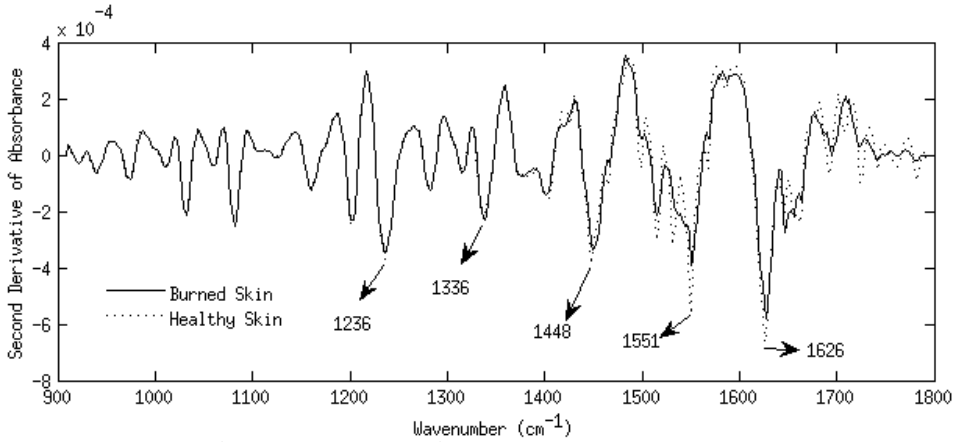


Fig. 2. Second derivative of averaged spectral data of healthy skin (dot line) and burned skin (continuous line)

Second derivatives of spectra from both groups were submitted to principal component analysis and the first ten PCs, which explained 99.4% of global variance, were used as input data for LDA classification. Finally, we

evaluated the performance of the classification (accuracy, sensitivity and specificity) of the method to discriminate healthy tissue from burned skin.

In this study, cutaneous wounds induced by thermal burns, and healthy skin were evaluated through ATR-FTIR spectroscopy associated to PC-LDA multivariate statistical analysis. A larger amount of proteins in ß-sheet secondary structure were observed in burned skin lesions in comparison with healthy tissue, and the method showed an overall accuracy of 92.7%, 93.5% of sensitivity and 92.3% of specificity in the discrimination of both tissue-samples. Thus, ATR-FTIR spectroscopy associated with PC-LDA is a useful tool to study cutaneous wounds induced by thermal stress and hopefully to assist the physicians in the discrimination of different burns degrees.

## 4. Acknowledgements

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