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A preliminary study on the importance of normalization methods in Infrared Micro-Spectroscopy for biomedical applications

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***Abstract* – Fourier transformed infrared (FT-IR) micro spectroscopy has become a reliable, non-destructive and automatic tool to analyze the chemical differences in biological samples associated with healthy or diseased states for diagnostic or biomedical research purpose. To extract useful information from the huge number of spectra usually recorded, statistical multivariate analysis is applied and careful data pre-treatment to reduce or eliminate possible sources of error is necessary. In this regard, a quantitative criterion to evaluate the performance of different methods applied in the most sensitive pre-processing step, normalization, is proposed here. This could constitute a promising approach for validating the significance of chemical variations in complex samples of biomedical interest.**

I. INTRODUCTION

Fourier transformed infrared spectroscopy (FT-IR) is a robust analytical tool for detecting and characterizing the molecular component of a material. When an infrared radiation interacts with a specimen, a fraction of the incident radiation is absorbed at a particular energy. In the recorded spectrum the characteristic absorption bands derive from the infrared-active vibrational modes of molecules present in the sample; therefore, important information on chemical content of specimen can be extracted. The amount of radiation absorbed is dependent upon Lambert-Beer's law:

$$A = \log\left(\frac{I_0}{I}\right) = \varepsilon\, c\, d \qquad (1)$$

where I and I_0 are respectively the intensity of transmitted and incident light, c is the sample concentration, d is the sample thickness and ε its molar extinction coefficient.
FT-IR spectroscopy was also combined with optical microscopy and the derived micro-spectroscopy allowed to generate infrared maps or images of sample areas providing spatially resolved chemical composition information of the analyzed region. In recent years this technique has been widely applied in the biomedical field and has resulted in a non-destructive, reliable, univocal and rapid tool for histologic analysis and diagnostic purposes [1]. The micro-FTIR spectroscopy is applied to carry out numerous investigations in order to extract information regarding the macromolecular contents and their distribution in tissues and cells samples.
From literature the FTIR spectroscopy is able to: differentiate between diseased and non-diseased states, especially cancer tissue from healthy tissue [2], identify the presence of pathological micro-calcifications in tissues [3], reveal the composition of biological calcifications [4], determine cell cycle stage [5] and monitor cell death [6].
By examining the wavenumbers values and the intensities related to absorption bands, cellular macromolecules can be detected and assigned, including carbohydrates lipids, proteoglycans, collagens, nucleic acids and proteins [7].
Therefore, finding slight modifications of the IR signals of these biomolecules could lead to important considerations about the analyzed sample. However, the biological tissues are heterogenous samples and it is complicated to extract the chemical information and detect the very small chemical changes in pathological conditions from the complex raw FTIR spectra [8]. In order to overcome these limitations and obtain meaningful information, it is necessary to process and analyze data using multivariate statistical methods. To improve the robustness and effectiveness of these applied statistical methods, pre-treatments of data are required. The pre-processing steps reduce noise and enhance requisite signals, correct the sloped or oscillatory baselines due to scattering effect, and remove the signals derived from atmospheric water vapor, carbon dioxide or other interfering compounds. Finally, a crucial pre-treatment of spectra is the normalization, which

is essential to overcome the confounding effect of varying sample thickness on the band intensity [9]. Although the pre-processing steps are fundamental to increase the interpretability of data, often it is not easy to identify the best approach among the available procedures for each step, especially in the case of complex samples such as tissues, and a criterium to guide the choice has not yet been established. Keeping this question in mind, this work focused on the critical step of normalization. This is the first time, to best of author's knowledge, that chemically homogeneous paraffine samples are used to evaluate the performance of different normalization methods [10] and that a χ^2-based metric is proposed to quantitatively identify a selection criterion among them. Furthermore, this work lays the foundations for establishing a quantitative criterion for validating the significance of chemical variations in complex materials identified by comparing very similar spectra.

II. METHODOLOGY

Pure paraffin sections with two thicknesses of 10 and 20 µm were cut by microtome and placed on CaF_2 slides; three replicates per sample were prepared.

FTIR measurements were performed using a Nicolet iN10 infrared microscope (Thermo Fisher) equipped with a Mercury-Cadmium-Telluride (MCT-A) nitrogen-cooled detector. Spectra were collected in transmission mode in the 4000 – 950 cm^{-1} region, using a nominal spectral resolution $\Delta\upsilon$=8 cm^{-1}. A fixed number of scans and points was considered for each thickness, with a minimum of 64 scans and 30 point registered for sample. Each spectrum was subjected to equal correction which consisted in vapour compensation and rubber band baseline correction [11]. The two regions of the spectrum, I and II, containing the signal (*Fig. 1*) were then subjected to normalization separately to better appreciate lower intensities bands [11].

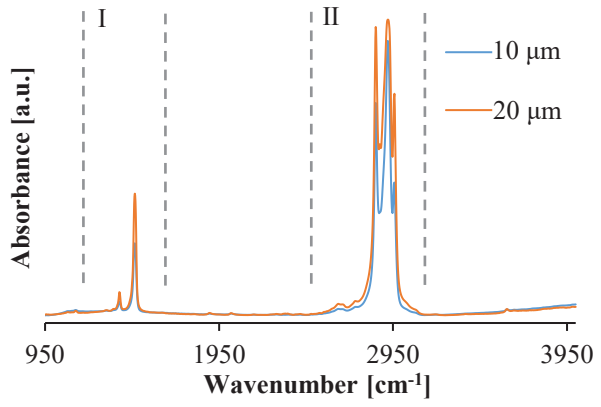

Fig. 1. FTIR spectra of pure paraffine collected using 10 µm (blu) and 20 µm (orange) thickness samples. Dotted lines highlight the regions of interest selected for standardization.

The four most common normalization technique [12] were then compared, namely Min–Max normalization (mM), vector normalization (VN), 1-norm (1-n) and standard normal variate (SNV). Representing the spectrum to be normalized as a vector 'A' whose element A_i is the amplitude at a given wavenumber and as vector A_N of element An_i the normalized spectrum, their relationship from the application of the above methods can be expressed as in table I:

*Table 1. FT-IR spectrum normalization methods [***Errore. Il segnalibro non è definito.***].*

Method	Expression
Min–Max normalization (mM)	$An_i = \frac{A_i - A_{min}}{A_{max} - A_{min}}$
Vector normalization (VN)	$An_i = \frac{A_i}{\sqrt{A_1^2 + A_2^2 + ... + A_N^2}}$
1-norm (1-n)	$\bar{A} = \frac{\sum_1^N A_i}{N}$ $An_i = \frac{A_i - \bar{A}}{\sum_i^N \lvert A_i - \bar{A} \rvert}$ i=1, 2, … N [a]
standard normal variate (SNV)	$An_i = \frac{A_i - \bar{A}}{SD}$ $SD = \sqrt{\frac{1}{N-1}\sum_i^N (A_i - \bar{A})^2}$

([a]) Where N is the number of wavenumbers considered

The performance of the above methods was assessed by a χ^2 metric according to the subsequent equation:

$$\chi^2 = \sum_i^{N_B} \frac{\left(\bar{A}_{10\mu m_i} - \bar{A}_{20\mu m_i}\right)^2}{\sigma^2_{10\mu m_i}} \qquad (2)$$

were $\bar{A}_{10\mu m_i}$ is the average absorbance over 30 spectra at the mean wave number υ_i for the 10 µm thick samples, $\bar{A}_{20\mu m_i}$ is the average absorbance at υ_i for the 20 µm thick samples, $\sigma^2_{10\mu m_i}$ is the variance at υ_i for the 10 µm thick samples chosen as reference.

A further normalization is applied to (2) and a reduced $\chi_d^2 \approx \chi^2/N_B$ is here used [13], where N_B is the (high) number of bins used to group frequencies υ in the spectral range of interest: the higher is χ_d^2 value, the smaller are the differences between the mean spectra from 10µm and 20µm thickness sample respectively. In other terms, the lower is the χ_d^2 value, the more effective is the normalization. Here $\sigma^2_{10\mu_i}$ of the i-th bin in (2) is calculated considering the non-normalized 10 µm thick sample as

$$\sigma^2_{10\,\mu m_i} = \frac{\sum_j^{N_\upsilon} \left(A_{10\,\mu m_j} - \bar{A}_{10\,\mu m_i} \right)^2}{N_\upsilon - 1} \qquad (3)$$

where the summation is extended to all spatial frequencies, as j=1, 2, .., N_υ is the number of spectral lines into the bin, with $N_\upsilon = R_\upsilon/N_B \cdot 1/\Delta\upsilon$ where R_υ is the spectral region of interest (cm^{-1}), N_B is the number of bins and $\Delta\upsilon$ is the spectral resolution (cm^{-1})

All the data processing and subsequent evaluation were performed using in house algorithms running under MATLAB R2017a software (The Mathworks Inc.)

III. DISCUSSION

Numerous studies have shown that FTIR is a powerful method for classifying tissues and cells based on differences in their chemical content. Since the variations that are considered significant are often extremely small, it is essential to ensure the reliability of the conclusions drawn from measurements, ensuring that these are effectively due only to sample characteristics by eliminating any possible influence due to external factors. The aim of this work was to rigorously address, using a reliable reference, the effect of the most important pre-processing step within this frame, normalization. For this purpose, we obtained slices of different thickness (10 µm and 20 µm) from a single block of pure paraffin, so that the chemical information contained in each sample was equal, given that each of the signals is generated by the same functional group in the same molecule. By applying the normalization procedures to spectra relative to these samples, it is expected to restore the correspondence in the chemical information so that the normalized spectra should be indistinguishable from each other in the limit of the experimental error.

The effect of the most common normalizations methods applied in literature to biological samples, mM, 1-norm, VN and SNV on paraffin spectra collected at 10 and 20 µm thickness respectively are shown in Fig. 2 for region I. Observing the mean spectra obtained after the treatments it can be noticed that all the applied techniques have drastically reduced the difference in the intensity of the considered signals, whit mM and SNV showing the best performances.

Table 2. χ_d^2 for different normalization methods of spectral region I (1540-1260 cm^{-1}).

	Raw	mM	VN	SNV	1-n
Region I	6.6	4.3	2.7	1.9	3.8

The χ_d^2 values reported in **Errore. L'origine riferimento non è stata trovata.** demonstrate quantitatively what was observed, allowing further discrimination between methods: the values calculated for the normalized sets are in fact all lower than that of the raw set from the sample at 10 µm thickness, chosen as a reference as the most used for FT-IR applications in the biomedical field. Based on this criterion, SNV normalization is in this case to be preferred.

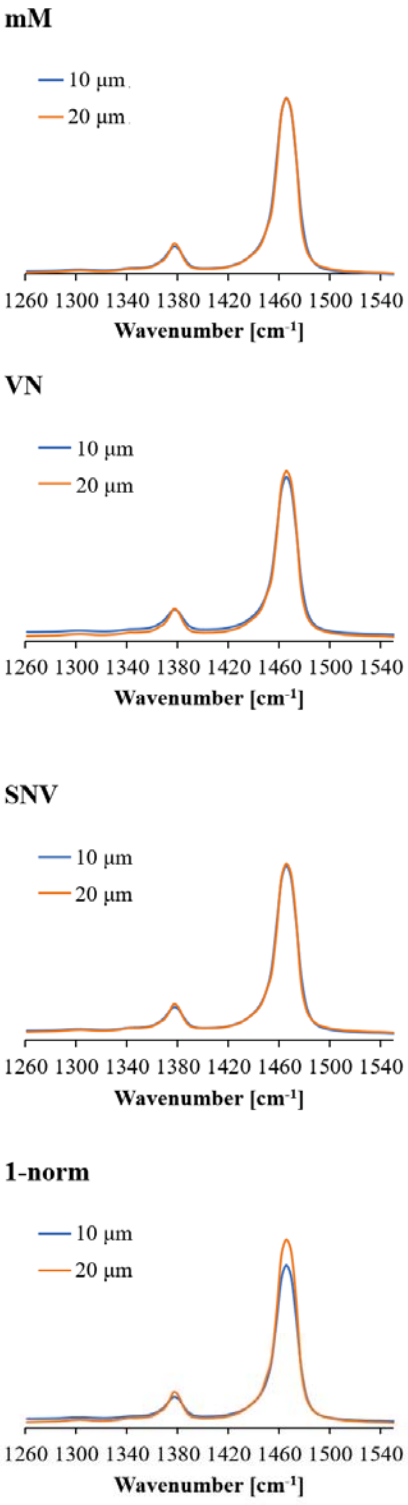

Fig. 2. Average spectra of the region I sets at 10 µm (blue) and 20 µm (orange) subjected to the four normalization

techniques

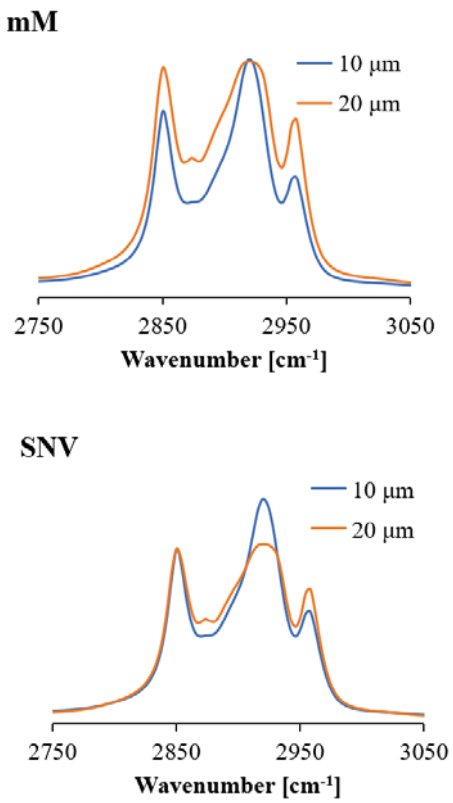

Fig. 3. Average spectra of the region I sets at 10 µm (blue) and 20 µm (orange) subjected to the four normalization techniques.

Table 3. Normalized χ_d^2 for different normalization methods of spectral region II *(2260-3300 cm^{-1}).*

	Raw	mM	VN	SNV	1-n
Region II	30.4	59.6	7.1	42.8	3.4

Applying normalization to region II (Fig. 2) significant differences in the normalized average spectrum were found. The agreement between the normalized average spectra is generally lower than in region I. Despite an apparent improvement in the agreement between spectra mM and SNV do not satisfied the criterion, being the respective χ_d^2 higher than that of the raw set (**Errore. L'origine riferimento non è stata trovata.** and **Errore. L'origine riferimento non è stata trovata.**). What observed is likely due to the different trend shape of the spectrum, made of less resolved bands and it is interesting to note that the simple observation "by eye" of the (mean) spectra would not have allowed to discriminate between the techniques. It is worth nothing here that, in all the considered cases, the application of the normalization algorithms on selected regions, I and II respectively, has given much better results than the direct application on the whole spectrum. Although this procedure may prevent a direct comparison between the reciprocal intensities of different zones within the same spectrum, it appears the best choice in cases where the interest is directed to the in-depth analysis of the content of a specific region.

IV. CONCLUSIONS

In this preliminary study we demonstrated objectively that the normalizations considered are not equivalent, since differences between normalized spectra from samples of different thickness may change noticeably depending on the applied method, especially in the case of spectra with convoluted bands and signals of significantly different intensity. It is still not possible to establish an "χ_d^2-score" on which to establish a "ranking" of the methods because of the limited number of samples considered in this feasibility study, on the other hand it is clear however that identifying the above mentioned score could provide a useful indicator of reliability for subsequent studies and analysis based on differences in spectral intensities in physiological and non-physiological conditions. The criterion here proposed is very promising for evaluating the performance of the various techniques by comparing their respective χ_d^2 with a threshold value related to the experimental variability measured at a refence thickness. Moreover, the above metric is quite simple to implement and not computationally expensive.
Further studies will be dedicated to clarifying the limits within which the differences among the χ_d^2 values are significant in considering the corresponding normalizations non-equivalent. The robustness of the method and the generality of the proposed criterion will also be assessed in view of possible applications to the study of more complex systems such as biological tissues.

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