



Evaluation of Pre-Fixed Biological Tissues Preparation Methods for ATR-FTIR Biospectroscopy

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Abstract

Fourier transform infrared (FTIR) spectroscopy in ATR (attenuated total reflection) mode is a powerful tool for studying biomedical samples, which can provide important structural information on the molecular composition. Currently, formalin fixation and paraffin preservation (FFPP) is the preferred source for the histological examination of tissue sections. There is lack of consensus with regard to a standard protocol for de-paraffinization of embedded sections in the field of FTIR spectroscopy for which several approaches have been used. The aim of the present study is to optimize the de-paraffinization procedure for biological samples FTIR spectroscopy. To this aim, Rat's lung tissue samples were paraffinized in blocks according to standard procedures. Different exposure, duration, and dewaxing timing protocols using any or combinations of n-hexane, xylene, acetone, and absolute ethanol have been applied on embedded sections. The results were evaluated with the comparison of the spectroscopic outcome from these methods with comparison to fresh tissues dried with other methods, as well as pure paraffin spectra. As a result, although n-hexane is an effective dewaxing agent for biological samples after a 24 hours exposure, xylene is a better choice with higher efficiency in less time (6_8 minutes). However, sections that was immersed in xylene for 8 minutes and then rinsed in acetone for 5 minutes showed amide I and II bands and DNA contents in FTIR spectra better than other strategies. Visualization of the sections has shown that the paraffin is not removed completely. The disappearance of peaks at 1426 & 2850_2950 cm^{-1} of the FTIR spectrum was used to ensure complete deparaffinization that happened with 15 min xylene embedding, which effects on other cellular structures and subsequently on the spectrums. However it is important to note that these processes are not instantaneous and two important properties of the dewaxing agents are its penetration rate and binding time which obey diffusion law, whereby the depth of penetration was proportional to the square root of time. According to this, we have also demonstrated that using pressure, sample proper thickness and higher surface in ATR spectroscopy play an important role in optimization of spectra & decreasing wave disturbances.

Keywords: ATR, FTIR, n-hexane, spectroscopy, tissue deparaffinization, xylene.

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1. Introduction

Applications of spectroscopic techniques in biological studies have increased a great deal, and particularly clinical investigations by spectroscopic means have attracted attention both by the clinical and non-clinical researchers [2]. The vibrational spectroscopic techniques, including FTIR spectroscopy, are potential tools for noninvasive optical tissue diagnosis. These techniques are relatively simple, reproducible, nondestructive to the tissue, and only small amounts of material (micrograms to nanograms) with a minimum sample preparation are required. In addition, the method is employed to find more conservative ways of analysis to measure characteristics within tissue and cells that would allow accurate and precise assignment of the functional groups, bonding types, and molecular conformations. [1, 3, 10]

Vibrational spectroscopy is used to study a very wide range of sample types and can be carried out from a simple identification test to an in-depth, full-spectrum, qualitative and quantitative analysis. Samples may be examined either in bulk or in microscopic amounts over a wide range of temperatures and physical states (e.g., gases, liquids, latexes, powders, films, fibers, or as a surface

or embedded layer). Every molecule has a unique fingerprint of vibrational frequencies, which makes Fourier transform infrared (FTIR) spectroscopy highly specific techniques for molecular identification. FTIR spectroscopy consists of the absorbance of frequencies of light by a molecule that contains the same vibrational frequencies within its molecular bonds. A beam of infrared light is passed through or reflected by a sample. [2, 3, 7]

If spectroscopy can be shown to be as good as, or better than the gold standard histopathology, then there is great potential for these techniques to be used as an alternative or an adjunct to the current cytology screening methods. The advantages would be higher accuracy, higher throughput and reduced workload for the cytologist/pathologist and higher accuracy and chance of earlier detection for the patient.

All pathologies are marked by fundamental biochemical changes at the molecular, cellular, and tissue level. The identification and further understanding of these changes would allow improved diagnosis and treatments, as well as overall management and disease survival. The potential of FTIR spectroscopy in molecular diagnostics relies on its ability to determine and characterize the unique fingerprint of a sample at the biochemical level. [23]

The three major spectroscopical sampling Modes are transmission, transfection, and Attenuated Total Reflection (ATR). Each mode offers convenience for some samples. ATR has grown into the most widely practiced

technique in infrared spectrometry. The reasons for this are fairly straightforward: the technique requires little or no sample preparation, and consistent results can be obtained with relatively little care or expertise. The technique is not fool proof, but it can be very forgiving. [4, 8]

ATR is a technique whereby the sample is placed in contact with a sensing element, and a spectrum is recorded as a result of that contact. Unlike many other sampling techniques used in infrared spectrometry, radiation is not transmitted through the sample; consequently, the sample does not have to be thin enough to allow transmission of the incident radiation, with no band having an absorbance greater than 2.0AU. Furthermore, samples can be measured in their neat state and do not require dilution to record the spectrum. The physical morphology of the sample is usually not a problem, as long as sufficient contact area between the sample and the sensing element can be maintained. Even highly irregular samples, such as powders and woven fabrics, will sometimes give good spectra, but sample contact area may be difficult to reproduce; hence, quantitative analysis may be difficult. [1, 10, 24]

Today Biospectroscopy is envisaged as an objective and robust tool to be used in cancer screening and other disease diagnosis. Despite the increasing popularity of the field, there are several challenges to the developing application of biospectroscopy with regards to sample preparation, instrumentation and data handling; these need to be addressed before

the technique can become a routine method in the clinical laboratory or the biological laboratory. As an example, surgically excised tissue may undergo one of two commonly used methods of preservation for long-term storage: paraffin embedding or flash-freezing. The choice between these two methods is based on the specific purpose of the resected tissue. Currently, formalin fixation and paraffin preservation (FFPP) is the preferred source for the histological examination of tissue sections. This method involves immersing tissue in an aqueous formalin solution. Hydrated formalin (methylene glycol, OH-CH-OH) is a coagulative protein fixative, which cross-links the primary and secondary amine groups of proteins, but preserves some lipids by reacting with the double bonds of unsaturated hydrocarbon chains. Following formalin fixation, the tissue is dehydrated through consecutive immersions in increasing grades of ethanol up to 100% ethanol. Displacement of water with ethanol preserves the secondary structure of proteins but denatures their tertiary structure. Furthermore, formalin or ethanol induces coagulation of the globular proteins present in the cytoplasm, which can result in the loss of structural integrity of organelles such as mitochondria. Another disadvantage is that ethanol precipitates lipid molecules that are not preserved through the primary fixation step. However, stabilization of intercellular proteins by formalin and ethanol localizes associated glycogen. Following dehydration, the alcohol is replaced by an organic solvent such as xylene, which is miscible with both

alcohol and molten paraffin wax. The tissue is then immersed in and permeated by molten paraffin wax. The infiltration of the wax into the intracellular spaces is promoted by the previous ethanol dehydration step that created pores in the plasma membrane of the cells. [8, 10]

Researchers working in the field of FTIR tissue diagnostics have employed a variety of methods for tissue preparation. It is important to note that the process of fixation is not instantaneous and two important properties of the fixative are its penetration rate and binding time. Fixative agents obey diffusion laws, whereby the depth of penetration was proportional to the square root of time.

It may be argued that, for FTIR studies, the removal of paraffin is not necessary at all as only discrete frequency ranges corresponding to the lipid hydrocarbon modes are affected. Although a number of studies have shown that non-dewaxed FFPP sections can be used

successfully for analysis but here we can't extract enough data from FFPP sections, thus we've determined a method for deparaffinization presently in the field of FTIR spectroscopy, there is lack of consensus with regard to a standard protocol for deparaffinization of paraffin embedded sections, and several approaches have been used. Here we tried to optimize the deparaffinization procedure for biological samples in the FTIR spectroscopy.[8,10-14]

2. Materials and Methods

2.1. Experimental Methods (Sample Preparation)

Rat's lung tissue samples were paraffinized in blocks according to standard procedures. Tissue sections are typically cut from fixed, paraffin-embedded tissue blocks using a microtome. Sections between 5 and 6 mm thickness were used. The tissue sections were reconstituted by a process of washing in

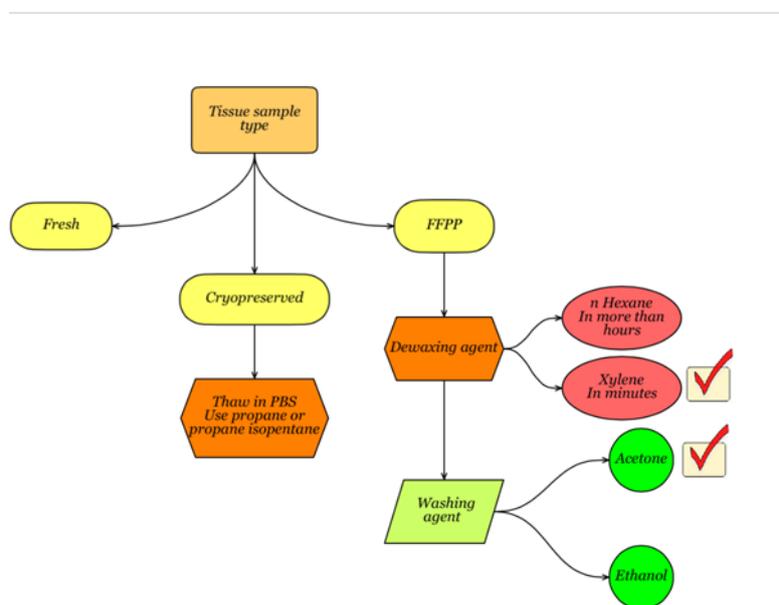


Figure 1. Summary of tissue sampling & used protocol.

solvents for increasing polarity & removing paraffin residue. Different exposure, duration, and dewaxing timing protocols using any or combinations of n-hexane, xylene and also hydrating agents such as acetone & absolute ethanol have been applied on embedded sections. (Fig. 1)

2.1. Infrared Spectroscopy

The infrared spectral results, were collected using MIRacle™ Single reflection ATR (PIKE technologies, WQF-510, Rayleigh Optics, China) containing a diamond crystal.

FTIR spectral maps were collected using a Beijing Second Optical Instrument Factory MainFTOS system Ver1.0 which can collect and process IR-ATR data in transmittance and absorbance modes.

The nitrogen pressure was fixed on 100

mmHg during whole steps of this study. It's notable that N₂ pressure was employed to omit the obstructive peaks such as water vapor, CO₂ & also for strengthening other desired peaks.

Small sample of resected tissue (volume <10mm³) was placed onto the ATR crystal. 100 scans were taken at a resolution of 4 cm⁻¹. Spectra were acquired in the spectral range of 400–4000 cm⁻¹. For each sample, a background spectrum was recorded before measuring the tissue's spectrum. Baseline correction and normalization on the maximum absorbance were performed. Data processing was performed using the Essential FTIR® software version 3.00.019.

3. Results and Discussion

Researchers working in the field of FTIR

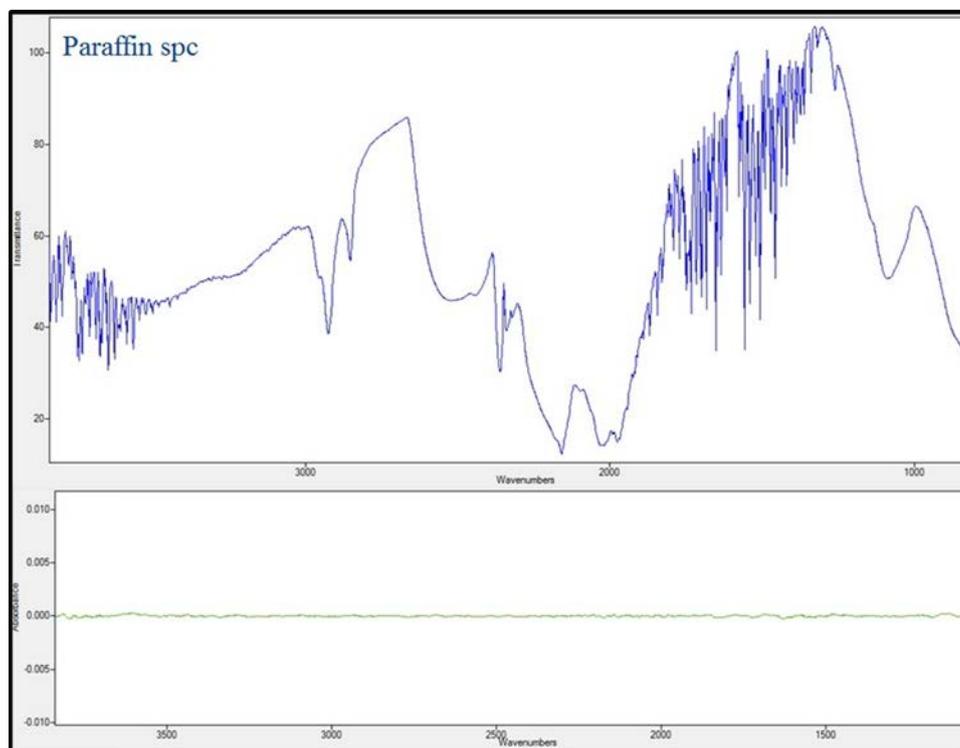


Figure 2. Top is the blank background & the bottom, is its absorbance.

have employed a variety of methods for tissue preparation which one of them is chemical fixation. Although many articles have been shown that non_dewaxed FFPP sections can be used successfully, but here we've shown this process is required.

At the first step, for calibration of ATR-FTIR instrument, blank background within each step of study were taken, then we tested background as a sample and as we expected, no IR peaks have been seen. (Fig. 2) Also, for insurance of instrument accuracy & validity,

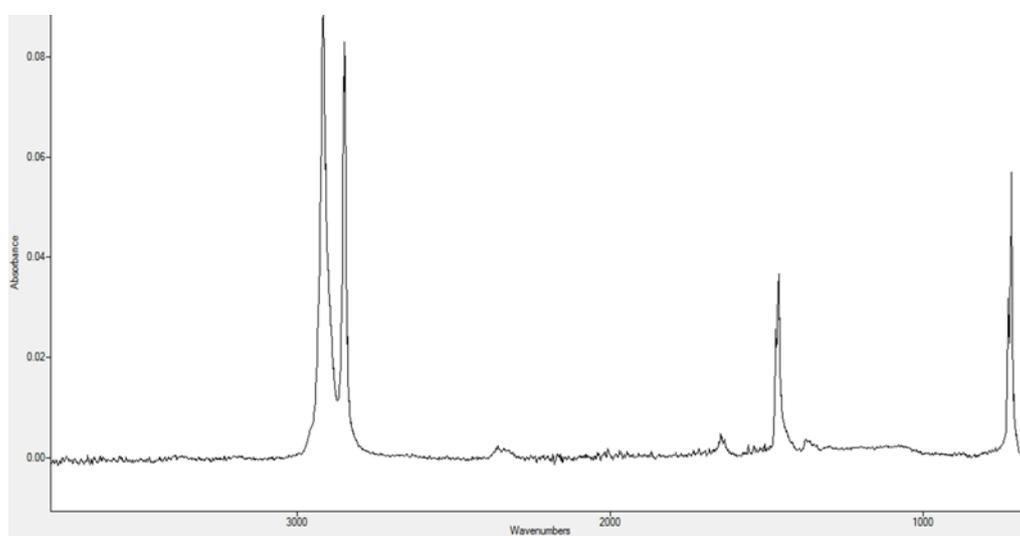


Figure 3. Nylon absorbance spectra.

we used nylon as a standard & it's strong peaks were basis of correctness performance of the instrument. Polyethylene spectra include C-H stretching (strong peaks at 2919 & 2851 cm^{-1}), bending (strong peaks around 1466 cm^{-1}) and rocking (medium strong peaks around 720 cm^{-1}) peaks. (Fig. 3)

Then , we defined the exact place of paraffin peaks as you can see in Fig. 4.strong peaks appear at 723,1463,2848,2913 & 2954 cm^{-1} that indicate C-H aromatic ,CH₂ & CH₃.

Why we decide to deparaffinize our samples? Fig. 5.a describes the reasons well; by extracting paraffin from sections and tissue rehydration, spectrums found more clarity & therefor accuracy and power of the instrument in the diagnosis & in all that biospectroscopy in order for it to be handled, improved.

For tissue deparaffinization, two types of dewaxing(n-hexane/xylene) and hydrating agents(acetone/ethanol) were applied.

In comparison of hydrating agents, it seems

that acetone has been more successful than ethanol. However, by the time the hydrating agent's effect gets better. So, as it's shown in Fig. 5.b we suggest acetone as a more proper hydrating agent for tissue sections.

For dewaxing agents comparison, since n-hexane in longer time has a better response, we chose 18, 24, 48 hours for the test. According to results(Fig. 5.c),although in 18 hours embedding ,paraffin peaks is ever exist, but sample's desired peak such as amides and DNA contents are also well shown. While in

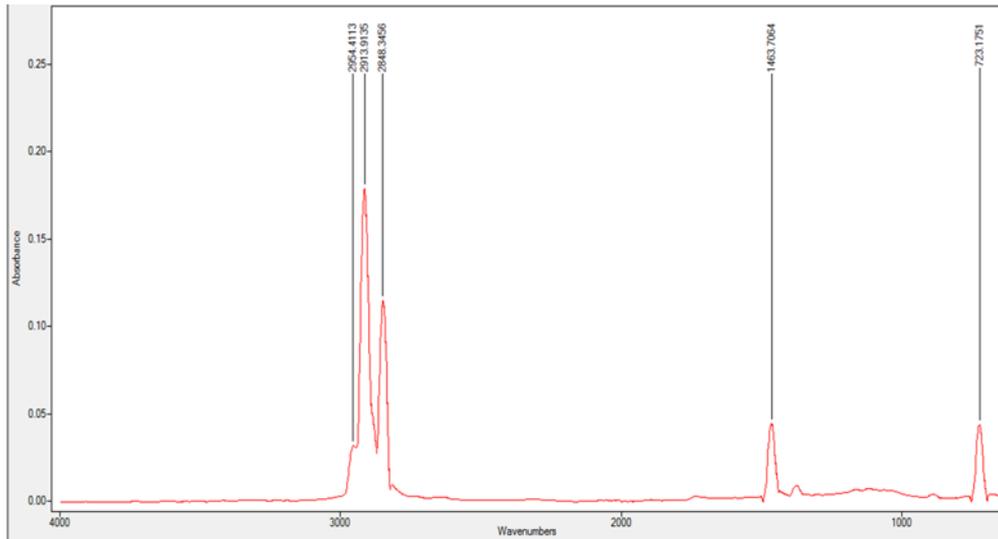


Figure 4. Paraffin absorbance spectra.

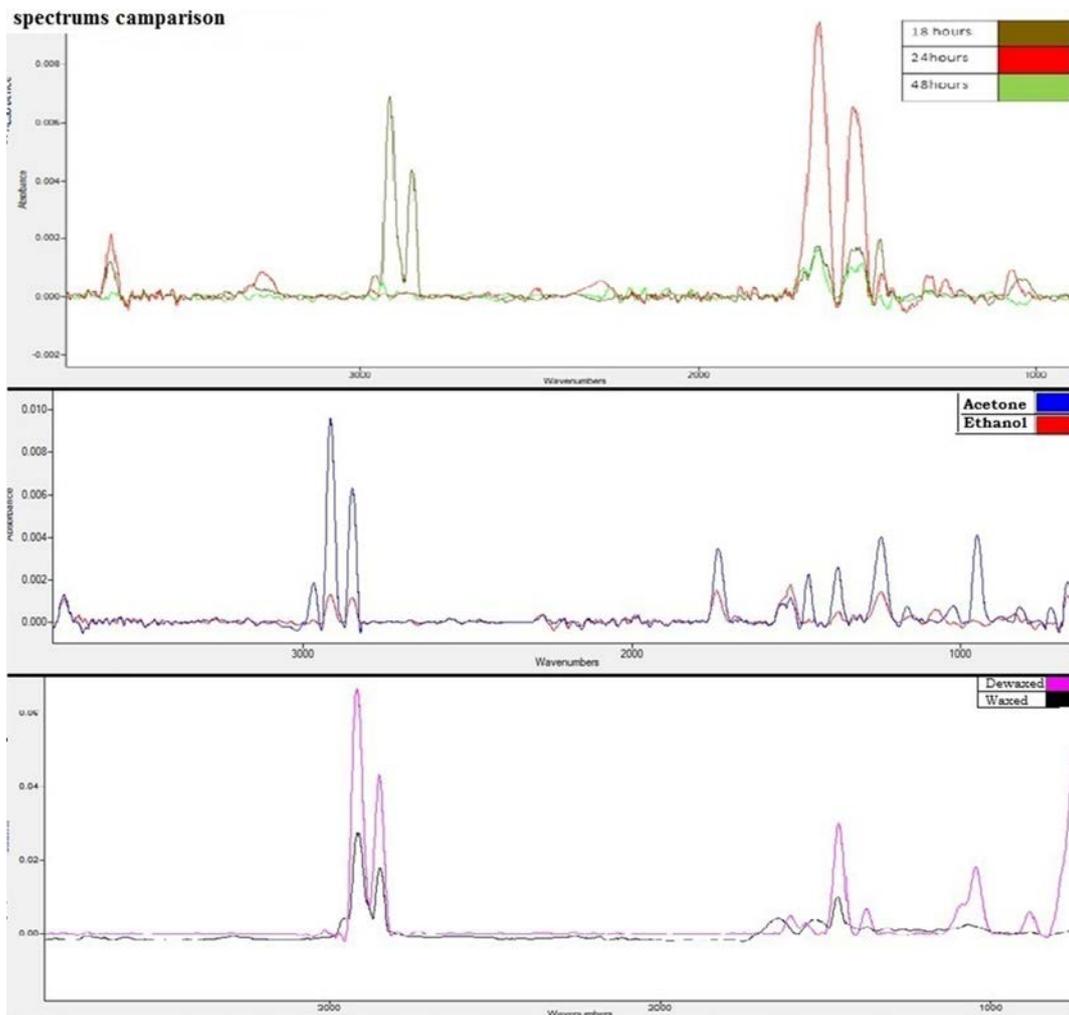


Figure 5. a. waxed (black) & dewaxed (pink) tissue comparison .b. ethanol (red) & acetone (blue) as hydrating agent comparison. c. n-hexane embedding time comparison.

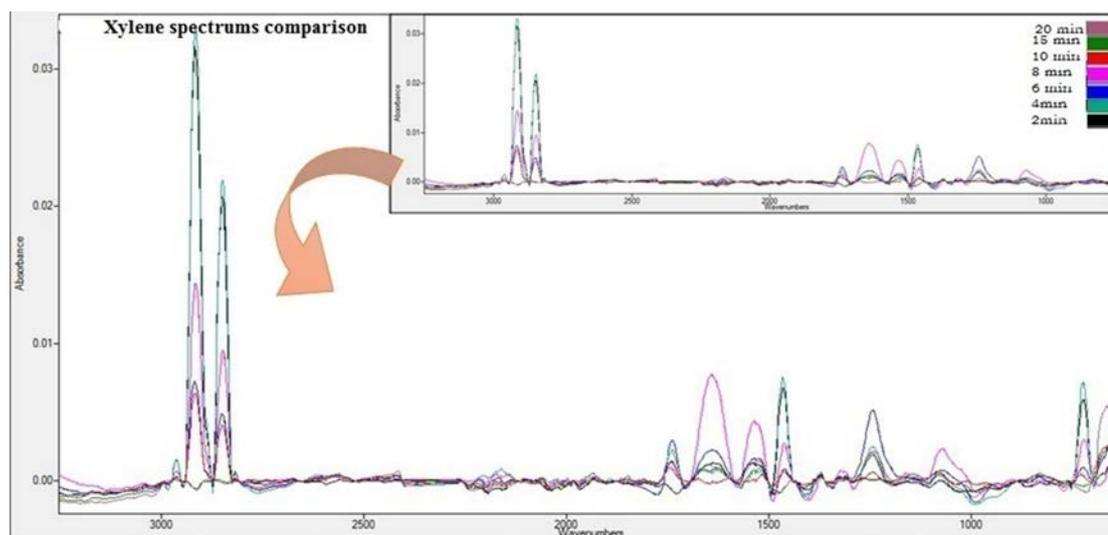


Figure 6. Xylene embedding time comparison.

48 hours embedding, paraffin is completely removed, but tissue peaks are decreased contemporaneous.

For the other agent, xylene, we chose shorter time (according to past researches) from 2 to 20 minutes embedding (2, 4, 6, 8, 10, 15 & 20 minutes). As Fig. 6 suggest, although the paraffin will be removed in longer time, but tissue peak's intensity are also decreased. In fact, noises of spectrums increase by the time. It seems that in the time range 6-8 minutes our desired spectrum is appeared.

For further study to prove repeatability, we again evaluated samples in 6, 7, 8 minutes embedding. The point is that, samples that belong to one paraffinized block, act the same in deparaffinization process and therefore in the spectrums, but in general these three minutes (6, 7 & 8) were not repeatable for all the tissue microtomes. (Fig. 7)

In next step, in the field of individual errors (operator's error), we evaluated sample preparation within a day & then between days with fixed taking of deparaffinization method.

We theorized that, repeatability is independent of time and maybe depends on sample deparaffinization methods. For answering this question, we take spectrum from each sample consecutive and between each time, sample area was cleaned & background collection was taken (Fig. 8). We repeated this process in several days.

Regardless of peaks intensity, it seems that spectrum's pattern that belongs to one paraffinized block is the same. In fact, there is no difference in content in one day/days but lack of repetition in times can relate to samples characteristics such as microtome's porosity, rate of paraffin penetration to intracellular space, lipophilicity of tissue and also existence of some tissue components like capillaries. More ever we can't ignore the effects of sample's thickness & holder pressure.

In general, although n-hexane is an effective dewaxing agent for biological samples after a 24 hours exposure, xylene is a better choice with higher efficiency in less



Figure 7. Xylene embedding comparison between days . a. 6 min b.7 min c.8 min.

time (6_8 minutes). However sections that was immersed in xylene for 8 minutes and then rinsed in acetone for 5 minutes showed amide I and II bands and DNA contents in FTIR spectra better than other strategies, visualization of the sections has shown that the paraffin is not removed completely. The disappearance of peaks at 1426 & 2850_{-2950} cm^{-1} of the FTIR spectrum was used to ensure complete deparaffinization that happened with 15 min xylene embedding, which effects on other cellular structures and subsequently on the spectrums.

We have repeated this process sequentially and as the Fig. 9 suggests, repeating is more effective in paraffin removing.

Notably, appearance characteristics can help in sample preparation process over all steps; in this process after the deparaffinization step, Sections should appear clear. If white patches are present, this in an indication that the paraffin wasn't removed well. Also, when the sections go from the xylene to the first alcohol or acetone in the hydration sequence, the sections should turn slightly opaque. If there were clear patches present, then the deparaffinization was incomplete which may cause with water existence. If the clearing xylene is contaminated with water it will appear milky-white.

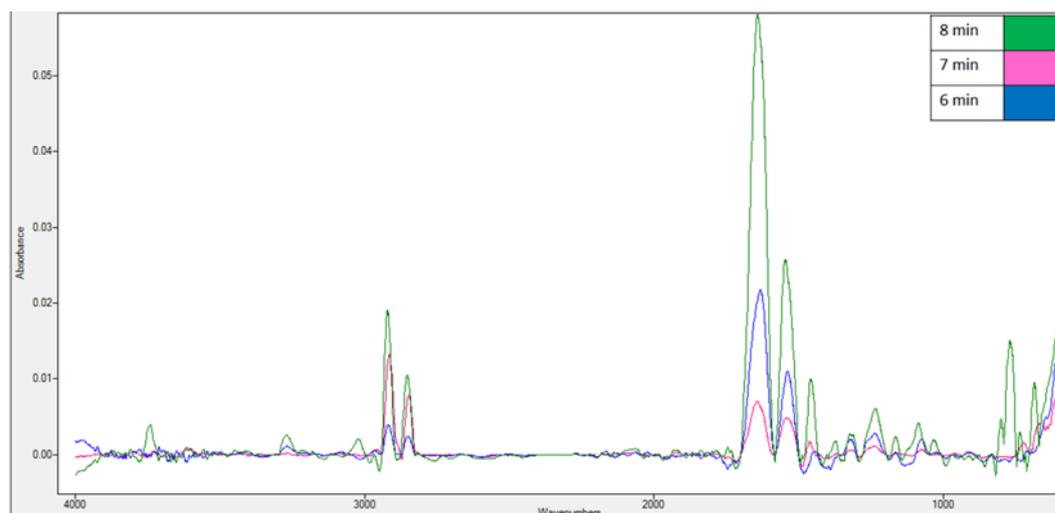


Figure 8. Xylene embedding comparison within a day.

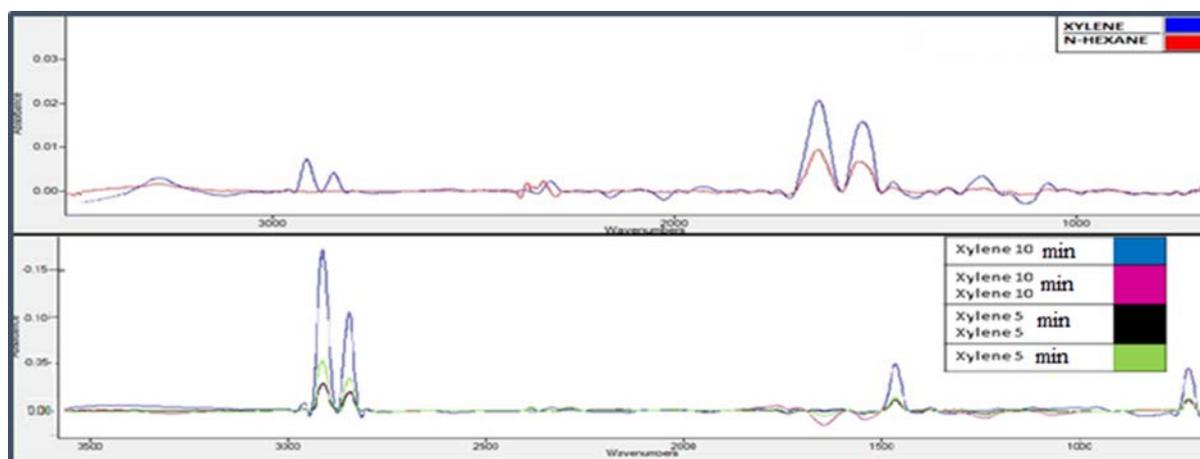


Figure 9. Top is 8 min xylene & 24h n-hexane embedding comparison & the bottom is xylene sequential embedding vs. one time embedding in 5 & 10 min.

4. Conclusion

Optical spectroscopy is becoming a very powerful diagnostic tool. However, to develop a cost-effective system for routine clinical uses, an enormous amount of research still needs to be conducted. The use of vibrational spectroscopic techniques for the mapping and imaging of cells and tissues is undergoing a rapid expansion in the range of techniques, sampling procedures, and applications that span from fundamental studies to clinical applications. The research results obtained from these rapidly evolving techniques are

providing many new insights into biochemical architectures and processes [16, 23].

Problem in diagnosis is very common with some other approaches (e.g., pathological observations) and the rate of misdiagnosis for IR analysis has been reported as being very low. IR is a rapid technique, because the spectral data are collected and interpreted within minutes [2, 5]. Usually, sample preparation is not obligatory prior to spectral analysis or is minimal. The potential advantages of using IR for disease detection include the following: no reagents are required, a profile of spectral alterations can

be determined, and the methods are suitable for automation. Sample preparation is minimal, techniques involved are relatively low cost, and data frameworks are available. It is expected that vibrational spectroscopy methods will be integrated into more frequent clinical use in the near future.[5,9]

Sample preparation is a key aspect of any experimental design and particularly so for spectroscopic analysis of cells and tissues. The continuing developments in tissue preservation for optimum detection of specific biomolecules using emerging bioanalytical approaches will shape the tissue repositories of the future[19-22]. These developments will also have an impact on biomedical vibrational spectroscopy, since this technology can play an important role in determining the biochemical basis underpinning disease progression.

Nevertheless, it is apparent that existing tissue banks have proven adequate for FTIR studies of tissue pathologies, providing high classification power. This is despite the fact that spectral artifacts exist as a result of sample processing. These spectral artifacts can be due to protein depolymerization or a change in the lipid to protein ratio for dried cryosections, or in the case of deparaffinized sections, due to residual paraffin, coagulation of proteins and loss of lipids. [6, 15-19]

We showed that deparaffinization using hexane for 24 h is an appropriate method for this purpose and this has wider implications in immunohistochemical pathology. However, this protocol can be time limiting and so less

rigorous protocols may be sufficient where spectroscopic markers for pathological assessment do not overlap with paraffin signals.

In summary, for spectroscopic analysis of tissue samples, fresh or carefully prepared frozen tissue would be considered ideal, but FFPP tissue can be used successfully if deparaffinized using hexane. Other dewaxing agents like xylene and protocols can also be used successfully if the paraffin signals do not overlap with the spectral regions of interest.

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