# Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopic analysis of regenerated bone

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## ABSTRACT

The cutting of bone is routinely required in medical procedures, especially in dental applications. In such cases, bone regeneration and new bone quality can determine the success of the treatment. This study investigated the main spectral differences of undamaged and healed bone using the ATR-FTIR spectroscopy technique. Three rabbits were submitted to a surgical procedure; a small piece of bone ($3\times3$ mm$^2$) was removed from both sides of their jaws using a high speed drill. After 15 days, the rabbits were euthanized and the jaws were removed. A bone slice was cut from each side of the jaw containing regions of undamaged and newly formed bone, resulting in six samples which were polished for spectroscopic comparison. The samples were analyzed by FTIR spectroscopy using a diamond ATR accessory. Spectral characteristics were compared and particular attention was paid to the proportion of phosphate to amide I bands and the width of the phosphate band. The results show that the ratio of phosphate to amide I is smaller in new bone tissue than in the undamaged bone, indicating a higher organic content in the newly formed bone. The analysis of the width of the phosphate band suggests a crystallinity difference between both tissues, since the width was higher in the new bone than in the natural bone. These results suggest that the differences observed in bone aging processes by FTIR spectroscopic can be applied to the study of healing processes.

**Keywords:** FTIR; bone, laser, healing processes

## 1. INTRODUCTION

The cutting of bone is routinely required in medical procedures, especially in dental applications. In such cases, the bone regeneration process and the new bone quality can determine the success of the treatment. The use of lasers as a surgical tool allows more precision for the surgeon, making it possible the cut of tissue where the access is limited. The laser also minimizes the mechanical damage in the surrounding tissue, and promotes hemostasis. For these reasons, the development of a laser technique for bone surgery offers an attractive alternative, replacing the mechanical tools normally used.[1],[2,3]

However, despite the fact that the laser equipment seems to be a more advantageous cutting tool, there is no consensus in the literature concerning the effectiveness of lasers when compared to cutting instruments commonly used (especially in relation to the initial stages of wound healing)[1], making it necessary more studies with a view to a better understanding of the differences promoted by both tools in the bone regeneration.

The Fourier Transform Infrared Spectroscopy (FTIR) technique is able to analyze compositional and structural changes of mineralized tissues[4]. The technique has already been successfully used to study the differences that laser irradiation promotes in dentin, enamel and bone[5],[6,7],[8]. It was observed that the laser can affect the mineralized tissues according to the irradiation conditions, especially the organic compounds.[6,8]

The bands of the bone tissues observed in IR spectrum are shown in Figure 1. The bone mineral matrix is formed by hydroxyapatite[4], as in dentin and enamel[9]. The main inorganic components observed in the bone IR spectrum are the phosphate (1300–900 cm$^{-1}$) and carbonate (1600-1300 cm$^{-1}$ and around 870 cm$^{-1}$). Related to the organic matrix, the main observed components are amide I (1680-1600 cm$^{-1}$), amide II (1580-1480 cm$^{-1}$), amide III and collagen (1200-

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1300 cm$^{-1}$). Water is also a bone component and it holds a strong absorption on IR. It presents two main bands on IR: one between 3700 cm$^{-1}$ to 2000 cm$^{-1}$ and another overlapped with the amide I, between 1680-1600 cm$^{-1}$.[7]

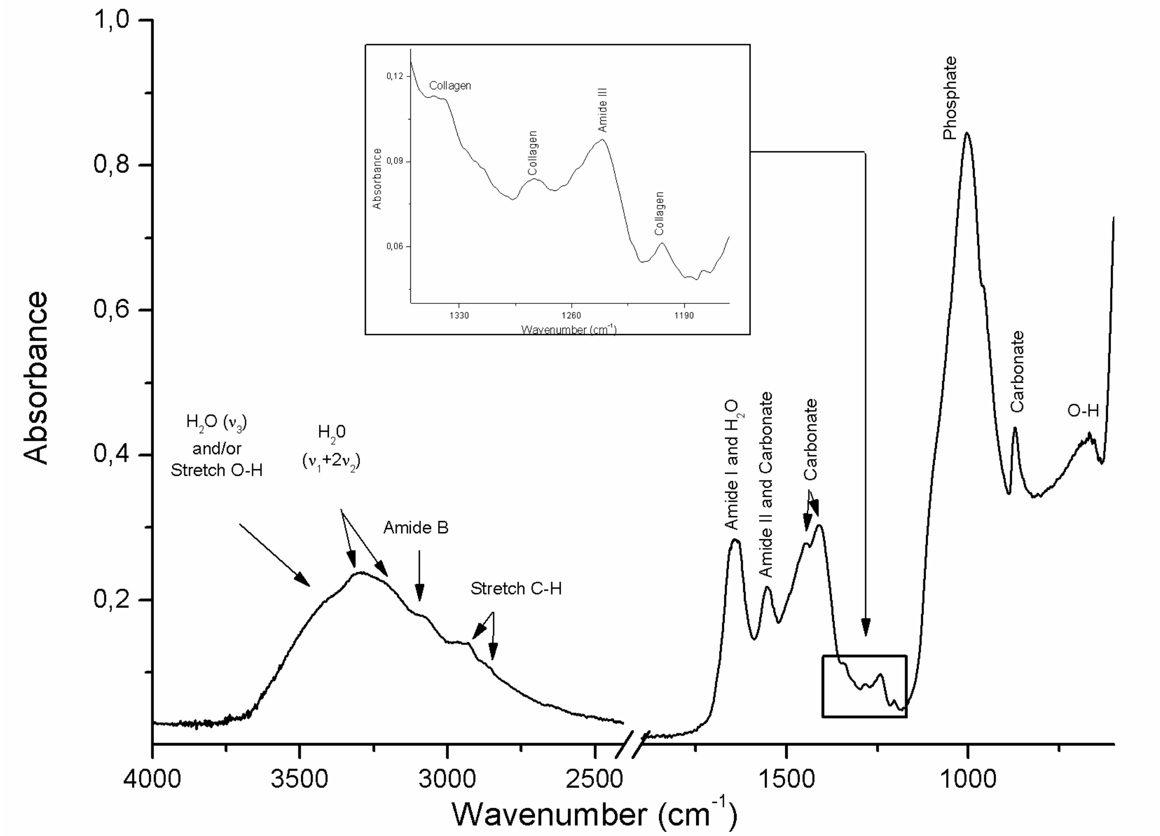


Figure 1 – FTIR spectrum of a mandibular bone tissue

It is known that some characteristics of bone IR spectrum can be related to the tissue age, such as the area of the phosphate band (900–1200 cm$^{-1}$) to the area of the amide I band, or the area of the carbonate band (850-890 cm$^{-1}$) to the phosphate (or amide I) bands[10]. Some of these relations may be used in the understanding of the physic-chemical aspect of the bone healing process, and to contribute for a better understanding of the differences promoted by laser in the bone regeneration.

## 2. OBJECTIVE

This is a preliminary study to investigate the potential of the ATR-FTIR technique in the study of bone healing process, in order to use this technique hereafter to study the bone healing process after laser cut. In this step, the main spectral differences of the undamaged and the healed bone after mechanical cut were investigated using the ATR-FTIR spectroscopy technique.

## 3. METHODS

This study was approved by the by Animal Ethics Committee of IPEN (6/CEPA-IPEN/SP). Three rabbits were submitted to a surgical procedure and a small piece of bone (3×3 mm$^2$) was removed from both sides of their jaws using a high-speed drill. After 15 days, the rabbits were euthanized and the jaws were removed. A bone slice was cut from each side of the jaw containing regions of undamaged and newly formed bone, resulting in six samples which were polished with sandpaper and diamond suspension solution for spectroscopic comparison.

The infrared spectra acquisition was made using an FTIR spectroscopy using a diamond ATR accessory (Varian 670 FTIR) via a diamond ATR imaging Golden Gate™ (Specac) and an FPA detector. The spectra range analyzed was from 4000 to 900 $cm^{-1}$, with a resolution of 4 $cm^{-1}$ and 64 scans per spectra. This range was the maximum range allowed with this equipment configuration, and it was chosen because it is where the organic components are mainly found[11]. The detector size was set between 64×64 pixels and the total image size was 550×600 $\mu m^2$. [12,13,14]

After the acquisition of the samples' spectral image, the data were computed in CytoSpec. Two regions of the image were selected: one of the undamaged tissue and another of the healed tissue. The average spectrum of each region was calculated. The analysis of band areas and band position was made using the OriginPro software. Spectral characteristics were compared and particular attention was paid to the proportion of phosphate to organic bands and to the width of the bands. To compare the different groups, a t test ($p<0.05$) was used.

# 4. RESULTS AND DISCUSSION

The main region of a spectrum of an undamaged bone and a new formed bone are shown in **Figure 2**. In both cases, it is possible to observe the main infrared bands reported in literature. Comparing the spectra of each group, it is possible to observe that the healing process did not promote any significant difference in bands' detection. The position of the bands also did not suffer any significant changes.

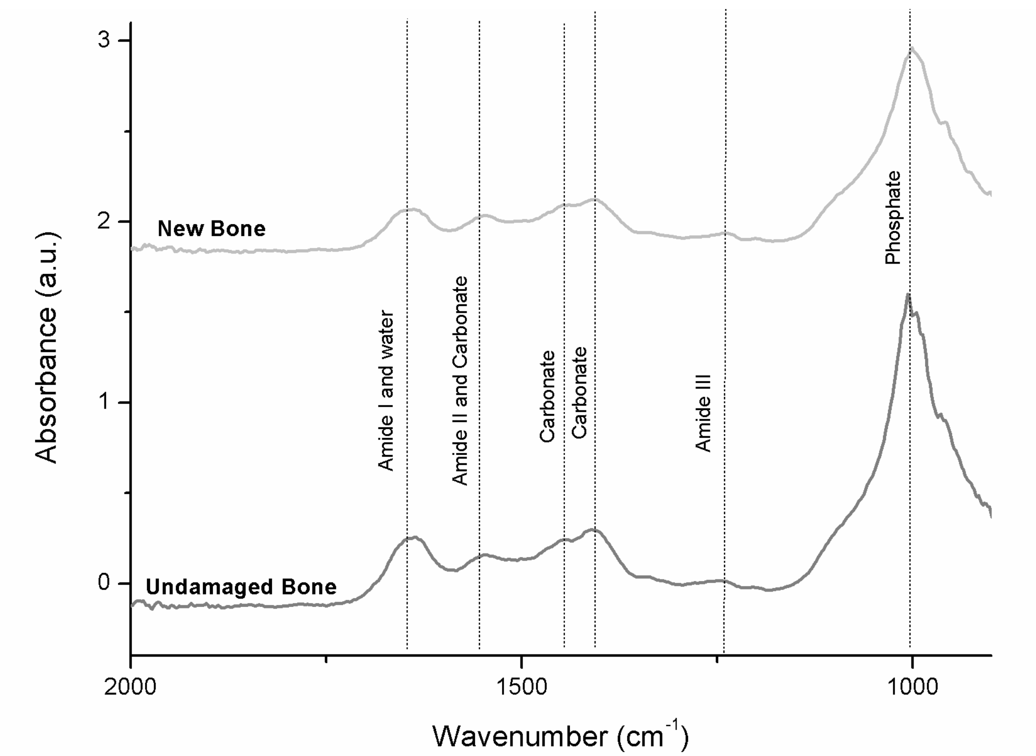


Figure 2 – Comparison of the FTIR spectrum of an undamaged bone region (in dark gray) and a new formed bone region (in light gray) from the same sample. It was not observed any significant changes in bands position.

To verify any difference between the new formed bone and the undamaged bone, the widths of the bands were compared. The bone width can be related to compositional and crystallographic characteristics[15]. To prevent those small differences in the band positions from being neglected, the spectra were analyzed one by one and the beginning and end of each band was set to determine the measure of the width.

The Figure 3 shows the average of the widths orbited by amide I, amide II, amide III, carbonate and phosphate of both tissues. No statistical difference was observed in any group, however it was noticed that difference between the widths of the new and the undamaged bone is greater in carbonate and phosphate bands.

The phosphate band width is related to the crystallinity of the material[12], so the results suggest that the new formed bone have different crystallinity when compared to the undamaged bone. The new formed bone shows a larger bandwidth when compared to the undamaged bone. In the literature, it was showed that a decreasing bandwidth of phosphate band at 960 $cm^{-1}$ indicates greater crystallinity[15], using Raman spectroscopy. So, the results obtained here suggest that the new formed bone have smaller crystallinity than the untreated/mature bone. This was an expected result, since the crystallinity is related to the bone maturity[15], and past 15 days of the surgery the healing process is not completed yet[1].

To verify changes in bone composition, the ratio between the area of the organic components (amide I, amide II and amide III) and the area of the phosphate (970 $cm^{-1}$) band were calculated. For each component, the average of the six samples was considered. The results are shown on Figure 4.

The phosphate band was used in the normalization because it is related to the inorganic matrix of the bone tissue[7]. The carbonate band (1600-1300 $cm^{-1}$) is also related to the inorganic components; however this band is overlapped with the amide bands (Figure 1)[16], which could impair the organic/inorganic ratio analysis.

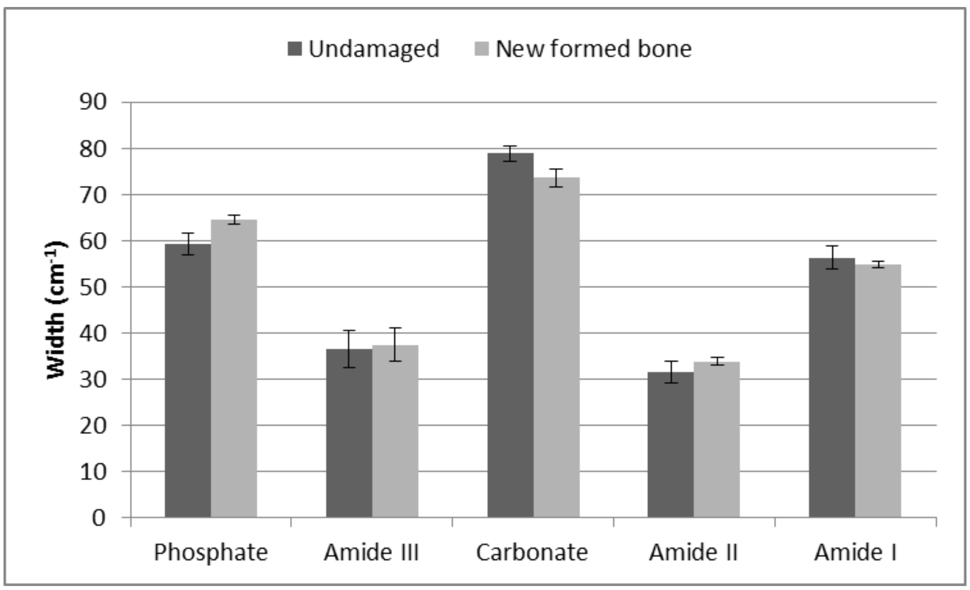


Figure 3 – Widths of the bands of the undamaged and the new formed bone.

It was not observed any statistical difference between the tissues proportion of amide I, amide II, amide III and carbonate. However, it is possible to note that the proportion of the areas of the amides and phosphate bands were always larger in the new formed tissue when compared to the undamaged tissues, which may indicate a higher level of organic components in the former than in the latter.

The highest difference between the undamaged and the new formed bone was in amide III: the difference between the groups ratio average was nearly 40%. The amide I and amide II bands did not present the same difference; in the first group the organic/inorganic proportion increased less than 2.5%; in the second one the increase was approximately 15%. Despite these bands are related with the organic matrix, only the amide III do not suffer effects of other components once the amide I band is overlapped with the water band[7] and the amide II band is overlapped with the carbonate band[17]. It is possible that in this study, the overlapping of the bands have affected the organic/inorganic ratio using the amide I and amide II.

Despite the fact that a new bone had already been formed 15 days after the surgery, the healing process had not completed yet. It is possible that the cell activity inside the new tissue was greater than in the undamaged bone, and as a consequence there was a higher proportion of organic components in the new formed bone than in the undamaged tissue, as shown in Figure 4. Similar results were observed in studies with different bone ages, in which younger bones present a smaller ratio between phosphate and amide I than older tissues[10].

The carbonate/phosphate ratio analysis suggests that the carbonate content is smaller in the new formed bone than in the undamaged bone (the differences between both values were around 22%). However, considering that: i) the carbonate band is overlapped with the amide II and collagen bands[16]; and ii) the others bands analysis has indicated a higher level of organic components in the new formed bone than in the undamaged bone, it is possible that the apparent decrease of carbonate is not necessarily related with a decrease of this component, but it is related with an increase of the organic bands.

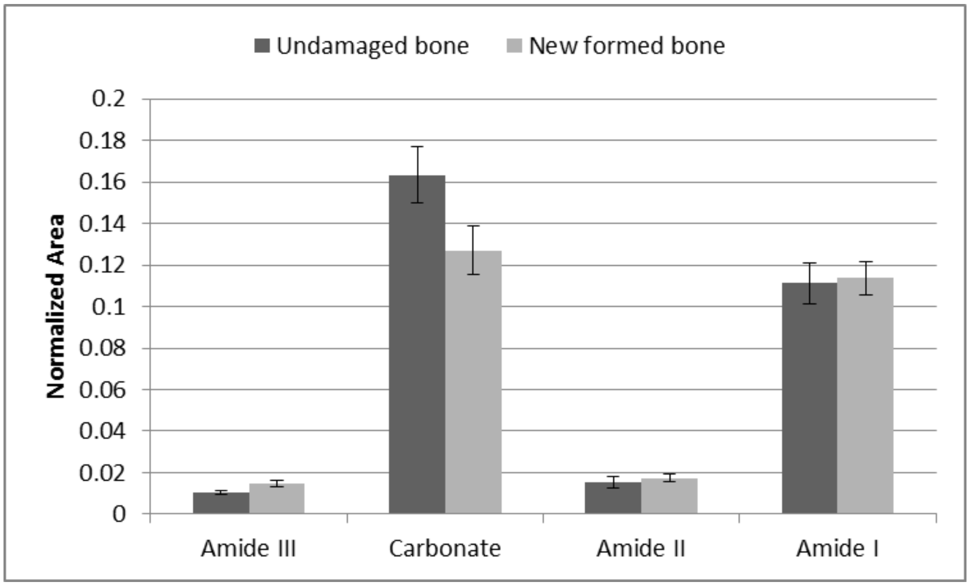


Figure 4 – Ratio between the phosphate and amide I area bands of the undamaged and the new formed bone.

The results of this study show that it is possible to use the FTIR spectroscopy to observe alterations in bone healing process, indicating that the FTIR is a potential tool to a better understanding of the changes promoted by different cutting tools. However, the comparisons of the results with a well-established technique (as histopathology analysis) may be crucial for a real comprehension of the data, and further studies will be necessary to a better understanding of the physic-chemical aspect of the healing processes.

## 5. CONCLUSION

The analysis of the results suggests that the differences observed in bone aging processes by FTIR spectroscopic can be applied to the study of healing processes. Despite the fact that a statistical difference was not observed, the results were compatible to the characteristics expected in the bone healing process and can be related to other results found in the literature. Thus, the technique has the potential to be used to complement the investigation of the main differences that the laser promotes in bone cut when compared to the usual cutting tools.

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