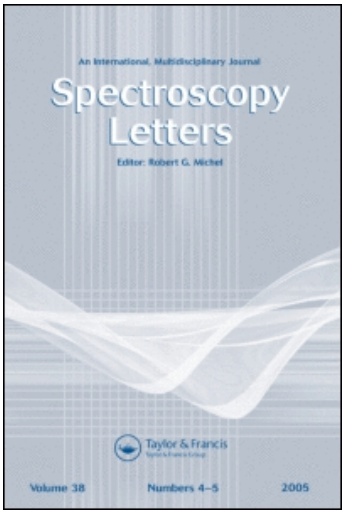

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Changes in Dentin Collagen After Sample Grinding and Heating

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ABSTRACT Changes to the structure of the organic matrix of the dentin tissue were determined after sample grinding and heating. Powder dentin measuring 25–38 μm and slices with a thickness of about 50 μm were employed. Spectra acquisition was conducted with a Fourier-transform infrared spectrometer. The thermal treatment was performed between 100°C and 300°C, with steps of 25°C. After grinding, two bands ($1283.5 \pm 0.5\,cm^{-1}$ and $1240.7 \pm 0.5\,cm^{-1}$) shifted to higher wavenumbers, while three bands ($1339.5 \pm 0.5\,cm^{-1}$, $1283.5 \pm 0.5\,cm^{-1}$, and $1202.7 \pm 0.5\,cm^{-1}$) shifted to lower wavenumbers after thermal treatment in the range 100–300°C; the band at $1283.5 \pm 0.5\,cm^{-1}$ shifted only $2\,cm^{-1}$. Thermal treatment produced a wavenumber shift in the opposite direction compared with the shift produced after grinding. The observed changes in the vibration modes of the structure indicate that sample preparation or sterilization involving grinding and heating must be carefully evaluated in order to preserve the natural characteristic of the collagen structure.

KEYWORDS collagen, dental tissues, FTIR, infrared spectroscopy

INTRODUCTION

The organic matrix of the dentin corresponds to 20 wt% of the tissue, and its majority component is collagen, which corresponds to 18 wt% dentin.[1] The collagen is a family of proteins found in the dentin matrix as well as in all multicellular animals. As a major organic component of the skin, bone, and dentin, the collagen proteins correspond to 25 wt% of the total protein mass in mammals.

The main feature of a collagen molecule is the three distinct polypeptide chains, which are twisted into a spiral around an individual axis with a typical width of 1.5 nm. The molecule chain is denominated as *alpha chain*. Currently nearly 25 different alpha chains of collagen have been found. The collagen proteins are rich in proline and glycine, which are amino acids that are important in the formation of the three-dimensional structure. The major types of collagen found in the skin, bone, and dentin are types I, II, III, V, and XI; type I is the main collagen observed in the mentioned tissues. These types of collagen polymerize in a fibril structure and are know as *fibrillar collagen*; there are other types of nonfibrillar collagen as well as collagen whose structure has not been determined.[2]

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In fibrillar collagen the molecules are parallel and bound together; their ends are separated by gaps of about 40 nm, while the interfibrillar zones are distant from each other by about 0.24 nm. The three-dimensional structure of fibrillar collagen is defined by electrostatic interactions, van der Waals forces, hydrogen bonds, hydrophobic interactions, and covalent bounds. These collective noncovalent bonds are strong enough to hold the molecular structure together, but they are susceptible to external interference such as heat, which partially breaks these bonds, leading to changes in the conformation of the molecule.[3] A kinetic model for the thermal denaturation of collagen can be found in the literature.[4,5] In these models, collagen denaturation is described as a first-order chemical reaction in which the protein must overcome a potential energy barrier known as activation energy.

Because freshly extracted teeth are by nature a potential source of cross-contamination to laboratory equipment and personnel, newly extracted teeth must be decontaminated. An ideal system for tooth sterilization must sterilize, but it should not alter the structure of the hard dental tissues.

Several methods for tooth sterilization have been examined: steam (autoclave), dry heating, liquid solutions that inhibit bacterial growth, ethylene oxide, and gamma radiation.[6–8] Changes in the natural features of the tissues can occur after these sterilization methods. To avoid interference with the targeted results, these changes must be carefully evaluated and minimized. Examples of changes in the tissue after sterilization are (a) mineral matrix demineralization and nanomechanical changes[9] after the use of solutions with low pH values; (b) denaturation of the organic matrix[3–5] after procedures involving temperature rise; and (c) creation of defects in the crystals with probable changes in the natural fluorescence, color, or paramagnetic radicals after ionizing radiation. Gamma radiation is considered an effective sterilization method since it does not modify the FTIR and UV/VIS/NIR spectra or tooth permeability,[7] but its use is strongly discouraged in research experiments on the paramagnetic radicals of the tissue.

After tooth sterilization, the second step to be carried out before the target experiment is sample preparation. This procedure can also bring about changes to the tissue features because grinding and cutting lead to mechanical stress and heat generation. The determination of physical and chemical changes by different techniques usually requires a specific procedure for sample preparation. In infrared spectroscopy, sliced samples resemble the whole tissue more closely,[10] but sometimes the sample must be powdered because even thin slices show limited transmission of infrared light in the regions of the spectrum where dentin absorbs strongly.

As a consequence of tissue susceptibility, changes in tissue composition and structure can occur during sterilization and sample preparation, mainly in the case of the organic matrix, which is thermally unstable. These two procedures, which are normally applied in several experiments, must be appropriately evaluated so that the natural tissue features are not modified and the introduction of errors and misinterpretation of the final results are avoided.

OBJECTIVE

The objective of this work is to determine which changes occur in the structure of the organic matrix of the dentin tissue after sample grinding and heating, which respectively simulate the common procedures of sample preparation and sterilization.

MATERIALS AND METHODS

Bovine incisor teeth used in this work were initially placed in 0.9 wt% sodium chloride immediately after extraction and kept in this solution at 5–10°C in a refrigerator until sample preparation and spectroscopic evaluation. The teeth were cut into slices of 0.5 mm using a diamond blade system, and then two different sample sets were obtained: slices with a thickness of about 50 μm and powder measuring 25–38 μm. The slices with an initial thickness of 0.5 mm were sanded manually with 1200-mesh silicon carbide sand paper and water, until a thickness around 50 μm was achieved. A total of 16 samples were selected for the control (seven) and heating (nine) experiments. The powder samples were obtained from dentin pieces that were manually ground in a mortar and pestle. The resulting powder was sieved, in order to select particle with sizes between 25 μm and 38 μm. A total of 14 samples weighing 5 mg were selected and mixed with 50-mg KBr. The final powder (dentin and

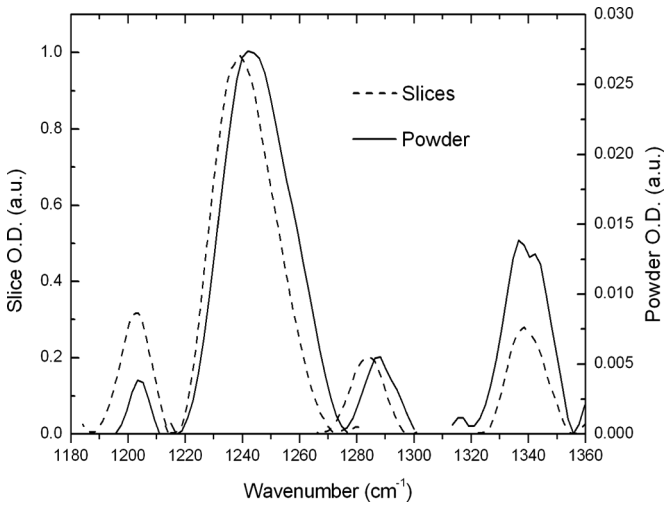

FIGURE 1 **Absorption bands of natural samples of bovine dentin in range 1180–1360 cm^{-1} in sliced and powdered forms. The bands at $1240.7 \pm 0.5\,cm^{-1}$ and $1283.5 \pm 0.5\,cm^{-1}$ obtained from slices shift to higher wavenumbers after grinding, while the other bands, namely $1202.7 \pm 0.5\,cm^{-1}$ and $1339.5 \pm 0.5\,cm^{-1}$, do not change significantly.**

KBr) was compressed (4 ton), to produce a pellet and allow evaluation in the spectrometer.

The spectra acquisition was conducted with a Fourier-transform infrared spectrometer (Nicolet 380; Thermo Fisher Scientific, U.S.A.). The spectra were analyzed in the region between $4000\,cm^{-1}$ and $400\,cm^{-1}$ and were registered in the transmission mode with a resolution of $0.5\,cm^{-1}$. Data analysis was conducted with the program Microcal Origin® (Version 6.0, OriginLab Corporation, USA); after spectra acquisition, the background was removed using a stretch line, producing bands as shown in Figures 1 and 2. Then the area was measured by integration, and peak position was located by the maximum absorbance value. Gaussian and Lorentzian functions were not employed because of the asymmetric shape of some bands.

The thermal treatment of the tissue was conducted in an oven with controlled temperature in the temperature range between 100°C and 300°C, with steps of 25°C. Each sample was heated for 30 min.

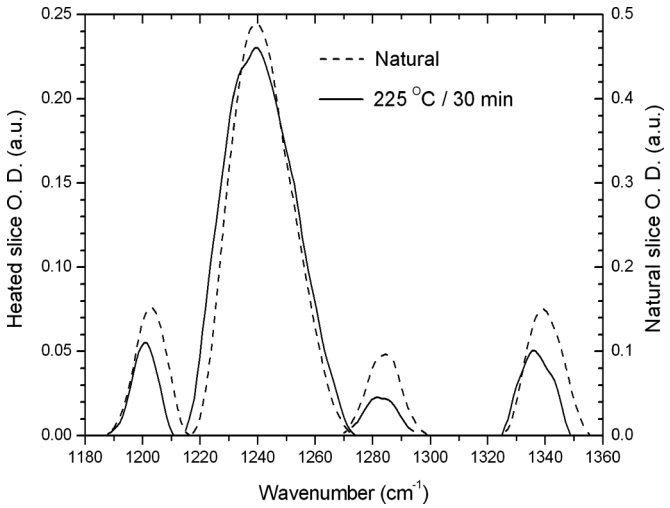

FIGURE 2 **Absorption bands of natural samples of bovine dentin in range 1180–1360 cm^{-1} in the sliced form after heating (225°C for 30 min). The bands at $1202.7 \pm 0.5\,cm^{-1}$, $1283.5 \pm 0.5\,cm^{-1}$, and $1339.5 \pm 0.5\,cm^{-1}$ shift to lower wavenumber after heating, while the band at $1240.7 \pm 0.5\,cm^{-1}$ does not change significantly.**

RESULTS

In Table 1, the mean position values of the observed bands are compared with bands from the rat skin and human bone.[11] The statistical error of the observed bands (0.1–0.01 cm^{-1}) is below the resolution of the spectrometer ($0.5\,cm^{-1}$), and the final error value used for the present results is $0.5\,cm^{-1}$. Apart from CH_2 wag and amide III, all the bands observed in the range 1100–1400 cm^{-1} are associated with the structure of collagen, so changes in this structure will influence these bands.[12]

The intensity of the bands present in the spectra of the powder samples are 10 times weaker than those of the sliced samples, because of the different quantity of material used in the two experiments. Two bands display a statistically significant shift to higher wavenumbers after grinding: the bands at $1283.5 \pm 0.5\,cm^{-1}$ and $1240.7 \pm 0.5\,cm^{-1}$, which had shifted to $1288.1 \pm 0.5\,cm^{-1}$ and $1245.2 \pm 0.5\,cm^{-1}$, respectively, after grinding. The two other bands did not change significantly after the two procedures.

The bands of the sliced samples and powdered samples are shown in Figure 1. There is a shift of two bands to higher wavenumbers. Figure 2 depicts the bands obtained after heating the sample to 225°C for 30 min and shows that two bands shift to lower wavenumbers. The positions of the bands after heating can be visualized in Figures 3, 4, 5, and 6 for all the temperature range (100–300°C), and they are also compared with the positions of the bands after grinding. The data corresponding to $T = 300$°C do not appear in the figures because this band value was too weak to be measured after heating.

In Figure 3, the band at $1202.7 \pm 0.5\,cm^{-1}$ shifts to lower wavenumber after progressive heating, but no

TABLE 1 **Position of Infrared Absorption Bands of Bovine Dentin Near 1400–1100 cm^{-1} as Observed in Figure 1: A Comparison With the Absorption Bands Obtained for Collagen From Rat Skin and Human Bone Can Be Made**

Bovine dentin (±0.5)		Rat skin (cm^{-1})[8]		Human bone (cm^{-1})[8]		
Powder	Slice	Powder	Slice	Powder	Slice	Assigned[8]
1338.5	1339.5	1334	1337	1334	1342	Collagen structure
Weak	Weak	—	1306	—	—	CH_2 wag
1288.1	1283.5	1277	1281	1276	—	Collagen structure
1245.2	1240.7	1233	1232	1238	1233	CONH (amide III) CN stretching and NH deformation
1203.6	1202.7	1206	1201	—	1204	Collagen structure

difference is observed after sample grinding (compared with the sliced sample). After heating, there is a difference that increases progressively with the applied temperature. The final shift for the band at $1202.7 \pm 0.5\,cm^{-1}$ after heating at a temperature of 275°C for 30 min is $3.9 \pm 0.5\,cm^{-1}$.

Figure 4 shows the behavior of the band at $1240.7 \pm 0.5\,cm^{-1}$ after heating and grinding. Heating up to 275°C does not alter the position of this band. After 300°C this band disappears, so its position could not be measured. After grinding, the band shifts $4.5 \pm 0.5\,cm^{-1}$ to a higher wavenumber, as observed in Figure 4.

Figure 5 represents the behavior of the band at $1283.5 \pm 0.5\,cm^{-1}$. This band shifts $2 \pm 0.5\,cm^{-1}$ to lower wavenumbers after heating to 275°C. Despite the difference observed after 275°C, the other treatment reveals differences only after 200°C and 250°C. The behavior after heating in the range 100–300°C is quite similar to that of the natural sample. After grinding, this same band shifts $4.6 \pm 0.5\,cm^{-1}$ to higher a wavenumber.

Figure 6 depicts the behavior of the band located at $1339.5 \pm 0.5\,cm^{-1}$ after heating and grinding. A shift of $3.8 \pm 0.5\,cm^{-1}$ to lower wavenumbers after thermal treatment up to 275°C is observed, but the position of this band does not shift significantly after grinding.

DISCUSSION

The bands observed in the range 1200–1400 cm^{-1} are assigned to the secondary structure of proteins. The behavior of these bands aids evaluation of the structure upon conformational changes and distinguishes molecules with the same composition but

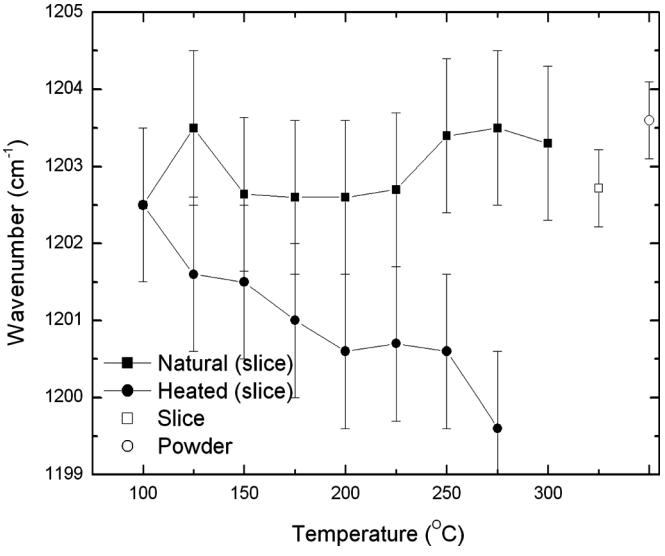

FIGURE 3 **Position of the band at $1202.7 \pm 0.5\,cm^{-1}$ after heating and grinding. Heating to 300°C shifts the band by $3.9 \pm 0.5\,cm^{-1}$ to lower wavenumber ($1199.6 \pm 0.5\,cm^{-1}$). The position of the band in the case of sliced samples does not significantly differ from its position in the ground sample ($1203.6 \pm 0.5\,cm^{-1}$).**

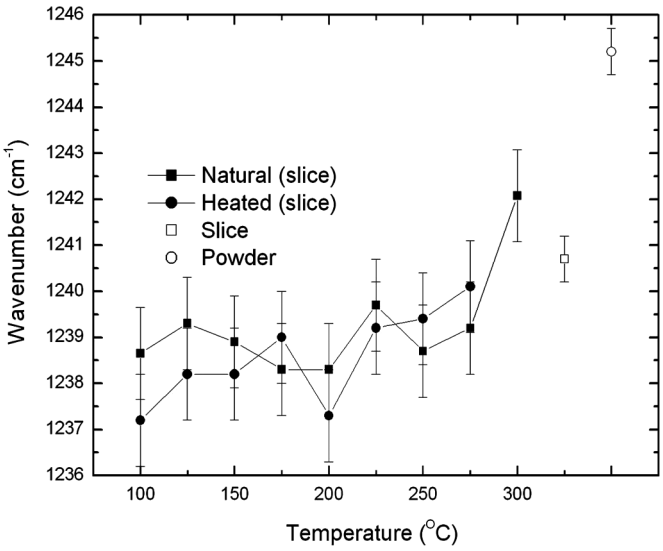

FIGURE 4 **Position of the band at $1240.7 \pm 0.5\,cm^{-1}$ after heating and grinding. Heating to 275°C does not alter the band. The band shifts $4.5 \pm 0.5\,cm^{-1}$ to higher wavenumbers ($1245.2 \pm 0.5\,cm^{-1}$) after grinding.**

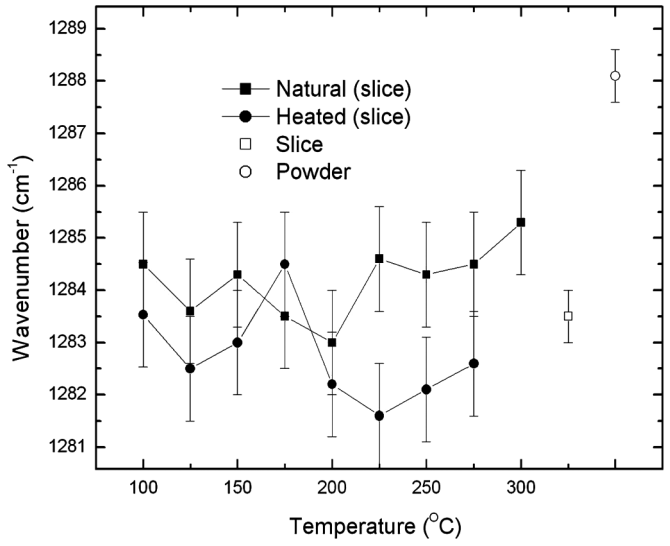

FIGURE 5 **Position of the band at $1283.5 \pm 0.5\,cm^{-1}$ after heating and grinding. The band shifts slightly ($\sim 2 \pm 0.5\,cm^{-1}$) after heating at three temperatures (namely 200°C, 250°C, and 275°C), and the shift does not depend on the applied temperature. The band shifts $4.6 \pm 0.5\,cm^{-1}$ to higher wavenumbers ($1288.1 \pm 0.5\,cm^{-1}$) after grinding.**

different structures.[12] The bands observed in this study are attributed to the helical structure of collagen and amide III;[11,13] all the bands (including those of amide III) are sensitive to collagen denaturation and conformational changes.

After grinding, two bands ($1283.5 \pm 0.5\,cm^{-1}$ and $1240.7 \pm 0.5\,cm^{-1}$) shift to higher wavenumbers.

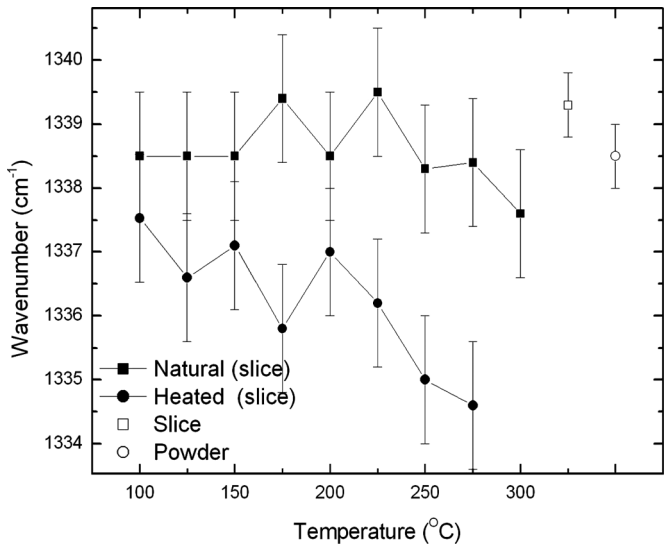

FIGURE 6 **Position of the band at $1339.5 \pm 0.5\,cm^{-1}$ after heating and grinding. The band shifts $3.9 \pm 0.5\,cm^{-1}$ to lower wavenumbers ($1335.5 \pm 0.5\,cm^{-1}$) after heating to 275°C; the band position in the sliced samples ($1339.5 \pm 0.5\,cm^{-1}$) does not differ from that of the powdered sample ($1338.5 \pm 0.5\,cm^{-1}$).**

After thermal treatment in the range 100–300°C, three bands ($1339.5 \pm 0.5\,cm^{-1}$, $1283.5 \pm 0.5\,cm^{-1}$, and $1202.7 \pm 0.5\,cm^{-1}$) shift to lower wavenumbers; the band at $1283.5 \pm 0.5\,cm^{-1}$ shifts only $2\,cm^{-1}$. To better understand the behavior of the shifts in wavenumber, this term can be changed to "frequency shift" and thereby be better associated with the vibration mode frequency.

The shift to lower frequencies is also observed in other denatured collagens[3] and upon collagen rehydration.[14] Several changes in the infrared spectrum of collagen upon denaturation are observed: shift of the amide A band from 3325–3330 cm^{-1} to around 3305 cm^{-1}; loss of the fine structure of amide I near 1640 cm^{-1}; and shifts of about 56–10 cm^{-1} to lower frequencies in the case of amide I and II bands.

The proposed model for the interlocking of collagen molecule and hydroxyapatite crystals is described as follows: hydroxyapatite occupies the spaces between the collagen fibrils and the existing spaces within the fibrils.[2,15,16] The thermal stability of the mineralized collagen is weaker than that of native collagen because, during mineralization, the hydroxyapatite crystals break the cross-links within each collagen fiber as well as the cross-links between collagen fibrils.[17,18]

Thermal treatment produces a shift in the opposite direction compared with the shift produced after grinding. These differences between heating and grinding shows that, despite the widely accepted idea that heat is produced by sample friction during grinding, the changes produced by these processes are not similar.

Three of the four structure vibration modes are susceptible to heating, two bands are susceptible to grinding, and the band at $1283.5 \pm 0.5\,cm^{-1}$ changes after both processes, but the shift after heating is slight. To understand and propose a model that explains the observed shifts after grinding and heating, it is necessary to describe what happens to the collagen-hydroxyapatite system during these two procedures.

Grinding of the sample leads to cleavage of the collagen fibril, and the final size depends on the selected powder size. In the present study, we selected powder sizes between 25 μm and 38 μm. It is supposed that fibrils located transversely across the granule are composed of approximately 150 units of collagen molecules that remain linked together by

the native noncovalent and covalent bonds. If the observed structural vibration modes are susceptible to the linear size of the molecular fibril, the observed frequency shift can originate from the chain cleavage and the reduction of its length to about 150 collagen molecules.

With the pressure between the mortar and pestle, the granules are submitted to high tensions, which possibly break the intermolecular bonds within the same granule; i.e., the proposed collagen fibril piece with 150 units can be cleaved into several other pieces in the same granule. Considering the different mechanical properties of collagen and hydroxyapatite, the tension and friction between the granules can easily separate the hydroxyapatite crystals from the collagen molecules and introduce a larger gap in the hydroxyapatite-filled regions. This larger gap and the probable distortion in the molecules will introduce changes into its vibration modes.

Thermal denaturation of collagen corresponds to the melting of its periodic organization. In their native state, collagen molecules are strongly oriented and packed. A temperature rise provides more freedom to the molecules, which exhibit random configurations, destroying the original periodic arrangement and unfolding the protein. In our system, after thermal denaturation up to 300°C, the noncovalent interactions between different atoms that are important in defining the three dimensional structure are broken, and the helical structure of the collagen is lost.[17,19] With heating (100–300°C), progressive structure denaturation produces an unfolded molecule with a different molecular length compared with the length of the natural molecule. The different length obtained after heating is the probable origin of the shifts in the frequency of the three structural vibration modes.

All the four analyzed bands corresponding to the structure of collagen shift to other frequencies after heating or grinding. Two of them shift to higher frequencies after grinding ($1283.5 \pm 0.5\,cm^{-1}$ and $1240.7 \pm 0.5\,cm^{-1}$), and three bands shift to lower frequencies after heating ($1339.5 \pm 0.5\,cm^{-1}$, $1283.5 \pm 0.5\,cm^{-1}$, and $1202.7 \pm 0.5\,cm^{-1}$). These observed changes in the vibration modes indicate that sample preparation or sterilization involving grinding and heating must be carefully evaluated, in order to preserve the natural characteristics of the collagen structure.

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