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SCIENTIFIC PAPER

CURVE-FITTING MICRO-ATR-FTIR STUDIES OF THE AMIDE I AND II BANDS OF TYPE I COLLAGEN IN ARCHAEOLOGICAL BONE MATERIALS

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Bone and alike materials, including ivory and antler, have an important place among prehistoric remains as they record a wealth of information on the past ways of life in the form of morphological and structural changes as well as in their chemical and isotopic composition. Such materials generally exhibit a high degree of hierarchy and essentially consist of collagen molecules and hydroxyapatite crystals at the nanoscale. The structure and the chemical composition of archaeological bone material may be modified by interactions with the burial environment. The present work focuses on the characterisation of structural modifications of the type I collagen, especially its secondary structure, preserved in archaeological bone remains induced by diagenesis. Therefore, the potential of ATR-FTIR combined with curve-fitting of the amide I and II bands is evaluated. Using this method, the conservation state of the collagen secondary structure has been determined for well preserved archaeological bone material from Neolithic layers of two stations (19 and 21) at the lake site of Chalain (Jura, France).

1 Introduction

Bone is a nanocomposite biomaterial consisting of a mineral phase (carbonated hydroxyapatite (carbHAP) representing 60-70% of the bone tissue) and an organic fraction (mainly collagen with an amount of 20-30% in the whole bone tissue and representing 90 % of the organic part). Bone also contains non-collagenous proteins, lipids and water (about 10%). The relative amounts of these fractions are slightly variable depending for example on animal and tissue age.¹ This variability is evidently increased in archaeological bone material. Indeed, ancient bones are modified by interactions with the environment during burial time. The ensemble of these alteration phenomena is called diagenesis.^{2,3}

Archaeological bones constitute an important part of archaeological remains and can largely contribute to the understanding of ancient

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societies as they can give evidence of human or faunal occupation. As biomaterials they register in their chemical and isotopic composition information on past diets, climatic and environmental conditions. They can also show technological skills on objects made of bone as well as their usage thanks to traces left on the surface of these objects. Thus, it appears necessary to develop analytical methods for a better characterisation of the state of conservation of bone remains and objects found in excavations.

FTIR micro-spectroscopy constitutes a technique of choice in structural analysis of bone material because it enables simultaneous investigation of the organic and mineral parts.⁴ Moreover it doesn't necessarily require destruction of the analysed material contrary to other analytical techniques like mass spectrometry analyses (measurement of the carbon and nitrogen ratios⁵), separative⁶ or thermal analyses⁷ currently used to determine the quality of collagen. Besides, FTIR micro-spectroscopy can provide spatially resolved information in the micrometer range on the modification of the chemical composition and of the structure of the mineralized collagen preserved in the archaeological bone.

Most of the studies conducted by FTIR on archaeological bone focused on the characterisation of the preservation state of the bone mineral part:⁸ semi-quantitative indices have been defined to estimate the bone crystallinity⁹ or the carbonate content.^{4,10} Another semi-quantitative index is used to characterise the presence of remaining organic matter in the archaeological bone¹¹. However, no information on the collagen structure in the bone material has been extracted from the spectra (Table 1).

Infrared spectroscopy is commonly used to study changes in the secondary structure of proteins¹²⁻¹⁴ and in particular in collagen¹⁵ by decomposing the amide bands. Many applications concern the field of medical research¹⁶⁻¹⁸. Some studies on historical¹⁹ and archaeological²⁰ bone material have already used a curve fitting procedure to extract more detailed information from the IR spectra. Concerning archaeological bones the investigations is generally focused on the remaining mineral fraction of fossils without any remaining organic matter.²⁰ However, in some special environments as found on waterlogged archaeological sites the organic part of bones can be well preserved.²¹

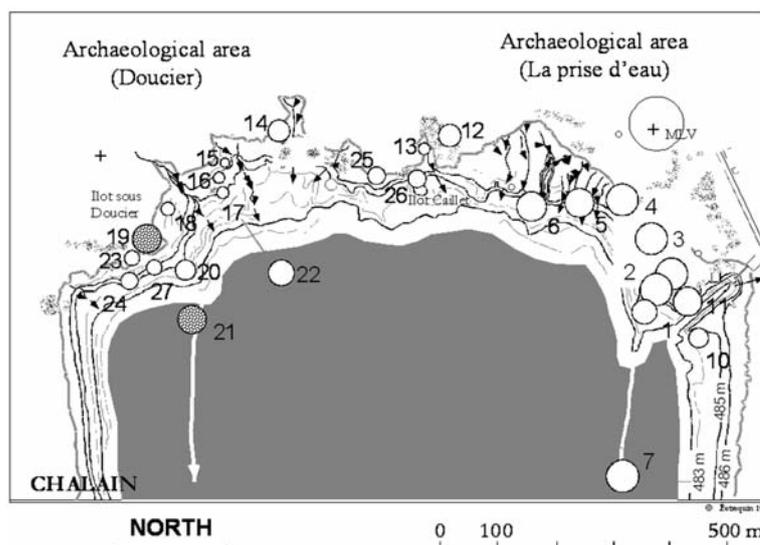


Figure 1: Map of the Neolithic lake site of Chalain (Jura) with both stations: Chalain 19 and Chalain 21 (subaquatic). © P. Pétrequin, reproduced with kind permission by the author.

Parameter	Definition	Evolution with alteration
Mineral crystallinity index	Splitting factor of the phosphate peaks at 567 cm ⁻¹ and 605 cm ⁻¹	Increases
Carbonate to phosphate ratio (C/P)	Peak intensity ratio of bands at 1415 cm ⁻¹ and 1030 cm ⁻¹	Increases
Organic to mineral ratio (N/P)	Peak intensity ratio of bands at 1660 cm ⁻¹ and 1030 cm ⁻¹	Decreases

Table 1: Measures of archaeological bone properties by FTIR spectroscopy (adapted from references [4] and [8]).

This study was conducted on relatively well preserved archaeological bone material from Neolithic layers of two stations (19 and 21, dated between 3850 and 2900 BC) at the lake site of Chalain (Jura, France).²² Station 19 is characterised by a fluctuating hydrology and sediment layers rich in organic matter, whereas station 21 is a subaquatic site (Figure 1). Bones from these stations provide material for a case study corresponding to a situation found relatively frequently in archaeology: fragments from wet environments directly coming from the excavation.

The present work aims to examine the microstructural modifications induced by diagenesis on the secondary structure of collagen preserved in the archaeological bone excavated in the Chalain lake sites by infrared micro-spectroscopy combined with curve-fitting. The IR spectroscopic results presented here are based on the decomposition and the reconstruction of the amide I band and on the study of the position of the amide II band component.

Station	Characteristics	Labotory reference	Nature	Dimensions of the bone cross-section analysed
Chalain 19	Fluctuating hydrology and sediment layers rich in organic matter	AB_CH19nb1	Unknown	1.5 x 0.5 cm ²
		AB_CH19nb2	Unknown	0.3 x 0.5 cm ²
		AB_CH19nb3	Left-humerus (wild boar)	0.6 x 0.8 cm ²
		AB_CH19nb4	Unknown	2.2 x 0.4 cm ²
Chalain 21	Subaquatic site	AB_CH21nb1	Left-radius (bovine)	0.3 x 0.5 cm ²
		AB_CH21nb2	Flat bone (deer)	1 x 0.5 cm ²

Table 2: Characteristics of the archaeological bone samples from the lake sites of Chalain.

2 Materials and Methods

2.1 Bone Materials and Burial Conditions

Six pieces of Neolithic animal bones originating from Chalain (Jura, France) were analysed. Among them, four samples (AB_CH19nb1-4) are coming from the Neolithic station 19. This station is characterised by a fluctuating hydrology (alternation between period of immersion and of emersion). All of them were recovered by a layer of limestone and were buried in anaerobic conditions. The sample AB_CH19nb1 belongs to a layer above that where the other bone samples were buried. Two samples (AB_CH21nb1-2) were excavated from the subaquatic station 21 (Table 2). In this station, pH measurements of the water were realised and have indicated values between 7.5 and 7.7.²³ These samples were not consolidated and fragments have been analysed in a previous study by different complementary analytical methods including optical and electron microscopy, proton-induced X-ray and gamma ray emission and X-ray diffraction.²⁴ As modern reference, a fragment of modern bovine bone (MBB) was analysed under the same conditions.

2.2 Sample Preparation

Bone sections were cut with a water-cooled diamond saw to obtain smooth surfaces. The sections were successively rinsed under ultrasound with water, distilled water and ethanol before being dried.

2.3 ATR-FTIR spectroscopy

A FTIR imaging system combining a spectrometer (Varian Excalibur FTS-4000, Varian) with a microscope (UMA 600, Varian) supplied with a Mercury Cadmium Telluride (MCT) detector was used to obtain FTIR spectra on the bone sections. Analyses were conducted in reflection mode by micro-Attenuated Total Reflection (ATR) by an ATR monoreflexion objective based on a germani-

um (Ge) crystal. For each point 128 scans were collected at a spectral resolution of 4 cm⁻¹ and spatial resolution obtained by the ATR objective is around 50 μm.

2.4 Infrared Data Analysis: Curve Fitting of the Amide I Profile, Estimation of the Collagen Cross-links and Study of the Position of the Amide II Component

Figure 2 shows the IR spectrum of modern bone reference (MBB) with the band assignments for each part of the spectrum. Specifically, the bands at about 1660 cm⁻¹ and 1550 cm⁻¹ can be mainly attributed to the (ν(C=O)) and the (ν(C-N)) vibrations and are called amide I and amide II, respectively. The shape of the amide I band is representative of the collagen secondary structure.¹⁴ A curve fitting treatment can be carried out to estimate quantitatively the relative proportion of each component representing a type of secondary structure (Figure 3). The fourth derivative function was calculated using the PeakFit™ software to determine the number of components in the amide I region for the curve-fitting process (Figure 3b). It is used to determine the number and positions of the bands corresponding to the different components in the amide I profile. This function was used instead of the second derivative function to increase the sensitivity of the peak detection.^{25,26} According to this band decomposition, the amide I profile of collagen contains six major components that can be linked, in analogy to other protein models, with aromatic ring vibrations, β-sheets (two compents), random coils, turns and α-helix where the α-helix is evidently the most intense component (Figure 3a) even if these structures are not found as it in type I collagen. Band shape was considered as Gaussian. A linear baseline was always used between 1600 and 1720 cm⁻¹. The position of the band components were fixed, whereas their bandwidths could be adjusted to perform the curve-fitting of the amide I profile. The amount of each secondary structure element is given in percentage terms, by dividing the area of

one amide I band component by the area of the sum of all amide I band component areas.¹⁹

Intermolecular cross-links could be investigated thanks to the amide I band decomposition. As described by Paschalis *et al* (2001), collagen cross-links provide the fibrillar collagen matrices

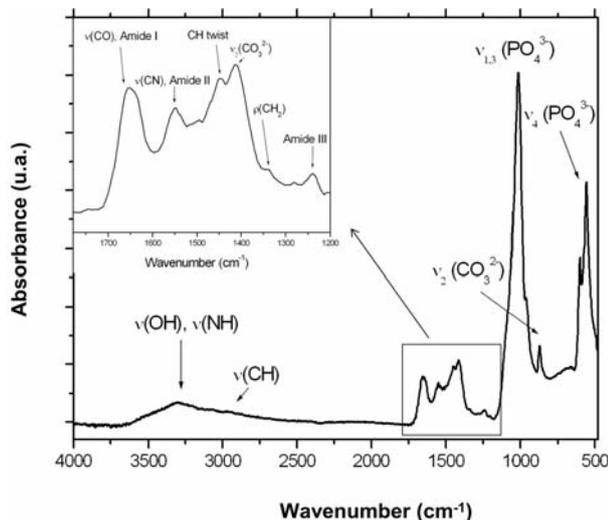


Figure 2: ATR-FTIR spectrum of a modern bone material (MBB) with bands assignments.

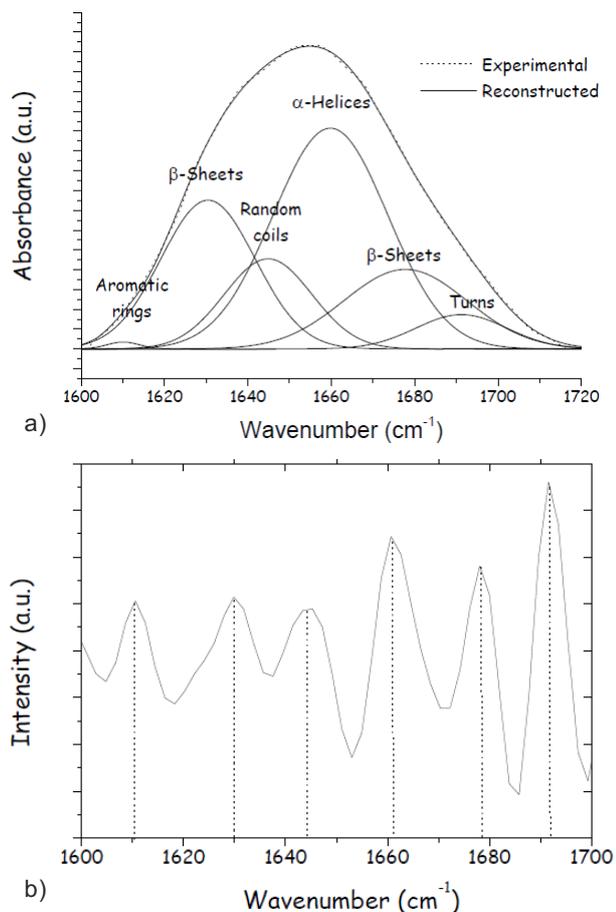


Figure 3: a) Curve-fitting analysis of the amide I profile on MBB in ATR-FTIR and b) fourth derivative function. Identification of the six components (aromatic rings, β -sheets (two components), random coils, α -helix and turns).

with properties such as tensile strength and viscoelasticity. These properties may be modified by diseases (osteoporosis for example) but also by interaction with the burial environment. A ratio was defined to estimate the cross-link content in the bone material: collagen cross-link structure was analysed as a band area ratio of the 1660-1690 cm^{-1} subbands.^{18,27} This index can be related to the ratio of non-reducible to reducible collagen cross-links in bone.

A larger region of interest from 1200 to 1800 cm^{-1} was also fitted to follow the shift of the amide II component position during bone diagenesis. Band shape was considered as Gaussian. A linear baseline was assumed. The best fit was obtained by using twelve gaussians located around 1240 cm^{-1} (amide III), 1270 cm^{-1} (non attributed), 1340 cm^{-1} ($\nu(\text{CH}_2)$), 1420 cm^{-1} ($\nu(\text{COO}^-)$, $\nu(\text{CO}_3^{2-}$, B)), 1450 cm^{-1} ($\delta(\text{CH}_3)$, $\nu(\text{CO}_3^{2-}$, B)), 1470 cm^{-1} (non attributed) 1500 cm^{-1} (non attributed), 1550 cm^{-1} (amide II and $\nu(\text{CO}_3^{2-}$, A)), 1620 cm^{-1} (β -sheets), 1640 cm^{-1} (amide I, random coils), 1660 cm^{-1} (amide I, α -helices), 1680 cm^{-1} (amide I, β -sheets).^{4,14,28} The position of the twelve band components were not fixed to perform the curve-fitting of the 1200-1800 cm^{-1} domain.

3 Results

3.1 ATR-FT-IR Results on Modern Bone

On a modern bovine bone section (MBB) twelve points were analysed across the cortical part from the periosteum to the endosteum in order to determine for the amide I band the number of components, their position and their relative area. Six components were obtained with the maxima of the fourth derivative function (Figure 3b). The six components are located at 1610 cm^{-1} (aromatic rings), 1630 cm^{-1} (β -sheets), 1645 cm^{-1} (random coils), 1661 cm^{-1} (α -helix), 1678 cm^{-1} (β -sheets) and 1692 cm^{-1} (turns), respectively. Box charts, represented in Figure 4, show the reproducibility of the decomposition conducted on modern bone. No significant variations were observed across the bone section as a function of the exact position of the measurement. Therefore, the mean modern bone profile was used as a reference profile for the analyses on archaeological bones: component positions have been fixed for curve-fitting and the ones obtained on archaeological bone specimens will be compared to the relative areas calculated for the modern reference.

Concerning the position of the component related to the amide II absorption, the mean value

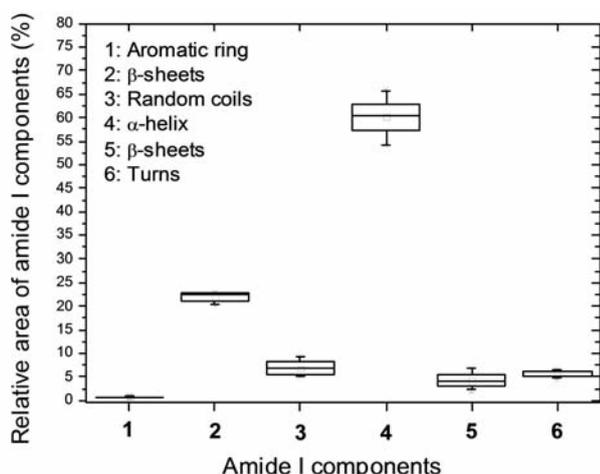


Figure 4: Box plot of the amide I decomposition in ATR-FTIR for modern bovine bone (with mean, maximum and minimum values and degree of dispersion).

obtained after measuring twelve points on the modern bone section is located around 1548 cm^{-1} .

3.2 FTIR Results on Archaeological Bone of the Lake Site of Chalain

Table 4 summarizes the relative areas obtained by decomposition of the amide I band for each of the archaeological bone samples and for the modern bovine bone. Besides, the number of analysed points for each sample is specified in both tables. According to these results, several observations can be made. Indeed, the areas of the amide I components for archaeological bone samples of the two stations exhibit significant differences with the modern one. First, even if the α -helix stays the major component, its relative area is decreased in all archaeological samples. Simultaneously, there is an increase of the fraction of the random coils (unordered structures). Concerning the cross-link

Components (Assignments)	Number of analysed points	1 (Aromatic ring)		2 (β -Sheets)		3 (Random coils)		4 (α -Helix)		5 (β -Sheets)		6 (Turns)	
		Mean (%)	Range (%)	Mean (%)	Range (%)	Mean (%)	Range (%)	Mean (%)	Range (%)	Mean (%)	Range (%)	Mean (%)	Range (%)
AB_CH19nb1	9	0.55	0.35-0.69	21.95	20.81-23.33	15.69	13.36-18.72	50.73	46.53-56.45	5.95	3.26-8.59	5.12	4.35-5.96
AB_CH19nb2	3	0.12	0.10-0.14	21.93	21.67-22.07	12.12	11.99-12.36	57.49	57.33-57.66	3.98	3.90-4.05	4.36	4.29-4.48
AB_CH19nb3	8	0.66	0.53-0.84	24.49	22.61-25.61	22.12	21.28-24.12	34.28	32.35-35.84	15.64	14.17-17.47	2.81	1.72-3.89
AB_CH19nb4	6	0.56	0.50-0.66	22.21	21.77-22.79	15.73	13.45-17.82	51.7	47.36-55.76	4.01	2.22-6.34	5.79	4.83-7.05
AB_CH21nb1	3	0.51	0.50-0.52	21.08	19.84-22.00	19.65	17.65-21.97	48.65	47.14-50.95	5.64	4.88-6.59	4.48	3.94-4.87
AB_CH21nb2	4	0.44	0.36-0.55	18.29	16.94-19.28	20.1	17.21-26.13	51.45	44.96-54.43	6.02	4.97-7.69	3.7	3.45-3.88
MBB	12	0.7	0.41-0.93	22.26	20.25-25.03	6.91	5.01-9.36	60.37	54.14-65.95	4.18	1.70-6.95	5.57	3.21-6.46

Table 3: Amide I band components relative areas obtained by ATR-FTIR spectrum decomposition on archaeological bone material from the site of Chalain and on a modern bovine bone reference.

content evaluated in the archaeological samples, no general trend has been observed (Table 4). In addition, the cross-links values obtained are around five to ten times higher than values measured in modern bones by other groups.²⁷ This difference may be related to the spectral decomposition and has to be investigated more in details in order to clarify this issue.

Concerning the position of the amide II band component, two groups of samples are evidenced. One of them is composed of the samples coming from the station 19 (except AB_CH19nb3). For these samples, the position of the component related to the amide II vibration is located at lower wavenum-

Samples	Number of analysed points	Cross-links ratio ($1660/1690\text{ cm}^{-1}$)	
		Mean	Range
AB_CH19nb1	9	10.10	7.88-12.98
AB_CH19nb2	3	13.20	12.81-13.44
AB_CH19nb3	8	13.17	9.02-19.24
AB_CH19nb4	6	9.06	7.12-10.88
AB_CH21nb1	3	10.94	9.83-11.97
AB_CH21nb2	4	13.94	12.05-15.05
MBB	12	10.60	8.84-13.00

Table 4: Collagen cross-linking in archaeological bone material from the site of Chalain and in a modern bovine bone reference.

Samples	Number of analysed points	Amide II component position
AB_CH19nb1	9	1547.6 ± 0.3
AB_CH19nb2	3	1549.7 ± 0.8
AB_CH19nb3	8	1551.7 ± 0.1
AB_CH19nb4	6	1550.9 ± 0.1
AB_CH21nb1	3	1551.7 ± 0.6
AB_CH21nb2	4	1551.6 ± 0.8
MBB	12	1547.8 ± 0.2

Table 5: Mean values of the position of the amide II band component obtained by curve fitting of the wavenumbers domain located between 1200 and 1800 cm^{-1} .

bers than 1551 cm^{-1} (Table 3). In this group, the value obtained for the sample AB_CH19nb1 (1547.6 cm^{-1}) is close to the value measured in the modern one MBB (1547.8 cm^{-1}). Samples AB_CH19nb1 and AB_CH19nb4 evidenced a value slightly higher (close to 1550 cm^{-1}). In the second group of samples, the amide II component is located at higher wavenumbers than 1551 cm^{-1} . This group is constituted of AB_CH19nb3 and of the two samples coming from the subaquatic station 21.

4 Discussion

4.1 FTIR Analysis

Some new criteria have been introduced thanks to IR data treatment to compare the collagen structure of archaeological bones coming from the station 19 and the station 21 with the modern reference. Indeed, the general feature of amide I components profile (the relative area of the α -helix and that of the random coils) seems to reflect a desorganisation in the collagen secondary structure.

For all archaeological specimens, in comparison with the modern reference, a decrease of the α -helix percentage and an increase of the unordered structures (random coils) have been observed and evidenced that the bone structure has been modified during burial. However, the α -helix structure is always the main component in all archaeological samples, which indicates that the collagen secondary structure is still quite well preserved.

Concerning the cross-link content, the highest values are obtained for samples AB_CH19nb3 and CH21nb2. However, this parameter seems difficult to discuss for other samples. Indeed, even in the modern reference, the variation observed is very high (8.84-13.00), so that the cross-links content does not seem to constitute a pertinent indicator of the conservation state of the collagen secondary structure.

The position of the amide II band component seems to be shifted toward higher wavenumbers in the archaeological bone samples. This phenomenon seems to reflect an alteration in the collagen structure and may be a useful indicator to detect either a modification in the collagen structure or a particular modification in the bone composition.

Before comparing the state of conservation of the archaeological bone samples in each station, it should be noted that several points have been measured on each sample but the number of points depend of the specimen size (Table 2). This parameter has to be taken into account to estimate

the homogeneity or the heterogeneity of the sample state of preservation.

4.2 State of conservation of archaeological samples from the Chalain 19 station

These complementary analyses conducted on the archaeological samples from the Chalain lake site have provided new information concerning the state of conservation of the collagen secondary structure. Thanks to these analyses, different alteration features have been characterised:

Sample AB_CH19nb2 presents a good state of preservation evidenced by a position of the amide II component located below 1551 cm^{-1} close to 1548 cm^{-1} (MBB) and an amide I profile similar to the modern one.

Sample AB_CH19nb1 appears slightly more degraded. Indeed, the state of conservation of the collagen seems to be more heterogeneous: even if the amide I profile is very similar to the profile measured in MBB, an inversion of the β -sheets/turns ratio has been noted.

Previous analyses performed on AB_CH19nb4 have shown that this sample presents a higher content of F which has been related to a degraded state of conservation of the organic phase.²⁹ The present study confirms these results. Indeed, some areas, where the collagen secondary structure appears less well preserved, have been evidenced within this sample.

Sample AB_CH19nb3 presents a particular state of preservation which was already suspected during the sample preparation. The mechanical properties reflecting the state of preservation of the micro- and the nanostructure,¹⁷ it was not surprising that a specific state of preservation was found by the results based on IR curve fitting for this sample: the proportion of α -helix is small and the position of the amide II component is shifted toward higher wavenumbers. These two results reveal a degraded state of conservation of the organic part. In addition, the shift of the amide II component can be related with the incorporation in the bone structure of CO_3^{2-} (type A, substituted to HO^-).³⁰ Indeed, these ions show IR absorptions at around 1550 cm^{-1} , which can be an overlap with the amide II band. Previous analyses conducted by micro-PIXE indicated for this sample a higher Ca/P ratio than in the other samples.²⁴ These results confirm the enrichment in calcite of this sample.

The four archaeological bone samples, AB_CH19nb1-4, were buried in an anaerobic environment enriched in calcite. The oxidation of the bone was also limited, which justifies partially the preservation of the organic matter in these samples. However, some specific alteration features have been characterised. It is also important to focus on the contiguous burial environment of these samples. More especially, sample AB_CH19nb1 was buried in an archaeological layer located higher in the stratigraphy than the other samples. The burial environment of this sample may be more acid which could justify a lower content of CO_3^{2-} in this sample. Basic conditions may alter the organic part. It could explain that the collagen secondary structure of AB_CH19nb3 and AB_CH19nb4 has been disturbed. This microscopic observations obtained by IR curve fitting were confirmed at nanoscale in AB_CH19nb4 by investigating the state of preservation of the mineral/organic organisation.²⁹ However, even if the collagen structure seems to be partially degraded in AB_CH19nb4, it is well preserved in AB_CH19nb2 which belongs to the same layer. The burial environment may be locally more basic close to AB_CH19nb4 and AB_CH19nb3 than close to AB_CH19nb2. Previous analyses assumed that the burial environment of AB_CH19nb4 may have been more reduced because of the presence of pyrite inclusions in this bone microstructure, whereas no pyrite was revealed in AB_CH19nb2²⁴. It can be also assumed that a reduced environment does not constitute good conditions for the preservation of the collagen secondary structure.

4.3 State of conservation of archaeological samples from the Chalain 21 station

Concerning the samples belonging to the sub-aquatic station 21, a higher fraction of unordered structures (about 17 to 26 %) than most of the bone sample of the station 19 has been determined in the two bone remains. Besides, they evidence a lower percent of α -helix (about 50%) than the Chalain 19 samples (except AB_CH19nb3). These samples were buried in a subaquatic station, so that the increase of the unordered structures can be related to the presence of hydrogen bonds induced by the interaction with water present in the burial environment³¹. However, the bending frequency of O-H at 1644 cm^{-1} characteristic of liquid water³² has to be taken into account and especially for archaeological bones coming from this subaquatic station. Indeed, this water contribution may modify the amide I profile and may induce an increase of the relative area of the

unordered structure at 1645 cm^{-1} . In addition, a shift of the amide II component was measured in these samples which can be related to an enrichment of CO_3^{2-} , as described previously. Hence, the pH values (7.5 and 7.7) measured in this sub-aquatic station close to the samples favour the preservation of the archaeological bone materials^{33,34} and are consistent with the presence of CO_3^{2-} . In particular, as flat bone, sample AB_CH21nb2 is more porous¹ than sample AB_CH21nb1 which can justify a more important interaction with the burial environment and a more important heterogeneity within this specimen. However, due to the changes of the subaquatic characteristics of this station over time, the interactions established between the bones and their environments are complex. So, the alteration features observed may be the result of different alteration processes which are difficult to relate with the burial conditions at one point.

5 Conclusions

The investigation of archaeological bone samples from the Neolithic lake site of Chalain and the comparison with modern bone permitted to evidence various modifications of the collagen secondary structure by diagenesis. These modifications have been partially related to the burial environment of each sample. Therefore, FTIR micro-spectroscopy combined with spectra decomposition by curve fitting seems to be a powerful approach in archaeological biomaterial studies. However, even if the results obtained are promising, the spatial resolution of the micro-ATR objective in the order of fifty micrometers is not high enough to examine local effects of diagenesis which could modify the bone structure at the microscale. Therefore, the use of a IR synchrotron source will enable to decrease the analysed area in order to obtain highly spatially resolved mapping to better characterise the sample heterogeneity.

Besides, thanks to this new characterisation approach of the conservation state of archaeological bone material, it will be possible to establish the conservation state of pieces displayed in museum by ATR-FTIR spectroscopy without any sampling. It can have interesting applications in bone material conservation science.

It is also possible to use FT-Raman micro-spectroscopy for the investigation of the conservation state of the collagen secondary structure in bone. Work is in progress to test its potential to determine pertinent indicators. First results on modern bone (Figure 5) show that it is possible to use a

similar procedure for curve fitting of the Raman amide I band analogously to IR amide I band analyses. However, two minor band components cannot be clearly attributed to structural features and FT-Raman spectra of archaeological bone samples exhibit a spectral spreading compared to modern bone (Figure 6).

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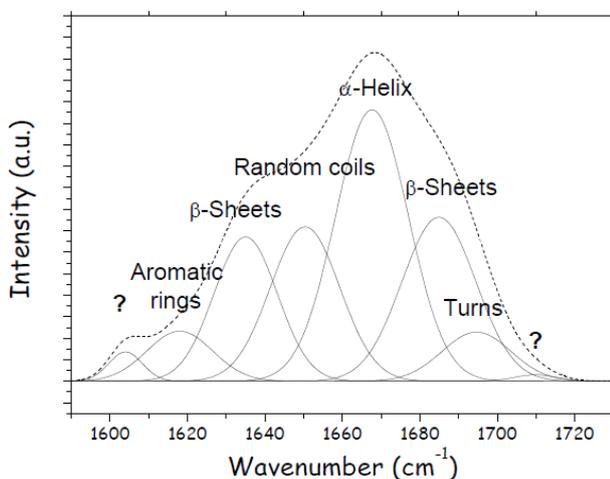


Figure 5: Curve-fitting analysis of the amide I profile of MBB in FT-Raman. Identification of the eight components (aromatic rings, two times β -sheets, random coils and α -helix, turns and two non-attributable minor components).

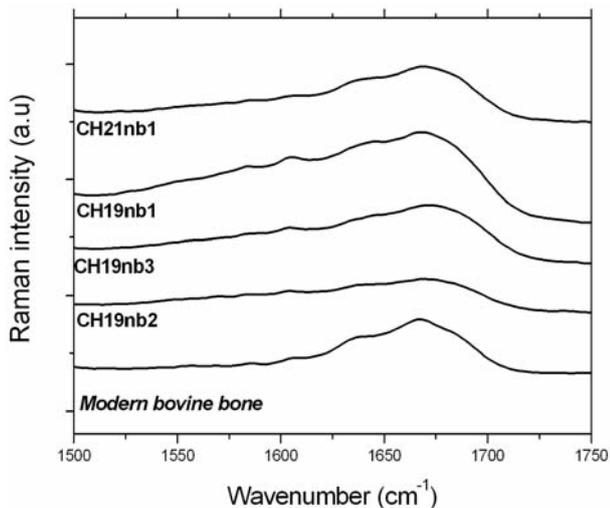


Figure 6: FT-Raman spectra of archaeological bone material from the Chalain site (AB_CH19nb1-3, AB_CH21nb1) in the amide I region.

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