Romanian Reports in Physics **71**, XYZ (2019)

NMR CHARACTERIZATION OF BONE COLLAGEN USED FOR ^{14}C DATING OF OSTEOLOGICAL MATERIAL

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Received September 5, 2019

Abstract. The degree of conservation of collagen used for radiocarbon dating was determined by evaluating the percentage of broken collagen chains as a result of its degradation caused by improper storage conditions. This paper proposes a method for determining the amino acid content of fragments that are lost through the Amicon Ultra Filter 30kDa filter used for collagen extraction to date osteological material. For this purpose, the 13C-NMR spectra obtained from the analysis of collagen extracted from four bones of different ages were used. The analyses were performed using a Bruker Avance III HD NMR spectrometer and TopSpin standard software for acquisition and analysis of NMR data.

Key words: collagen, NMR spectrometry, radiocarbon dating.

1. INTRODUCTION

^{14}C dating technique using Accelerator Mass Spectrometry (AMS) is the most widely and effective method used for determining the age of bone remnants discovered in archaeological sites. Age is determined from the isotopic ratios $^{14}C/^{12}C$ or $^{13}C/^{12}C$ measured by AMS in type I bone collagen extracted from the organic fraction of the bone.

The organic fraction of the bones is composed of proteins (mostly collagen) and lipids. Over the time, depending on the conditions of conservation, they can incorporate external carbon from biogenetic sources leading to a younger age when bones are dating by radiocarbon.

For the removal of these contaminants and the extraction of pure collagen, the osteological material is pre-treated using various HCl solutions for demineralization of the bone and the decomposition of the organic phase; for the extraction of collagen a special Amicon Ultra Filter 30kDa filter is used. The

collagen is then lyophilized, dried and graphitized, and the resulting graphite is introduced into the source of AMS facility for measuring the carbon isotopic ratios.

To be sure that in the sample there are no external carbon contaminants, such as bacteria or microorganisms, the individual amino acids of collagen are separated and dated. Hydroxyproline is the amino acid that responds best to this requirement because it is present in mammalian bone collagen in a high percentage (10%) and it is not found in significant amounts in other animal proteins [1].

However, if the collagen is degraded due to the bones storage condition over the time, then the collagen chain may be fragmented, it might pass through the Amicon filter, and the amount of collagen that remains is not enough to obtain graphite to be dated with good accuracy; in some cases, we might end up with a small quantity that also cannot be dated.

A solution to make possible the dating of such samples is to recover the broken fragments and the dating to be done on the amino acids extracted from them. Because HPLC (High Performance Liquid Chromatography) separation is time consuming, it is useful to know the quantities of amino acids that have passed through the filter and to evaluate if enough graphite to be dated from each of them will be obtained.

Bone diagenesis (all post-mortem physical and chemical processes that produce an alteration of the inorganic phase of hard biological tissue) can be characterized by spectroscopic and chemical analyzes such as NMR spectrometry (Nuclear Magnetic Resonance), X-ray diffraction and infrared spectrometry [2 - 5].

Studies on the conservation of organic fraction using NMR spectrometry are very few or almost missing [6, 7, 8].

The present paper proposes to determine the quantities of each amino acid found in significant proportions in bone collagen fragments by nuclear magnetic resonance (NMR) analysis. From the obtained results the degree of collagen degradation extracted from bone samples of different ages was determined.

2. EXPERIMENTAL

Studies using NMR were performed for collagen extracted from four bones samples from two archaeological sites dated to different periods. The amino acid content of the solution passed through the Amicon Ultra Filter 30kDa filter used for collagen extraction was analyzed by NMR.

To identify the amino acids in the NMR spectra obtained for the archaeological samples, collagen extracted from a contemporary bone and standards of the amino acids of interest were used.

2.1. SAMPLE DESCRIPTION AND PREPARATION

Fragments from each sample were processed using an adaptation of the bone pre-treatment procedure described in [9, 10]. The surface of the bone was mechanically removed with a DremelTM tool before any chemical pre-treatment and 2–3 g of bone was crushed to a size of 0.5 to 2 mm with a grinder mill. To remove the mineral part of the bones, the powder was then first treated with 15 mL of 0.5M HCl solution at room temperature for 30 minutes, washed in MilliQTM water to neutral pH, followed by the gelatinization phase, with 15 mL 0.2M HCl for 16-18 hours, at 80°C and 500 rpm. For the extraction of collagen the samples were filtered through the SFCA filter and the special Amicon Ultra Filter 30kDa MWCO (Molecular Weight Cut Off), which retains only the molecules with a molecular mass greater than 30kDa. The collagen was recovered using a Pasteur pipette in an Eppendorf tube; after that it was frozen before being freeze drying at -45°C for 16-18 hours. The collagen was weighed on an analytical balance with 2 decimal places. The solution after the second filtration was recovered and evaporated using the Genevac Ez-2 evaporator.

Each freeze-dried collagen sample was dissolved in 0.5 mL or 1mL D_2O / H_2O (1: 9), depending on the mass of the sample and each solution was transferred to a 300 mm long and 5 mm diameter Duran tube and subjected to NMR analysis. Similarly, collagen was extracted from the contemporary bone. The masses of collagen extracted from the osteological material and the one used for MRI analysis, as well as the volume of deuterated water in which it was dissolved, are presented in Table 1.

Table 1

Description of bone samples, collagen mass used in NMR spectrometry and the volume of deuterated water in which samples were dissolved

Sample code	Type of sample	Mass of collagen extracted (mg)	Mass of collagen used (mg)	Volume D_2O/H_2O (mL)
CC	Contemporary animal collagen	46.90	46.90	0.5
CDE 1	Household object from animal bone	116.90	53.15	0.5
CDE 2	Fragment of animal bone	137.46	44.35	0.5
CDT 1	Fragment of human bone	68.86	66.85	1.0
CDT 2	Fragment of human bone	106.82	46.03	0.5

For NMR analysis, the dry residue from the solution passed through the Amicon filter was dissolved in 1 mL D_2O / H_2O solution (1: 9), then filtered

through a 0.22µm PVDF filter (polyvinylidene difluoride) and transferred in a 300 mm long and 5 mm diameter Duran tube and subjected to NMR analysis.

The volume remaining after collagen filtration and the amount of residue resulting after its evaporation to dryness for each sample are presented in Table 2.

Table 2

The volume of the residue and the final mass evaporated of the studied samples.

Sample code	Residue volume (mL)	Evaporated final mass (mg)
CDE1	2.5	35.03
CDE2	3.5	73.76
CDT3	4.0	65.35
CDT4	4.0	65.13

The deuterated water solution used in the NMR analyzes was made of deuterium oxide for magnetic resonance spectrometry (99.9%) from Merck and MilliQ ultrasonic water in a ratio of 1: 9.

2.2. NMR SPECTROMETRY

NMR analyzes were performed on a 400 MHz Bruker Avance III HD; for the acquisition and analysis data TopSpin software was used, *i.e.* the standard software provided by Bruker [11]. 13C-NMR spectra were obtained using the standard data acquisition parameters, namely 100.60 MHz frequency at room temperature.

The choice of 13C-NMR spectrum was justified by the fact that the carbon resonances corresponding to Cα and Cβ peaks are specific to each studied amino acid and thus it is possible to easily identify the type of amino acid present in the collagen. In order to obtain simpler spectra but with sufficient information for the study, the C13DEPT45 method of obtaining ^{13}C spectra was adopted, selecting only the CH, CH2 and CH3 signals.

Carbon spectra were acquired at 100.60 MHz, using 30° pulses, collecting 1024 scans with a length of 65536 data points with a relaxation delay of 1.0 seconds, for amino acids standards and contemporary collagen. Collagen bone and fragments of collagen spectra were acquired at 10000 respectively 5000 scans, with the same length and relaxation delay as the standards.

3. RESULTS AND DISCUSSIONS

This study consisted in the quantitative determination of the amino acids contained in the collagen chain in significant proportions, namely Glycine (Gly), Alanine (Ala), Proline (Pro), Hydroxyproline (Hyp) and Threonine (Thr).

In order to identify the amino acids corresponding peaks, an amino acid mixture and collagen extracted from a raw bone fragment from a contemporary animal were analyzed. Once identified, to calculate the amino acid content of the old samples, the integral of each amino acid peaks were normalized to those obtained for contemporary collagen. In order to determine the degree of collagen degradation for the studied samples, as well as to evaluate the amino acid content of the residues, the amount of amino acids in the spectra recorded for the two types of samples, collagen and residues, was compared.

3.1. IDENTIFICATION OF THE AMINO ACID PEAKS STUDIED IN THE 13C-NMR SPECTRUM OF CONTEMPORARY COLLAGEN USING AMINO ACID STANDARDS

The peaks corresponding to the amino acids of interest in the 13C-NMR spectrum obtained for the collagen extracted from the contemporary bone (Figure 1 below) were identified by comparison with those obtained from mixing their standards (Figure 1 above).

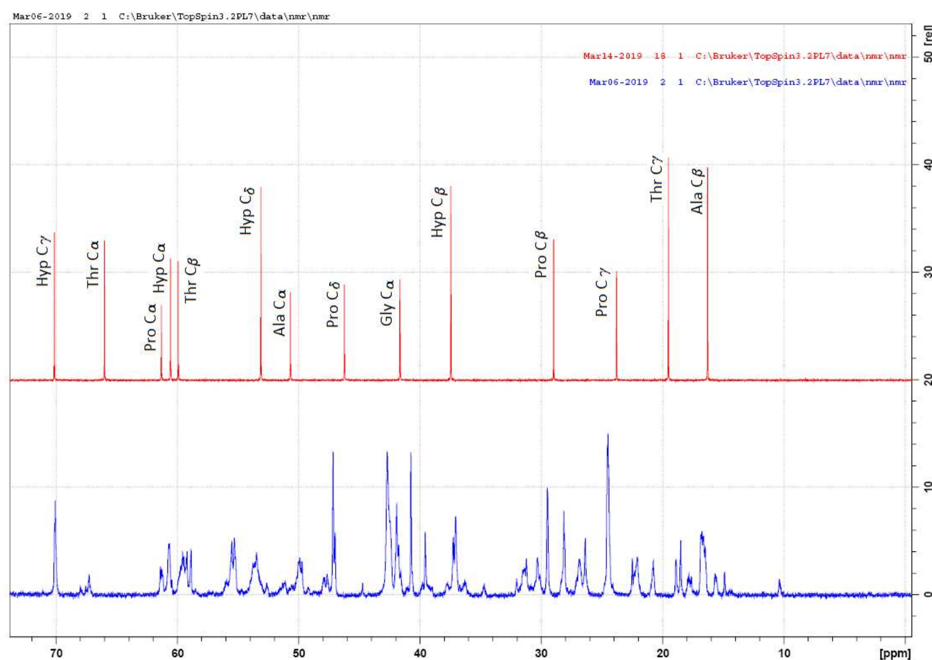

Fig. 1 - The positions of the peaks of the studied amino acids. Above: Mixture of amino acid standards, Below: contemporary bone collagen.

The peak positions of the five amino acids in the spectra obtained for contemporary collagen and the mixture of standards are presented in Table 3.

In the second column of Table 3 are presented the intervals of the chemical shift corresponding to the amino acids of interest, on which the integral of the peaks was performed, for the quantitative determination of the amino acid content of the studied samples. The position of these peaks ranges from the amino acid standard, as well as the deviation of these peaks are shown in the last columns of Table 3.

Table 3

Spectral data for the amino acid standards mixture and the contemporaneous collagen

Aminoacid	Integration range (ppm)	Chemical shift of standards (ppm)	Chemical shift dispersion from standard (%)	Chemical shift position
Gly Cα	41.34-43.07	41.93	-	centered
Ala Cα	50.81-51.54	49.96	-	centered
Ala Cβ	16.17-16.98	16.81	-	centered
Pro Cα	60.94-61.55	61.34	-0.03	left
Pro Cβ	29.14-29.76	29.47	0.35	right
Pro Cγ	24.06-24.83	24.51	0.41	right
Pro Cδ	46.77-48.08	47.17	0.89	right
Hyp Cα	60.39-60.93	59.59	0.36	right
Hyp Cβ	36.70-37.47	37.07	-0.36	left
Hyp Cγ	69.75-70.31	70.08	-	centered
Hyp Cδ	52.88-54.13	53.46	-	centered
Thr Cα	66.98-67.66	67.29	1.37	right
Thr Cβ	58.66-60.07	60.59	-	centered
Thr Cγ	20.46-20.98	18.89	4.35	right

From this table it can be observed that most of the positions of the amino acid peaks obtained for the standard mix are centered in the intervals taken into account, or deviate very little (below 1%) from the center. These results confirms that the positions of the collagen amino acids are correctly identified.

3.2. AMINO ACID CONTENT OF COLLAGEN BONE EXTRACTED FROM OLD SAMPLES

The 13C-NMR spectra obtained for the collagen extracted from the old samples that we studied are shown in Figure 2.

The spectra obtained from the old collagen contain all 35 peaks, as well as the contemporary samples, but in different quantities.

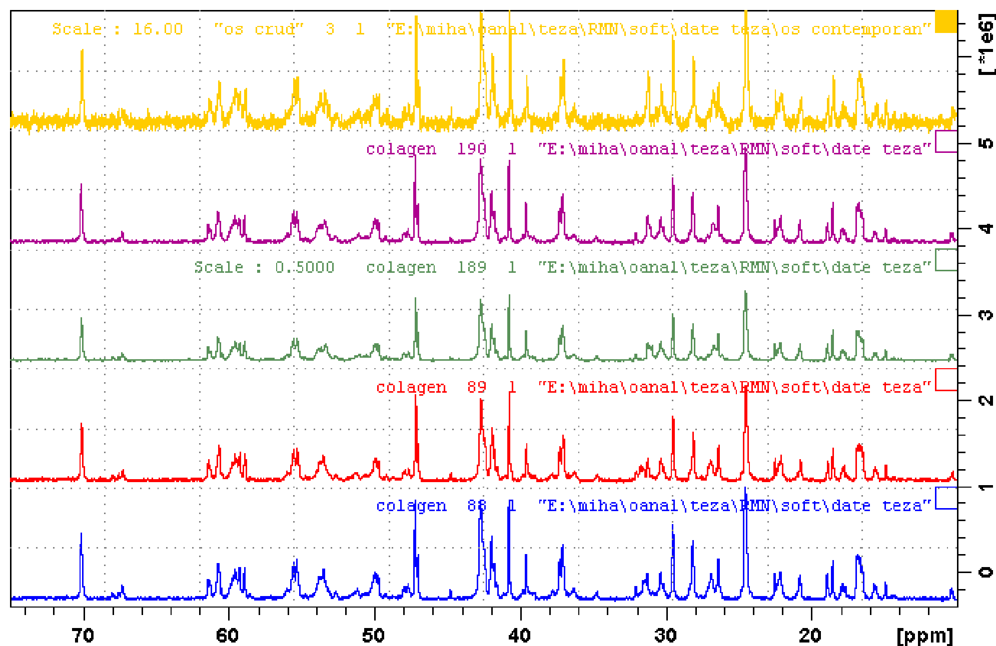

Fig. 2 - 13C-NMR spectra obtained for collagen: from contemporary collagen (yellow) and collagen extracted from old bones samples.

To find out the amino acid content of each sample, the corresponding peaks were integrated and normalized to the integrals obtained for contemporary collagen, assuming that it is not altered. The results are presented in Table 4.

Table 4

Amounts of amino acids contained in collagen extracted from the samples from two archaeological sites obtained from NMR analyzes

	Integration interval	Raw collagen (mg)	Collagen extracted in filter (mg)			
			CDE1	CDE2	CDT1	CDT2
Integral 1	130.97-130.13	0.33	0.08	0.06	0.11	0.08
Integral 2	129.73-129.09	0.55	0.49	0.44	0.57	0.44
Integral 3	128.98-128.60	0.27	0.38	0.30	0.48	0.3
Integral 4	70.31-69.75	1.46	1.66	1.40	2.04	1.43
Integral 5	67.66-66.98	0.37	0.42	0.32	0.42	0.29
Integral 6	61.55-60.94	0.77	0.83	0.74	0.98	0.72
Integral 7	60.93-60.39	1.22	1.42	1.28	1.81	1.29

Integral 8	60.07-58.66	2.82	3.23	2.73	4.08	2.87
Integral 9	56.21-55.82	0.26	0.38	0.31	0.42	0.31
Integral 10	55.81-54.96	2.25	2.45	2.06	2.98	2.1
Integral 11	54.13-52.88	2.22	2.58	2.21	3.30	2.24
Integral 12	51.54-50.81	0.45	0.56	0.45	0.71	0.48
Integral 13	50.67-50.27	0.28	0.31	0.26	0.42	0.26
Integral 14	50.26-49.54	1.30	1.66	1.39	2.16	1.45
Integral 15	48.08-46.77	2.53	3.13	2.65	3.77	2.63
Integral 16	43.07-41.34	6.85	7.82	6.56	9.69	6.66
Integral 17	40.92-40.42	1.38	1.41	1.21	1.76	1.17
Integral 18	39.68-39.16	0.77	0.98	0.81	1.23	0.88
Integral 19	37.47-36.70	2.22	2.53	2.03	3.36	2.28
Integral 20	36.43-36.03	0.33	0.35	0.32	0.48	0.27
Integral 21	31.54-30.93	1.22	1.23	0.83	1.47	1.04
Integral 22	30.62-29.87	1.09	1.29	1.03	1.99	1.24
Integral 23	29.76-29.14	1.67	1.75	1.46	2.14	1.48
Integral 24	28.45-27.77	1.36	1.64	1.34	2.00	1.4
Integral 25	26.99-25.97	1.97	2.20	1.78	2.83	1.93
Integral 26	24.83-24.06	3.85	4.16	3.50	5.07	3.54
Integral 27	22.60-21.76	1.51	1.73	1.43	2.16	1.5
Integral 28	20.98-20.46	0.58	0.67	0.58	0.83	0.56
Integral 29	19.03-18.67	0.35	0.53	0.37	0.60	0.39
Integral 30	18.66-18.18	0.58	0.67	0.60	1.04	0.73
Integral 31	18.05-17.39	0.54	0.69	0.59	1.02	0.67
Integral 32	16.98-16.17	2.59	2.95	2.45	3.74	2.56
Integral 33	15.77-15.25	0.44	0.50	0.45	0.54	0.4
Integral 34	14.98-14.64	0.23	0.26	0.21	0.32	0.24
Integral 35	10.45-10.06	0.29	0.23	0.18	0.32	0.22
Sum		46.90	53.15	44.35	66.85	46.03

3.3. EVALUATION OF THE DEGREE OF COLLAGEN DEGRADATION FROM CONTENT ANALYSIS OF AMINO ACIDS THAT WERE NOT RETAINED IN THE SPECIAL AMICON ULTRA FILTER 30KDA FILTER

Depending on the degree of collagen degradation, part of the collagen chain breaks down and fragments of it can pass through the Amicon Ultra Filter

30kDa filter used to extract the collagen. By determining the collagen content passing through the filter in 0.2M HCl solution it is thus possible to determine the degree of collagen degradation extracted from bone material.

The experimental results obtained from the analysis of this solution treated according to section 2.1 are presented in Figure 3 and Table 5.

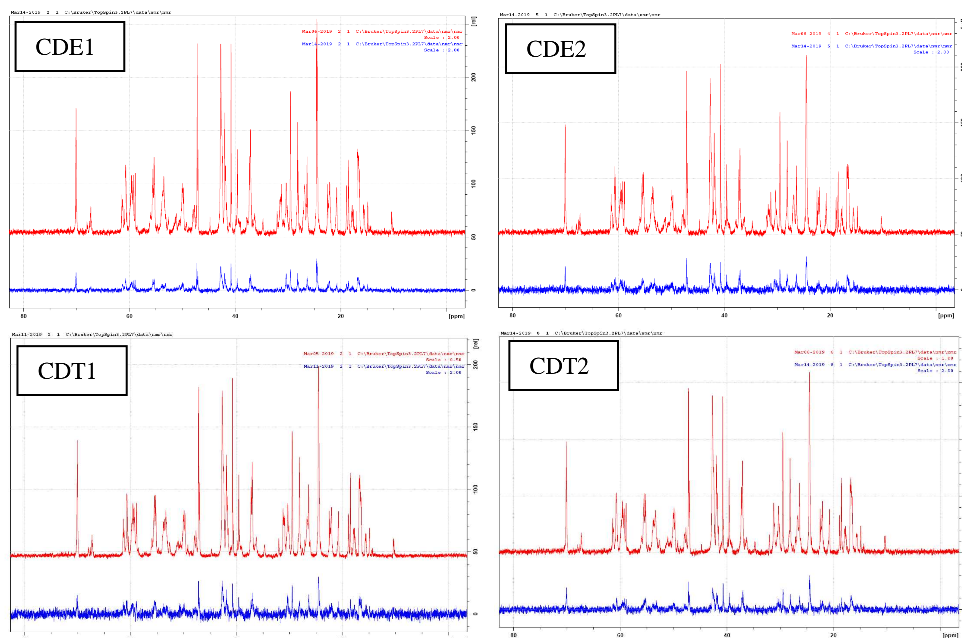

Fig. 3 - The 13C-NMR spectra of collagen fragments from old bone samples.

Table 5

Amounts of amino acids resulting from collagen fragments that passed through the Amicon Ultra Filter 30kDa filter

	Integration interval (mg)	Raw collagen (mg)	Collagen fragments (mg)			
			CDE1	CDE2	CDT1	CDT2
Integral 1	130.97-130.13	0.33	0.01	0.00	0.00	0.02
Integral 2	129.73-129.09	0.55	0.11	0.04	0.08	0.11
Integral 3	128.98-128.60	0.27	0.10	0.06	0.01	0.02
Integral 4	70.31-69.75	1.46	0.42	0.24	0.18	0.27
Integral 5	67.66-66.98	0.37	0.11	0.06	0.01	0.03
Integral 6	61.55-60.94	0.77	0.26	0.11	0.12	0.10
Integral 7	60.93-60.39	1.22	0.42	0.24	0.13	0.18
Integral 8	60.07-58.66	2.82	0.89	0.42	0.30	0.42

Integral 9	56.21-55.82	0.26	0.07	0.06	0.00	0.04
Integral 10	55.81-54.96	2.25	0.68	0.34	0.27	0.35
Integral 11	54.13-52.88	2.22	0.63	0.36	0.20	0.25
Integral 12	51.54-50.81	0.45	0.12	0.07	0.02	0.02
Integral 13	50.67-50.27	0.28	0.17	0.06	0.04	0.05
Integral 14	50.26-49.54	1.30	0.40	0.21	0.14	0.17
Integral 15	48.08-46.77	2.53	0.91	0.44	0.32	0.45
Integral 16	43.07-41.34	6.85	2.18	1.09	0.82	1.03
Integral 17	40.92-40.42	1.38	0.44	0.22	0.18	0.24
Integral 18	39.68-39.16	0.77	0.24	0.16	0.12	0.15
Integral 19	37.47-36.70	2.22	0.73	0.40	0.31	0.39
Integral 20	36.43-36.03	0.33	0.15	0.04	0.05	0.04
Integral 21	31.54-30.93	1.22	0.03	0.04	0.02	0.01
Integral 22	30.62-29.87	1.09	0.66	0.34	0.25	0.29
Integral 23	29.76-29.14	1.67	0.51	0.27	0.19	0.25
Integral 24	28.45-27.77	1.36	0.49	0.26	0.15	0.19
Integral 25	26.99-25.97	1.97	0.54	0.30	0.20	0.23
Integral 26	24.83-24.06	3.85	1.16	0.61	0.48	0.57
Integral 27	22.60-21.76	1.51	0.51	0.24	0.17	0.22
Integral 28	20.98-20.46	0.58	0.17	0.09	0.09	0.07
Integral 29	19.03-18.67	0.35	0.15	0.08	0.00	0.03
Integral 30	18.66-18.18	0.58	0.19	0.08	0.08	0.09
Integral 31	18.05-17.39	0.54	0.14	0.04	0.05	0.10
Integral 32	16.98-16.17	2.59	0.83	0.43	0.33	0.41
Integral 33	15.77-15.25	0.44	0.17	0.10	0.05	0.06
Integral 34	14.98-14.64	0.23	0.07	0.03	0.01	0.06
Integral 35	10.45-10.06	0.29	0.08	0.03	0.05	0.05
Sum		**46.90**	**20.23**	**10.32**	**7.54**	**9.34**

Dividing the sum of the peak integrals obtained from the 13C-NMR spectrum for the collagen fragments to the sum of the peak integrals obtained from both the collagen retained in the filter and the one that passed through it, it resulted that 27.57%, 18.87%, 10.13% and 16.87% of the total collagen into CDE1, CDE2, CDT1 and CDT2 samples was broken. From these ratios it turned out the degradation degree of collagen extracted from the old bones.

Although radiocarbon dating of collagen and individual amino acids showed that CDE1 and CDE2 samples belong to the same historical period [12], 13C-NMR analysis of collagen and collagen fragments indicated that collagen of sample CDE1 was more degraded than that of the sample CDE2. One possible explanation is that the first sample, representing a household object made from an animal bone fragment, was subjected to aggression through its use, while the second sample is just a buried bone fragment.

Regarding the samples from the second archaeological site, the results showed that the collagen is less damaged, which was somehow expected, as they are much younger. CDT1 collagen is shown to be the best preserved, supporting the assumption that these human remains have been treated with myrrh. Another explanation for the slightly different conservation degree of CDT1 and CDT2 samples may be that the bone fragments from which the samples were extracted were not of the same type (*e.g.* femur, tibia, skull, etc.) and the amount of collagen in them differs, which explains the difference between the proportions of each type of amino acid in the sample.

To verify that the quantities of individual amino acids that could be separated from the collagen fragments that passed through the filter can be dated by ^{14}C, the quantities of the most representative amino acids from the studied samples were calculated (Table 6).

Table 6

Amounts of datable amino acids contained in the collagen and collagen fragments extracted from the studied samples

	Collagen (mg)				Collagen fragments (mg)			
	CDE1	CDE2	CDT1	CDT2	CDE1	CDE2	CDT1	CDT2
Gly	1.411	1.213	1.755	1.170	0.439	0.219	0.181	0.239
Ala	0.810	0.708	0.960	0.659	0.348	0.160	0.094	0.110
Pro	7.331	6.159	8.917	6.254	2.164	1.051	0.756	0.952
Hyp	8.192	6.925	10.514	7.236	2.196	1.241	0.825	1.092
Thr	4.173	3.422	5.096	3.552	1.154	0.564	0.309	0.469

For dating the separated amino acids from the collagen fragments that have passed through the filter, a quantity of more than 2 mg is required from each of them. From Table 6 it can be seen that only two amino acids can be separated from the CDE1 sample that can provide a sufficient amount of graphite to be dated by AMS.

4. **CONCLUSIONS**

This paper presents a method for characterizing the degree of collagen deterioration obtained from the osteological material to be radiocarbon dated using NMR spectrometry.

The method consists of comparing the amino acid content of collagen broken chains with that of the whole collagen chains. The presence of a high content of independent amino acids in the collagen fragments which passed through the 30 kDa filter shows a high degree of collagen degradation and thus a smaller amount of graphite resulting for dating. By separating the individual amino acids from the collagen fragments, which in the classical procedure are eliminated, some of the carbon can be recovered and added to the collagen extracted through the filter.

From the analysis of four old bone samples, the amino acid content of collagen and their percentage in the collagen fragments was obtained. The results showed, as expected, that the degree of collagen in the four samples is higher in older bones and it is efficient to recover some amino acids from the broken collagen chains to be dated using AMS.

Acknowledgements. The authors would like to thank Gh. Custurea, C. Şova, C. Talmaţchi, Gh. I. Cantacuzino, D. Căprăroiu, P. V. Diaconescu, A. Ioniță, M. Năstase, Gh. Olteanu, F. G. Petrică and C. Simion, for providing the household objects, human and animal bones fragments and to the RoAMS team from for the previous AMS measurements and results.

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