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COMPARATIVE RADIOCARBON DATING STUDY OF INDIVIDUAL AMINO ACIDS ISOLATED FROM ARCHAEOLOGICAL BONE COLLAGEN TOWARDS BULK COLLAGEN

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Abstract. Radiocarbon dating of archaeological bones may sometimes provide erroneous data when ^{14}C contaminants from the surroundings where they were buried in the post-depositional phase or from the environment in storage conditions were not eliminated in the pretreatment stage. To solve this problem, a way out may be the dating of individual amino acids. In this paper, various amino acids isolated from collagen were separated using the high performance liquid chromatography (HPLC) method and subsequently dated by accelerator mass spectrometry (AMS). A comparative study of the data obtained for individual amino acids towards bulk collagen was performed.

Key words: radiocarbon dating, amino acids, HPLC, graphitization, AMS.

1. INTRODUCTION

Dating technique with ^{14}C was developed by Willard Frank Libby, for which he received the Nobel Prize in Chemistry in 1960, and revolutionized archeology, paleontology, geology and other fields of science. The introduction of Accelerator Mass Spectrometry (AMS) has increased the sensitivity and precision of ^{14}C dating of various artifacts, relics and cultural goods and reduced the amount of samples, allowing to solve dating problems that have not been possible until then.

Nevertheless, ^{14}C dating remains a challenging and complex process because of some archaeological samples (*e.g.* bones, in this particular case) are found in a bad state of preservation and contain little or contaminated collagen with exogenous carbon both in inorganic and organic form or even derived from microorganisms and bacteria. Often, a combination of all situations occurred.

There is an intense effort all over the scientific world to separate the exogenous from endogenous carbon which are present in samples for a more reliable dating. In this respect, several studies have been made on various chemical pre-treatment

procedures to determine some indicators that define whether a bone is suitable for radiocarbon dating. One of these is the C/N atomic ratio of the raw bone that can provide an indication of the general state of preservation. The values of C/N atomic ratio of collagen are typically 2.9–3.6 [1] or 3.1–3.5 [2]. Higher values indicate contamination with exogenous carbon compounds, extensive diagenesis, the presence of a high proportion of humics and, on the other hand, a lower value indicates that the sample contains too little carbon to be dated with good accuracy or in some cases, can not be dated [1–5].

Marom *et al.* argue that there are situations when the C/N atomic ratio has a normal value, but the ^{14}C age is not the one expected, due to contaminants having the same C/N atomic ratio as the sample. They suggested that through dating of a particular amino acid or pairs of amino acids separated from bone collagen, one can obtain more accurate dating results, even if the sample is assumed to be inappropriate for dating, considering the C/N atomic ratio [6]. The idea of dating some individual amino acids isolated from bone collagen existed since 1980 [7], where dating the hydroxyproline, was considered the „golden standard" for archaeological bone samples, because it is a major component, most resistant in time and accounting for about 10% from collagen mass [6].

This paper is a study on dating some amino acids extracted from collagen coming from bones in various states of preservation, that until now were less studied compared to hydroxyproline (Hyp). The most abundant amino acids in collagen have been investigated: glycine (Gly – 33%), proline (Pro – 12%), alanine (Ala – 10%), hydroxyproline (Hyp – 10%) and threonine (Thr – 2%) [8]. Because of the glycine could not be completely separated from proline (Pro), their mixture is the one that has been studied.

Amino acid separation was carry out by High Performance Liquid Chromatography (HPLC) using a Shimadzu system Prominence™. The extraction of collagen and amino acids was performed at RoAMS Laboratory–IFIN-HH.

Radiocarbon dating was done using the 1 MV Tandetron™ Accelerator Mass Spectrometry (AMS) of IFIN-HH, produced by HVEE (High Voltage Engineering Europe), and in Hungary at Institute for Nuclear Research of the Hungarian Academy of Sciences, Hertelendi Laboratory of Environmental Studies, Debrecen, using the Mini Carbon Dating System (MICADAS) type Accelerator Mass Spectrometer, developed by Ion Plus of Eidgenössische Technische Hochschule Zürich (ETHZ).

2. EXPERIMENTAL PART

2.1. SAMPLE DESCRIPTION AND PREPARATION

Four bone samples found in different conservation states of preservation from two archaeological sites were studied. The description of analyzed samples, as well

as the expected age based on artifacts found in archaeological context with the osteological material, are presented in Table 1.

Table 1

Bone samples from archaeological sites studied in this research

Sample	Type of sample	Expected age
1	Bone awl	11^{th}–12^{th} c.
2	Astragalus	11^{th}–12^{th} c.
3	Human bone fragment (M4)	15^{th} c.
4	Human bone fragment (M2)	15^{th} c.

Samples no. 1 and 2 were provided by the National History and Archeology Museum of Constanţa and come from the archaeological site "Head of the Hill", Constanţa county.

In the same excavation, some fragmentary pottery typical for the 11^{th}–12^{th} centuries were discovered alongside Byzantine coin, stone tools, human bone, bone and iron household items and few fragments of early medieval pottery specific for the 4^{th}–7^{th} centuries. The material has been stored at the Museum of National History and Archaeology in Constanţa [9].

The human bone samples no. 3 and 4 come from the National Museum Complex "The Princely Court in Târgoviște". Bone remains have been found in the old chapel church built around 1415. Based on archaeological material collected, mostly from the first half of the 14^{th} century first half 15^{th} century plus background information materialized graphics and photographic documentation can make the following statements: the chapel church, according to the latest research, the church is built in the middle of 15^{th} century [10].

2.2. COLLAGEN EXTRACTION AND HYDROLYSIS

The surface of the bone was mechanically removed with a Dremel™ before any other interventions and 2–3 g of raw bone was crushed to a size of 0.5 to 2 mm with a grinder mill. For a complete demineralization, the chunks were then treated in 0.5 M HCl at room temperature for 30 minutes in order to discard carbonates, fulvic acids and other contaminants soluble in acid medium. Further the sample is washed in ultrapure MilliQ™ water up to neutral pH and the final step is gelatinization overnight at 80°C in 0.2 M HCl using a thermomixer at 500 rpm. The gelatin solution was filtered off using a Corning™ SFCA (Surfactant-Free Cellulose Acetate) syringe filter membrane with 0.2 μm pore size. The filtrate was transferred into a pre-cleaned Vivaspin ™ 15, 30 kDa MWCO (Mollecular Weight Cut Off) and centrifuged at 4500 rpm for 30 min. The gelatin fraction remained having a molecular weight >30 kDa was frozen before being freeze dried at –45°C [11].

Prior to chromatography, it is necessary to break the protein chain from bone collagen down into its constituent amino acids by hydrolysis. For this, aliquots of

collagen were treated with an excess of 6M HCl in a nitrogen atmosphere at 105°C for 24 h, which was then removed using a vacuum evaporator Genevac EZ-2 HCl compatible. The dried residue was then reconstituted in water up to a concentration 10 mg/ml. This hydrolysate solution was filtered through 0.2 µm pore size PTFE syringe filters and then injected onto the chromatographic column [12].

2.3. SEPARATION OF AMINO ACIDS

Amino acids were analyzed using a HPLC Shimadzu system equipped with two delivery pump units (LC-20AD), an auto-sampler (SIL-20A), a UV/VIS photodiode array detector (SPD-M20A), and a fraction collector (FRC-10A). Amino acid separation was performed on a Primesep A column 22 × 250 mm™, particle size 5 µm (SIELC Technologies, Prospect, Height, Illinois, USA). To determine the elution order and retention time for each amino acid, standards solutions of 10 mg/ml concentration were used. The reference standards of amino acids were purchased from Sigma Aldrich.

The injection volume of hydrolyzed collagen was 500 µl, the flow rate of the mobile phase was set at 6 ml/min and the mobile phase was ultrapure MilliQ™ water. The column temperature was kept at 25°C. The collection times were different depending on the analyzed amino acid. The UV/VIS photodiode of the detector working at 205 nm wavelength. To obtain enough material for each amino acid to be graphitized and implicitly measured at the 1 MV Tandetron Accelerator of IFIN-HH (RoAMS), 3 to 6 injections of hydrolyzed collagen were made. The amino acid fractions collected were subsequently evaporated to dryness using the Genevac system.

After each injection, the Primesep A column was washed with 0.3% o-phosphoric acid for 3 h to make all the mixed-mode interaction sites available and washed with ultrapure MilliQ^{TM} water at a flow rate of 6 ml/min or at least 4 h before the next injection [12].

2.4. SAMPLES GRAPHITIZATION

The freeze-dried samples were weighed into a tin capsule and burned using an Elemental Analyzer (EA), CN mode on the Elementar vario MICRO cube, and automatically determine the percentage of C, N and C/N mass ratio which is then converted to atomic ratio. The CO_2 from sample combustion is then adsorbed on the zeolite trap of the Automated Graphitization Equipment (AGE 3). Finally, the pure CO_2 from each sample was thermally released into a reactor using pre-conditioned iron as catalyst, and hydrogen gas as reducing agent both for the iron conditioning and for the CO_2 reduction. The final sample which will be directly measured into de AMS unit was a homogeneous mixture of carbon and iron in an approximately 1/5 ratio [13–15].

2.5. RADIOCARBON ANALYSIS

Depending on the obtained amino acid quantity either the AMS facility from IFIN-HH, Romania or from Hertelendi Laboratory of Environmental Studies, Hungary was used. The large enough quantities of amino acid and bulk collagen were dated on our AMS system and the small samples were dated in Hertelendi Laboratory.

The AMS facility from IFIN-HH, based on a Cockcroft-Walton type 1 MV Tandetron was built by HVEE, Netherlands as a multi-element system for measuring ^{14}C, ^{10}Be, ^{26}Al and ^{129}I [16–20]. Used for radiocarbon dating, this facility is able to measure graphite samples into quantities up to 1 mg, with an accuracy of less than 1% [21].

The facility from Hertelendi Laboratory of Environmental Studies is based on a MICADAS (Mini Carbon Dating System) type radiocarbon accelerator mass spectrometer. This system was developed to allow not only the measurement of graphite samples, but also the direct measurement of CO_2 from burning samples. The sensitivity of MICADAS allows the measurement of quantities up to 50 μg of total carbon, with an accuracy of 5 ‰ [22, 23].

3. RESULTS AND DISCUSSIONS

The $^{14}C/^{12}C$ ratios determined by the AMS at RoAMS Laboratory were background corrected using synthetic graphite purchased from Merck and normalized to the Oxalic Acid standard (Oxa II). At the Hertelendi Laboratory amino acids were used as standards; glycine, in the source measurements using the graphitized samples, and alanine for the gas source. For background process the IAEA-C9 fossil wood reference material was used. The ^{14}C content expressed as $^{14}C/^{12}C$ ratio was also corrected with isotopic fractionation of carbon species measured by AMS [24].

The Conventional Radiocarbon Age, CRA was reported in BP years (years before present, the present being considered 1950); to determine the age in calendar years, the OxCal v4.3.2 program and the IntCal13 atmospheric calibration curve were used [25].

3.1. SAMPLE NO. 1 – BONE AWL

Approximately 50 mg of bulk collagen was extracted from this bone fragment; one part was used for direct dating, and the other part was hydrolyzed and injected into the HPLC column to separate the individual amino acids. Two fractions were collected: one which is very well separated – Hyp, and the second representing a mix of Pro and Gly (Fig. 1). The amounts of the three samples dated by AMS, collagen and the two collected fractions are shown in Table 2.

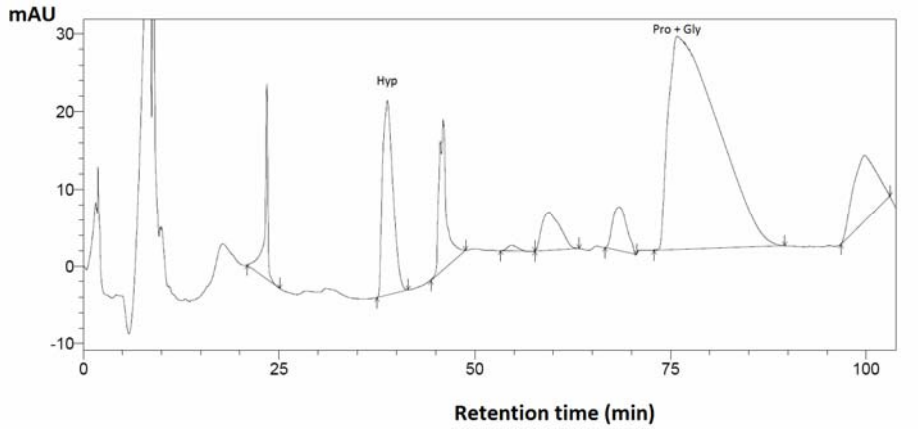

Fig. 1 – Chromatogram of sample 1. Amino acids separated from bone collagen hydrolysate measured by AMS.

The percentage content of carbon, nitrogen and the C/N atomic ratio measured after burning samples in EA confirm that the bone sample was well preserved (Table 2). The value of 3.19 for C/N atomic ratio obtained for collagen falls within the range of 2.9–3.6 which is considered as indicator that the collagen is well preserved [1]. The C/N atomic ratio for Hyp is close to the theoretical value of 4.99. This indicates that the Hyp fraction is very pure after the separation and no exogenous carbon was introduced during procedures. However, the Gly+Pro mix cannot be characterized by this ratio because the proportion of the two amino acids is unknown. Even if the theoretical C/N atomic ratio is between 2 (corresponding to Gly) and 4.9 (corresponding to Pro), it is not possible to check the degree of contamination in the isolation process of the amino acid mixture.

Table 2

Experimental data obtained after graphitization and AMS analysis for sample no. 1

Sample name	Weight (mg)	N (%)	C (%)	C/N	Radiocarbon age (years BP)	Calibrated age (cal AD)	Probability for $\sigma = 2$ (%)
Collagen	3.38	15.99	43.72	3.19	1354 ± 39	612 – 720 741 – 767	85.1 10.3
Hyp	10	10.19	43.87	5.02	1398 ± 39	575 – 679	95.4
Pro + Gly	7	17.10	34.34	2.35	1523 ± 44	423 – 620	95.4

For bulk collagen a radiocarbon age of 1354 ± 39 BP was obtained, and by calibration using the IntCal13 atmospheric curve, a calendar age in the main interval of calAD 612–720 was generated with a probability of 85.1% and having a secondary interval calAD 741–767 with a probability of 10.3 % (see Fig. 2). The obtained overall interval shows that this household object is of the same age as some ceramic fragments dated by the archaeologists to the 4th–7th centuries.

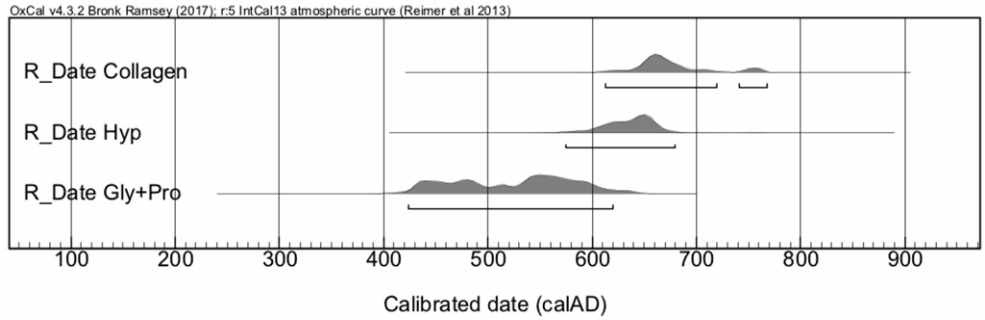

Fig. 2 – Calibrated ^{14}C dates for sample no. 1 – bone awl, produced with OxCal v4.3.2 and IntCal13 atmospheric calibration curve.

The dating of the Hyp fraction isolated from collagen can led to a more reliable age, due to its purity and its characteristic of being found in large amounts only in mammalian bones [1, 7, 26]. This 575–679 calAD range with a probability of 95.4% overlaps better with the age estimated by the archaeologists for the ceramics found at the same site.

The calendaristic age obtained for the acid mixture, namely 423–620 calAD, does not seem to be a reliable value, because the time interval is far too large and earlier.

3.2. SAMPLE NO. 2 – ASTRAGALUS

Approximately 35 mg of collagen was extracted from the sample no. 2; one part was directly dated by AMS, and the rest was used to isolate individual amino acids. The sample quantities from collagen and amino acid used for AMS analysis are shown in Table 3.

Table 3

Experimental data obtained after graphitization and AMS analysis for sample no. 2

Sample name	Weight (mg)	N (%)	C (%)	C/N	Radiocarbon age (years BP)	Calibrated age (cal AD)	Probability for $\sigma = 2$ (%)
Collagen	3.30	15.96	43.20	3.16	1378 ± 37	595 – 693 748 – 762	93.6 1.8
Thr	1.20	10.90	47.48	5.09	1467 ± 45	434 – 455 469 – 488 534 – 657	2.6 2.6 90.1

The only amino acid obtained with a very good resolution and in a sufficient amount (1.20 mg) was Thr (Fig. 3).

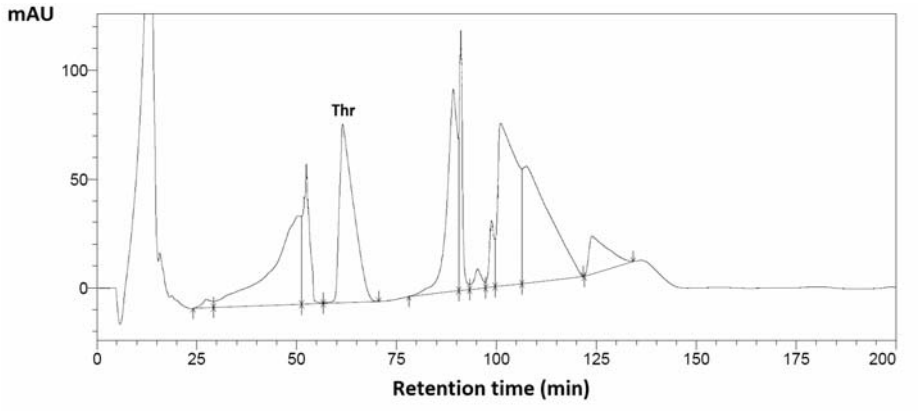

Fig. 3 – Chromatogram of sample no. 2. Amino acid separated from bone collagen hydrolysate measured by AMS.

The 3.16 value for the C/N atomic ratio of bulk collagen is a good indicator of preservation. Thus, the radiocarbon age of 1378 ± 37 BP can be considered trustworthy. Using the IntCal13 atmospheric calibration curve, a calendar age of calAD 595–693 with a probability of 93.6% was generated, and also the interval calAD 748–762 with a probability of only 1.8% was found (Fig. 4).

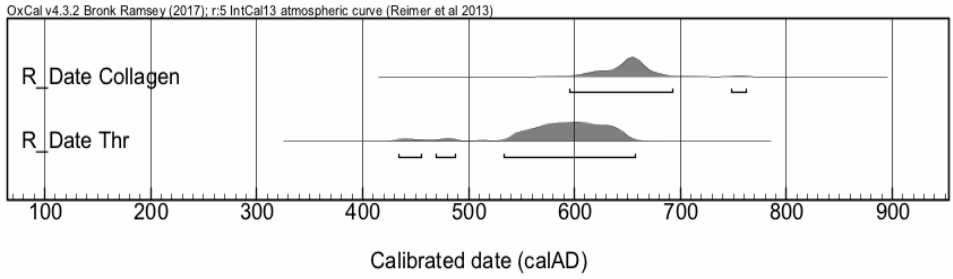

Fig. 4 – Calibrated ^{14}C dates for sample no. 2 – astragalus produced with OxCal v4.3.2 and IntCal13 atmospheric calibration curve (Reimer *et al.*, 2013).

By comparing this result with the ceramics dated to the 4th–7th centuries from the same archaeological site, we can say that the age obtained for bulk collagen is reliable.

Dating amino acid Thr did not give a reliable age of the tibia fragment despite the very good resolution and quantity obtained on HPLC system. The explanation might be that the value of the atomic C/N ratio obtained for this amino acid is by far too high than the theoretical value, namely 4. The value of 5.09 indicates an externally carbon Thr contamination with ^{14}C "dead carbon" which could be introduced due to "column bleed" [27]. By calibrating with IntCal13 atmospheric curve, the obtained calendar age intervals were the following: calAD 534–657,

calAD 434–455 and calAD 469–488 with probabilities of 90.1%, 2.6% and 2.6%, respectively. Those ages are higher than the age obtained by bulk collagen dating.

The results obtained in this study on the two bone samples from the archaeological site "Head of the Hill", Constanța county led to the conclusion that the bone awl can be dated to calAD 575–679 and the astragalus fragment to calAD 595–693; therefore, the two objects were dated to the same historical period.

Dating Hyp led to a level of confidence a little better than the direct radiocarbon dating of collagen. In the case of Thr, due to problems with amino acid separation by HPLC, the data obtained by AMS could not be used for dating.

3.3. SAMPLE NO. 3 – HUMAN BONE FRAGMENT (M4)

From the first bone fragment from the "The Princely Court in Târgoviște", 104.63 mg of collagen was extracted. The chromatogram of hydrolyzed collagen is shown in Figure 5.

Four individual amino acids were separated with a good resolution and the experimental data are shown in Table 4. Collagen, Thr and Hyp were analyzed at the RoAMS laboratory and the other two amino acids Pro and Ala, being in small quantities (1.87 mg and 0.47 mg respectively) were measured at Hertelendi Laboratory.

The analysis of the C/N atomic ratios for the samples measured at RoAMS shows that the collagen is well preserved, according to the criteria from [1]. The value obtained for Thr is low in comparison to the theoretical value, which is 4. The C/N atomic ratio obtained for the Hyp, close to the theoretical one, shows that the separation and graphitization of this amino acid were well accomplished. For Pro and Ala the values are not available (N/A).

The time intervals for the five samples using the IntCal13 atmospheric calibration curve are shown in Fig. 6 and Table 5.

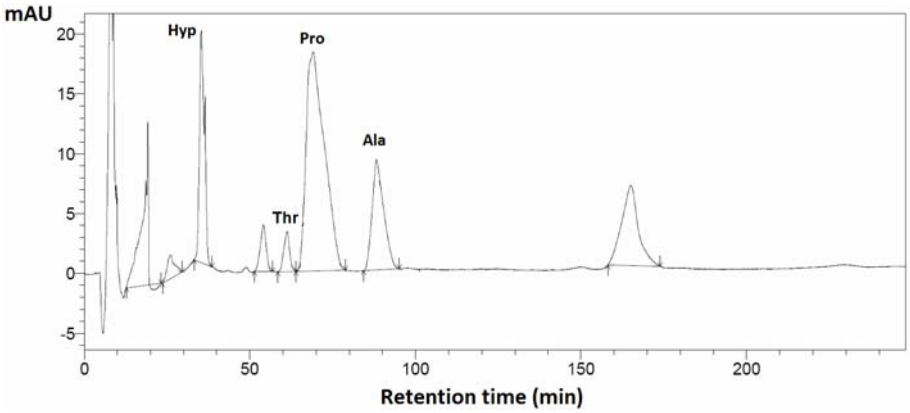

Fig. 5 – HPLC chromatogram of sample no. 3 in which the individual amino acids measured by AMS are indicated.

Table 4

Experimental data obtained after graphitization and AMS analysis from sample no. 3

Sample name	Weight (mg)	N (%)	C (%)	C/N	Radiocarbon age (years BP)
Collagen	3.60	13.56	37.05	3.19	614 ± 33
Pro*	1.87*	N/A	N/A	N/A	603 ± 18
Thr	3.32	18.04	39.53	2.56	713 ± 37
Ala*	0.47*	N/A	N/A	N/A	677 ± 18
Hyp	3.37	11.42	49.21	5.03	557 ± 38

* indicates that the sample was analyzed at Hertelendi.

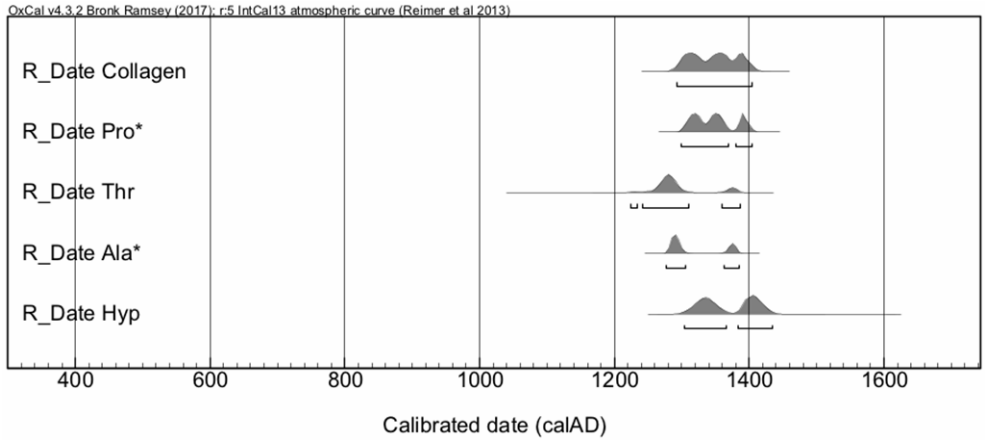

Fig. 6 – Calibrated ^{14}C dates for sample no. number 3 produced with OxCal v4.3.2 and IntCal13 atmospheric calibration curve. * denotes samples measured at Hertelendi Laboratory.

Table 5

Calibrated ^{14}C dates for collagen and amino acids from sample no. 3

Sample name	Calibrated age (cal AD)	Probability for σ = 2 (%)
Collagen	1293 – 1404	95.4
Pro*	1300 – 1368 1381 – 1404	74.7 20.7
Thr	1225 – 1234 1242 – 1310 1360 – 1387	1.7 79.9 13.8
Ala*	1276 – 1306 1363 – 1385	66.3 29.1
Hyp	1303 – 1366 1383 – 1434	49.4 46.0

The calendar data obtained from the radiocarbon ages for each amino acid are relatively close and they fall in the range calAD 1225–1434, which is approximately

the same interval obtained for collagen. This interval is larger and from these ranges it cannot be accurately stated whether the bones from the grave can be dated before or after the chapel church was built, namely around 1415 AD. More reliable intervals, given by the data obtained in Hyp and Pro analysis are calAD 1303–1434 and calAD, respectively 1300–1404.

3.4. SAMPLE NO. 4 – HUMAN BONE FRAGMENT (M2)

From the second bone fragment received from "The Princely Court in Târgoviște" 98.5 mg collagen was extracted. Further on, 3.34 mg collagen was direct dating and the rest was hydrolyzed and separated into amino acids by HPLC. Ala and Hyp were separated with good resolution. The Pro could not be completely separated from Gly, and consequently, they were collected together as an amino acid mixture (Fig. 7).

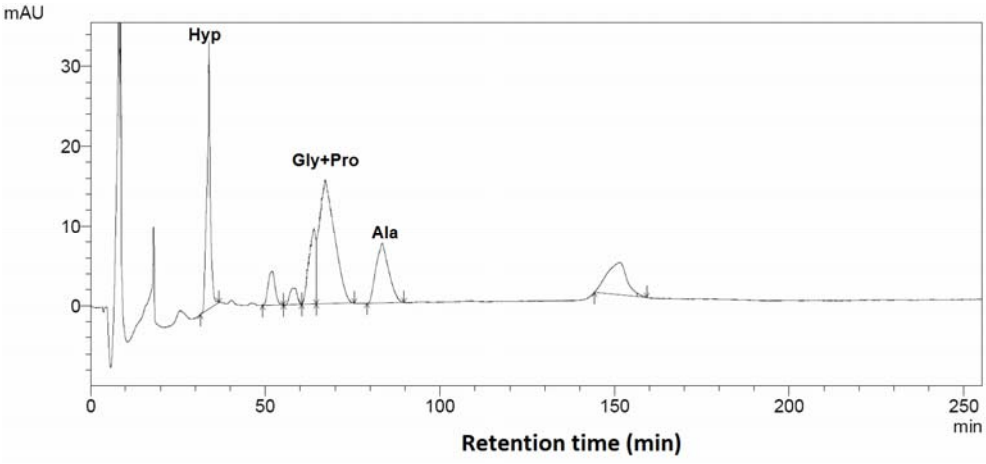

Fig. 7 – HPLC chromatogram of sample no. 4 in which the individual amino acids measured by AMS are indicated.

The amounts of each collected amino acid are shown in Table 6. Due to the very small amount (0.11 mg and 1.11 mg) Ala and Pro were measured only at the Hertelendi Laboratory where, for analysis of Ala, the gas source of the AMS facility was used.

The analysis of the C/N atomic ratios for the bulk collagen and amino acids samples which was made at the RoAMS laboratory shows that, as for the other bones, collagen is well preserved. The amino acid mixture of Pro and Gly cannot be characterized by the C/N ratio because the proportion of the two amino acids is unknown. For Ala and Hyp the values are not available.

Table 6

Experimental data obtained after graphitization and AMS analysis for sample no. 4

Sample name	Weight (mg)	N (%)	C (%)	C/N	Radiocarbon age (years BP)	Calibrated age (cal AD)	Probability for σ = 2 (%)
Collagen	3.34	14.81	40.15	3.16	709 ± 31	1255 – 1308 1361 – 1387	83.2 12.2
Mix Gly+Pro	4.05	16.30	38.92	2.79	580 ± 39	1298 – 1421	95.4
Ala*^	0.11	N/A	N/A	N/A	888 ± 92	990 – 1279	95.4
Hyp*	1.11	N/A	N/A	N/A	713 ± 19	1264 – 1295	95.4

* indicates that the sample was analyzed at Hertelendi and the symbol ^ indicates that the gas source was used.

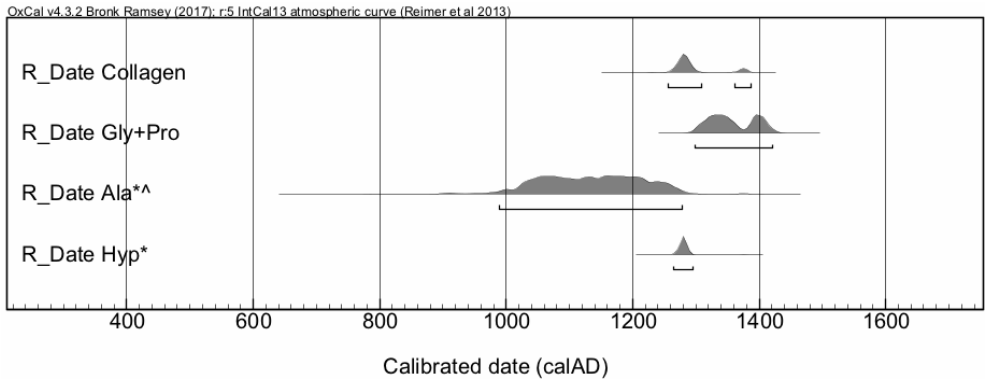

Fig. 8 – Calibrated ^{14}C dates for sample no. 4 produced with OxCal v4.3.2 and IntCal13 calibration atmospheric curve (Reimer *et al.*, 2013). * denotes samples measured at Hertelendi and ^ denotes samples measured direct using CO_2.

From these results, only dating of the Gly-Pro mixture may confirmed that the bones could have been buried in the church built in 1415. However, considering the other data and the fact that the mix cannot be characterized by C/N, this hypothesis is not well sustained.

From dating bulk collagen and Hyp, almost identical ranges were obtained in both laboratories, namely calAD 1255–1308 for collagen, and calAD 1264–1295, respectively for Hyp. From these results, it can be said at first glance that these bones can be most likely dated to calAD 1264–1295. By comparing these results with the data for the first bone fragment (M4), it can be said that the two bone remains are indeed from two periods differing by about 50–100 years.

A much older, and more extensive range was obtained by dating Ala, *i.e.* 990–1279 calAD with a probability of 95.4%. This might be due to the very small sample quantity (0.11 mg) directly measured in the gas ion source [27].

4. CONCLUSIONS

In this study we performed AMS measurements of the ^{14}C content in the bone collagen and in some individual amino acids constituents to establish the age of a two set of samples.

The first set consisted of a domestic object, bone awl (sample no. 1) and a fragment of astragalus (sample no. 2), while the second set (sample no. 3 and sample no. 4) contains human bones fragments discovered in two graves, that were found in an old church chapel.

The direct measurement of collagen by AMS showed that the two samples from the first set belonged to a much earlier period than most of the objects found at the same archaeological site. Dating of amino acids separated by HPLC confirmed that the period obtained by direct collagen analysis was correct.

In the case of second set, the data obtained for bulk collagen showed that the two human bone remains belong to the same historical period, with a difference of about 50 –100 years.

All radiocarbon measurements by AMS for amino acids and bulk collagen from this paper have been provided approximately the same or much more reliable data than bulk collagen analysis when the amino acids separation was well accomplished.

However, in the case of some amino acids, some erroneous data were obtained due to the very small quantities, the bad separation or due to contamination during the separation process.

Contaminants caused by the HPLC “column bleed” will be studied in a future work using well known-age samples and thus, specific amino acid-specific corrections will be determined.

This study complements the work of researchers at IFIN-HH regarding the characterization, by other methods, of cultural heritage artifacts from Romania [28–30].

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