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Changes in Raman spectra of bone tissue as an indicator for absolute dating

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Diagenetic changes in bone tissue are accompanied by transformations of its Raman spectra. The results of the analysis of the prospects for using such changes for absolute dating of archaeological material are presented in the present work. The study was performed on animal bone samples collected during archaeological studies of the cultural layer of ancient cities in central Russia. The studies were performed with using visible excitation radiation. The effect of photoluminescence of chromophores in bone tissue was reduced by photobleaching methods. Changes in the intensity of the main peaks of the mineral and organic parts of bone tissue are compared with the value of the postmortem time of material. The effectiveness of using the obtained dependencies as a chronometric tool for dating archaeological osteological material is shown for the case of short and medium duration of occurrence in the cultural layer. The historical age was clarified for a sample with an erroneous dating established by the traditional method.

Keywords: Raman spectroscopy, bone tissue, diagenesis, absolute dating.

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Introduction

The capabilities of Raman spectroscopy (RS) to determine the structural specifics of substances and materials have determined its relevance not only in fundamental research and in the development of industrial technologies [1], but also for many other applications, including biomedical research [2,3]. Cultural heritage here is not an exception. Here RS spectroscopy is successfully used in the study of materials used in the creation of paintings and ancient manuscripts [4]. First of all, attention is paid to the paints and pigments that make up their composition. Pigments are also often the subject of research in the study of archaeological artifacts [5,6]. There are known examples of RS analysis of archaeological objects made of glass, clays, copper alloys and other materials, including organic substances jcite7-9. Bone products have been ignored by spectroscopists, which leaves a niche for individual scientific research, although Raman spectroscopy has been used to study the remains of ancient humans and hominids [10,11], as well as paleontological bones [12].

Archaeological research is the main method of providing historical science with material sources of information about human life and society in the Middle Ages and earlier periods. The description of the events of Modern Times (XVIII–XIX centuries) is based mainly on preserved written sources and objects of cultural heritage. But archaeological artifacts often complement and clarify, and sometimes reject, the existing historical vision of this period. In any case, the age dating of artifacts is a priority task.

In archaeological practice, relative and absolute age dating methods are used. The first ones include stratigraphic and comparative-typological methods, as well as cross-dating. Based on many years of practice and a large amount of processed material, they are successfully used by archaeologists, but in many cases they allow for careful and wide age dating intervals. Absolute natural science methods are very helpful in clarifying the conclusions reached. The most well-developed among them are dendrochronology and radiocarbon analysis. Other isotopic and thermoluminescent methods are also used. In principle, all natural scientific age dating methods are based on determining the change in time of any parameter of the studied object. During the time when bones are buried, diagenetic transformations occur in them, which are mainly associated not only with the destruction of the organic component (collagen), but also affect the mineral part (hydroxylapatite). These changes are reflected in RS spectra, which makes it possible to use it to determine the postmortem interval. Absolute age dating based on bovine bone tissue is at an early stage of development, but it has already shown interesting results for estimating the age of artifacts [10–12], as well as determining the safety of bones as a source of material for isotopic or DNA analysis [13].

Unfortunately, almost all the studies in this area are related to the analysis of individual most expressive artifacts, whereas for general use a continuous time dependence of the target parameters is required. Only one study is known providing a similar dependence for samples from Italian regions, covering the time interval from modern times to 4000 BC [14]. However, for the study of Russian archaeological sites, the same tool is needed based on the same appropriate initial data. The soil and climatic specifics of the bones location affect diagenetic processes, and thus such studies cannot be deployed in the practice all over Russia. Therefore, our research is limited to the material collected during archaeological excavations within Vladimir region. At the initial stage of the study, we determined the relative intensities of the most expressive peaks in Raman spectra of bones from the construction horizons, which are the most powerful and confidently identified in the cultural layer of ancient cities in the selected region (XII−XIV and XVII−XVIII centuries). These results, together with the data for modern bones, allowed us to establish a general trend and range of changes in the target parameters.

During archaeological excavations of the 2025 field season, additional samples of osteological material were obtained, the RS spectroscopy of which allowed to construct time dependences of characteristic relative intensities to a depth of XI centuries with an interval of one century (with some gaps), presented in this work. The conducted studies have shown a significant influence of the fluorescent background on feasibility of the chosen approach, and the data from solving this problem are also reflected in the given article.

1. Materials and methods

A multifunctional microscope NTEGRA Spectra (NT-MDT, Zelenograd, Russia) with exciting laser radiation with a wavelength of 473 nm and power of 4 mW was used in the study. A sounding beam of linearly polarized radiation from an LM473 solid-state laser was focused on the surface of the samples by means of Mitutoyo M Plan Apo 100 lens with a numerical aperture of 0.7 into a spot with a diameter of 0.4 μm. The RS response from the samples was recorded with the accumulation of three samples during exposure for 10 s each. The selected frequency of exciting radiation ensures high availability of the developed age dating method, as it is often used in the basic configuration of RS systems, and the RS efficiency is achieved 7.6 and 25.6 times greater than when using radiation with a wavelength of 785 and 1064 nm, respectively [15]. The processing of the obtained spectra, the calculation of relative intensities, and the approximation of their time dependences were performed using OriginPro software package.

Fragments of pig bones collected during archaeological excavations in the historical centers of the cities of the Vladimir region were selected as samples for analysis: Murom, Suzdal, and Alexandrov, which are well-known administrative and cultural centers of Northeastern Russia. Murom is considered to be the most ancient. The first chronicle mention of it dates back to the 9th century. This factor, as well as the active archaeological field research currently being conducted here, have determined the prerequisites for obtaining the most representative sample to the greatest historical depth. Therefore, the main pool of examined bones, attributed by experts to the 19th, 17th, 14th, 12th and 11th centuries, was selected from the studies of the cultural layer of Murom (excavations 2025, head of work — Val.V. Beylekchi). The time gaps in this sample were filled in, if possible, with samples from the excavations in Alexandrov (XVIII century, works 2023 Head — O.V. Danilov) and Suzdal (XV−XVI century, studies of 2008 Head — D.A. Kabaev), the osteological material of which is in the funds of the State Center for the Preservation, Use and Restoration of Cultural Objects and Cultural Heritage (Vladimir). For each century-long period provided with material, one sample was selected for study, from the surface of which the RS spectra were registered at three arbitrary points.

The presence of archaeological samples from excavations located in different cities in the sample is acceptable in this case due to the close values of climate and soil parameters. First of all, the influence of acidity and the hydrological regime of soil on bone tissue diagenesis is noted [16]. Acidic soils are more aggressive than alkaline ones. The known soil hydrogen index in the Murom, Suzdal, and Alexandrovsky districts of the Vladimir Region are 5.9, 5.5, and 5.4, respectively, i.e. they all relate to slightly acidic environments. All samples were found in anthropogenic pits embedded in dense continental loam, filled with gray-brown loam with varying degrees of humus content. This prevents formation of the flow hydrological regime, which leads to additional leaching of the mineral component of bone tissue. Given the climatic conditions common to all excavations with average temperatures $-8.5\,^\circ$C in January and $+18.8\,^\circ$C in July, as well as the average annual precipitation of 550−600 mm, it is suggested that we deal with a single hydrological replenishment regime with cyclic humidification and drying, as well as freezing and thawing. This regime is accompanied by a general increase in the physical destruction of all components of bone tissue because of mechanical stresses. Thus, the uniformity of conditions of the samples staying in the cultural layer allows to neglect their influence when determining the time dependences of the relative intensities of characteristic peaks in their RS spectra for the selected historical and geographical region. It should be noted that heat treatment of bones (boiling) changes the initial conditions of bone tissue diagenesis. As our preliminary studies have shown, the relative intensity of peaks of the organic component in boiled bones of modern period decreases by 35 % compared to the raw samples. In this paper, the samples selected in the approximation of the exclusion of thermal treatment like cooking (large bones of limbs and lower jaw) are analyzed.

2. Results and discussion

2.1. Suppression of fluorescence background

The effectiveness of the study of biological objects and organic compounds is often limited by background fluorescence (e.g., see [17]). The strong fluorescence in Raman spectroscopy analysis of bone tissue is due to the presence of carotenoids and heme [15] in it, which are part of the blood, the remnants of which remain in the studied samples, despite heat treatment like cooking or prolonged exposure to soil deposits. The absorption band of these chromophores is in the blue and green regions of the spectrum, which means that the wavelength of the exciting laser radiation used in this study is within its limits. Therefore, the preliminary preparation of samples aimed at quenching this fluorescence became a must.

There are different approaches to solving the problem of fluorescence of chromophores in bone tissue, e.g., long-term vacuum drying or extraction of carotenoids with ethyl alcohol [15], treatment with 30 % hydrogen peroxide solution [18] or photo discoloration [19]. The last two seem to be the most beneficial, as they require less time compared to the others.

In case of using H_2O_2, Raman spectra suitable for analyzing the state of bone tissue were obtained after 40 min exposure in solution. During photobleaching, a similar result was achieved when exposed to laser radiation focused by $\times 100$ lens of the main measuring head (intensity $25 \cdot 10^7\,\mathrm{W/m^2}$) for10 min, while the noise level corresponded to 0.35 of the initial one. However, in this case, the required fluorescence decay was achieved only for the analyzed point, and when moving to the next one, the procedure had to be performed anew. As a result, the total pre-preparation time of the sample becomes comparable or longer than that during chemical treatment.

As part of the overall task to ensure a prompt absolute age dating of osteological material collected as a result of archaeological research, the pace of research is a critical factor. But here, it is equally important, and often mandatory, that the analysis be performed without destroying or changing the structure of the samples. This requirement becomes especially important with the possible extension of the developed age dating method to individual bone finds. Based on this, the method of bone fluorescence quenching due to photobleaching acquires priority importance, and its effectiveness was studied when the sample was exposed to laser radiation in Raman spectroscopy.

In order to increase the area of the sample's exposed surface, a replaceable focusing head with a $\times 10$ lens was used. This provided exposure of the bone with a laser beam focused into a spot with a diameter of $40\,\mu$m (intensity $25 \cdot 10^5$,W/m^2). Within this range, when using $\times 100$ lens, it is possible to register RS spectra from a significant number of points. Bones dated 11th and 19th centuries were used as samples for a step-by-step analysis of the photobleaching effectiveness. (M-11, M-19). In the entire set of finds, confidently dated by archaeologists, these samples were in a buried state for the longest and shortest time, which means that they differ the most in the degree of diagenetic changes. The bone surface area was treated with laser radiation for 60 min. Every 10 minutes of the sample's exposure the RS spectrum was registered again. Relative fluorescence levels A_f versus exposure time t_e were shown in Fig 1. At the end of treatment, the noise level was recorded at 0.37 and 0.43 of the initial level for the ancient and younger samples, respectively. In both cases, this is comparable to the noise level achieved during the preparatory irradiation using the main measuring head. The experimentally obtained data are well approximated by exponential dependencies $A_{f\mathrm{XI}} = 0.31 + 0.7\exp(-0.04t_e)$, $R^2 = 0.9973$ and $A_{f\mathrm{XIX}} = 0.39 + 0.6\exp(-0.04t_e)$, $R^2 = 0.9992$. In both cases, certain dependencies show that a further increase in exposure time will lead to a slight additional suppression of fluorescence. At the end of treatment of M-19 sample, the residual fluorescence level slightly (by 5 %) exceeds the result for M-11 sample, and comparable ratios are observed at all stages of the analysis. This shows that this method may be effectively used in photobleaching for archaeological bones, regardless of the time they were in a buried state.

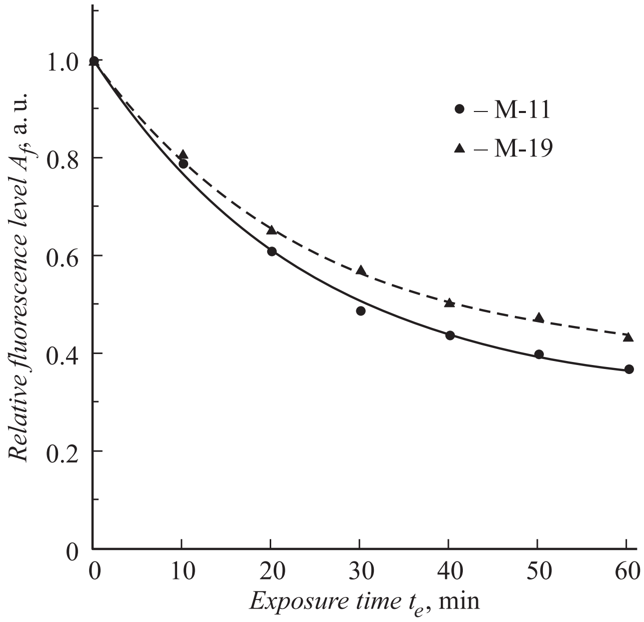

Figure 1. Relative fluorescence level versus exposure time for the samples of XI (solid line) and XIX centuries (dashed line).

2.2. The relative intensities of the bone tissues RS spectra peaks versus time

For the analyzed samples of archaeological osteological material, the RS spectra were registered in the range from 200 to 2000 cm^{-1}. In this spectral band (Fig. 2) there are three characteristic peaks, the relative intensity of which is associated with changes in bone tissue caused

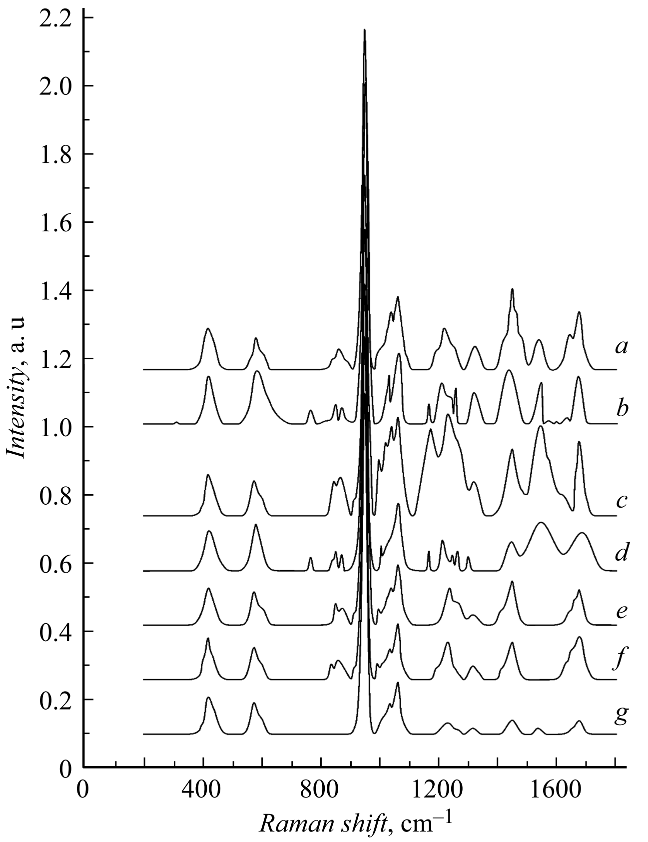

Figure 2. Spectra of the bone tissue archeological samples dating back to XIX (*a*), XVIII (*b*), XVII (*c*), XIV (*d*), XII (*e*), XI (*f*) and X centuries (*g*).

by the processes of diagenesis and therefore promising for use as indicators for absolute age dating. Two of them are related to the organic component of bones. First, it is a peak of about 1450 cm^{-1}, reflecting deformation fluctuations of methylene groups (CH_2) in the side chains of collagen amino acids [20]. Its intensity serves as an indicator of the collagen content in ancient bone samples when assessing their suitability for analysis by various methods, such as radiometric or genetic method [21]. The second highlighted peak is located near 1670 cm^{-1}. It corresponds to the valence vibrations of C=O bond in the amide group (amide band I) [22]. One of the chemical transformations that occur during bone diagenesis is deamidation. It is accompanied by the destruction of amide bonds of the polypeptide chain, as well as the loss of the amide functional group by amino acids that make up the collagen [23].

The third characteristic peak in the studied samples' Raman spectra was registered near 1060 cm^{-1}. It is caused by the RS response of the mineral component of bone tissue (hydroxylapatite), i.e. calcium carbonate ions contained in it [24]. The relative intensity of this peak also changes as the postmortem time interval becomes longer. However, the main effect here is caused not by monotonously degrading diagenetic transformations, as with organic bone matrix, but, on the contrary, by an increase in the crystallinity of hydroxylapatite. The presence of carbonates in its structure negatively affects the long-range crystal order. This dependence is clearly visible when comparing biological, geological, and synthetic apatite. Thus, high crystallinity of the bone mineral fraction is associated with a lower content of calcium carbonate ions [25]. The diagenesis of buried bones is accompanied by the dissolution of poorly ordered bioapatite, with small-sized crystallites dissolving faster, and by redeposition on large crystallites, leading to their growth in a more stable configuration depleted in CO_3^{2-} [26,27]. In Raman analysis, this process results in higher peaks of carbonate ions compared to the response from phosphates.

To determine the relative intensities of the peaks selected to solve the problem set in this paper, their amplitude intensities were normalized by the value of a similar parameter for a peak of about 950 cm^{-1}, which is associated with symmetrical stretching vibrations in PO_4^{3-} phosphate ions in hydroxyapatite. In the Raman spectra of bone tissue, there are several noticeable peaks corresponding to the calcium phosphate vibrations, but this peak has the largest amplitude.

Certain values of the relative peak intensities around 1060, 1450, and 1670 cm^{-1} for the prepared set of archaeological bone samples allowed to construct their time dependences (Fig. 3). The relative intensity of the vibration peak of I_{1450}/I_{950} methylene groups in modern bone is 0.309, and it decreases to 0.105 for the sample of the eleventh century (M-11). The latter value shows that the selected parameter can be confidently used to estimate the postmortem time after the death of an animal within the Northeastern Russia's most interesting development period according to the researchers.

It was the eleventh century that is considered by the researchers as the period when this region had been systematically and intensely studied by Russian settlers. Earlier urban cultural strata are present in isolated admin-

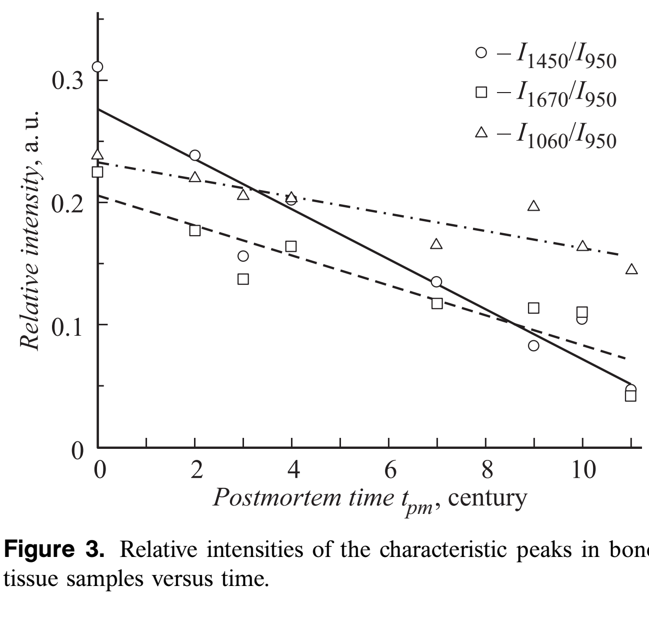

Figure 3. Relative intensities of the characteristic peaks in bone tissue samples versus time.

istrative centers of Vladimir region and adjacent territories. Despite the fact that Murom is just such a city [28,29], anthropogenic deposits of the tenth and ninth centuries are rarely found during archaeological excavations in it, and samples from them were not present among the specially selected reliably dated osteological material, used in the research, the results of which became the basis of this study.

Relative intensity of the peak around 1670 cm^{-1} in the band of amid I (I_{1670}/I_{950}) varies from 0.225 for a modern bone to 0.109 for M-11 sample. This behavior of the studied indicator proves that it may be used for absolute age dating purposes simultaneously or instead of I_{1450}/I_{950}. A narrower range of values was found for the relative intensity I_{1060}/I_{950}. Over a thousand years of the postmortem time interval, this indicator, characterizing the crystallinity of hydroxylapatite, decreased from 0.238 to 0.166. On the one hand, this limits the effectiveness of age dating within the selected historical period, on the other hand, it allows us to extend the developed method to more ancient osteological archaeological finds.

Currently, the absolute age dating method based on the Raman spectra of archaeological osteological material is mainly focused on plotting their time dependencies using data obtained from the study of bone tissue samples with a known occurrence time in the cultural layer. Testing the results from the field and/or desk material in order to determine or clarify its temporal attribution is expected at a further stage of research. However, the possibilities of the developed method in this area have already been significantly demonstrated by the Raman spectroscopy of sample C-15-16, which is part of the initial sample. This bone was found during archaeological excavations in Suzdal in a utility pit dated XV−XVI centuries. (according to the work report [30]). The sample C-15-16 was added to the set of material collected during excavations of the cultural layer of Murom to fill in the gap corresponding to the specified period. The relative intensities of the characteristic peaks in its spectrum determined by Raman spectroscopy were as follows: $I_{1450}/I_{950} = 0.045$, $I_{1670}/I_{950} = 0.042$, $I_{1060}/I_{950} = 0.145$. These values are significantly lower not only than expected but also obtained for the sample of the eleventh century.

This result required a more thorough study of the report, taking into account the inventory and planographic context surrounding the investigated find. It turned out that the utility pit in which C-15-16 bone was found cuts through another, more ancient object buried in the mainland, identified as the underground of an above-ground residential building that existed in ancient Russian times. The experience of archaeological research suggests that in such conditions it is often very difficult to identify the exact boundary between the fillings of intersecting pits. Thus, part of the ancient osteological material was attributed to XV−XVI centuries. It should be noted that the term „Old Russian“ is used to define the historical period from IX to XII centuries, and in archaeological practice is applied to the attribution of material, a more accurate dating of which is difficult. Cultural deposits of the tenth century are found during the archaeological study of Suzdal territory [28,31]. A comparison of historical data and the results of RS spectroscopy allowed to attribute the indicators determined for sample C-15-16 to the postmortem time interval of 11 centuries.

The set of samples available for the study allowed us to obtain a discrete set of indicators corresponding to the centuries of deposition of osteological material in the cultural layer. Using the analysis results to estimate the historical age of any new animal bone finds requires continuous dependencies. Therefore, the data was approximated for each of the selected relative intensities. In [14], an exponential dependence was used for these purposes, which showed good results for bone samples with a historical age of up to several thousand years. However, in the initial interval of up to a thousand years, the resulting curve has an obviously quasi-linear section. The bone tissue samples, the results of the analysis of which are presented here, have an age dating that falls within this time interval. Therefore, a linear relationship was used to approximate the data obtained during their study. The following expressions were obtained for the considered relative intensities:

$$I_{1450}/I_{950} = 0.275-0.020t_{pm},\ R^2 = 0.8901, \quad (1)$$

$$I_{1670}/I_{950} = 0.206-0.012t_{pm},\ R^2 = 0.8370, \quad (2)$$

$$I_{1060}/I_{950} = 0.232-0.007t_{pm},\ R^2 = 0.8138, \quad (3)$$

where t_{pm} — postmortem time interval (in centuries).

In Fig. 3 the approximating dependencies are shown by solid line for I_{1450}/I_{950}, dashed line — for I_{1670}/I_{950} and dotted line — for I_{1060}/I_{950}. The steepness of the lines slope clearly confirms that among the selected indicators in the medium-term historical period, the use of the relative peak intensity of about 1450 cm^{-1} is most effective for the age dating, and the relative peak intensity of about 1060 cm^{-1} makes it possible to extend the developed dating method to even more ancient bone samples.

Conclusion

The results of the conducted work provide researchers with an additional tool for absolute age dating of osteological archaeological material based on the use of Raman spectroscopy. The obtained empirical dependences for changes in the relative intensities of peaks corresponding to collagen (about 1450 and 1670 cm^{-1}) can be used to estimate the historical age of bones starting from the Ancient Russian period, and the peak reflecting the crystallinity of hydroxylapatite (about 1060 cm^{-1}) — for even earlier finds. It has been demonstrated that animal bones with such a postmortem time interval can be successfully analyzed using excitatory laser radiation of the visible range when performing a making photobleaching procedure. This greatly raises the availability of the developed approach in the natural science age dating, which is especially important in the absence or limitations of clothing and ceramic materials that hinder the traditional typological dating of the cultural layer, which is often observed during archaeological exploration and expertise. In such cases, the role of natural science methods becomes of higher value. Today, the most common among them are dendrochronology and radiocarbon analysis. Raman spectroscopy can boast a number of benefits compared to them. Dendrochronology can be applied only if there are samples of wood with a well-preserved transverse structure of annual rings among the archaeological material. The radiocarbon analysis is a more universal tool, but the cost of expertise using it is at least twice as high as with Raman spectroscopy. Obviously, at present research stage, the absolute error in determining the historical age of bones cannot be better than the error in age dating of original archaeological material and is estimated as a century for the entire considered historical interval (up to 1000 years). In such conditions, the developed method is comparable to traditional methods of archaeological dating. Reducing the error of empirical dependencies is possible by involving more samples in the study, including those with a historical age that can be more accurately determined based on the archaeological context (known catastrophic events, building horizons, numismatic material). This is possible with continued and expanded collaboration with practicing archaeologists. However, the results already obtained make it possible to introduce the developed age dating method into the field practice and office analysis of the collected material.

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Conflict of interest

The authors declare that they have no conflict of interest.

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