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IR SPECTROSCOPY OF THE FLOUR FROM BONES OF EUROPEAN HARE

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The general objective of the research was to study the biostructure of flour from bones of Europeanhare (*Lepus europaeus*)with the aim of valorizing it by its use in non food activities. In the study of biostructure obtained from bone flour we used IR absorption spectroscopy. The studies allowed us to characterize this biostructure obtained from bone flour at the moment of obtaining, after 7 days of obtaining and 14 days, respectively. The IR spectra of the obtained samples present bands of characteristic frequency without essential differences between them. The differences that appear are referring to the intensity and profile of the bands with insignificant displacement of the characteristic bands.

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1. Introduction

The infrared absorption spectroscopy is a technique used in investigating matter (in crystalized and uncrystalized state), being applicable to molecular systems in any aggregation state. A link in a molecule may have elongation vibrations, ν or valence vibration (along the link) and deformation vibration, δ, which deforms the valence angle.

In the composition of bones there is the fundamental substance and the fiber system. The fundamental substance (almost 35%) includes osein (scleroprotein insoluble in cold water, but soluble in warm water), with granular or filamentous structure, and osteomucoid (a sulphatic proteinosaccharide), both being linked. It also contains mineral substances (~ 65%) such as: phosphates, carbonates and small quantities of calcium fluoride and chloride, hydroxiapatites with Ca, Mg, P, F, S [1]. The mineral phase consists of calcium and phosphorous and is characterized as a weekly crystalline hydroxyapatite, even though the molar ratio calcium/phosphorous is smaller than 1,67, the characteristic value for to the hydroxyapatite with the empirical formula $Ca_{10}(PO4)_6(OH)_2$. The mineral phase of the bone is initially deposited in relationship with the collagen fibers and is localized in a specific way in the "gaps" between the fibrils of collagen [2]. The bones of the hare are a group of subproducts important as volume, but they are not so much processed and valorized directly as food products (accompanied by edible material) [3]. Without the presence of meat or fat, the bones of hare become subproducts valorized for technical use [4].

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Being part of the group of subproducts, the bones take 16-20% of the volume of the body of hare, physical composition placed second after the muscle tissue (edible raw material). The data regarding the differentiation criteria of animal bones are few and unedifying [5], as the information regarding evaluation of: obtaining bone flour (zootechnics, food industry and legumiculture); obtaining of collagen, gelatin, concentrate food from bones intended for pets feeding; realizing art pieces from bones; manufacture of glues from bones; producing compost from bones (US) [6]. The bone flour is the result of grinding calcinated bones, while from the small tearing of the raw bones it is obtained a feed. The bone flour is a valuable raw material, supplying energy, vitamins, minerals, with a high degree of digestibility. Depending on humidity, the bone powder might be stable even at room temperature and the agglomeration/aggregation would not take place during storage of bone powder. Besides the physical-chemical modifications, a low content of humidity allows the hare bone powder to be resistant to microbial deterioration (humidity content around 2% is not enough for microbial development). There are data that support the fact that lipids may not be present in the composition of bone. However, the determinations indicate that lipids exist in small quantities (complex lipids) [7].

The aim of the study was the preliminary analysis of the bone flour and comparing the modifications that occur during 14 days due to the water in the environment.

2. Materials and methods

Bone Preparation. Hare bones were soaked in NaOH solution (0,8%) bone:NaOH ratio was 1:2 at 90°C for 1 hour. The bone residues were rinsed with deionized water and were dried in an oven at 100°C (≈ 12 h). The decrease of dimension of dried bone samples was realized using a homogenizer at 15.000 rpm for 4 min. The bone powder was kept in vacuum conditions at -20°C until further use.

*Proximate Analysis.*Hare bones were analyzed in order to quantify the protein content based on the determination of total nitrogen using the Kjeldahl method [8]. The humidity content was achieved gravimetrically after drying in oven at 105°C for 12 h (according to AOAC, 2000) [9]. The raw lipids were analyzed using the Soxhlet extraction, using petroleum ether as solvent. The ash content was quantified after calcination (dry sample at 550°C for 16 h according to AOAC, 2000) [9]. The extraction of heavy metals was achieved with concentrated sulfuric acid and hydrogen peroxide 50% with a Digestal type mineralizer. The calcium content was measured by atomic absorption spectroscopy, and the phosphorous was determined colorimetrically.

Sample Preparation. The solid substances, soluble and insoluble, were prepared in the form of tablets in potassium bromide. The sample (1mg) is ground with anhydrous KBr (200 mg) and the mix is pressed in the form of a very thin disc, practically transparent. The compression is done with a hydraulic press at approximately 10^9 N/m^2, simultaneously taking out the air from the sample with a vacuum pump. The spectrum quality can be affected by the quality of the tablet.
The analysis was run with JASCO 660 PLUS spectrophotometer in optical area 4000 cm^{-1}- 400 cm^{-1}. The work parameters were: resolution 4 cm^{-1} and scan number 20. The FTIR spectra were processed with the help of a specialized program from the SpectraManagerseries [10].

3. Results and discussion

After the analysis it was observed that the humidity values for the bone powder of the hare was 2.03 according to table 1.

Table 1.Major component of harebones powder

Proximate parameter	***The wild haresbones (Sample 1) Content (%)***
Moisture	2.03
Crude fat	5.52
Protein	14.93
Ash	77.52
	Mean±SE was estimated from 3 replications.

There is the possibility to observe fat residues in the bone powder, the raw lipid content in the bone tissue being almost 6%. The unsaturated fatty acids are susceptible to oxidative degradation (oxidation); but the bone dust has a low risk of autoxidation [11, 12], because the lipid content is relatively low [13].

The environment conditions influence the impurities in the samples according to figure 1. At 1463 cm^{-1}, specific bands appear for CO_3^{2-} associated to the fact that the bone flour absorbs CO_2 from the atmospheric air. The bands at 1090 cm^{-1}, 1030 cm^{-1} and 960 cm^{-1} correspond to PO_4^{3-} vibrations.

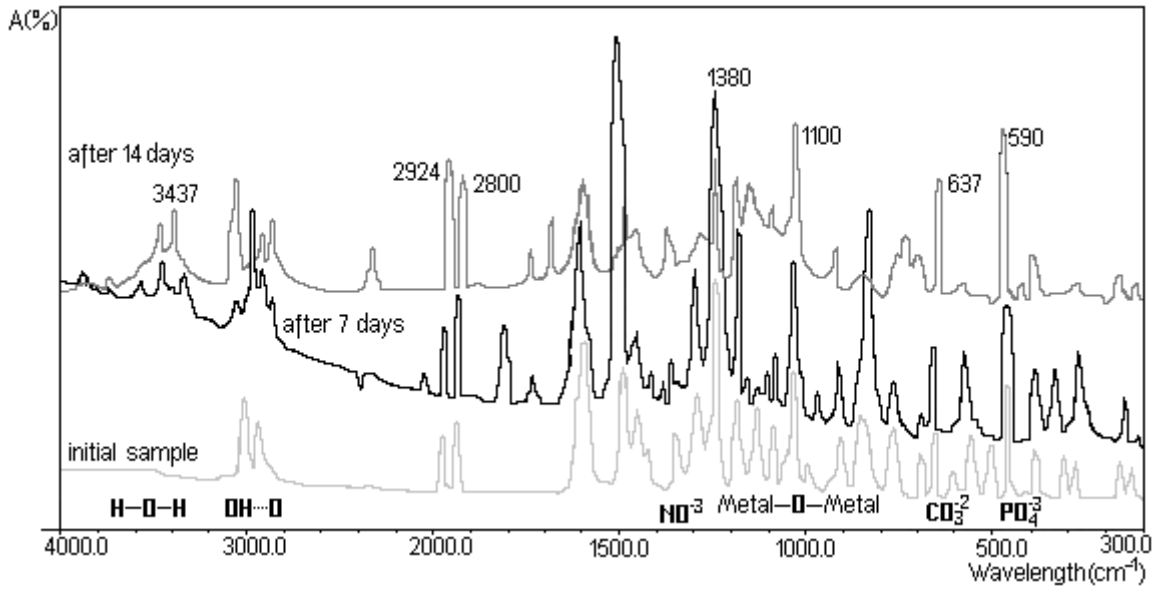
The spectrum presents characteristic bands at 980 cm^{-1} associated with the Metal-OH vibration and at 1100 cm^{-1} which corresponds to the –Metal-O-Metal vibration. Their presence is due to the existence of macroelements in the field hare bone flour, which belongs to the structure of hydroxyapatite. In the analyzed systems, transformation processes occur, such as the decomposition of tetracalcic phosphate $Ca_4(PO_4)_2O$:

$$Ca_4(PO_4)_2O \rightarrow Ca3(PO_4)_2+CaO$$

In the case of collagen, (formed of ~33% glycine and 22% proline sau hydroxyproline) we have the following peaks: at 3432 cm^{-1} the H-O-H group (elongation) and in the aliphatic zone 2924 and 2857 cm^{-1} the C-H- group (elongation vibration). In the area 1654 cm^{-1} the elongation vibration C=O and at 1541 cm^{-1} the deformation vibration combination N-H and elongation C-N. At 1541 cm^{-1} is present the deformation vibration for the CH_3 groups and at 1242 cm^{-1} the elongation vibration C-N and deformation N-H. In the IR spectrum of the hare bone flour, there are bands characteristic to the crystalline and amorphous domains (1370, 1427 cm^{-1} and 890, 2900 cm^{-1}).

By determining the ratio between the absorbances of these bands, information is obtained regarding the crystalizing index. This ratio provides data that correlate with crystallinity, in the case of bands at 1427 cm^{-1} and 891 cm^{-1} there are modifications with the passing of time [14], so it is indicated that this ratio to be used when in the system there are no polymorphous forms. The band at 2900 cm^{-1} and, in a small amount, the one at 891 cm^{-1}, are representative for the IR absorbtion of OH groups, in the crystalline domains, as well as in the amorphous ones. The FTIR spectra of the hare bone flour in the 4000–2500 cm^{-1} and 1800–400 cm^{-1} spectral domains, present specific bands for the hydrogen bridges of the OH···O groups at 3425 cm^{-1}–3437 cm^{-1} in the studied samples (in the analyzed samples, peaks appear after 7 and 14 days). In the area 3561 cm^{-1} free OH groups appear from the surface of the analyzed samples after 7 and 14 days, respectively. The peak at 1380 cm^{-1} indicates the existence of the NO^{3-} group in the hare bone flour, the presence of impurities being more evident than in the flour sample analyzed immediately after obtaining (peak present in all the analyzed samples regardless of the time moment). Based on the literature data [15] there can be observed specific vibration frequencies for hydroxyapatite,

localized in the high frequency domain associated to the H-O-H groups at 3425 cm^{-1} from water by hydrogen bonds.



Fig, 1.IR spectra of the studied biostructure (1) immediately after obtaining bone flour, (2) after 7 days, and (3) that after 14 days.

At 3561 cm^{-1} there can be observed the presence of free OH groups from the surface of the particles (intense peaks in the analyzed sample after 7 and 14 days, respectively). In the 2800–2924cm^{-1} vibration areas we can identify minimum absorbtion bands associated to the CH_2 groups from the organic part (peaks in the initially analyzed sample, but also after 7 and 14 days, respectively). At 637 cm^{-1} there are specific bands appearing for the CO_3^{2-} group originating from the bicarbonate (peaks in the initially analyzed sample, but also after 7 and 14 days, respectively). Vibration frequencies PO_4^{3-} are localized in the low frequency domain at 1090 cm^{-1}, 1030 cm^{-1} and 590 cm^{-1} and appear in the initially analyzed sample, but also after 7 and 14 days, respectively. In the case of hare bone flour, relevant modifications in the analyzed spectrum could be due to the water that comes in contact with the sample during the 14 days. The water is the main absorbent of solar light from the atmosphere and is the reason for the transformations that occur in the spectrum of bone flour during the 14 days. The water absorbtion spectrum is very complex [16]. The water molecule can vibrate in a number of different ways. In the gas state, the vibrations imply symmetric elongation combinations (v1), asymmetric elongation (v3) and bending (v2) of covalent bonds with different absorbtion intensity according to table 2. The elongation vibrations refer to the vibrations of unique bonds, or the combined movements of several types of vibrations. The rotations in the gas state are complex and are combined with the elongation vibrations. The rotations in liquid state are completely dominated by the hydrogen bonds. The variations in the environment around each liquid water molecule give rise to a considerable modification with changes in vibration. Thus, the vibrations in a molecule that donates hydrogen are bigger than in a molecule that accepts hydrogen, but both act in the sense of accumulating hydrogen bonds. The strength of the hydrogen bond depends on the nature of the hydrogen bond/anti-bond, the ones with strong hydrogen bonds having relatively high vibrational frequencies [17]. In water, in the liquid state, the molecular elongation vibrations occur at high frequency. With the increase in temperature (hydrogen bonds are getting weak, the covalent O-H bonds making them to vibrate at higher frequencies), the intermolecular vibrations occur by going to lower frequencies, and the molecular bending vibrations give narrow peaks at low and strong frequencies.

Table 2. Assignment of infrared region of electromagnetic vibrational absorption spectrum

No.	***Wavenumbers cm^{-1}***	***Relativeintensity***	***Assignment***
1.	500	m/w/w/m/w/m/m/m	hydrogen bond bend
2.	583	s/w/w/w/m/m/w/m/m	hydrogen bond stretch
3.	595	w/w/s/w/s/w/w/w/w/w/w/m/w	L_1, librations
4.	686	s/w/w/w/m/w/w/w	L_2, librations
5.	1645	w/s/w/w/m/w/w	v_2, bend
6.	2150	w/w/m/w/s/w	$v_2 + L_2$
7.	2277	m/w/w/w/s	v_1, symmetric stretch
8.	2490	m/s/w/w/w	v_3, asymmetric stretch
9.	2660	w/w/s/w/w/w/w	$av_1 + v_2 + bv_3$; a+b=1
10.	2800	w/w/w/w/w/m/w/w/w/w/w/s/w/w/m	$av_1 + bv_3$; a+b=2
11.	2830	w/s/m/w/w	$av_1 + v_2 + bv_3$; a+b=2
12.	1031	w/w/s/w/m/w	$av_1 + bv_3$; a+b=3
13.	1196	w/m/s/m/w	$av_1 + v_2 + bv_3$; a+b=3
14.	1353	s/m/s/m/m/m/m/m/m	$av_1 + bv_3$; a+b=4
15.	1515	s/m/m/w/w	$av_1 + v_2 + bv_3$; a+b=4
16.	1650	s/m/m/m/m/s/m/m/m/m/w/w/w/w/w	$av_1 + bv_3$; a+b=5
17.	1946	m/s/w	$av_1 + bv_3$; a+b=6
18.	2227	w/m/w/m/w/w/	$av_1 + bv_3$; a+b=7
19.	2494	w/m/w	$av_1 + bv_3$; a+b=8
s–strong; m–mediu; w–weak.			

Further information may be gleaned about strength on a bond, relying on empirical guideline called Badger's rule. This rule states that strength of a bond correlates with frequency of its vibrational mode (increase in bond strength leads to corresponding frequency increase and vice versa).These differences between the elongation and bending vibrations are due to the intermolecular hydrogen bonds at low temperatures, that tend to reduce the intermolecular bending, favoring at the same time the elongation vibrations [18]. With the increase of temperature, the intensity of the elongation bands decreases. This divergent behavior of the bending and elongation vibrations allows the occurrence of combined bands that can be identified in the hare bone flour spectrum.

4. Conclusions

In some states of the EU, the meat processing industry was not subjected to the environment and dangerous emissions regulations. The reason is one of the actual desiderates: orientation toward improving performances regarding environment protection, by using solutions of valorizing the non-food sub-products.

The humidity in the bone powder, according to table 1, was relatively low. The lipids exist in small quantities, probably being complex lipids, which can be eliminated only by soaking the bones in alkaline solution. According to the information in table 1, there is the possibility to observe fat residues in the bone powder, the raw lipid content being almost 6%. The protein content in the bone powder was approximately 14% (the alkaline solution was efficient in solubilizing the meat tissue and the bone proteins). Nevertheless, the alkaline solution was not efficient enough to completely remove the proteins, because a part of the proteins was left in the bone powder. The major component in the analyzed bone powder was the ash content, which proved to be 75%. The ash from the bone powder represents quite a lot, which means that the bone product is important for obtaining a high purity of the bone component. Regarding the spectrum, the only differences appear due to the water in the environment which accumulates in the hare bone powder during the 14 days of observation.

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