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AGE DETERMINATION OF FOSSILS COLLECTED FROM KARABÜK, SAFRANBOLU, BULAK RIVER AREA BY USING AMINO ACID RACEMIZATION METHOD

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Abstract. Amino acids present in the proteins of living organisms are mostly in L forms at the time they are synthesized. Over a very long period of time after the death of living organisms, due to the effect of high temperature, pressure and other environmental factors a chemical transformation of L form to its enantiomer, D form occurs. This conversion reaction is known as racemization and it continues until D/L ratio is equal to 1. Thus, the age of any death body can be predicted by determining the D/L ratio of amino acids. The aim of this study is to determine the age of fossils collected from Karabük, Safranbolu, Bulak River area by using amino acid racemization method. Five fossil samples were used and D/L alanine ratios in the samples were used to determine their ages. As a result of the study it was observed that the age estimations by amino acid racemization method were quite lower than the age predictions during fossil identification step. The reason of this difference is thought to be related to the lack of data in the literature, which is used in constructing calibration curve.

1. Introduction

Chirality was firstly defined by Louis Pasteur in 1848, as a result of separating the two stereoisomers from $C_4H_8NNaO_6$ (ammonium sodium tartrate) [1-3]. Stereoisomerism can be possible as a result of containing a center of asymmetry that causes two different structural configurations. Stereoisomers of an amino acid, which contains an asymmetric carbon atom, a center of asymmetry, is expressed by prefixing D or L to the name of the amino acid [4].

Racemization is the name of the process that is responsible of the conversion of one enantiomer of a compound to the other [5]. Scientific studies have revealed that amino acids exhibit racemization depending on time. For example, the racemization of the amino acids found in teeth starts as the tooth is formed, but racemization in a bone starts after the animal has died, when no connection has left between the

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bone and the body fluids. On the other hand, the racemization starts right after the formation of the shell in an eggshell or a mollusc shell [6].

Amino acids present in the proteins of living organisms are mostly in L forms at the time they are synthesized [7]. Over a very long period of time, due to the effect of high temperature, pressure and other environmental factors racemization takes place and it continues until D/L ratio is equal to 1 [6]. Thus, the amount of the increase in the D form of an amino acid gives clue about the time has passed after its formation [8,9]. Recent studies have shown that the age determination by using the D/L ratio of an amino acids is as precise technique as radiocarbon analysis [10]. Although the age determination by amino acid racemization is common in the literature, in Turkey unfortunately it is not widely used.

In this study it is aimed to determine the age of fossils collected from Karabük, Safranbolu, Bulak River area by using amino acid racemization method.

2. Materials And Methods

2.1. Study Area

The study area was Karabük, Safranbolu, Bulak River area (41 ˚ 15 15.91" N, 32 ˚ 39 47.26" W) as given in Figure 1.

Figure 1. Study area

The study area is known to be a funnel-shaped area extending between Bolu and Kastamonu including Karabük, Safranbolu. This area is defined as Karabük - Safranbolu Tertiary basin and it is filled with Eocene to Post - Eocene deposits. The Karabük region is known to contain shallow marine and fluvial deposits [11].

2.2. Fossil Samples

The fossil samples, which are used in this study, were collected from 41 ° 15 12.5" N, 32 ° 39 46.7" E coordinates (523 m). The collected samples are given in Figure 2. Fossil samples were identified by Prof. Dr. Muhittin GÖRMÜŞ (Ankara University) by an interview in 2013. According to the identification, the fossil samples were contain examples of *Nummulites* sp., *Assilina* sp., some gastropods, some bivalves and gastropod inner molds.

Only five fossil samples, which were enough to continue for further analysis, were selected. The selected samples are given in Figure 3.

2.3. Sample Preparation

Selected fossil samples were washed firstly with running tap water, then distilled water (dH_2O) to clean the surface of the samples. Washed fossil samples were dried and ground to obtain a fine powder. Possible apolar contaminants in samples were removed in a Soxhlet apparatus at 40 °C for 3 hours by using petroleum ether (Merck, Germany) [12].

2.4. Determination of the Nitrogen Content of the Samples

One gram of ground fossil sample was transferred into a digestion flask, and 20 mL of 0.1 M H_2SO_4 (Merck, Germany) was added. Seven grams of K_2SO_4 (Emir Kimya, Turkey) was added to the digestion flask as a catalyser. The digestion flask was heated until white fumes have appeared. The mixture was continued to be heated for an additional 90 min [13].

The pH was increased by adding 45% NaOH (Emir Kimya, Turkey) into the mixture. The ammonia formed in the digestion flask was removed by bringing the mixture to the boiling point and distilled ammonia was collected into a trapping solution (15 mL HCl (Merck, Germany) + 70 mL of dH_2O) [13].

Three grams of bromothymol blue (Merck, Germany) was added to the trapping solution and titrated by 0.1 M NaOH (Emir Kimya, Turkey) until the colour change.

FIGURE 2. Different types of fossils collected from the study area

FIGURE 3. Fossil samples selected to be used in the study

The amount of nitrogen found in the samples were determined by using following formula.

moles of acid = molarity of acid x volume used in flask
moles of base = molarity of base x volume added from buret
moles of ammonia = moles of acid - moles of base

Since the moles of ammonia is equal to the moles of nitrogen, grams of nitrogen can be calculated as follows.

grams of nitrogen = moles nitrogen x atomic mass
(Atomic mass N = 14.0067)

2.5. Preparation of Samples for HPLC analysis

Ground fossil samples were weighed to contain 20 mg of nitrogen as it is given Table 2. 6 M HCl (Merck, Germany) was added and the samples were hydrolysed for 24 hours at 110 °C. At the end of the process, the samples were attached to a freeze dryer (Christ) and HCl was totally evaporated at 0.12 atm and - 82 °C. At the end of the drying process, the residue was dissolved in dH_2O. The precipitated silicate compounds were separated from free amino acids by centrifugation at 10,000 x g for 5 minutes. After centrifugation the pH was set to 9.0 and precipitated metal hydroxides were removed through filtration. The solution was then neutralized and freeze dried [12].

2.6. Choosing the Reference Amino Acid for the Study

TABLE 1. D/L ratios for alanine

Age of Samples	Ala
2200	-
2800	-
3110	-
3240	-
4630	-
5460	-
6850	-
11200	0.112
12400	0.131
15600	0.158
18600	0.192
20200	0.209
22600	0.228
25400	0.246
28600	0.289
30400	0.321
32500	0.343
36900	0.381
44600	0.465
46800	0.483
54300	0.510
62200	0.586
65000	0.613
72400	0.652

Alanine was chosen as a reference amino acid for the study. The D/L ratios for alanine concerning the ages of samples determined by the radiocarbon method are given in Table 1 [12].

The ages of fossil samples are expected to be higher than 11200 and alanine has a wide range of detection for samples having age over 11200 according to the data given in Table 1, thus alanine was chosen for the study.

2.7. Determination of D/L Alanine Ratio by HPLC

Freeze dried sample was dissolved in citrate buffer (pH=2.2) and the D/L Alanine ratio was determined by the method, which was previously defined by Csapó et al. [12].

2.8. Statistics

All samples were tested in triplicates. The statistical analysis was done using a non-parametric method, Kruskal-Wallis one-way analysis of variance, with a significance level of 0.05. All statistical analysis were conducted by using R Studio, version 3.3.2 [14].

3. Results And Discussion

3.1. Determination of the Amount of Sample Containing 20 mg Nitrogen

As a result of titration process the amount of samples containing 20 mg nitrogen was found as given in Table 2. The data given in Table 2 are mean values of triplicates and the difference between the results of triplicates were found to be statistically similar ($p > 0.05$).

Table 2. Amount of samples containing 20 mg nitrogen

Sample Number	Amount of samples containing 20 mg nitrogen
Sample 1	1.42 g
Sample 2	1.42 g
Sample 3	1.05 g
Sample 4	1.11 g
Sample 5	1.33 g

3.2. Calibration Curve for D/L Alanine Ratio

For preparing the calibration curve to identify age according to D/L ratio the data given in Table 1 are used and the calibration curve ($R^2 = 0.98$) given in Figure 4 is obtained.

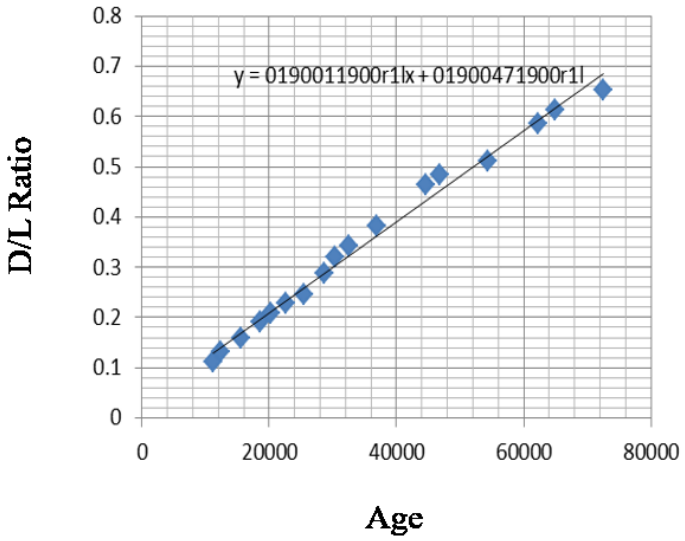

FIGURE 4. Calibration curve for D/L ratio

3.3. D/L Alanine Ratios of Samples

D/L Alanine ratios of samples obtained from HPLC analysis are given as mean values of triplicates in Table 3. The difference between the results of triplicates were found to be statistically similar ($p > 0.05$).

TABLE 3. D/L Alanine ratios of samples

Sample Number	D/L Alanine Ratio
Sample 1	0.996
Sample 2	0.999
Sample 3	0.996
Sample 4	0.997
Sample 5	0.998

3.4. Age Estimations

By using data given in Table 3 and the calibration curve given in Figure 4 the ages of the fossil samples were estimated as it is given in Table 4.

TABLE 4. Estimated age of samples

Sample Number	Estimated Age
Sample 1	107466
Sample 2	107800
Sample 3	107466
Sample 4	107577
Sample 5	107688

The fossil samples used in the amino acid racemization method, which are collected from Karabük, Safranbolu, Bulak River area and the age of these fossil samples were confirmed to be between 30 and 35 million years at the time of identification. But unfortunately the ages of the samples estimated through amino acid racemization method we found to be between 107466 and 107800, which are quite far from the expectations.

The reason of this difference could possible related to constructing calibration curve with the data in the literature, which are for ages between 2200 and 72400, where the ages of our samples are far beyond the upper age limit in the literature.

4. Conclusion

The results of this study has a great importance, because with this study we have added D/L Alanine ratios for fossil samples having ages between 30 and 35 million years to current literature. These observations should be confirmed again by supporting the ages of the same fossil samples by radiocarbon method.

It is also important to fill the D/L Alanine ratio gap between samples having ages between 72400 years and 30 - 35 million years, so that for further studies it could be possible to construct more accurate calibration curves, which may give much more precise estimations.

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