PERSPECTIVES IN PROTEIN AND AMINO ACID GEOCHEMISTRY

PERSPECTIVAS EN GEOQUÍMICA DE PROTEÍNAS Y AMINOÁCIDOS

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Abstract

Recent advances in protein and amino acids are presented. In spite of the interest on using protein and amino acids geochemistry for dating purposes decreased for some years, a better understanding of error sources and diagenesis processes allowed to obtain a powerful tool for geological use. Errors can be grouped in three different clusters: analytical error, sample depending error –intrashell, intragenus, intergenus and microenvironment– and palaeoenvironment depending error-thermal history, sediment geochemistry, moisture, diagenesis, etc. All these errors can be calculated and/or estimated. Amino acid geochemistry study has been focused on Quaternary dating: relative (Aminostratigraphy) and absolute dating (Aminochronology) of lacustrine, fluvial, marine deposits and mammal remains have been obtained. Protein preservation has been analyzed to ascertain fossil DNA preservation potential.

Key Words: organic geochemistry, proteins, amino acids, stratigraphy, geochronology, Quaternary.

Resumen

En este trabajo se presentan los avances más recientes en el uso de la geoquímica de proteínas y aminoácidos. A pesar de que el empleo de la geoquímica de aminoácidos y proteínas para datación disminuyó durante algunos años, un mejor conocimiento de las fuentes de error y de los procesos diagenéticos le han permitido revelarse como una herramienta muy válida para su empleo en Geología. Los errores se pueden agrupar en tres grupos diferentes: error analítico, error de la muestra –intraconcha, intragénero, intergénero y microambiental– y error paleoambiental-historia térmica, geoquímica del sedimento, humedad, diagenesis, etc. Estos tres errores se pueden calcular y/o estimar. El estudio de la geoquímica de aminoácidos se ha centrado en la datación del Cuaternario: se han obtenido dataciones relativas (Aminostratigrafía) y absolutas (Aminocronología) tanto de depósitos lacustres, fluviales y marinos como de restos de mamíferos. Se ha analizado la preservación de las proteínas para predecir la preservación de DNA fósil.

Palabras clave: geoquímica orgánica, proteinas, amino ácidos, geocronología, Cuaternario.

Introduction

The amino acid analysis of fossils was initiated 50 years ago (Abelson, 1954) although it didn't became widespread until the last 1960s and early 1970s when the development of faster analytical methods occurred. Initial works from these period came from Bada (1972, 1973), Bada and Prostch (1973), Hare (1969, 1971, 1974), Hare and Mitterer (1966), Hare and Hoering (1967). The initial controversies about this method mostly appeared because the behavior mechanism of the racemization kinetic, which was supposed to be a first order chemical reaction that allowed to obtain indirect and immediate datings, was poorly known.

Recent works (Wehmiller, 1984, 1993, 1995 and Wehmiller *et al.*, 1992, 1995) have provided enough criteria to evaluate the real possibilities of this method.

Amino acids and dating

In almost all living beings all amino acids are L-amino acids, that is, the amino group is placed at the "left side" of the molecule. Organisms incorporate amino acids as part of protein molecules into their skeleton. In mineralized skeletal components as shells, enamel teeth and bones, amino acids are located in intracrystalline and intercrystalline positions, being the former less prone for leaching. Amino acids in collagen, a non-full mineralized structure, are less protected.

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After the organism's death the racemization process starts and L-amino acids change into D-amino acids until the D/L ratio equals 1, that is, when the racemic equilibrium is reached. Nevertheless, amino acids with more than one C atom at their molecule, as isoleucine, can undergo a process called epimerization, which consists on the transformation of the L-enantiomer (L-isoleucine) into a different D-enantiomer (D-*Allo*-isoleucine) not present in living beings. In this case the equilibrium is reached at a D-*Allo*/L-Ile ratio value of 1.3.

Anyway the racemization/epimerization process can be considered as a first order reaction kinetics which is temperature controlled. The equation of this process is:

$$\text{L-enantiomer} \underset{K_D}{\overset{K_L}{\rightleftharpoons}} \text{D-enantiomer}$$

$$\ln\left(\frac{1 + D/L}{1 - D/L}\right) - C = (1 + K')\,K_L\,t$$

ln (D/L): amino acid enantiomers ratio.

C: method induced racemization

t: time $K' = K_D/K_L$

K_L: racemization reaction equilibrium constant

In the Biomolecular Stratigraphy Laboratory of the Madrid School of Mines, eight amino acid pairs of enantiomers are usually determined: alanine, valline, proline, D-*Allo*-isoleucine and L-isoleucine, leucine, aspartic acid, glutamic acid and phenylalanine. In some cases (dentine samples) L-hydroxyproline is also identified.

Up to now we have used a Goodfriend (1991) and Goodfriend and Meyer (1991)'s protocol for sample preparation method. For the racemization ratios measurements a Hewlett Packard 5890 ser. II Gas Chromatograph with a HP 6890 autosampler and NPD detector has been employed. The main problem of this method is the high gases consumption and a complex, time consuming, sample preparation method. Also, the requested sample weight is too large: 80 mg. Now we have changed to a Hewlett Packard HP 1100 HPLC analyzer with fluorescence detector that decreases the sample preparation time and allows to use very small samples (0.02 mg). The sample preparation method is largely automatized according to Kaufman and Manley's (1998) protocol.

Method constraints (Sources of error)

According to Murray-Wallace (1995) there are 6 factors (F) affecting the method reliability (Fig. 1): F1-Analytical error; F2-Intrafossil variation; F3-intraspecific variation; F4-Genus effect; F5-Small scale environmental influence; F6-big scale environmental influence.

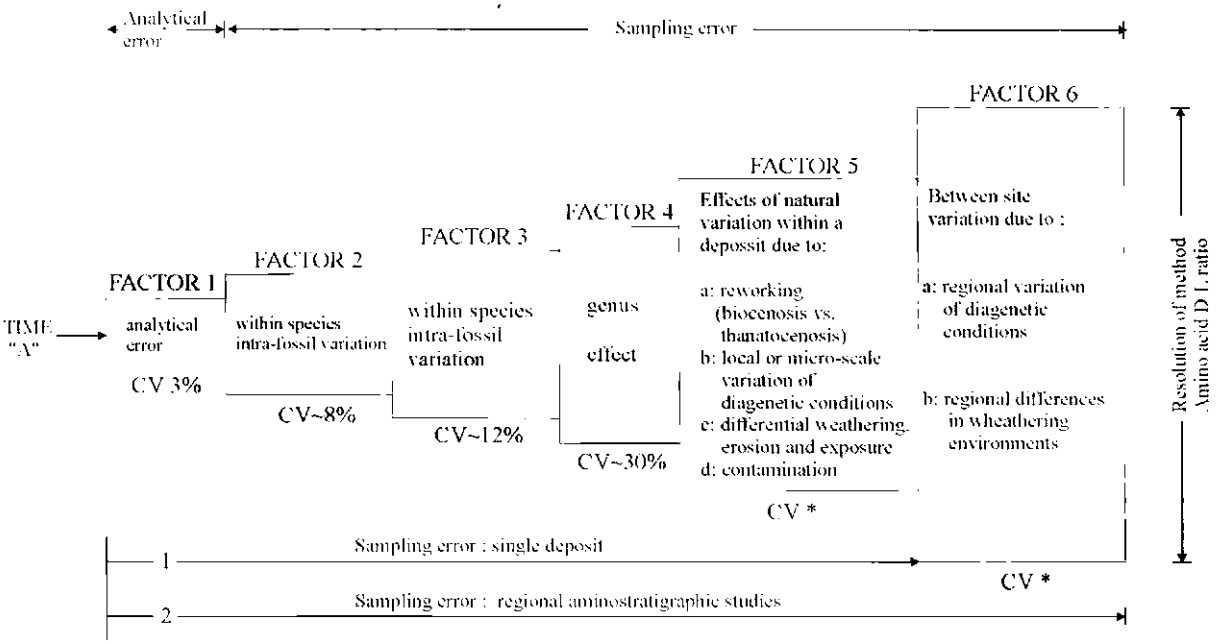

Figure 1: Sources of error in amino acid racemization dating (Murray-Wallace, 1995).

F1: It is an easy work to face this problem which can be solved according to usual laboratory practice standards.

F2: Some authors (Wehmiller, 1993) have reported intrafossil variations on mollusk shells. This means that slight amino acid racemization ratio differences, which appeared between samples taken from the umbo and from the ventral border, are due to the different internal shell structure. To avoid the differences in our experiments (Torres *et al.*, 1999a), Figure 2 and table 1, our sampling protocol recommends to obtain the samples from the same shell area (umbo). On gastropods, samples are obtained from the last whorl near the aperture or the parietal callus if present. Recently a very interesting phenomenon (Torres *et al.*, 2000a) has been observed on mammal teeth: a very high racemization ratio variation has been found on samples taken across the tooth root. This can be explained as a diagenesis-linked process which we avoided by drilling through the central part of the whole root, equidistant from the cement layer and the pulp cavity.

D/L Asp	m	s	n	min	max	CV	R
Panopea	0.18	0.04	13	0.12	0.25	22	0.68
Ostrea	0.25	0.03	7	0.19	0.19	12	0.81
Glycymeris	0.24	0.03	6	0.20	0.20	13	0.83

Table 1: Elemental statistics from intrashell amino acid racemization in samples from a Holocene spit bar in Huelva (Spain) (Torres *et al.*, 1999a). m = mean; s= standard deviation, n = number of analysis, min = minimum value, max = maximum value, CV= variation coefficient, r= correlation coefficient.

We have studied this F2 variation on recent material (Holocene) from a spit bar near Huelva, southwest of the Iberian Peninsula. (Torres *et al.* 1999) where a high variability of the aspartic acid racemization ratio was found between samples taken near the umbo and the ventral border of different mollusk shells: *Panopaea* sp, *Glycymeris* sp. and *Ostrea* sp., being noticeable that the highest racemization ratio values appeared in samples recovered from the ventral border of the shells. Obviously this study cannot be made on small shell representatives, or (such as) ostracods.

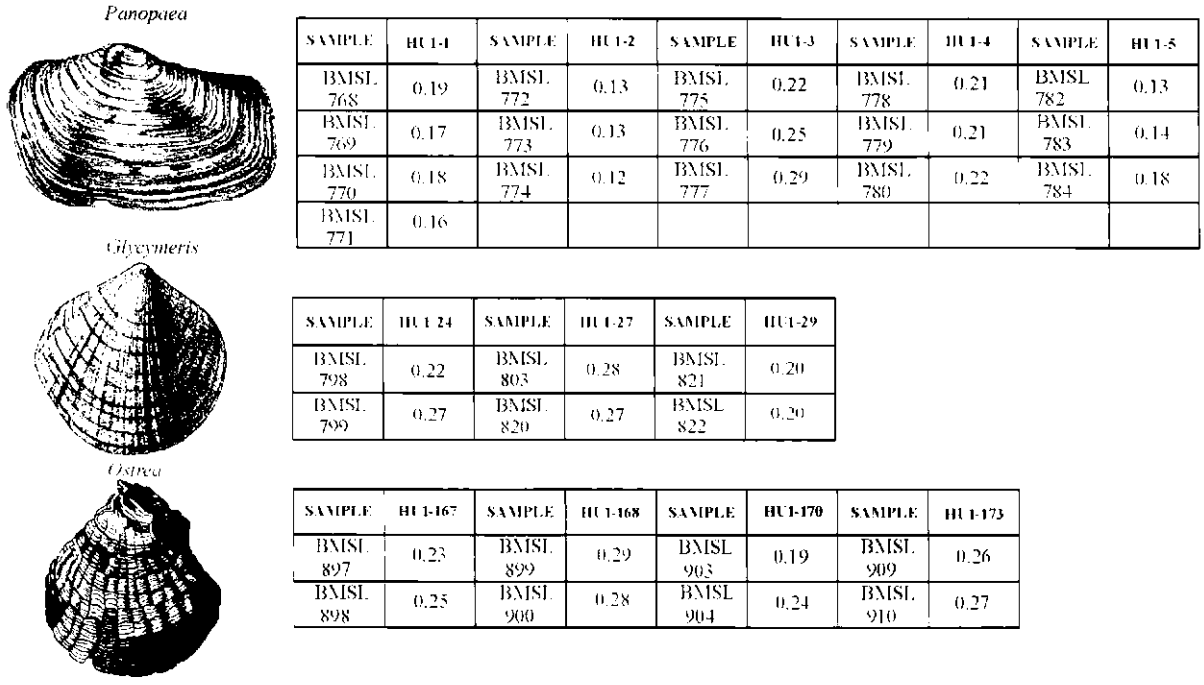

Figure 2: Intrasample variation in Holocene pelecypoda shells (modified from Torres *et al.*, 1999a).

F3: In spite of in most cases an absolute isochrony can be assumed for the whole sample set picked from a single bed, a variation in the racemization ratios obtained from different shells can be expected. This fact makes necessary to analyze at least five samples from each bed, but in most cases the laboratory experience will act as procedure guideline to determine the highest admissible standard deviation value and the requested minimum number of samples to be analyzed. When "statistical representative samples" such as ostracods or small mollusks are analyzed the measured racemization ratios are quite similar because of a great number of individuals needed (e.g. a standard ostracod sample consists of two thousand valves), which makes the standard deviation smaller.

F4: The genus effect has been noticed since the method began to be applied and can be a very important source of error. In fact, different genus representatives can never be analyzed together. In figure 3 the variation of racemization/epimerization ratios of leucine, glutamic acid and D-*Allo*/L-Ile isoleucine of different pelecypod, gastropod and algae representatives appear. It is possible to notice that there are differences between the reached racemization ratios of the different genera but being generally greater between markedly different "genus", as "Opercula" and "Chara" are.

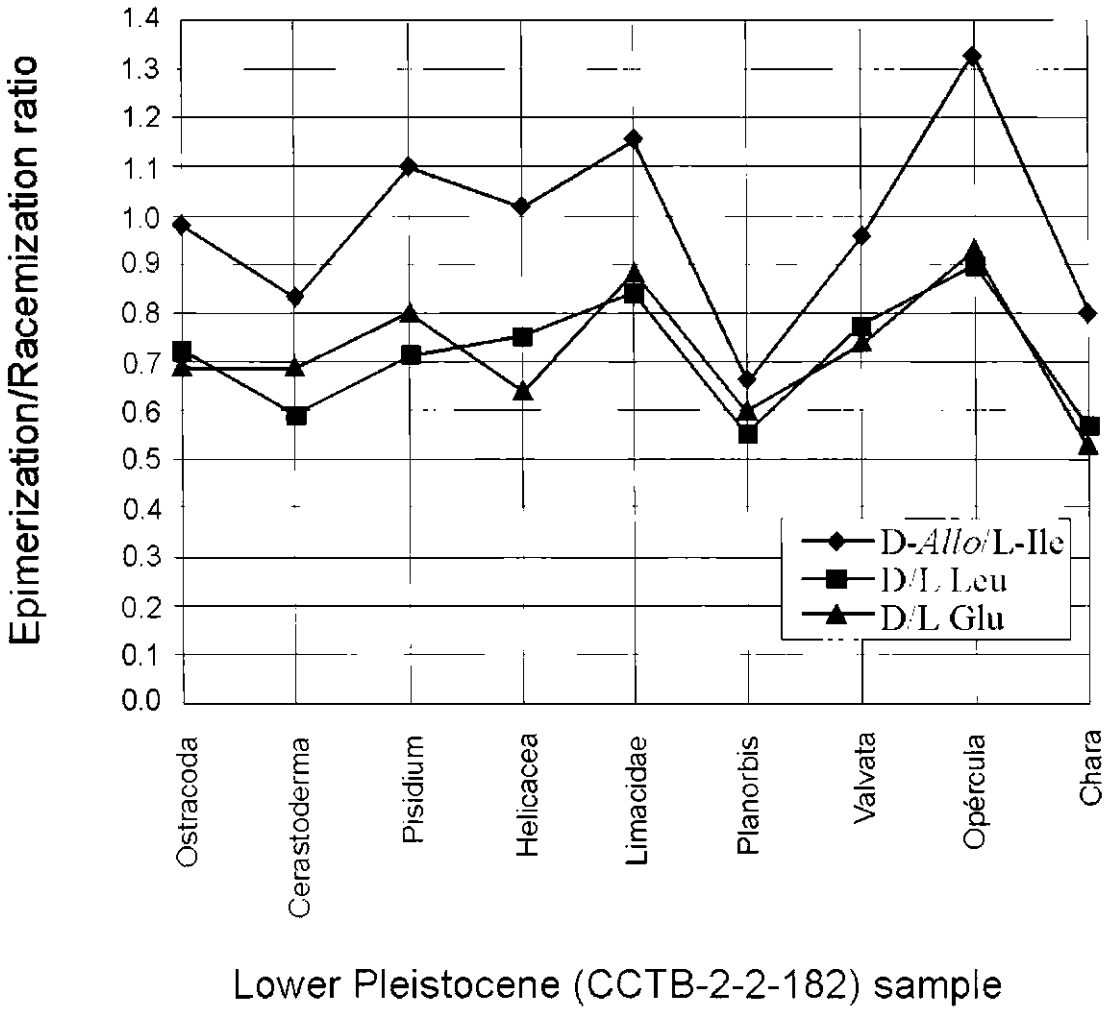

Figure 3: Epimerization/racemization ratios of isoleucine, leucine and glutamic acid from different genus representatives sampled at the same strata (Lower Pleistocene) in Cúllar-Baza Basin (Granada, Spain) (Torres, 1999).

The aforementioned differences appear to be more marked in D-*Allo*/L-Ile epimerization ratios. Nevertheless the genus effect diminishes when old samples are analyzed and the measured racemization ratios do not diverge in a markedly way as they do in very young samples. This can be clearly observed in the D-*Allo*/L-Ile epimerization histogram of different marine gastropod and pelecipoda genera from a raised beach of Cabo de Huertas (Alicante, East

of Spain) dated *ca.* 120 ka (own unpublished data).

F5: Small scale environmental variation. This factor comprises a wide variety of local environmental aspects such as: moisture, metallic cations, buffered environment, reworking, erosion and exposure to external agents, burial depth and contamination.

Moisture has been demonstrated to be one of the most important factors affecting amino acid racemization/epimerization: a total lack of moisture can inhibit this process and differential permeability in fossil bearing bed loci, or differential leaking, in caves, can explain high ratio values variability. pH values of interstitial water can also explain some differences.

Metallic cation presence, associated to detrital or chemical mineral grains (Mg^{++} and Cu^{++}) can produce amino acid chelation and, thus, a higher amino acid racemization rate can be produced.

Reworking has been used as a factor explaining high racemization values variance and can affect strongly the method reliability in samples from recent deposits: reworking factors are of very different origin: bioturbation by burrowing organisms can displace fossils from a bed to another making it older or younger. Anyway, a statistical analysis of an adequate number of analyzed samples can allow us to discard spurious results. Erosion of former deposits and further deposition of fossil remains into modern ones either by wave or river action is a well known phenomenon. In any case this seems not to be very important in old deposits, but in recent ones can be an important source of error making necessary to analyze a big number of samples and to discard erratic results.

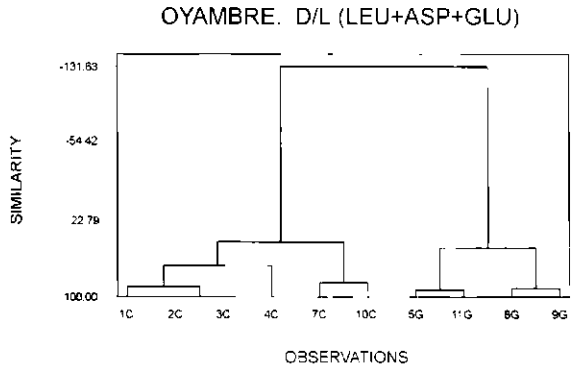

Figure 4: Similarity analysis of racemization ratios of Oyambre old beach of Eem age. C are pelecipoda (*Cardium* sp.) samples; G are gastropoda (*Murex* sp.) samples (Garzón *et al.*, 1996).

Also, reworking as an error source can never be discarded. Garzón *et al.* (1996) described a very paradoxal case in Oyambre (Cantabria, Spain) raised beach. Two marked clusters of amino acid racemization ratios were described (figure 4). The highest racemization ratios values appeared on *Cardium* sp. shells while the lowest, on gastropod (*Murex* sp.) samples. However, according to our experience (Torres *et al.*, 2000b), gastropods show higher racemization ratios than pelecypods. Thus, this fact can be explained in terms of reworking although due to the well known fragility of *Cardium* sp. shells, we finally conclude that gastropods (*Murex* sp.) shells were introduced in the sand beach bed by digging.

Burial depth can seriously affect the final amino acid analyses result. Very shallow buried fossils located in open air sites, which is very common in marine terraces, can suffer erosion, recent contamination and, the most important, non homogeneous exposition to solar heating. According to this fact and to our own experiences, raised beaches are the most difficult sites to be dated through amino acid racemization analysis being necessary to take samples, when possible, from trenches as deep as possible. In Mediterranean old shoreline deposits, green and magenta patches are very frequent coating on fossils and sediments recovered from holes 10 cm depth, which are interpreted as remains, even still living, of simple organisms that can proliferate under extreme environmental conditions. The amino acids of these organisms, either L or D amino acids, can be a very important source of error.

In brief: small scale environment variation-linked uncertainty can be corrected by means of careful statistical analysis of analytical results which, in some cases, will make necessary to analyze a large set of samples. The full sedimentological, taphonomical and diagenetical understanding of the sample bearing bed seems to be fundamental.

F6: Big scale environmental influence lies on two main factors: thermal history and diagenetic conditions. Because racemization is a thermal controlled first order chemical reaction, the regional thermal histories of the sampling sites play a very fundamental role in the final dating results. As an simple example, the estimated method range in Alaska an be estimated in > 4 Ma while in New Guinea is > 200 ka. According

to our laboratory experience, important racemization ratio differences can be expected between samples from the Mediterranean Border, Atlantic Coast, Balearic and Canary Islands and Outback. It is necessary to work with local models which can be exported from one zone to another. In figure 5 the calculated ages from different amino acid racemization ratios in samples from Guanche localities (Canary Islands) and raised beaches appear. In the lower part of the XY plot the calculated age from specific algorithms made from the Canary Islands thermal history appear while in the upper part the calculated ages are represented when the Outback of the Iberian Peninsula are used. In this case very marked differences in calculated ages appear, being source of unacceptable wrong values. Not all amino acid behave in the same way with thermal history differences and, therefore, it is difficult to export age calculation models from one area to another.

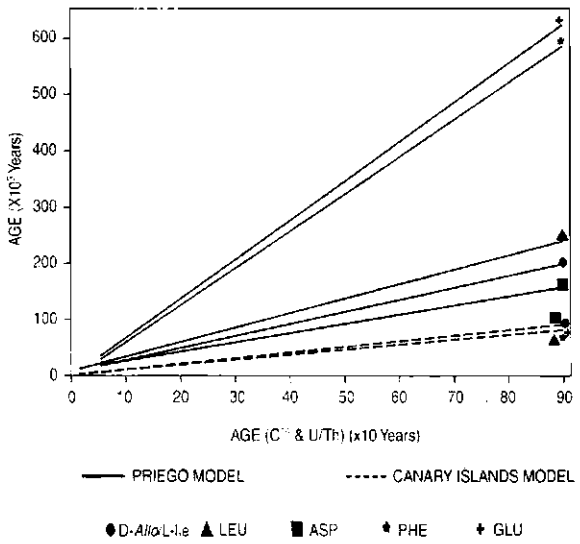

Figure 5: Comparison of results after the application of age calculation algorithms of Central Spain and the Canary Islands (dashed lines) to samples from the latter locality (García-Alonso *et al.*, 1996).

Same difficulties will appear linked to diagenetical history of the sites: racemization rates and, even, protein preservation through time, can be used together if samples come from different sedimentolodical and/or diagenetical environments. Caves with very homogeneous sedimentological and diagenetical conditions: stable thermal history, constant moisture, buffered environment etc. has allowed the Biomolecular Stratigraphy Laboratory to date an important number of localities where tooth protein was still well preserved. However, a total lost of proteins has been observed in fossil bones at open air sites or in shallow lacustrine environments under arid climate.

Which amino acid can be used

One of the first questions to be solved is which amino acid can be used for dating purposes. According to their racemization rates amino acids can be described as "fast" or "slow" depending on their activation energy values. The lower energy activation value appears in aspartic acid racemization process (*cf.* Torres *et al.*, 2001 and Goodfriend and Meyer, 1991) being the most adequate to date recent samples. In fact, (*cf.* Goodfriend, 1992; Goodfriend *et al.*, 1992) it was proposed for dating secular or even decadal events.

Glutamic acid, phenylalanine, isoleucine, leucine and proline with higher activation energy racemize with more paucity, being adequate for old samples. Furthermore, not all amino acids show similar reliability. Isoleucine seems to be the most reliable amino acid probably because D-*Allo*isoleucine is not present in the Nature and tardive contamination processes can be easier detected. Anyway we have analyzed the different amino acid reliability in samples from raised beach deposits from Cabo de Huertas (Alicante, Spain) and Garrucha (Almería, Spain). Marine terraces constitute one of the most problematic deposits to be dated because a high number of source errors converge on them: shallow burial, carbonate (and amino acid?) vertical migration to form caliches, differential (usually high) sun heating and contamination from the rizosphere, interstitial extremophyle organisms, guano from sea birds, etc. To analyze the reliability of some amino acids, Torres *et al.* (2000b), leucine, aspartic acid and glutamic acid racemization values from samples of a pelecipod shell (*Glycymeris* sp) were plotted against D-*Allo*isoleucine/L-Isoleucine epimerization values as a funcion of burial depth (figure 6). D/L Leu values were found to well correlate with D-*Allo*/L-Ile values for all burial depths. On the other hand, for both Glu and Asp, a good correlation with D-*Allo*/L-Ile values was found only for deep buried samples. This fact suggests that samples recovered from shallow or intermediate burial depth (less than 25 cm) may be diagenetically altered with respect to Asp and Glu.

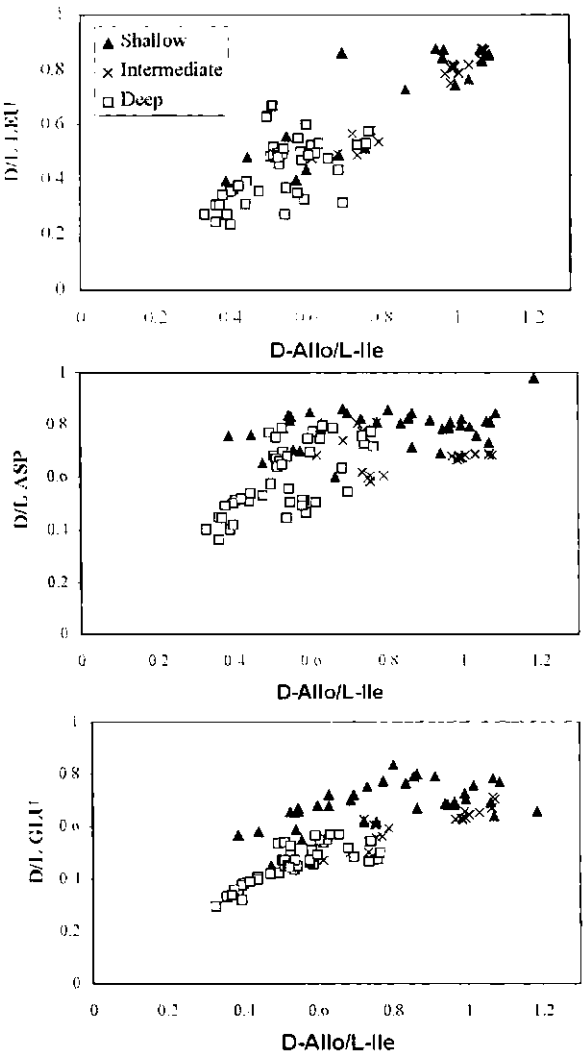

Figure 6: XY plots of D/L Asp, Glu and Leu values vs. D-Allo/L-Ile values in *Glycymeris* sp. samples from some raised beaches of the Cabo de Huertas (Alicante) and Garrucha (Almería) (Torres *et al.*, 2000b).

Because a correlation between age and terrace elevation can be established, it seems to be evident that amino acids from samples taken from the higher terraces will show the higher racemization/epimerization values, assuming a lack of neotectonic. The calculation of the Spearman's rank correlation between the aforementioned four amino acids racemization ratios and their rank position (elevation) demostrate that the order of reliability is: D-*Allo*/L-Ile>Leu>Glu>Asp. This means that in favorable environments, where homogeneous conditions have been maintained through time, as lacustrine deposits and caves, all the identified amino acids can be used for dating purposes while in samples from less favorable paleoenvironments, as marine terraces shelters, the best results are obtained through D-*Allo*/L-Ile and D/L Leu determination. Anyhow, our experience indicates that the best way to use this method is to handle the racemization ratios of the eight amino acids that we usually identify and check through statistics the lack of contamination or even the rightness of the genus, or even material, identification. In Figure 7 the cluster analysis calculated using five D/L ratios measured on different materials, recovered from a fossiliferous bed in Venta Micena (Granada, Spain) appears. This analysis reveals two amino acid racemization ratios clusters: fragments of gastropods (mostly Lymnaeaciidae) and opercula (from Bithyniidae). In this case there is a Gf sample (fragment of gastropod), which was formerly "wrong" classified, that according to the cluster where it was grouped, must be identified as opercula (O). This cluster analysis also reveals the importance of the intergenus-linked error. These samples, which are almost one million years old, still maintain the former differences between the proteins, made of different bonded amino acids, linked to their mineral structure (shell) building.

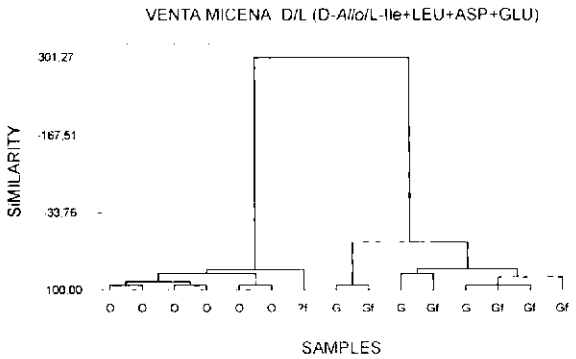

Figure 7: Cluster analysis of the amino acid racemization ratios (D-Allo/L-Ile, D/L Leu, D/L Asp, D/L Glu) from Gastropoda and Opercula remains recovered from Venta Micena (Granada, Spain), a Lower Pleistocene fossiliferous locality (Torres *et al.*, 1997).

Aminostratigraphy

Taking into account the problems involved in the immediate application of amino acid racemization rates in age calculation a first step could be the definition of an aminostratigraphical scale for local use. These scales must be based on amino acid racemization rates of similar genus (mollusca, ostracoda...) and are related only to a limited geographical realm whose thermal (palaeoclimatological) history had seemed to be

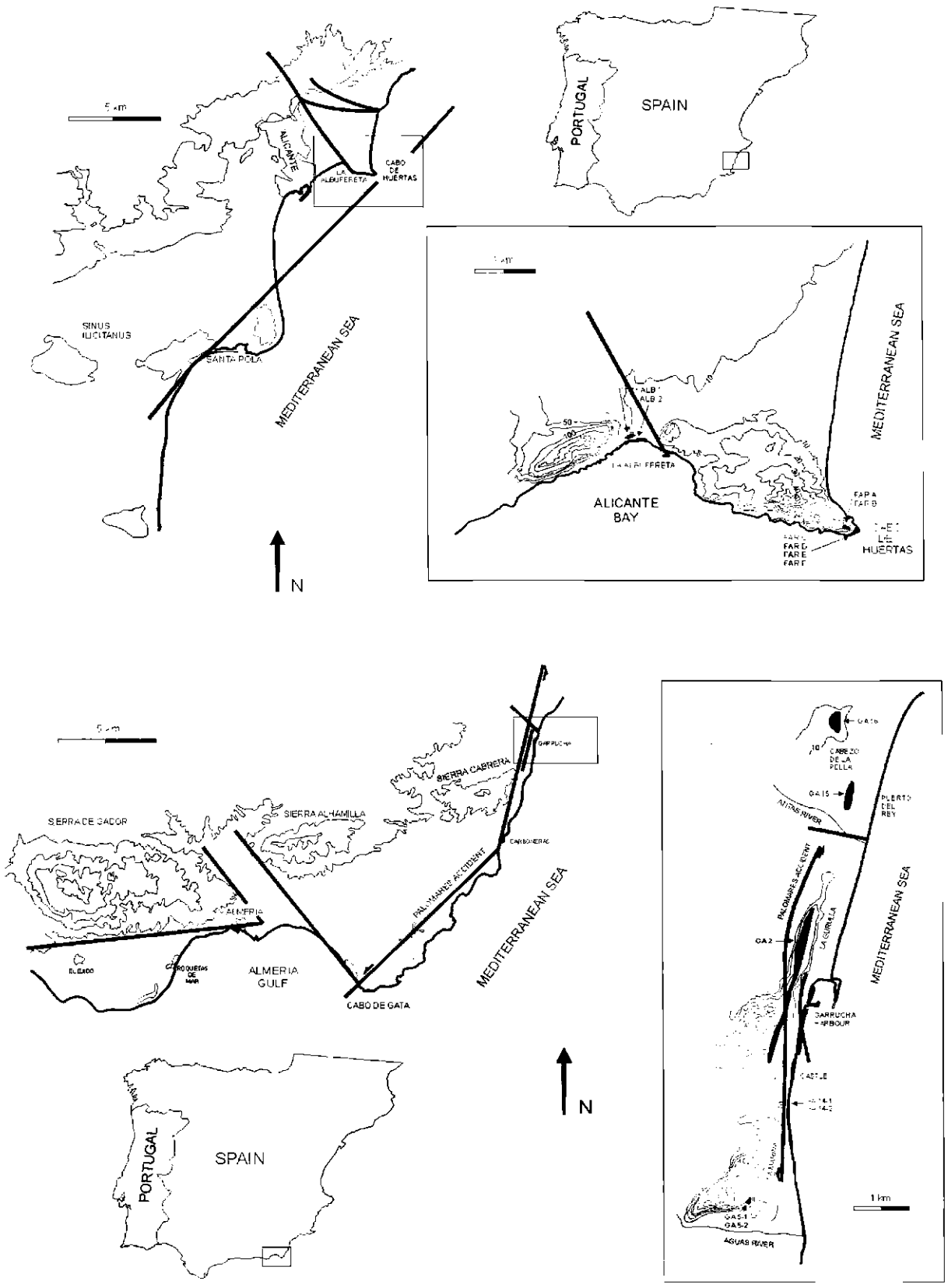

Figure 8: Geographical situation of marine terraces of Cabo de Huertas (Alicante) and Garrucha (Almería) (Torres *et al.*, 2000b).

similar during Pleistocene times as well as with a similar sedimentological environment. We widely use aminostratigraphy where algorithms for age calculations are not available. Therefore, aminostratigraphy is a powerful tool for geological correlation. Higher, lower or similar racemization ratios compared with the ones from the standard site indicate that the studied sample is older, younger or isochronous, respectively.

Thus, aminostratigraphy consists of arranging geological sites into a sequence on the basis of observed clusters of racemization ratios, established using representatives of one same zoological genus. This method is an excellent tool for the correlation of sea level oscillation-linked deposits: marine and fluvial terraces indicative of warm and stable climate periods (Kaufman, 1992; Hearty *et al.*, 1992; Miller and Mangerud, 1985), and is especially useful for the determination of neotectonics affecting Pleistocene deposits (Dumas *et al.*, 1988). In some cases, and mainly where almost continuous sedimentation has taken place, as in Cúllar-Baza continental Basin (Granada, Spain), it is necessary to produce numerical-age results, the most common being age-calibrated (^{14}C or U-series) results (Dumas *et al.*, 1988; Goodfriend, 1987; Hearty *et al.*, 1992; Wehmiller, 1993).

The analysis of Pleistocene raised beaches is one of the most satisfactory applications of aminostratigraphy. We have employed this method in the relative dating of marine terraces of the Cabo de Huertas (Alicante) zone (Figure 8), and Garrucha (Almería). According to the racemization ratio values it has been possible to establish both the aminostratigraphy of each of these zones and a whole aminostratigraphycal scale for the Mediterranean Border of the Iberian Peninsula, which probably is still incomplete. Our scale has been based on the D-*Allo*Isoleucine/L-Isoleucine epimerization ratios of *Glycymeris* sp., the most common pelecypod found in the marine Pleistocene record of the Iberian Peninsula. The results of this study demonstrated that the present height above the sea level of the marine terraces can never be used as a stratigraphic argument and that neotectonics played a very important role raising, sinking and tilting terraces.

According to the published data, *cf.* Torres *et al.* (2000b) (Figure 9), five high-sea-level events (aminozones) can be distinguished:

1. Uppermost Cabo de Huertas terrace (FAR-A)
2. La Marina (GA5-1,2) and La Gurulla (GA2)
3. Cabezo de la Pella (GA16)
4. Tyrrenian Upper terraces: Garrucha Castle (GA14-2, GA17) Puerto Rey (GA15) and upper Cabo de Huertas (FAR-C).
5. Tyrrenian lower terraces: Garrucha Castle (GA14-1) and lowermost Cabo de Huertas (FAR-D) terrace.

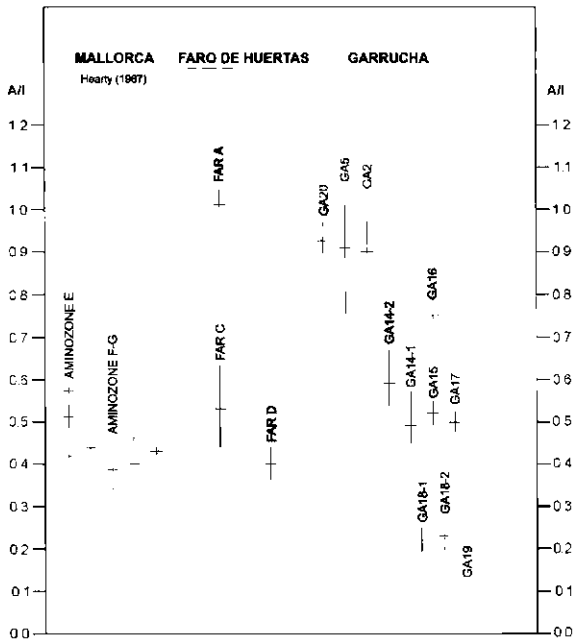

Figure 9: Aminostratigraphy of Iberian Mediterranean Border marine terraces (Hearty, 1987's aminozones are added) (Torres *et al.*, 2000b).

The aminostratigraphy of Pleistocene bears of the Iberian Peninsula, a very important challenge for us, has been established too. In this case the analytical method development was a "sweating, blooding and tearing" process that needed more than six years to be accomplished. The final success was reached after the adoption of a modified dialysis protocol published in Marzin (1990). The aim of this process is to analyze only homogeneous well preserved collagen proteins. Probably the good results obtained can be linked to the special good environmental conditions of caves where stable thermal history, constant moisture presence in sediments, buffered ($CaCO_3$) and low contamination opportunities favored the consistent accomplishment.

According to the results obtained (figure 10), it is possible to define an unquestionable

Middle Pleistocene *Ursus deningeri* V. Reich. aminozone, while *Ursus spelaeus* Ros. Hein. localities grouped in two very different sub amino zones, where the younger one (with lower aspartic acid racemization ratios) indicates that the last *Ursus spelaeus* representatives were coeval with brown bear (*Ursus arctos* Lin.). This aminostratigraphiycal scale will be very useful for archaeology and paleontology. Also confirms the Sima de los Huesos (Atapuerca, Burgos) man and bear remains age.

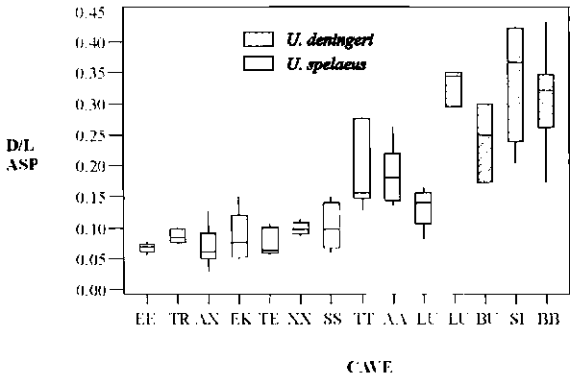

Figure 10: Aminostratigraphy of Iberian *Ursus deningeri* and *Ursus spelaeus* localities (Torres *et al.*, 2001).

Aminochronology

The transformation of the amino acids racemization or epimerization ratios into datings must be made through the use of calibrated methods: that is the analyzed samples must be correlated with datings obtained from, mainly, radioactive methods (^{14}C, U/Th or ESR). The steps to do this were: method validation, first age calculation ecuations definition, refining of calculation equations and application to the Venta Micena (Orce, Granada) site where very old human remains were found in a very rich mammal paleontological bearing bed.

After setting up the analytical method in our laboratory, we worked to obtain an external validation of the method, in order to control any kind of systematic error that might introduce a bias in the results. Three samples from an interlaboratory amino acid racemization exercise (Wehmiller, 1984) were also analyzed: ILC-A (*Saxidomus* sp. *ca.* 50 ka), ILC-B (*Mercenaria* sp. between 100 and 250 ka) and ILC-C (*Mercenaria* sp. ca 1.000 ka). These samples were analyzed by 11 laboratories in an exercise of interlaboratory control. The lack of bias and the validity of the process fit provided by our laboratory were confirmed (Table 2).

		D/L [SAMPLE (POWDER)]		
Amino acid		**ILC-A**	**ILC-B**	**ILC-C**
D-Allo/L-Ile	ILR	0.212 ± 0.072	0.54 ± 0.0162	1.215 ± 0.030
	LAB	0.180	0.650	1.245
D/L Pro	ILR	0.278 ± 0.068	0.595 ± 0.210	0.81 ± 0.26
	LAB	0.195	0.507	0.786
D/L Leu	ILR	0.196 ± 0.042	0.497 ± 0.098	0.833 ± 0.086
	LAB	0.182	0.444	0.849
D/L Asp	ILR	0.378 ± 0.056	0.705 ± 0.056	0.894 ± 0.158
	LAB	0.373	0.728	0.914
D/L Phe	ILR	0.239 ± 0.040	0.583 ± 0.059	0.873 ± 0.178
	LAB	0.220	0.608	0.885
D/L Glu	ILR	0.203 ± 0.022	0.432 ± 0.034	0.849 ± 0.070
	LAB	0.185	0.426	0.832

ILR Inter Laboratory Results (confidence intervals were calculated 2σ.

LAB Laboratory E.T.S.I. Minas.

Table.2: Comparison of D/L ratios between the inter-laboratory results exercise and the results obtained by the Biomolecular Stratigraphy Laboratory of Madrid School of Mines (Torres *et al.*, 1997).

Samples of molluscs from U/Th method dated Pleistocene fluvial travertine terraces in the Priego area (Torres *et al.*, 1994), were collected, prepared and analysed. The age of the sampled terraces ranged between 6 and 113 ka. In order to calculate the induced racemization during sample preparation, seven samples of alive gastropods were also collected, prepared and analysed. Likewise, D/L ratios of old samples, 100-250 ka and *ca.* 1000 ka, were used.

Spurious results were observed during interpretation of chromatographic analysis of the samples from Priego: anomalous D/L ratios in samples from the same stratigraphic level (travertine terrace). All the observed deviations appeared as an excess of D amino acids and seemed to be related with the observed *algae-fungi* mat covering some of the sampled gastropod shells. Some bacteria have D amino acids in their cellular walls (Leive and Davis, 1980) and algae and fungi mats are usual bacteria dwellers. By the time Venta Micena was sampled, we had already modified our recovery process in order to reduce the danger of contamination: the samples were obtained from deeper parts of the strata, where there is no influence by the atmosphere and sunlight. In order to avoid the influence of the spurious values, a previous single regression analysis of the D and L aminoacid pairs in the samples was performed. Table 3 shows the average ratios of racemization of Priego samples.

Age average (U/Th)	D-*Allo*/L-Ile	D/L Leu	D/L Asp	D/L Phe	D/L Glu
Today	0.000 ± 0.000	0.008 ± 0.0002	0.051 ± 0.008	0.019 ± 0.011	0.016 ± 0.012
6.000		0.041 ± 0.008	0.215 ± 0.051	0.029 ± 0.044	0.028 ± 0.022
12.500	0.149 ± 0.078	0.071 ± 0.018	0.257 ± 0.019	0.079 ± 0.009	0.070 ± 0.006
20.000		0.013 ± 0.009	0.346 ± 0.040	0.121 ± 0.031	0.105 ± 0.013
105.000	0.237 ± 0.016	0.423 ± 0.026	0.594 ± 0.026	0.479 ± 0.040	0.252 ± 0.031

Table 3: Average D/L ratios of mollusc samples from U/Th-dated travertine terraces in Priego (Cuenca, Central Spain) (Torres *et al.*, 1997).

Finally, prediction models were calculated using a set of 30 U/Th-dated samples from Priego: 21 freshwater gastropods, 6 terrestrial gastropods and 3 freshwater pelecipoda, as well as three samples of an inter-laboratory comparison exercise (Wehmiller, 1994). The racemization models are based on a first order reversible reaction (Bada and Protsch, 1973; Schroeder and Bada, 1976; Bada, 1985), the algorithms being based on a time square root ($\sqrt{t}$) adjustment (Goodfriend, 1987). This would appear to be justified since its use stabilizes the variance of the error that, in the case of time (t) adjustment, becomes progressively greater with growing age samples.

The models obtained are as follows:

LEU $\sqrt{t} = 1.17 + 11.38 \times \ln\{[1 + (D/L)]/[1 - (D/L)]\}$
(±0.62) (±0.88)
Correlation coefficient = 0.9774
(n = 33)

ASP $\sqrt{t} = -2.17 + 10.02 \times \ln\{[1 + (D/L)]/[1 - (D/L)]\}$
(±1.02) + (±0.85)
Correlation coefficient = 0.9740
(n = 32)

PHE $\sqrt{t} = 0.99 + 10.26 \times \ln\{[1 + (D/L)]/[1 - (D/L)]\}$
(±0.78) (±0.74)
Correlation coefficient = 0.9798
(n = 34)

GLU $\sqrt{t} = 2.16 + 12.44 \times \ln\{[1 + (D/L)]/[1 - (D/L)]\}$
(±0.65) (±0.82)
Correlation coefficient = 0.9816
(n = 36)

The algorithm selected for the isoleucine epimerization model, following the adjustment of different models, was the one commonly proposed by numerous authors (Mitterer, 1975; Goodfriend and Mitterer, 1988; Goodfriend, 1991). In this case the best fit was obtained through time (t) adjustment (c.c. 0.9872) rather than time square root (c.c. 0.9533). The model is as follows:

A/I $t = -34.99 + 267.14 \times \ln\{0.565/[0.565 - (A/I)/(1 + A/I)]\}$
(±25.8) + (±20.32)
Correlation coefficient = 0.9872
(n = 20)

Although the final equilibrium state is affected only by temperature, for the D-*Allo*/L-Ile epimerization reaction, it was demonstrated the existence of a relationship, in Foraminifera, between racemization percentages, age and the current mean annual temperature (CMAT) (Wehmiller, 1984). In our study, we have determined the CMAT for the areas of Priego (Torres

et al., 1994) and Redueña (Llamas *et al.*, 1995), which are climatologically similar, with a CMAT of 11-14 °C. Thus, it has been considered that the same models would be applicable. As regards the type of fossils under study, the initial results pointed to the existence of differences between amino acid kinetics depending on the genera of mollusca analysed. These differences were more marked in the oldest samples analysed. In 10 samples of ancient freshwater gastropoda (*Planorbis* sp. and *Radix* sp.) from Priego (Torres *et al.*, 1994) this effect was very marked for glutamic acid but negligible for leucine. A poor correlation between the racemization ratios of different amino acids might be explained in terms of early diagenesis (Goodfriend, 1991), e.g.: the loss of the most easily hydrolyzable amino acid from the terminal protein chains, aspartic acid, might be reflected in lower D/L ratios, this apparently being uncorrelated with the higher D/L ratios of the less easily hydrolyzable amino acids, such as isoleucine.

10 individual samples from the Priego area were dated according the D/L leucine ratios, obtaining an average value of 733±140 ka. For the Priego area a Current Mean Annual Temperature (CMAT) between 11 and 14°C was obtained. These values were coherent with those corresponding to this geographical location, and the CMAT was used according to the Wehmiller (1993) criteria. From our point of view, temperature might affect only the final D-*Allo*/L-Ile equilibrium stage. According to these results, other algorithm families were adjusted using only *Planorbis* sp. and *Radix* sp. remains, the adjusted models being as follows:

LEU $\sqrt{t} = 0.94 + 11.81 \times \ln\{ [1 + (D/L)]/[1 - (D/L)]\}$
(±0.63) (±0.70)
Correlation coefficient = 0.9870
(n=32)

ASP $\sqrt{t} = - 3.34 + 12.38 \times \ln\{ [1 + (D/L)]/[1 - (D/L)]\}$
(±2.31) (±2.24)
Correlation coefficient = 0.9050
(n=29)

PHE $\sqrt{t} = 0.48 + 15.64 \times \ln\{ [1 + (D/L)]/[1 - (D/L)]\}$
(±2.88) (±3.71)
Correlation coefficient = 0.8304
(n=34)

GLU $\sqrt{t} = 0.33 + 21.93 \times \ln\{ [1 + (D/L)]/[1 - (D/L)]\}$
(±1.44) (±2.48)
Correlation coefficient = 0.9476
(n=37)

In this case the best model found for D-*Allo*/L-Ile epimerization was the square root of time ($\sqrt{t}$) adjusted (c.c. 0.9495). Time (t) adjustment had a lower correlation coefficient value (c.c. 0.9359).

A/I $\sqrt{t} = - 0.02 + 21.85 \times \ln\{0.565/[0.565 - (A/I)/(1 + A/I)]\}$
(± 1.42) (± 2.31)
Correlation coefficient = 0.9495
(n=21)

Finally, at Venta Micena, only 21 analytical results were obtained from the 23 original samples, 2 being lost during the sample preparation process. The remaining samples could be classed into two groups: *Opercula* and *Gastropoda*. The former were 2-3mm sized, subcircular-ellipsoidal shaped dishes of aragonite, probably of *Bithynia* sp. Three different genera were grouped into *Gastropoda*.

First, a cluster analysis was performed with GC analysis results, three different groups being identified. One of these groups, which differed widely from the other two, included four samples with very low racemization rates, this disagreeing with the other two. These results were interpreted as being due to the influence of contamination, and were discarded. A second cluster analysis of the 17 remaining samples, revealed the existence of two groups: *Opercula*, along with an unidentified *fragmenta*, and Gastropoda (2 *Radix* sp., 1 *Bulimus* sp. and 6 unidentified gastropod *fragmenta*). In the second group a *fragmenta* sample presented very low racemization ratios, with the exception only of D- *Allo*/L-Ile. This result has been interpreted as a diagenetic effect and was also discarded prior to performance of the final cluster analysis. All the analyses were carried out on the basis of four amino acids (D-*Allo*/L-Ile, leucine, aspartic acid and glutamic acid) since some results for phenylalanine were missing. When the clustering was accomplished using complete results, phenylalanine included, the grouping obtained was the same.

Final age calculation was accomplished according to the first set of models for 7 samples grouped into *Opercula*, and according to the sec-

ond set of models for 9 samples included in the *Gastropoda* group (Table 4). The unidentified *fragmenta* was included in the *Opercula* group, this giving rise to a slight difference with respect to previously published data (Torres *et al.*, 1995a).

	D-*Allo*/L-Ile	D/L Leu	D/L Asp	D/L Phe	D/L Glu	Global
Opercula	778 ± 66	1174 ± 149	947 ± 18	909 ± 78	1062 ± 138	976 ± 65 ka
Gastropoda	1511 ± 318	1111 ± 258	591 ± 77	809 ± 144	1012 ± 107	1009 ± 130 ka

Table 4: Age calculations of Venta Micena samples (Torres *et al.*, 1997).

Taking into account the fact that the results obtained are independent measurements of the same parameter over a time interval, it is possible to calculate a global dating for each group (Table 4); it may be observed that age calculations for the *Fragmenta* group show a higher scattering than those for the *Opercula* group. Prior to computing a "general average dating", the former global results from (Table 3), were analyzed for each sample group, *Opercula* and *Gastropoda*. The test of the homogeneity of variances for the two groups shows that both are different, according to the Levene tests (p-value 0.002). The test for the equality of the means, taking into account the non-homogeneity of variance, demonstrates that the non-equability hypothesis cannot be rejected (p = 0.65).

To estimate the global mean from the means of the two sample groups, *Opercula* (1001 ka) and *Gastropoda* (933 ka), a weighted average has been calculated using the inverse of variances (1043 and 4212 ka^2) as weighting factors. The result obtained (Torres *et al.*, 1997) was 983 ka, with a variance of 836 ka^2 (983 ± 58 ka for the 95% confidence interval).

Recently (Torres *et al.*, 2001) have obtained the age calculation algorithms for D/L aspartic acid ratio of Pleistocene bears (Figure 11). For this purpose we have analyzed cave bear samples from three different localities:

1. A bone sample from Eirós cave (EE) was ^{14}C (AMS) dated (Grandal d´Anglade and Vidal Romaní, 1997) resulting 24,090 ± 440 a BP.

2. In the *U.spelaeus* bearing bed from La Lucia (LU) cave some small stalactites appeared, and a thin flowstone 2-3 cm thick sealed the bone bearing bed. The two calcite samples were U/Th dated ca. 77 ka.

3. A combination of electron spin resonance (ESR) and U-series dating methods on the remains of the Sima de los Huesos (BB) bear gave a probable date of 320 ± 4 ka Bischoff *et al.*, 1997).

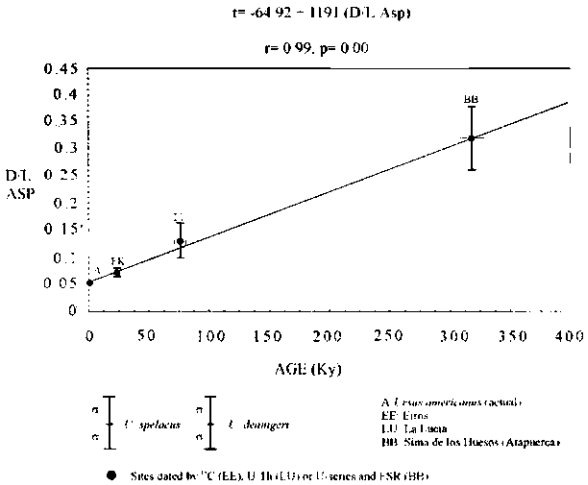

Figure 11: XY plot of average D/L asp ratios and radiometric age of the three sampled bear localities.

First of all, we want to state that the use of a previous dialysis process allowed a noteworthy accuracy in our analytical results, that was not achieved in all the former analyses carried out on non-dialyzed samples. In the first sets of samples we obtained racemization ratios of total (both free and bound) amino acids, that were too erratic to be seriously considered. The effect of L-hydroxyproline peak was also important because in some analyses (typically those carried out with an old Chirasil L-Val column), it overlaps the L-asp peak, and bizarre aspartic acid racemization ratio values were obtained. In our opinion the use of dialysis and a newly purchased Chirasil L-Val column and NPD detector could be the key to success.

The next step was to establish a statistical relationship between the mean D/L aspartic acid racemization values and radiometric ages from the three radiometrically dated localities: Eirós (EE- *U.spelaeus*), La Lucia (LU-*U.spelaeus*)

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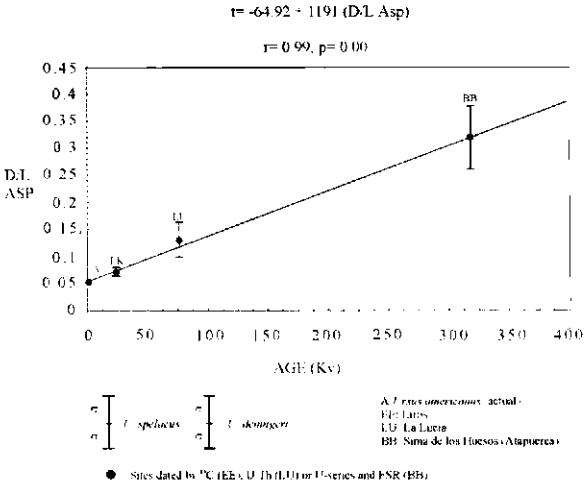

Figure 11: XY plot of average D/L asp ratios and radiometric age of the three sampled bear localities.

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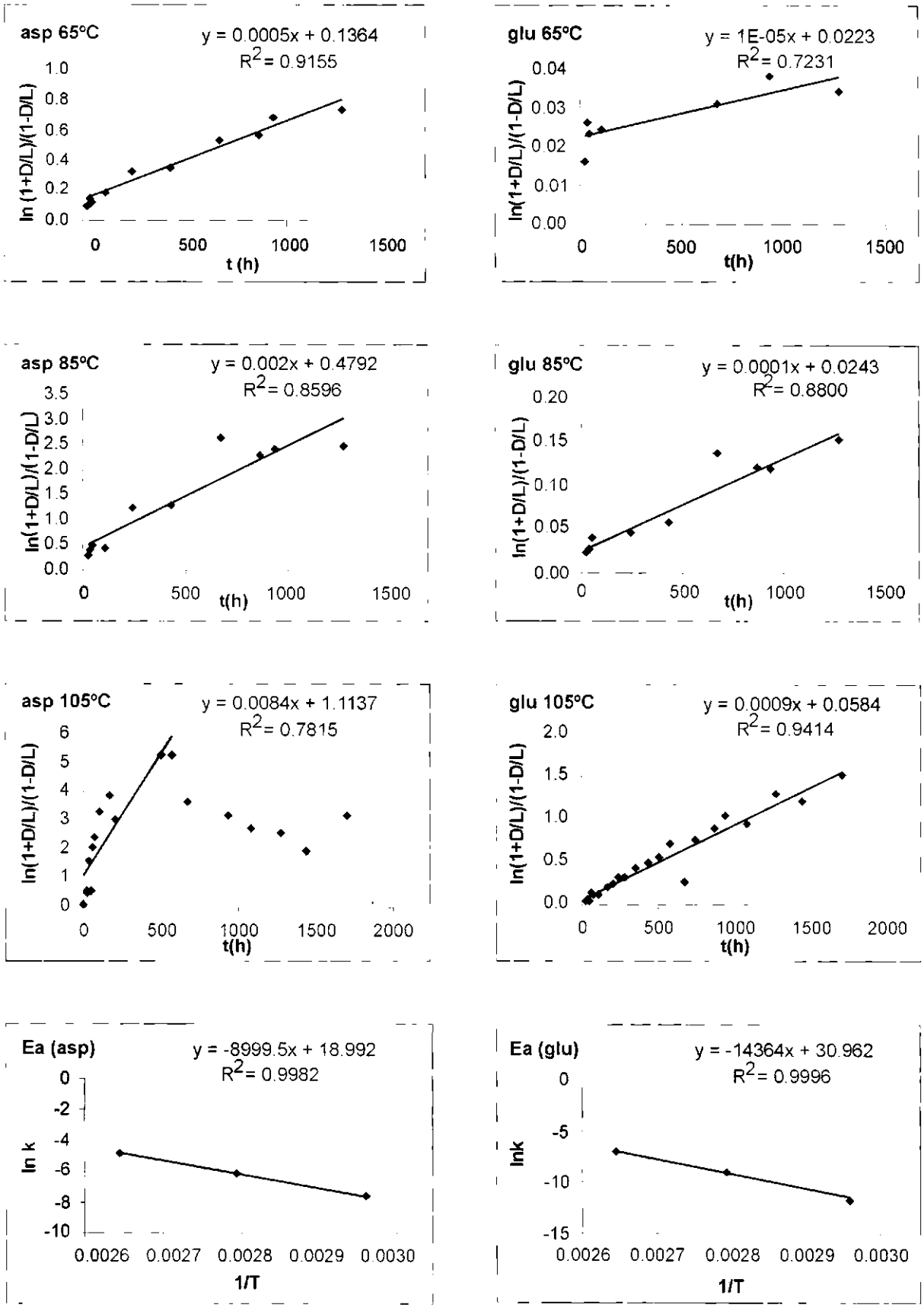

Figure 12: XY plot of $2k_L t = C + \ln \frac{1 + D/L}{1 - D/L}$ for aspartic and glutamic acids from the 65 °C, 85 °C and 105 °C kinetic experiments carried out on actual *Ursus americanus* Pallas dentine. XY plot of ln k_1 against 1/T (K^{-1}) for the estimation of the activation energies for aspartic and glutamic acids (Torres *et al.*, 2001).

and Sima de los Huesos (BB-*U.deningeri*). A simple linear correlation model was used. The resulting equation is:

t = -64.92+1191(D/L Asp)

where t is time (in years) and D/L the mean aspartic acid racemization value. The correlation coefficient value is extremely high (r = 0.998) and significant (p = 0.002).

We have decided to adopt the more convenient easier to handle linear model because, according to many authors (Masters and Bada, 1977; Mitterer and Kriasakul, 1989), for lower racemization ratios, comprised between 0.00 and 0.40 (as in our case) a linear racemization behaviour can be assumed. This linear D/L Asp ratio against radiometric age relationship cannot be considered an exception since a similar behaviour has been published for amino acids racemization in wool textiles (Csapò *et al.*, 1998).

The radiometric ages and mean aspartic acid racemization values appear in a XY plot, Figure 11; the origin ordinate value (0.05) is the induced aspartic acid racemization ratio measured in modern *U. americanus* dentine collagen (Torres *et al.*, 1999), which supports the correctness of the obtained correlation algorithm. We can conclude that there is good consistency between all of them. The age of *U. spelaeus* from La Lucia cave, deduced from U/Th dating of bear-bearing bed related speleothems, was 77 ka. According to the average aspartic acid racemization ratio, it could be slightly older, approaching 90 ka.

A linear aspartic acid racemization trend was found when average racemization ratio was regressed against radiometric ages of each locality. This means that the obtained mathematical equation can be confidently used for numeric age calculation. However, it is necessary to consider that local taphonomical conditions could play a very important role either in intersample variation or in intrasample racemization ratio variation.

Kinetics

Some experiments of dentine amino acids kinetics were made to determine their activation energy and to compare this results from the obtained from geological data. In our opinion it was necessary to verify the supposed one-way sense of the racemization process because some authors (Kimber *et al.*, 1986; Kimber and Griffin, 1987) described an "apparent kinetic reversal" in the >1000 Dalton peptide fraction in bivalve shells (*Ostrea* sp.). The "racemization kinetics" must be taken, at least, as the result of two different processes: one reversible (racemization) and another irreversible (hydrolysis) affecting proteins and peptides. The existence of a peptide hydrolysis resistance in the peptide bonds where hydrophobic amino acids are linked to aspartic acid exists has been reported, and since most of the racemization takes place at the terminal position of the peptide chains, an apparent racemization reversal can be produced. In our kinetic experiments, Figure 12, this effect was evidenced by taking into account that the obtained k_L value for an adjusted model:

$$2k_L t = C + \ln \frac{1 + D/L}{1 - D/L}$$

corresponds to a first order reversible kinetics model (FOK) where $k_D/k_L=1$ and D/L is the racemization ratio. The value obtained was k_L = 0.0042 h^{-1} for aspartic acid in samples from the first 576 h of experiment (excluding the "kinetics reversal" interval). In the experiment dentine powder was mixed with quartz sand and water soaked. Under nitrogen atmosphere was heated in a controlled stove at 105 °C .

Racemization rates in other amino acids (e.g., glutamic acid) are lower than in aspartic acid. The calculated k_L for glutamic acid it is k_L = 0.00045 h^{-1} for the 1704 h experiment.

Two additional kinetic experiments were carried out heating the dentine samples at 65 °C and 85 °C for 1272 h, in test tubes with quartz sand in the oven (full moisture conditions); the apparent rate constant k_L values are summarized in Table 5. From the plot of ln k_L versus T^{-1} (K^{-1}) following the Arrhenius relationships we have deduced that the activation energy for aspartic acid is 17.88 kcal/mol (R^2 = 0.9982) and for glutamic acid is 28.54 kcal/mol (R^2 = 0.996). No "kinetics reversal" has been observed for the glutamic acid at any heating temperature, nor for the aspartic acid at 65 °C and 85 °C. In any case, the best method for amino acid racemization dating is to analyze a set of different amino acids making it possible, through similarity analysis, to determine apparent kinetics reversal and to correct it.

The kinetic experiment has also shown the influence of moisture, also observed in bones

Temperature (°C)	Time (hours)	k_L Asp (h^{-1})	k_L Glu (h^{-1})
65	1272	$2.5 \cdot 10^{-4}$	$5.0 \cdot 10^{-6}$
85	1272	$1.0 \cdot 10^{-3}$	$5.0 \cdot 10^{-5}$
105	1704	-	$4.5 \cdot 10^{-4}$
105	576	$4.2 \cdot 10^{-3}$	-

Table 5: Apparent rate k_L constants of aspartic acid and glutamic acid from bear dentine kinetic experiments (Torres *et al.*, 2001).

(Hare, 1980) in the racemization rate of dentine amino acids. Only taking into account the samples that were heated up to 100 h, k_L values obtained range from $k_L = 0.0008\ h^{-1}$ for the dry samples to $k_L = 0.0170\ h^{-1}$ - 20 times greater- for the samples heated in the stove (full moisture conditions). The value obtained for the heating block samples (moisture saturated atmosphere) is an intermediate one: $k_L = 0.0070\ h^{-1}$, due to the formation of a condensation water ring in the upper part of the test tube.

Protein and amino acid preservation

One of the most exciting recent possibilities of amino acid analysis in fossils is to help in the search of fossil DNA. The search of fossil DNA is a painful and expensive process that sometimes ends with the determination of recent DNA, in most cases of human contamination origin. The previous determination of amino acids content, better if the presence of intact collagen molecules can be assured, can avoid to start working with rejectable material.

Protein and amino acid decay is a process which starts very soon after the organism death. We also have studied amino acid preservation in marine shells (Torres *et al.*, 1995b). In this case we analyzed free and bonded amino acids together. Samples came from the Gulf of Cadiz at the SW coast of Spain. A-samples from present day beach; B- samples from a spit bar 14-C dated 2235-2175 BP and C-samples from an offshore vibrocore dated *ca.* 10000 BP.

In Fig.13 we have represented the D+L aspartic acid peaks areas (:1000). The 350 (:1000) area corresponds to a standard of 0.0025 mg of total aspartic acid content in 80 mg of total weight sample. In A-samples there are large amounts of aspartic acid and no differences between *Chlamys* sp. and *Anomia* sp. analyses has been found. The spit bar samples (B) show an important decrease in aspartic acid content, mostly in *Glycymeris* sp. and *Cardium*, sp. fewer

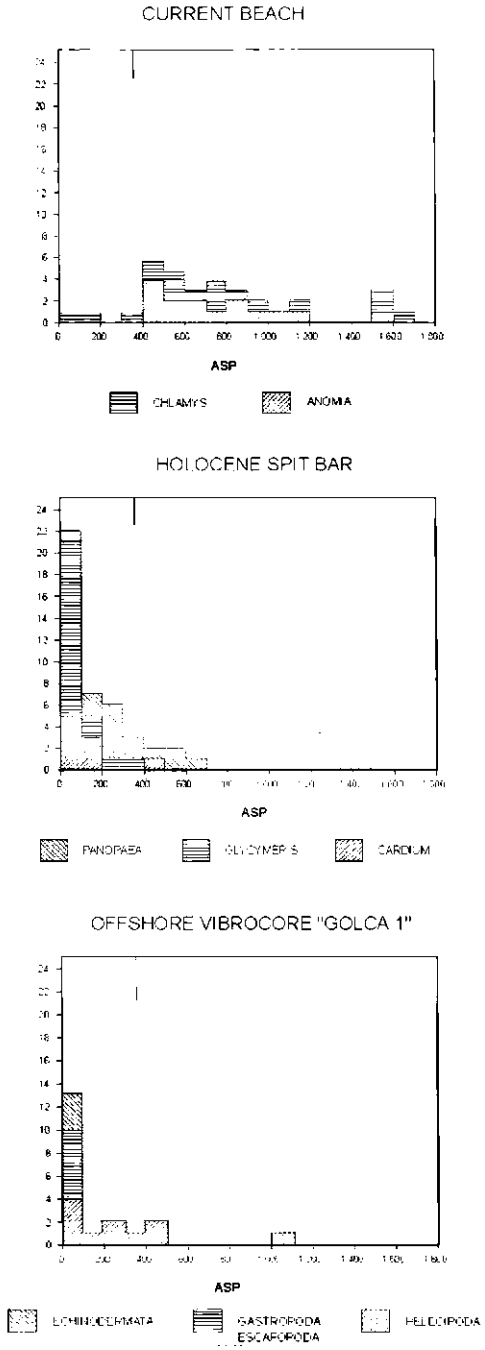

Figure 13: D+L aspartic acid chromatogram peak areas (:1000) of marine faunal remains from the Gulf of Cadiz area: current beach, Holocene spit bar (14-C dated in 2235-2175 BP) and vibrocore samples dated *ca.* 14,000 year old. The vertical tip indicates an aspartic acid concentration of 0.0025 mg in a standard 80 mg sample (Torres *et al.*, 1996).

in *Panopaea* sp. remains. Samples from vibrocore (C), mostly gastropoda and echinoderma (sea urchins), have only a little amount of amino acids whereas the highest values correspond to *Venus* sp. shells.

On mammal, fossil bear, dentine there is also a progressive collagen loss because of the collagen' triple helix deterioration and the molecule break down. Notwithstanding the peculiar favorable environmental conditions in caves (Fig. 14): stable thermal history allowed a surprisingly through-time good preservation of collagen. We worked on bear teeth dentine amino acid analysis with a previous 3500 Dalton dialysis in the way proposed by Marzin (1990) and Torres *et al.* (1999b). After the chromatographic analysis, because of the high accuracy of the volume injection reached with the HP 6850 autosampler, it has been possible to calculate in a quantitative way the total amount of bonded amino acids included in collagen molecules, since free amino acids were eliminated after dialysis.

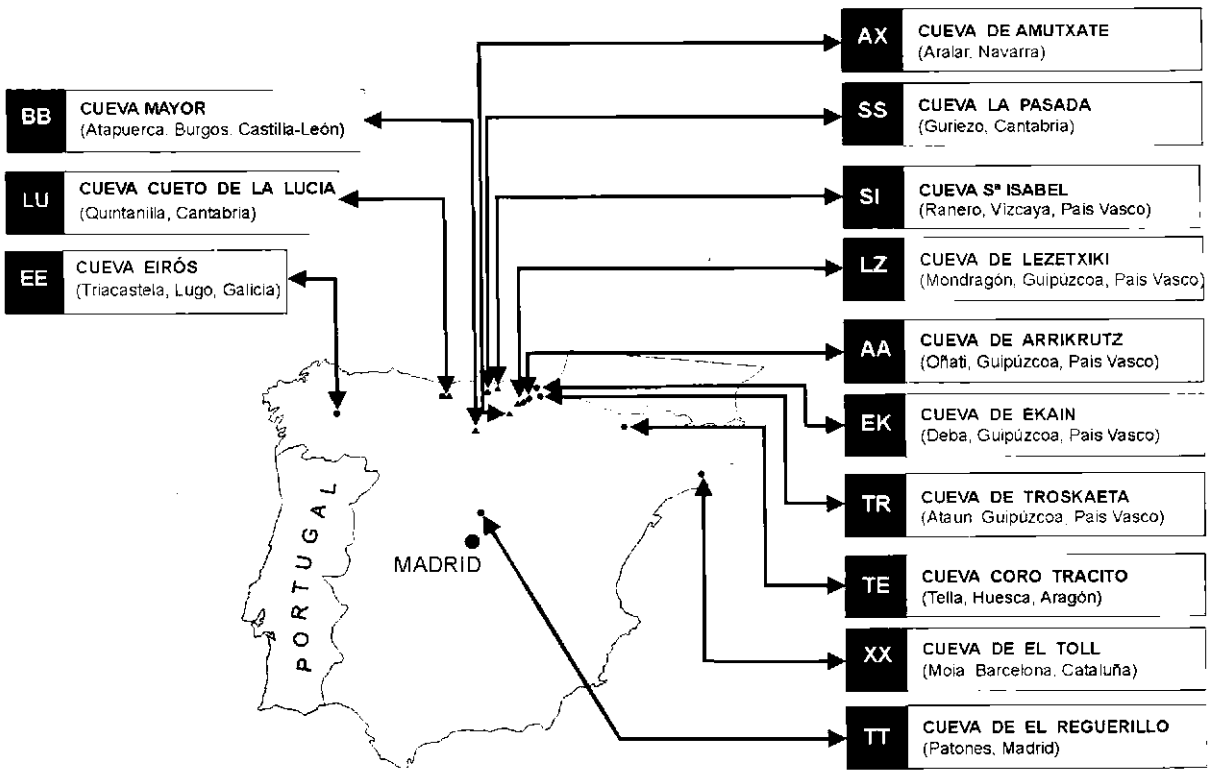

Figure 14: Geographical situation of sampled caves.

Results appear in Fig.15, where it is possible to observe a net differentiation between the oldest samples (Middle Pleistocene *ca.* 300 ka) from *Ursus deningeri* von Reichenau and the youngest ones of *Ursus spelaeus* Rosenmüller-Heinroth (Upper Pleistocene) and *Ursus arctos* Linneo (Holocene). This differentiation was produced because there is a dramatic decrease in the total amount of bonded amino acids (ASP) with older geological age. In fact other samples from Lower Pleistocene age bear (*Ursus etruscus* G. Cuvier) recovered from an open air site (Venta Micena, Granada) did not contained any bonded amino acid whereas samples of gastropoda and ostracoda of the same locality still contained amino acids amounts, large enough to be analyzed. This can be interpreted not only in terms of ageing of a no-all mineralized molecule but also because of paleoenvironmental influence because Venta Micena fossil bearing consisted of calcitic-dolomitic mudstones deposited in a shallow saline lacustrine environment under extreme hydrological stresses.

Conclusion

The marked improvements in chromatography analytical devices have allowed a more reliable case of racemization/epimerization analyses for dating purposes.

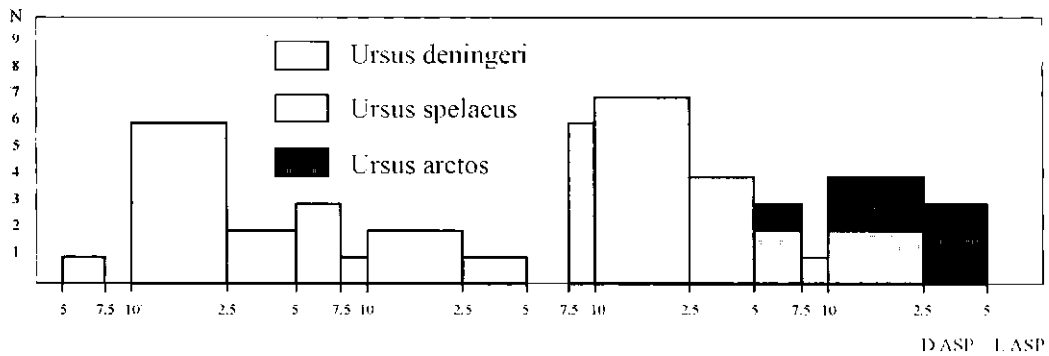

Figure 15: D+L aspartic acid peak areas histogram from bear teeth samples of different localities of Spain. Ursus deningeri samples are of Middle Pleistocene age. Ursus spelaeus samples are of Upper Pleistocene age. Ursus arctos samples are of Holocene age. The scale is semilogarithmic (Torres, 1999).

Anyhow, the sample-linked error, as well as, still remains other types of error, being necessary to know with detail the taphonomical and sedimentological conditions of the sampled site.

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