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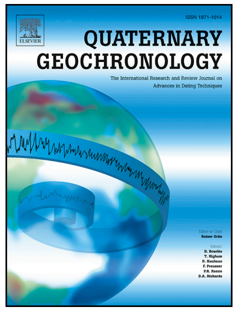

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Protein diagenesis in archaeological gastropod shells and the suitability of this material for amino acid racemisation dating: *Phorcus lineatus* (da Costa, 1778)

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Abstract

The inter- and intra-crystalline fractions of the topshell *Phorcus lineatus* recovered from modern specimens and shells from archaeological sites in Northern Spain covering Neolithic, Mesolithic, and Upper Magdalenian periods were examined for amino acid composition and racemisation over time. The main loss of proteins from the inter-crystalline fraction occurred within the first 6,000 years after the death of the organism. In contrast, the intra-crystalline fraction isolated by bleaching—with a different protein composition to that of the inter-crystalline fraction—appeared to behave like a closed system for at least 12.6 ka, as reflected by the lack of a significant decrease in amino acid content. However, changes in the relative

composition of the amino acids present in these shells occurred during this period. The concentration of aspartic acid remained almost constant with age within the intra-crystalline fraction and its contribution to the total amino acid content also remained the same. Good correspondence was obtained between Asx D/L values in unbleached and bleached samples and age, thereby allowing the dating of archaeological sites and the determination of chronometric age.

Key-words: *Phorcus lineatus*; inter- and intra-crystalline proteins; amino acids; microstructure; archaeology

Highlights:

- Inter- and intra-crystalline protein fractions of *P. lineatus* shells differ (amino acid proportions).
- The main loss of proteins (85-90%) from the inter-crystalline fraction occurs within the first 6 ka.
- The intra-crystalline protein fraction behaves like a closed-system.
- Asx D/L in unbleached and bleached *P. lineatus* specimens can be used for chronological purposes over ~ 13 ka.
- The percentage of aspartic acid remained constant in intra-crystalline proteins for over ca. 13 ka.

1. Introduction

Dating archaeological sites is crucial for interpreting changes in past human behaviour and for reconstructing environmental conditions. In recent decades, radiocarbon dating has become the most common approach for the chronological assessment of archaeological sites (Taylor, 1987; Stuiver and Brazuinas, 1993; Bronck Ramsey et al., 2004; Reimer et al., 2013). However, the limitations of the method (e.g. expense, time-constraints) makes it difficult to date large numbers of samples. In addition, radiocarbon dating is not suitable for dating samples older than 50 ka (Walker, 2005; Chiu et al., 2007; Reimer et al., 2013). In this context, it is therefore necessary to develop cheaper and faster methods for the chronological analysis of archaeological deposits. Amino acid racemisation (AAR) is one of the most used alternative methods to radiocarbon dating, as it is a faster and less expensive technique, allowing the dating of archaeological sites (Masters and Bada, 1977; Wehmiller, 1977). While AAR dating goes beyond the time range of the radiocarbon method, it has also been employed for dating Holocene sites (e.g. Bateman, 2008; Ortiz et al., 2009, 2015; Demarchi et al., 2011). Moreover, many studies have demonstrated that AAR is a satisfactory tool for dating a range of material from palaeontological and archaeological sites, such as teeth and shells (Helfman and Bada, 1976; Wehmiller, 1977; Julg et al., 1987; Bateman, 2008; Torres et al., 2013, among others).

Shell middens are unique archaeological deposits composed of large amounts of shells that were discarded by humans in the past after use or consumption of their content (Waselkov, 1987; Stein, 1992; Colonese et al., 2011; Gutiérrez-Zugasti et al., 2011). Understanding shell midden formation/transformation/erosion, as well as changes in subsistence strategies and settlement patterns of human groups, usually requires a large number of dates to be obtained. In this regard, AAR can be helpful for chronological purposes in this type of context, as a large number of samples can be analysed from a single horizon, thus facilitating the identification of time-averaging and the time over which a certain site formed (Kowalewski et al., 1998), or potential anthropogenic heating (Demarchi et al., 2011).

In Atlantic Europe, previous studies have centered on the use of the limpet *Patella vulgata* Linnaeus, 1758 for dating Palaeolithic, Mesolithic and Neolithic shell middens (Bateman, 2008; Ortiz et al., 2009, 2015; Demarchi et al., 2011). Recent studies of modern and archaeological *P. vulgata* in northern Spain have shown the potential of inter- and intra-crystalline proteins in the shells of this limpet for AAR geochronology (Ortiz et al., 2009, 2015; Demarchi et al., 2013a,b). In the studies of Demarchi et al. (2013a, b), artificial diagenesis was induced in the whole protein content (inter- and intra-crystalline proteins) and in the isolated intra-crystalline protein fraction (IcP) of modern *Patella* shells. The extent and racemisation of various amino acids provided data on protein diagenesis in modern limpets, showing that the IcP fraction behaves like a closed system and is thus suitable for geochronological purposes. Ortiz et al (2015) revealed the patterns of protein degradation in fossil *P. vulgata* representatives collected from several archaeological sites of diverse ages (ca. 34 ka cal BP to 5.8 ka cal BP) in Northern Spain, by examining the amino acid content and D/L values in the whole protein content and the IcP fraction separately. The main protein leaching from the inter-crystalline fraction was observed to occur within the first 6 ka after the death of the organism. In contrast, the IcP fraction, which has a distinct protein composition to that of the inter-crystalline fraction, appeared to behave as a closed system for at least 34 ka. Notwithstanding, Asx D/L values appeared to be suitable for geochronological purposes even when considering the whole protein fraction, likely to be due to rapid initial leaching of the inter-crystalline matrix (Ortiz et al., 2015).

In contrast, the inter-crystalline fraction of *Glycymeris* sp. shells does not seem to behave as a closed system, with the inter- and intra-crystalline proteins probably being similar (Demarchi et al., 2015, Ortiz et al., 2017). In spite of the high intra-sample variability, the extent of racemisation in unbleached *Glycymeris* sp. shells should be used with caution for AAR dating (Torres et al., 2014; Demarchi et al., 2015; Ortiz et al., 2017).

In a recent study, *Phorcus turbinatus* shells were subjected to AAR analysis in Ksâr 'Akil site (Lebanon). IcPs provided a robust fraction for AAR dating, showing closed-system behaviour (Bosch et al., 2015). However, the poor resolution of the D/L values obtained on multiple amino acids hampered the usefulness of AAR for chronological applications within this site, at least between 43 to 30 ka BP.

Therefore, further research is required to clarify the processes of protein preservation and degradation and the concomitant success of AAR for dating archaeological localities using molluscs. The quality of the archaeological record, as well as the range of species available and their abundance, makes northern Spain an excellent area to test dating methods such as AAR. Although the limpet *P. vulgata* is one of the most common species in archaeological sites in northern Iberia, other molluscs are also present, including the topshell *Phorcus lineatus* (da Costa, 1778) (syn. *Osilinus lineatus*). This mollusc, also known as toothed or thick topshell, is commonly found in archaeological sites of a range of ages in northern Spain (Table 1; González-Morales, 1982; Bailey and Craighead, 2003; Gutiérrez-Zugasti, 2009, 2011; Álvarez-Fernández, 2011), thereby allowing the analysis of long-term chronological sequences. Previous mineralogical studies (by SEM and X-ray diffraction) of *P. lineatus* shells (Fig. 2) have shown that they have a very thin calcite outer layer (with foliated and prismatic structures) and an inner nacreous aragonite layer (Mannino et al., 2003; Mannino and Thomas, 2007; Gutiérrez-Zugasti et al., 2015). These studies showed that the inner aragonitic structures of archaeological *P. lineatus* shells remained unaltered and well preserved over 8,000 yr time range, probably not undergoing recrystallization or post-depositional isotopic exchange (Mannino, 2000; Mannino et al., 2003; Mannino and Thomas, 2007), therefore likely to be suitable for AAR dating.

Therefore, here we provide the background to the successful application of AAR of *P. lineatus* for geochronological purposes. We report the systematic study of the behaviour of the whole protein content (inter- and intra-crystalline proteins) and the IcP fraction (bleaching tests) separately within this species. To this end, we did the following:

- Tested the patterns of diagenetic reactions and robustness of whole protein content and IcP fraction during artificial diagenesis (leaching tests at high temperature).
- Compared the diagenetic patterns in archaeological representatives within the IcP fraction and the whole-shell proteins. Shell specimens from archaeological sites of known ages (Fig.1, Table 1) covering the Upper Magdalenian (16.3-13.5 ka cal BP), Azilian (13.5-10.7 ka cal BP), Mesolithic (10.7-6.3 ka cal BP), and Neolithic (ca. 6.3-5.7 ka cal BP) periods were selected for analysis
- Evaluated the potential for artificial diagenesis at high temperature in order to mimic diagenesis in archaeological sites, by comparing results from heated and archaeological shells.
- Tested the suitability of AAR for dating purposes.

2. Material and methods

A total of 101 shell samples of *P. lineatus* were selected from 19 stratigraphic levels belonging to 8 archaeological sites located in the regions of Asturias and Cantabria (Northern Spain) and radiocarbon-dated to the Upper Palaeolithic, Mesolithic and Neolithic periods (Fig. 1; Table 1). Shells were stored at the Museum of Archaeology of Asturias (MAA) and the Museum of Prehistory and Archaeology of Cantabria (MUPAC). For comparative purposes, 5 modern specimens (collected alive) were recovered from Cue beach (Asturias), located close to the archaeological sites (Fig. 1).

Between 5 and 8 *P. lineatus* shells (analytical samples) from each archaeological level were analysed for amino acid content (Table 2). In the laboratory, shells were carefully sonicated and cleaned with water to remove sediment. Peripheral parts of the shells, approximately 20–30%, were removed after chemical cleaning of the sample with 2 M HCl.

For all shell samples, we drilled a small disc in the aperture—a procedure that has been shown to reduce intra-shell variability (cf. Murray-Wallace, 1995). Approximately 5–20 mg of carbonate was extracted from each shell and subjected to AAR analysis of total protein content (inter- and intra-crystalline proteins) and the isolation of IcP through bleaching (Penkman et al., 2008; Demarchi et al., 2013a). Samples from the aperture of modern specimens were also used to measure the amino acids in the total protein fraction and in the IcP fraction after leaching (heating at 140ºC over a range of time intervals).

2.1 Leaching

Leaching was performed following the protocol described in Canoira et al (2003) and Torres et al (2017). A set of 20 modern shell samples were placed in borosilicate glass ampoules, together with 2 g of quartz sand (deeply pre-cleaned by oven baking at 600°C for 6 h). Next, 120 ml of ultraclean water (HPLC-grade) was added with a syringe. The top of the ampoule was fitted into rubber tubing connected to a vacuum-N_2 line, being alternately exposed to vacuum and N_2, a procedure repeated three to four times to flush out all the air, following Kriausakul and Mitterer (1978), Goodfriend and Meyer (1991) and Canoira et al. (2003). The ampoule was later sealed under nitrogen. The ampoules were placed in a rack and put in an oven at 140ºC.

Two ampoules with quartz sand were removed at the following intervals: 1, 2, 4, 6, 8, 24, 48, 72, and 240 h. The ampoules were opened and dried. Shell samples were separated, washed with distilled water, sonicated, and vacuum-dried. They were then analysed for total amino acid content and IcP fraction after bleaching.

After heating, 100 mL of the supernatant water was also analysed for the amino acids leached into the water (THAAw).

2.2 Bleaching

Powdered shell samples (from archaeological levels and leaching experiment) were used to isolate IcPs. The shell particles measured less than 500 µm, following Demarchi et al. (2013a, p. 151), a size for which bleaching is likely to be most effective. We exposed these samples to 12% sodium hypochlorite (NaOCl) for 48 h—a time reported to be the optimal bleaching period for some molluscs (Penkman et al., 2008; Demarchi et al., 2013a).

For each fraction, 50 μL of NaOCl per mg of powdered shell was added to accurately weighed subsamples at room temperature. To ensure the complete penetration of the oxidising agent, the vials containing the powders and the bleach were shaken every 24 h. The bleach was then removed, and the powders were rinsed five times in ultrapure water and once in HPLC-grade methanol, with centrifugation for 4 min between each rinse to minimise the removal of powder. Finally, the samples were air-dried overnight.

2.3 Amino acid analysis

Amino acid concentrations and racemisation/epimerisation ratios were quantified using a HPLC, following the sample preparation protocol described in Kaufman and Manley (1998) and Kaufman (2000). This procedure involves hydrolysis, which was performed under an N_2 atmosphere in 20 μL/mg of 7 M HCl for 20 h at 100ºC. The hydrolysates were evaporated to dryness in *vacuo* and then rehydrated in 10 μL/mg of 0.01 M HCl with 1.5 mM sodium azide and 0.03 mM L-*homo*-arginine (internal standard).

Samples were injected into an Agilent HPLC-1100 equipped with a fluorescence detector. Excitation and emission wavelengths were programmed at 230 nm and 445, respectively. A Hypersil BDS C18 reverse-phase column (5 μm; 250 x 4 mm i.d.) was used for the analysis.

Derivatisation was achieved before injection by mixing the sample (2 μL) with the pre-column derivatisation reagent (2.2 μL), which comprised 260 mM isobutyryl-L-cysteine (chiral thiol) and 170 mM o-phtaldialdehyde, dissolved in a 1.0 M potassium borate buffer solution at pH 10.4. Eluent A consisted of 23 mM sodium acetate with 1.5 mM sodium azide and 1.3 mM EDTA, adjusted to pH 6.00 with 10 M sodium hydroxide and 10% acetic acid. Eluent B was HPLC-grade methanol, and eluent C consisted of HPLC-grade acetonitrile. A linear gradient was performed at 1.0 mL/min and 25ºC, from 95% eluent A and 5% eluent B upon injection to 76.6% eluent A, 23% eluent B, and 0.4% eluent C at min 31; and then with a progressive gradient at 1.07 mL/min and the following percentages: 46.2% eluent A, 48.8% eluent B, and 5.0% eluent C at min 95. As a laboratory routine, we separated glycine (Gly) and the D and L peaks of the following amino acids (Fig. 1-Supplementary Data): aspartic acid and asparagine (Asx); glutamic acid and glutamine (Glx); serine (Ser); alanine (Ala); valine (Val); phenylalanine (Phe); isoleucine (Ile); leucine (Leu); threonine (Thr); arginine (Arg); and tyrosine (Tyr).

2.4 Data screening of the AAR analyses

A total of 108 powdered samples taken from the aperture of archaeological *P. lineatus* shells were analysed for amino acid content. The same 108 samples were also used for the bleaching experiment. Of these samples, 14 results (12.9% of the data- 3 in El Penicial, 3 in Bricia-A, 3 in Bricia-C, 1 in El Mazo-101, 1 in El Toral III-21, 2 in Mazaculos II-A2, and 1 in level 24 of La Riera) were excluded because Asx and Glx D/L values fell off the covariance trend (cf. Kaufman, 2003, 2006; Laabs and Kaufman, 2003) (Supplementary Data) and/or because of abnormally high D/L values, characterised by Asx D/L and Glx D/L values falling outside the 2σ range of the group (cf. Hearty et al., 2004; Kosnik and Kaufman, 2008). These samples also showed a low amino acid content. A similar percentage of the data set from bleached samples was also excluded following the same rejection criteria exposed above, coinciding in most cases with the outliers identified for unbleached samples. Most of the samples with high D/L values may have been subjected to anthropogenic heating (3 in El

Penicial, 1 in Bricia-A, 1 in El Mazo-101, 1 in El Toral III-21, and 1 in level 24 of La Riera), as they showed a similar pattern to that of heated ostrich eggshells (Brooks et al., 1991; Crisp, 2013) and suspected burned *P. vulgata* shells (Demarchi et al, 2011), i.e., the sum of total amino acid concentrations were considerably lower, especially for Asx, Ser, Thr and Arg. Each result and the samples rejected are shown in the Supplementary Data. The data used in the following sections are only from the screened samples and do not include outliers. None of the 20 powdered samples of modern *P. lineatus* shells that were analysed for total amino acid content or the same 20 samples that were bleached for the isolation of the IcP fraction were rejected.

3 Modern *P. lineatus* shells

3.1 Amino acid concentration and composition

The total concentration of amino acids in modern shells was ca. 386 nmol/mg, whereas in the IcP fraction registered *ca*. 16 nmol/mg (Table 3). Thus, the latter accounted for around 4.2% of the total proteins in these shells (Fig. 3A).
The amino acid composition of the inter- and intra-crystalline proteins in modern shells also differed, as the percentage of the individual concentration of Asx ([Asx]) was higher in bleached (40%) than in unbleached samples (14%) (Table 3), the relative proportion of Ala and Gly being higher in the latter (Fig. 3C).

3.2 Amino acid D/L values

The mean Asx, Glx, Ala, Ser D/L values and D-aIle/L-Ile values in bleached and unbleached modern *P. lineatus* shells are shown in Fig. 3B. We selected these amino acids because they account for a considerable percentage of the amino acid content in modern shells (Fig. 3C). Asx, Glx, and Ala were the amino acids with highest D/L values in unbleached samples. In contrast, Asx, Glx, Ala, and Ser D/L and D-aIle/L-Ile values were higher in bleached samples than in unbleached ones, the differences between Ser and Asx being significant.

3.3 Discussion

The IcPs in modern *P. lineatus* shells accounted for a small fraction with respect to the total protein content (ca. 4.2%) (Fig. 3A). Acidic amino acids were not abundant in the whole shell, representing only 22%. The relatively low percentages of acidic amino acids found in *P. lineatus* may be explained by the aragonitic composition of the shell (Mannino et al., 2003; Mannino and Thomas, 2007; Gutiérrez-Zugasti et al., 2015), as the presence of acidic and Asp-rich proteins is usually linked to calcitic structures (Gotliv et al., 2005; Marin et al., 2012). In the aragonite-dominated shells of *Margaritifera falcata* and *Bithynia tentaculata,* Asx and Glx account for a low percentage (ca. 15-25%) of the amino acid content, although in *Corbicula fluminalis* is ca. 45% (Penkman et al., 2008).
In contrast, acidic amino acids accounted for 45-50% of the IcP fraction (Fig. 3C). Similarly, in some other mollusc shells, the percentage of Asx has been reported to increase after bleaching (Penkman et al., 2008). This may be explained by the strong binding of acidic amino acids to the mineral matrix observed in ostrich eggshell (Demarchi et al., 2016).

Moreover, we observed that the relative composition of other amino acids (Ala, Gly, Leu, Val) in inter- and intra-crystalline fractions was dissimilar. This observation indicates that the inter- and intra-crystalline protein compositions differ, thus potentially affecting the AAR rates (Fig. 3B) (Penkman et al., 2008; Crisp, 2013; Demarchi et al., 2013a).

It is worth noting that, coinciding with previous studies, the D/L values were higher in the IcP fraction of modern *P. lineatus* than in the whole shell. The differences found in the concentration of amino acids and D/L values between the inter- and intra-crystalline proteins were in agreement with the findings of Sykes et al. (1995) and Penkman et al. (2007, 2008), who observed distinct racemisation rates in these fractions in a variety of mollusc shells. These results could be due to the removal of certain proteins and amino acids (mainly free amino acids-FAA) from the inter-crystalline matrix of the shells during bleaching (cf. Penkman et al., 2007, 2008). According to Penkman et al. (2008), the loss of FAA—which tend to be more highly racemised than the total hydrolysable amino acids (THAA) (Mitterer and Kriausakul, 1984)—in the inter-crystalline fraction during diagenesis produces a decrease in D/L values for the THAA of the whole shell. In this regard, the higher concentration of free amino acids (which are the most highly racemised) within the IcP fraction (Fig. 3A) may explain the higher D/L values obtained in the intra-crystalline fraction compared to whole shell (Fig. 3B). Other processes should also be taken into account, such as the different contribution of the distinct amino acids to the proteins entrapped within the biomineral, as some amino acids may be more tightly bound to the mineral (Demarchi et al., 2016). Also, the position of each amino acid in the protein chains can cause a variation in degradation rates (Kriausakul and Mitterer, 1980; Wehmiller, 1980, 1993; Mitterer and Kriausakul, 1984), i.e., the rate of racemisation differs depending on the sequence position of amino acids, with most amino acids racemising only when in a terminal position.

4 Behaviour of shell proteins during artificial diagenesis

4.1 Amino acid concentrations vs. heating time

The total concentration of amino acids decreased with heating time in unbleached samples (Fig. 4). In contrast, the total concentration of amino acids remained similar during artificial diagenesis in bleached *P. lineatus* shells.

After 6 h leaching at 140ºC, approximately 60% of total amino acid content of unbleached shells remained, while almost all of amino acids in bleached shells were retained after 240 h of leaching at 140ºC (Table 2).

4.2 Amino acid composition vs. heating time

- ***Unbleached***

The proportion of Asx remained similar over time in the 140ºC experiment (Fig. 5A, Table 5 Supplementary Information), as did that of Phe, Ile, Leu, and Thr. Only after heating at 140ºC for 240 h did the percentages of Glx and Val increase, while that of Arg decreased; this amino acid is very labile. The percentages of Ser and Gly decreased with heating time after 1 h, whereas that of Ala increased.

- ***Bleached***

The variation of the percentages of each amino acid in the IcP fraction with heating time did not precisely reproduce what was observed in unbleached shells (Fig. 5B, Table 6 Supplementary Information), although in most cases the pattern was the same, i.e., the percentage of Ala, Glx, and Val increased, while that of Ser decreased. In contrast to unbleached samples, the percentage of Gly increased, while that of Thr decreased.

4.3 D/L values vs. heating time

The D/L values increased with time in unbleached and bleached shells (Fig. 6). As expected, the highest D/L values for all amino acids were observed after heating for 240 h, thereby indicating that rates of racemisation/epimerisation in *P. lineatus* shells are regulated by temperature and time.

- ***Unbleached shells***

Asx showed the most rapid racemisation rate, followed by Phe, Glx, and Ala (Fig. 6).

- ***Bleached shells***

The IcP fraction in modern *P. lineatus* shells showed higher D/L values than the inter-crystalline one (Fig. 6). In this case, Ala and Phe showed the most rapid racemisation rates, followed by Asx and Glx, although Asx showed the highest D/L values before 8 h.

4.4 Discussion

The heating experiment at 140ºC confirmed the distinct protein composition of the inter- and intra-crystalline fractions, as the percentage of each amino acid in unbleached and bleached samples differed (Figs. 5A, 5B). Moreover, contrary to the unbleached samples, the IcP fraction remained almost constant, indicating that this fraction remained closed over time during isothermal heating. In this regard, according to Demarchi et al. (2013a, p. 154), the total concentration of amino acids in unbleached samples would eventually reach the concentration levels detected in bleached powders, as prolonged leaching would isolate the IcP fraction. However, this was not observed here, i.e. after 240 h heating at 140ºC the amino acid concentration in unbleached samples was still 14 times higher than that in the IcP fraction (Table 3). Nevertheless, it is likely that leaching would occur over geological timescales (Miller and Hare, 1980; Collins and Riley, 2000; Bright and Kaufman, 2011); after ca. 6 ka cal B.P., the amino acid concentration in unbleached samples was reduced by around 85-90% (see Section 5).

The leaching experiment also showed that, upon isothermal heating, 40% of proteins from the unbleached samples were lost (Table 3). However, only a small amount of the total amino acids lost in the unbleached samples were found in the water (4.9%). Therefore, the main loss of amino acids from the inter-crystalline fraction is likely to be due to decomposition (the processes leading to the chemical degradation of the molecular structure of the amino acids), either within the shell or once leached into the water.

5 Amino acids in archaeological shells

5.1 Amino acid composition of the whole shell

The total amino acid concentration of unbleached *P. lineatus* shells (representing the amino acids that comprise inter- and intra-crystalline proteins) was higher in modern specimens than in archaeological ones (Fig. 7), and more variable; the total amino acid content decreased by around 85-90% from modern to archaeological shells. However, the concentrations were similar in archaeological *P. lineatus* shells of diverse ages (Neolithic, Mesolithic, and Upper Magdalenian), even in the oldest samples analysed in this study. It is worth noting that [Asx] and [Glx] were higher in modern *P. lineatus* shells, while archaeological samples showed similar concentrations of these amino acids (Asx and Glx).

Similar percentages for all amino acids (considering [Asx], [Glx], [Ser], [Ile], [Leu], [Phe], [Val], [Ala], Gly], [Arg] and [Thr]) were obtained in archaeological levels of different ages. However, the percentage of each respective amino acid varied in a different way with respect to that of the modern shells (Fig. 8A). The percentage of Asx increased sharply with age (Fig. 8A), i.e. for modern specimens it was around 14%, whereas for the Neolithic ones (Mazaculos II-A2) it was 35%, remaining similar for Mesolithic and Upper Magdalenian ones. The percentage of Glx, Val and Leu also increased from modern to archaeological samples. In this regard, samples older than ca. 6 ka cal BP (Neolithic) showed similar proportions of Asx. In contrast, the percentage of Ser, Ala, Phe, Gly and Arg showed a rapid decrease in *P. lineatus* shells from modern to Neolithic age, after which the percentage remained almost constant. It should be noted that the percentage of Ile and Thr remained almost the same.

5.2 Amino acid composition of the IcP fraction

In contrast to that observed in unbleached samples, the concentration of amino acids in bleached *P. lineatus* shells (representing the amino acids that comprise only IcPs) was similar for modern and archaeological representatives of distinct ages (Fig. 7). The same results were obtained for [Asx].

Similar percentages were obtained for Asx, Glx, Ile, Leu, and Phe in modern and archaeological shells (Table 2). In contrast, the percentage of Ser decreased sharply with age (Fig. 8B), from modern (ca. 15%) to Neolithic (ca. 8%) specimens, remaining similar for Mesolithic and Upper Magdalenian ones. The percentage of Arg and Thr also decreased from modern to archaeological samples. The percentage composition of Ala, Val, and Gly showed a rapid increase from modern to Neolithic *P. lineatus* shells, after which the percentage of these amino acids remained almost constant.

5.3 Interpretation of amino acid concentration trends

5.3.1 Whole shell amino acids

Significant protein leaching is likely to have occurred from the inter-crystalline fraction during the ca. 6,000 yr cal BP after the death of *P. lineatus*, as the total amino acid content decreased ca. 85-90% over this time and then stabilised. After this decrease, the amino acid content in *P. lineatus* shells of Mesolithic and Upper Magdalenian ages (up to ca. 12.6 ka cal BP) remained almost the same (Fig. 4), whereas the contribution of each amino acid to the total content differed (Fig. 8A, B). Thus, there was an increase in the relative proportion of Asx, Glx, Val, and Leu with age, while the relative composition of other amino acids such as Ser, Ala, Phe, Gly and [Arg] decreased with age.

Of note is the decrease in the percentage of Ala with age in the whole shell (Fig. 8A), as an increase in the relative concentration of this amino acid is commonly observed upon artificial diagenesis in molluscs (Penkman et al., 2008; Demarchi et al., 2011, 2013b) and eggshells (Miller et al., 2000; Crisp, 2013). In contrast, the percentage of Ala increased in the IcP fraction of *P. lineatus* (Fig. 8B). This increase is assumed to be caused by the decomposition of other amino acids, such as Asx and mainly Ser, into Ala (Bada and Miller, 1970; Bada et al., 1978; Bada and Man, 1980; Walton, 1998).

We also observed a decrease in [Ser]/[Ala] values in unbleached *P. lineatus* samples, thereby indicating a general pattern of increased protein degradation with age, as also interpreted from the increase in D/L values (Fig. 9A) and a decrease in amino acid concentrations with age (Fig. 7).

Therefore, the different behaviour of Ala in unbleached *P. lineatus* shells may be explained by the loss of free Ala from the inter-crystalline matrix. Also, a different decomposition rate of amino acids in this species compared to other taxa cannot be ruled out. In fact, Ala accounted for ca. 25% of the total amino acid content in unbleached *P. lineatus*, whereas in other molluscs and eggshells it represents less than 15% (Penkman et al., 2008; Demarchi et al., 2011, 2013b, Crisp, 2013), thus indicating differences in protein composition. Moreover, the total amino acid content reduced considerably from modern to archaeological shells (ca. 85-90%), thereby revealing a considerable loss of amino acids. In this regard, the biomineral might also play an important role due to differential mineral binding of amino acids (Demarchi et al., 2016).

5.3.2 IcP amino acids

The IcPs accounted for around 4% of the total protein content of modern shells (Fig. 4). This percentage increased sharply with age (up to 60–70% over 6 ka), with apparently limited degradation of the proteins in this fraction (the concentration of amino acids remained constant with age in bleached samples), thereby indicating that there was an important preferential break-down and loss of inter-crystalline proteins. Similarly, [Asx] remained constant with age.

In contrast, the percentage of the distinct amino acids in bleached samples did not vary with age in a similar way to unbleached samples. This observation confirms that the composition of the intra- and inter-crystalline fractions differed.

Moreover, the amino acid percentages in unbleached samples showed a different pattern after leaching (Fig. 5A) and in archaeological sites (Fig. 8A). These observations can be attributed to the IcP fraction becoming more representative in archaeological *P. lineatus* shells (60-70% with respect to the total proteins) after 6 ka due to amino acid loss from the inter-crystalline fraction by leaching and decomposition. This finding coincides with reports by Penkman et al. (2008), who observed that the proportion of intra-crystalline amino acids within the whole shell increases as the sample ages.

The differences found in the concentration and composition of amino acids and D/L values between the inter- and intra-crystalline proteins are in agreement with Sykes et al. (1995) and Penkman et al. (2007, 2008), who observed distinct racemisation rates in these fractions in a variety of mollusc shells. In leaching experiments (140ºC for 24 h to 240 h) on unbleached and bleached *B. tentaculata* and *P. vulgata* shells, Penkman et al. (2008) and Demarchi et al. (2013a) reported that only a small percentage (1–4%) of the total amino acid content leached from the IcP fraction, in contrast to a higher percentage (ca. 40%) from unbleached shells under the same conditions. While inter-crystalline proteins are more susceptible to decomposition or leaching, the IcP fraction has been found to behave like a closed system in various mollusc shells, including those of *P. lineatus*. Similarly, Bosch et al. (2015) concluded that the IcP fraction of the topshell *P. turbinatus* approximates a closed-system.

The results observed in unbleached and bleached archaeological *P. lineatus* shells confirmed that the inter- and intra-crystalline fractions of this species differ in protein profiles, thus showing distinct racemisation rates and compositions. IcPs seemed to remain in a closed system, as the total concentration of amino acids remained similar with age, although percentages of some amino acids varied. It should be highlighted that the concentration and percentage of Asx (which is the amino acid commonly used for dating recent samples) remained constant with age in bleached samples.

6 Aminochronology

To establish the aminochronology of the archaeological samples here, we used only Asx, because it is the amino acid that shows the fastest racemisation. Furthermore, due to their low D/L values, other amino acids were not suitable to discriminate between archaeological sites of these ages.

6.1 Asx D/L values vs. age -unbleached

In general, topshells from archaeological sites showed Asx D/L values consistent with their age (Table 4; Fig. 9A), i.e. in the Neolithic site (Mazaculos II-A2) shells had the lowest Asx D/L values, followed by those belonging to the Mesolithic (shell midden and level 29 of La Riera, level 1.3 of Mazaculos II, El Penicial, Bricia-A, La Trecha, Arenillas, El Mazo and Toral), Azilian/Magdalenian (level 27 of La Riera), and Magdalenian (level 24 of La Riera, Bricia-C) periods. To select the best fit for the amino-age estimation algorithm, we compared the correlation coefficients (r^2) for various approaches. We used the relationship between D/L Asx values vs. age because it provided the highest correlation coefficient.

6.2 Asx D/L values vs. age -bleached

As with the Asx D/L values of unbleached samples, D/Ls also increased with age in the bleached fraction (Table 4; Fig. 9B). Asx D/L values in the bleached samples showed a strong correspondence (r^2= 0.88) with age.

Of note, the Asx D/L values were similar in unbleached and bleached samples, although in unheated modern specimens they were lower in the former (Table 4).

6.3 Aminochronological considerations

The mean Asx D/L values of 106 bleached and unbleached *P. lineatus* shells from the archaeological levels (after the rejection of samples with abnormally high D/L values) increased with age (Figs. 9A and 9B). Asx D/L values were similar in unbleached and bleached samples. This observation could be attributable to the considerable loss of amino acids in the inter-crystalline fraction over time, thereby producing a significant contribution of the IcP fraction to the whole protein content. Thus, good correlations were obtained for Asx D/L values in the inter- and intra-crystalline fractions *versus* age.

However, the extent of racemisation of some amino acids (Asx, Glx, Ala, Val) within the IcP fraction of *P. turbinatus* specimens from Ksâr 'Akil (Lebanon) revealed intralayer variability of the D/L values comparable with intra-horizon variability. Therefore D/L values could not be used to resolve the chronology within the site at the timescale relevant in that study, between 30 and 43 ka BP (Bosch et al., 2015). The low coefficients of variation for Asx D/L values in both bleached and unbleached *P. lineatus* samples (> 7% in most of cases) can explain the good resolution observed for discriminating the chronology of archaeological sites.

A general increase in Asx D/L values with radiocarbon age was observed (Figs. 9A and 9B) up to 13 ka cal BP. We propose that the palaeoclimatic variations that occurred after the accumulation of the archaeological remains did not significantly affect the amino acid racemisation rate of *P. lineatus* shells, in contrast to that observed from limpet shells of the Solutrean and Gravettian periods (Ortiz et al., 2015). The sites studied here were formed after the Last Glacial Maximum (LGM), when climate amelioration occurred and was maintained throughout the Holocene with no significant temperature variations during the last 13 ka cal BP, with the exception of Younger Dryas (Bard, 2002; Peck et al., 2008).

This study indicates that Asx D/L values of unbleached and bleached *P. lineatus* shells are useful for dating *P. lineatus* shells from archaeological levels in this region (Figs. 9A and 9B).

7. Conclusions

In summary:

1.-The protein composition of the inter- and intra-crystalline fractions of the topshell *P. lineatus* differs, as shown by differences in the percentages of amino acids present. IcP amino acids accounted for 4% of the modern total amino acid content, but this percentage increased to 60-70% after ca. 6 ka BP.

2.-The percentage of Asx remained constant with age (in archaeological samples over ca. 13 ka cal BP) within IcPs. However, in inter-crystalline proteins, the percentage of this amino acid increased sharply in the first ca. 6 ka after the death of *P. lineatus* and then stabilised. The relative composition of other amino acids in the inter-crystalline fraction decreased with age (Ser, Ala, Phe, Gly, and Arg), whereas the percentage of Glx, Val and Leu increased. In contrast, within IcPs, Ala and Gly increased. The different protein composition of the inter- and intra-crystalline fractions, the closed system behaviour of the IcP fraction, and the differential mineral binding of amino acids may explain these differences.

3.-The IcP fraction behaved like a closed system, as the concentration of amino acids did not vary significantly after heating at 140ºC. The main loss of inter-crystalline proteins occurred through decomposition, and only a small fraction was leached into water (ca. 4.9%).

4.-The main leaching of inter-crystalline proteins in *P. lineatus* shells occurred within at least the first 6,000 yr cal BP after the death of the organism. This is evidenced by the considerable decrease (85-90%) in the total amino acid content in archaeological samples with respect to modern representatives. However, the total amount of amino acids in the IcP fraction remained virtually intact for at least 12.6 ka, thereby confirming that this fraction approximates a closed system.

5.-Differences in the amino acid content of inter- and intra-crystalline proteins, which undergo racemisation at different rates, may be produced because the products of diagenesis are likely to remain in the IcP fraction. Likewise, the preferential removal of certain proteins and amino acids from the inter-crystalline matrix over time might cause this fraction to degrade faster than the intra-crystalline one. Although Asx D/L values were higher in unbleached samples, there was good correspondence between the Asx D/L values in inter- and intra-crystalline proteins. However, other amino acids, such as Glx, showed lower levels of racemisation in the inter-crystalline proteins. We consider that the age of archaeological levels can be established through analysing unbleached samples; however, bleaching provides important information and complements the interpretation of the results obtained from the inter-crystalline fraction.

6.-Asx D/L values in unbleached and bleached *P. lineatus* shells were comparable and showed a good correspondence with age. They can both therefore be used for the age calculation of archaeological localities.

7.-In brief, AAR is a satisfactory tool for dating *P. lineatus* from archaeological sites covering the Upper Magdalenian to Neolithic periods, i.e. from ca. 13 to 5.5 ka cal BP.

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Figure captions

Figure 1. Geographical location of the caves studied. 1-La Trecha, 2-Arenillas, 3-Mazaculos II, 4-El Mazo, 5-El Toral III, 6-La Riera, 7-Bricia, and 8-El Penicial. Cue beach is also plotted.

Figure 2. A) Photograph of an archaeological *P. lineatus* shell from level 108 of El Mazo. B) Cross-section of a shell showing the different layers, and the sampling area.

Figure 3. A) Concentration (nmol/mg) of Asx, Glx, Gly, Ala, Ser, Val and Ile in unbleached and bleached samples of modern *P. lineatus* shells (errors are shown in Table 1 Supplementary Information). B) D/L values of Ala, Ile, Asx, Glx, and Ser in unbleached and bleached samples of modern *P. lineatus* shells (errors are shown in Table 2 Supplementary Information). C) Relative amino acid composition of unbleached and bleached *P. lineatus* shells (errors are shown in Table 3 Supplementary Information).

Figure 4. Variation of total amino acid concentration (nmol/mg) in unbleached and bleached samples of modern *P. lineatus* shells in response to heating at 140ºC.

Figure 5. Percentage of each amino acid in unbleached (A) and bleached (B) samples of *P. lineatus* shells after heating at 140ºC (Tables 5 and 6 Supplementary Information).

Figure 6. Asx, Glx, Ala and Phe D/L values in unbleached and bleached samples of *P. lineatus* shells heated at 140ºC versus heating time (h).

Figure 7. A) Total amino acid concentration of the unbleached and bleached modern and fossil *P. lineatus* shells.

Figure 8. Percentage of each amino acid in unbleached (A) and bleached (B) samples of *P. lineatus* shells from modern and archaeological sites (Tables 7 and 8 Supplementary Information). The same colour code was used for all the levels of the same period, and localities are plotted in the age order indicated in the legend.

Figure 9. Best-fit relation between Asx D/L values obtained in (A) unbleached and (B) bleached samples of *P. lineatus* shells versus age.

Tables

Table 1. Archaeological levels studied and the periods assigned. Calibrated ages (yr cal) were converted using the Radiocarbon Calibration Program 7.1 (CALIB 7.1) (Stuiver et al., 2017) with the calibration dataset IntCal13 (Reimer et al., 2013). Reservoir effect was corrected using data from Monge Soares et al. (2016).

Cave	Archaeological level	Age (^{14}C yr cal BP)
Cue beach	-	Modern
Arenillas (ARE)	Shell midden	Mesolithic [1] 7,975±23 (OxA-X-2488) 8,227±58 (OxA-27154)
Bricia (BRI)	Shell midden (Level A)	Mesolithic [2,3] 7,680±150 (GaK 2908)
	Level C	Upper Magdalenian [2] aar 13,934 ± 1,949 [5]
Mazaculos II (MAZ)	Level A2	Neolithic [4] 5798±121 [4]
	Shell midden Level 1.3	Mesolithic [4] 8,490±40 (UGAM-9081) 8,529±49 (OxA-26953)
La Riera (RIE)	Shell midden	Mesolithic [6] 7,375±185 (GaK-3046)
	Level 29	Mesolithic [6] 9,722±379 (GaK-2909)
	Level 27upper	Azilian/Magdalenian[6] 12,510±195 (BM-1494);
	Level 24	Upper Magdalenian [6] 12,660±545 (GaK-6982)
El Mazo (EMA)	101	Mesolithic [1] 7,927±42 (OxA-30780) 8,112±52 (OxA-30806)
	113	Mesolithic [1] 8,032±43 (OxA-28403) 8,385±18 (OxA-28404)
	120	Mesolithic [1] 8,255±50 (OxA-28405) 8,436±38 (OxA-30976)
	105	Mesolithic [1] 8,209±86 (OxA-30535) 8,402±19 (OxA-30977)
	108	Mesolithic [1] 8,899±91 (OxA-28411) 9,193±63 (OxA-26954)
El Penicial (PEN)	Surface shell midden	Mesolithic [2,7] 9,760±250 (GaK 2906)
La Trecha (LTR)	Level 1	Mesolithic [8] 8,303±72(URU0083)
El Toral (TOR)	Level 17	Mesolithic [9] 7370±40(UGAMS 5403)
	Level 21	Mesolithic [9] 7510±40(UGAMS 5400) 7620±30(UGAMS 5401)
	Level 13C	Mesolithic [9] 9530±20(UGAMS 5404)

1.-Monge Soares et al. (2016); 2.-Clark (1976); 3.-Jordá (1957, 1958); 4.-González-Morales (1982); 5.-Ortiz et al. (2009); 6.-Straus et al. (1978); Straus and Clark (1986); 7.-Vega del Sella (1914); 8.-González-Morales et al. (2002), 9.-Rigaud and Gutiérrez-Zugasti (2016).

Table 2. Percentage of Asx and Glx content with respect to the total amino acid content of unbleached and bleached samples of modern and archaeological *P. lineatus* shells.

Period	Localities	N	%Asx unbleached	%Asx bleached	%Glx unbleached	%Glx bleached
	Modern	5	14.3 ± 0.4	40.4 ± 2.1	7.5 ± 0.8	8.4 ± 1.0
N	MAZ-A2	5	31.6 ± 1.9	37.6 ± 0.9	8.6 ± 0.5	9.1 ± 0.8
M	TOR-17	5	31.0 ± 2.7	35.2 ± 2.5	8.9 ± 0.7	9.2 ± 0.5
	RIE-SM	5	32.3 ± 2.2	37.1 ± 1.9	9.8 ± 3.8	8.6 ± 0.5
	TOR-21	5	32.4 ± 3.2	35.1 ± 2.4	9.8 ± 0.4	9.7 ± 1.6
	BRI-A	5	30.7 ± 4.9	38.5 ± 0.4	8.1 ± 0.2	9.1 ± 0.5
	ARE	5	30.0 ± 0.2	36.6 ± 1.0	8.3 ± 0.2	8.9 ± 0.5
	EMA-101	5	31.2 ± 1.6	35.6 ± 0.5	9.7 ± 1.6	9.0 ± 0.8
	EMA-113	5	30.1 ± 1.7	35.2 ± 1.1	8.5 ± 0.4	8.8 ± 0.3
	LTR	5	29.6 ± 4.4	37.1 ± 1.4	9.2 ± 0.7	9.6 ± 0.7
	EMA-105	5	30.1 ± 2.1	36.5 ± 0.9	9.0 ± 0.9	8.9 ± 0.1
	EMA-120	5	32.4 ± 1.7	35.1 ± 1.6	8.3 ± 0.4	8.8 ± 0.2
	MAZ-1.3	5	32.0 ± 0.2	37.3 ± 0.8	8.5 ± 0.2	9.0 ± 0.2
	EMA-108	5	31.8 ± 2.8	35.2 ± 3.6	8.5 ± 1.4	9.4 ±0.9
	TOR-13C	5	31.3 ± 1.5	37.5 ± 1.1	9.6 ± 0.4	8.9 ± 0.3
	RIE-29	5	30.5 ± 5.2	36.5 ± 1.1	8.3 ± 0.3	8.4 ± 0.3
	PEN	8	30.3 ± 0.7	37.2 ± 0.8	8.4 ± 0.2	9.7 ± 0.6
AZ/UM	RIE-27	5	28.9 ± 1.9	35.4 ±1.5	8.5 ± 0.3	8.7 ± 0.2
UM	RIE-24	5	30.3 ± 1.4	35.3 ± 1.5	8.6 ± 1.8	9.4 ± 1.0
	BRI-C	8	32.5 ± 0.3	36.9 ± 1.1	8.5 ± 0.3	9.5 ± 0.9

N:Neolithic; M: Mesolithic (Asturian); Az: Azilian; UM: Upper Magdalenian.

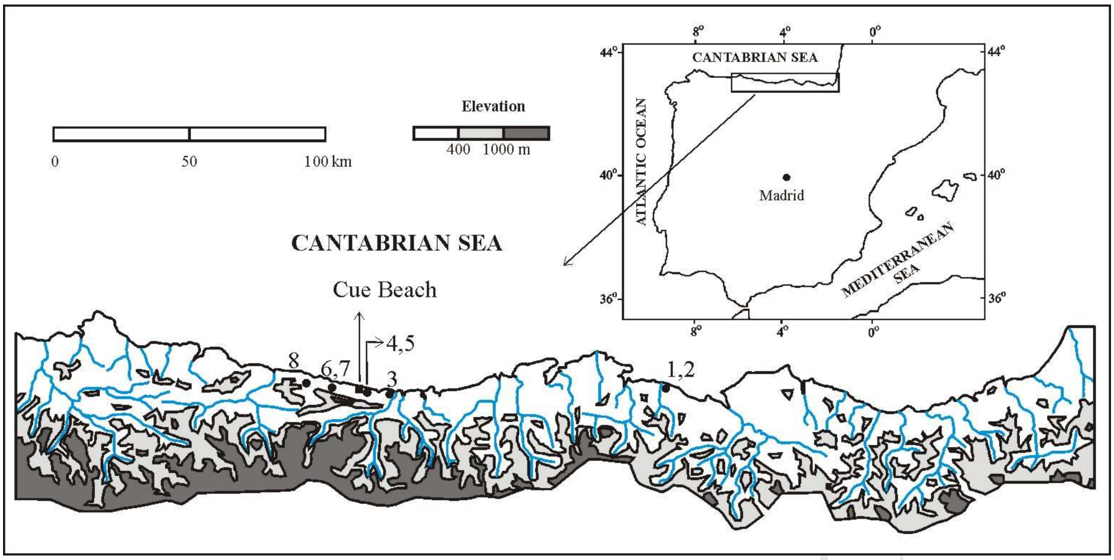
Table 3. Loss of amino acids from bulk unbleached and bleached *P. lineatus* powders (experimental samples) after 24h of heating at 140ºC (n= number of samples). Total concentrations (nmol/mg) were calculated using [Asx], [Glx], [Ser], [Ala], [Val], [Ile], [Leu], [Phe], [Gly], [Arg] and [Thr].

Loss of amino acids after 24h heating at 140ºC	Unbleached	Bleached
Initial [total] concentration in shell unheated (pmol/mg) (n=5)	385.733	16.130
[total] THAA after heating (pmol/mg) (n=2) for 24h	231.393	16.092
[total] THAA in water, heated (pmol/mg equiv.) (n=2)	18.903	-
Overall loss in shell (%) from the original	40.0	0.23
Loss into water by leaching (%) from the original	4.9	-
Loss by decomposition (%) from the original	35.1	-

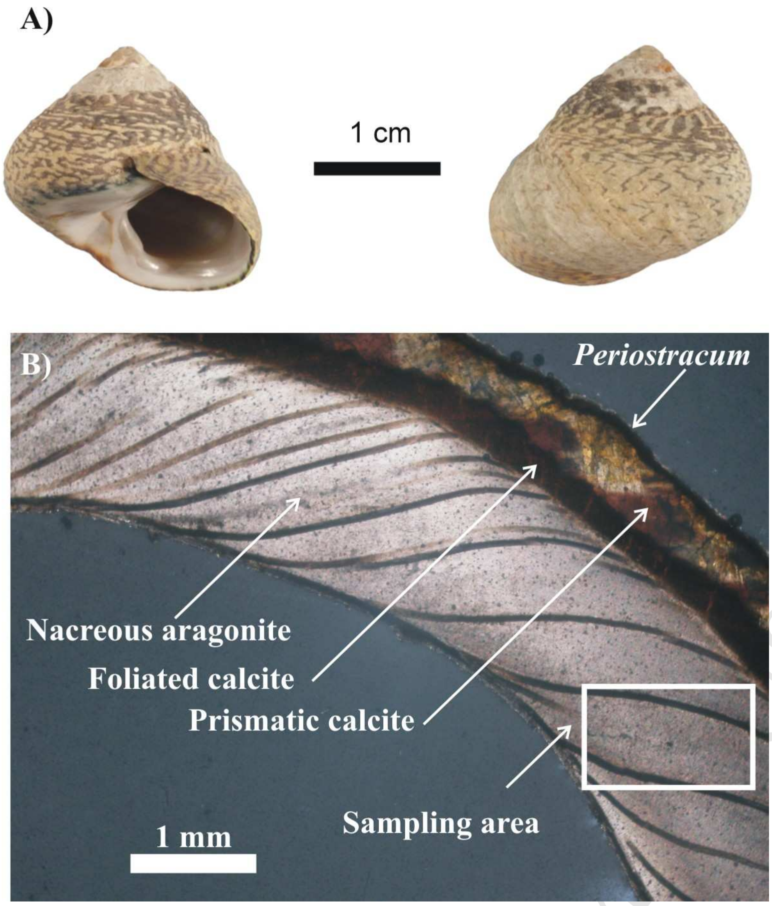
Table 4. Asx D/L values in unbleached and bleached samples of modern and archaeological *P. lineatus* shells.

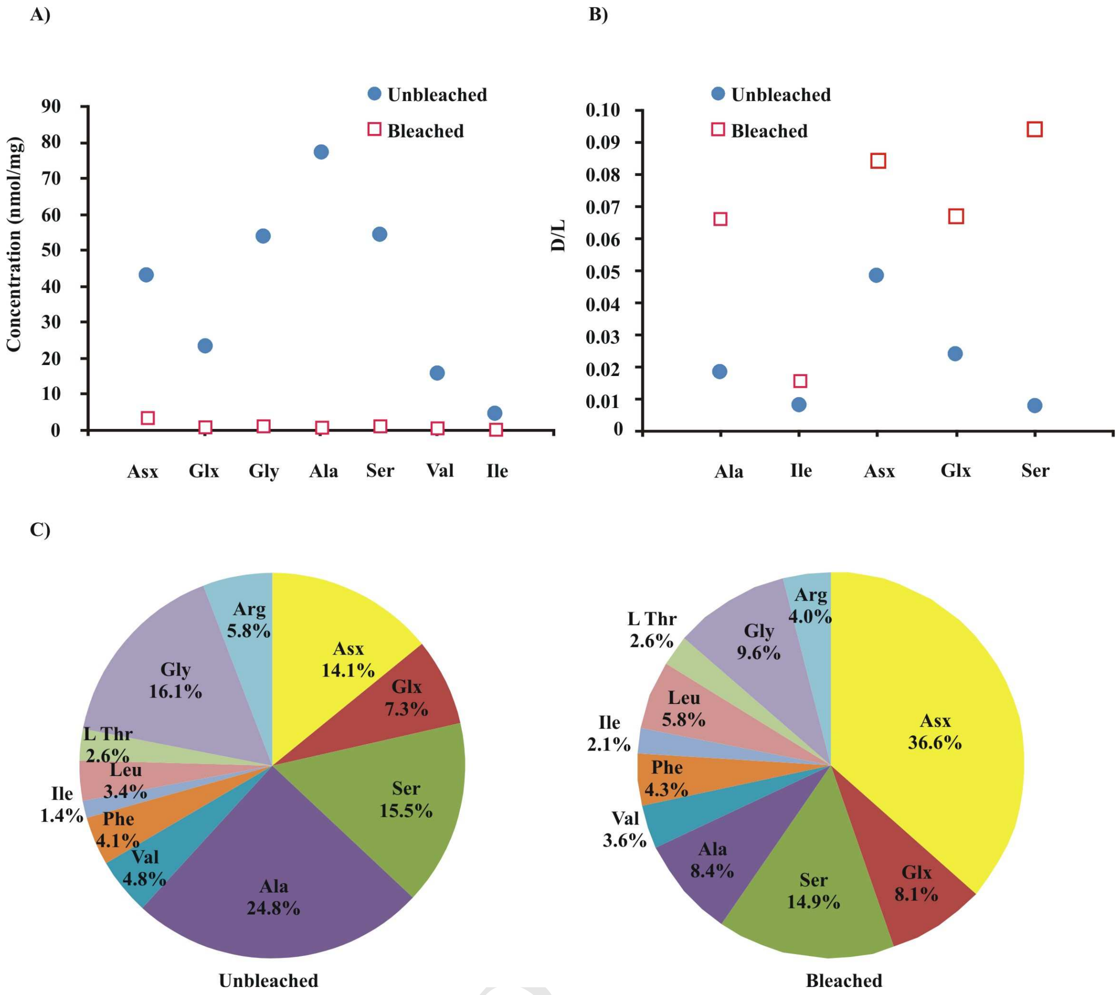
Period	Localities	D/L Asx unbleached	D/L Asx bleached
	Cue beach	0.048±0.001	0.084±0.004
N	MAZ-A2	0.177±0.022	0.180±0.012
M	TOR-17	0.225±0.011	0.208±0.009
	RIE-SM	0.223±0.021	0.228±0.015
	TOR-21	0.219±0.019	0.199±0.009
	BRI-A	0.237±0.018	0.240±0.023
	ARE	0.224±0.011	0.208±0.019
	EMA-101	0.235±0.009	0.227±0.033
	EMA-113	0.227±0.009	0.197±0.010
	LTR	0.235±0.013	0.235±0.011
	EMA-105	0.219±0.016	0.207±0.011
	EMA-120	0.228±0.008	0.199±0.006
	MAZ-1.3	0.218±0.021	0.201±0.007
	EMA-108	0.233±0.010	0.216±0.020
	TOR-13C	0.236±0.017	0.239±0.001
	RIE-29	0.251±0.020	0.257±0.017
	PEN	0.244±0.019	0.246±0.018
AZ/UM	RIE-27	0.268±0.013	0.268±0.028
UM	RIE-24	0.285±0.002	0.284±0.017
	BRI-C	0.299±0.030	0.294±0.027

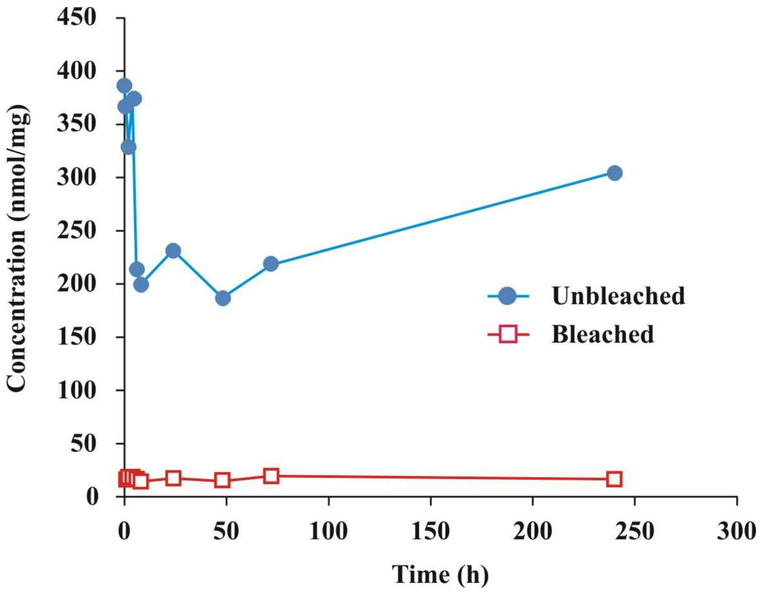
N:Neolithic; M: Mesolithic (Asturian); Az: Azilian; UM: Upper Magdalenian.



Elevation
0
50
100 km
400
1000 m
CANTABRIAN SEA
Cue Beach
8
6,7
4,5
3
1,2
8º
4º
0º
44º
40º
36º
CANTABRIAN SEA
ATLANTIC OCEAN
Madrid
MEDITERRANEAN SEA

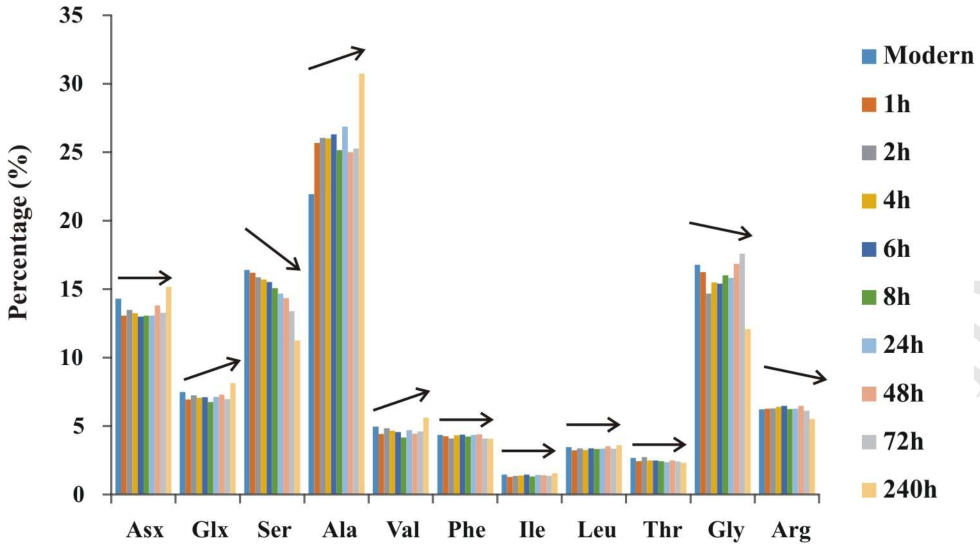

A)
1 cm
B)
Periostracum
Nacreous aragonite
Foliated calcite
Prismatic calcite
Sampling area
1 mm


A)
Unbleached
Bleached
Concentration (nmol/mg)
90
80
70
60
50
40
30
20
10
0
Asx Glx Gly Ala Ser Val Ile
B)
Unbleached
Bleached
D/L
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Ala Ile Asx Glx Ser
C)
Arg 5.8%
Asx 14.1%
Glx 7.3%
Ser 15.5%
Ala 24.8%
Val 4.8%
Phe 4.1%
Ile 1.4%
Leu 3.4%
L Thr 2.6%
Gly 16.1%
Unbleached
Arg 4.0%
Gly 9.6%
L Thr 2.6%
Leu 5.8%
Ile 2.1%
Phe 4.3%
Val 3.6%
Ala 8.4%
Ser 14.9%
Glx 8.1%
Asx 36.6%
Bleached

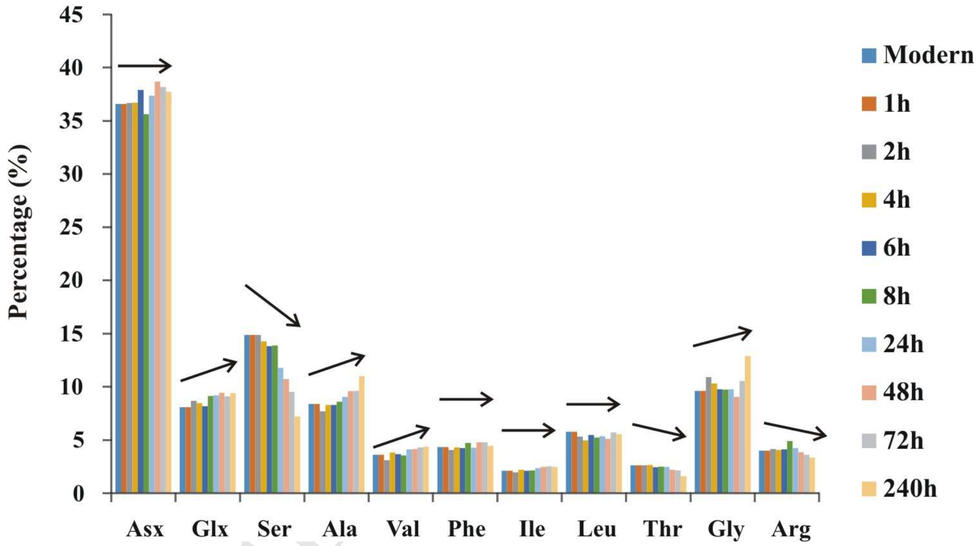


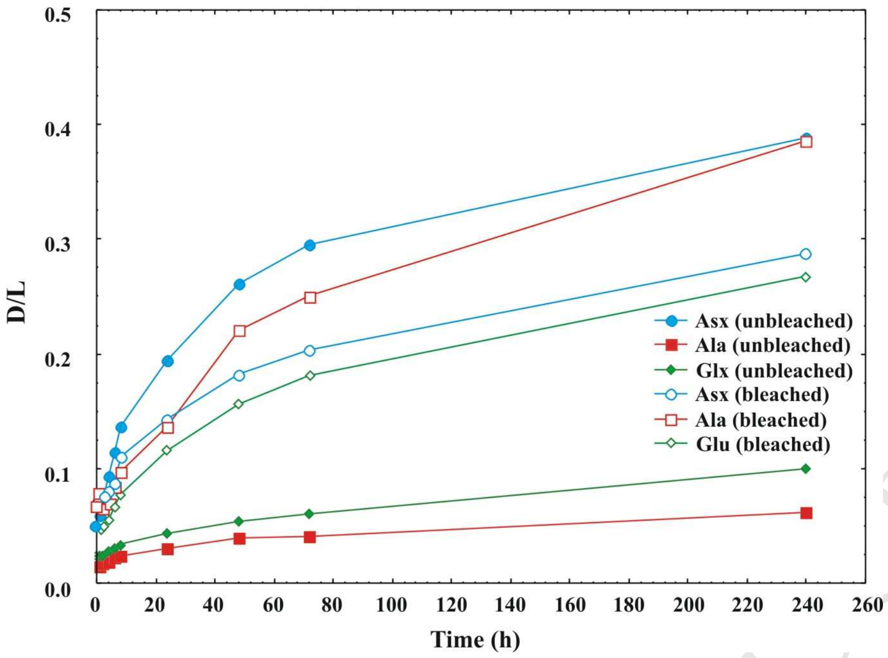
450
400
350
300
250
200
150
100
50
0
Concentration (nmol/mg)
0
50
100
150
200
250
300
Time (h)
Unbleached
Bleached

A) unbleached (whole shell)

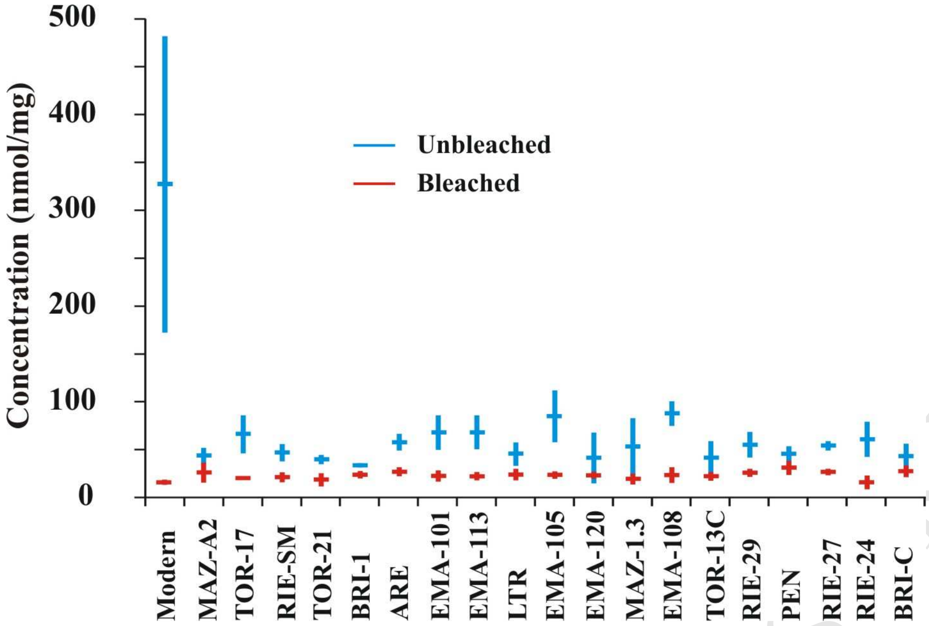


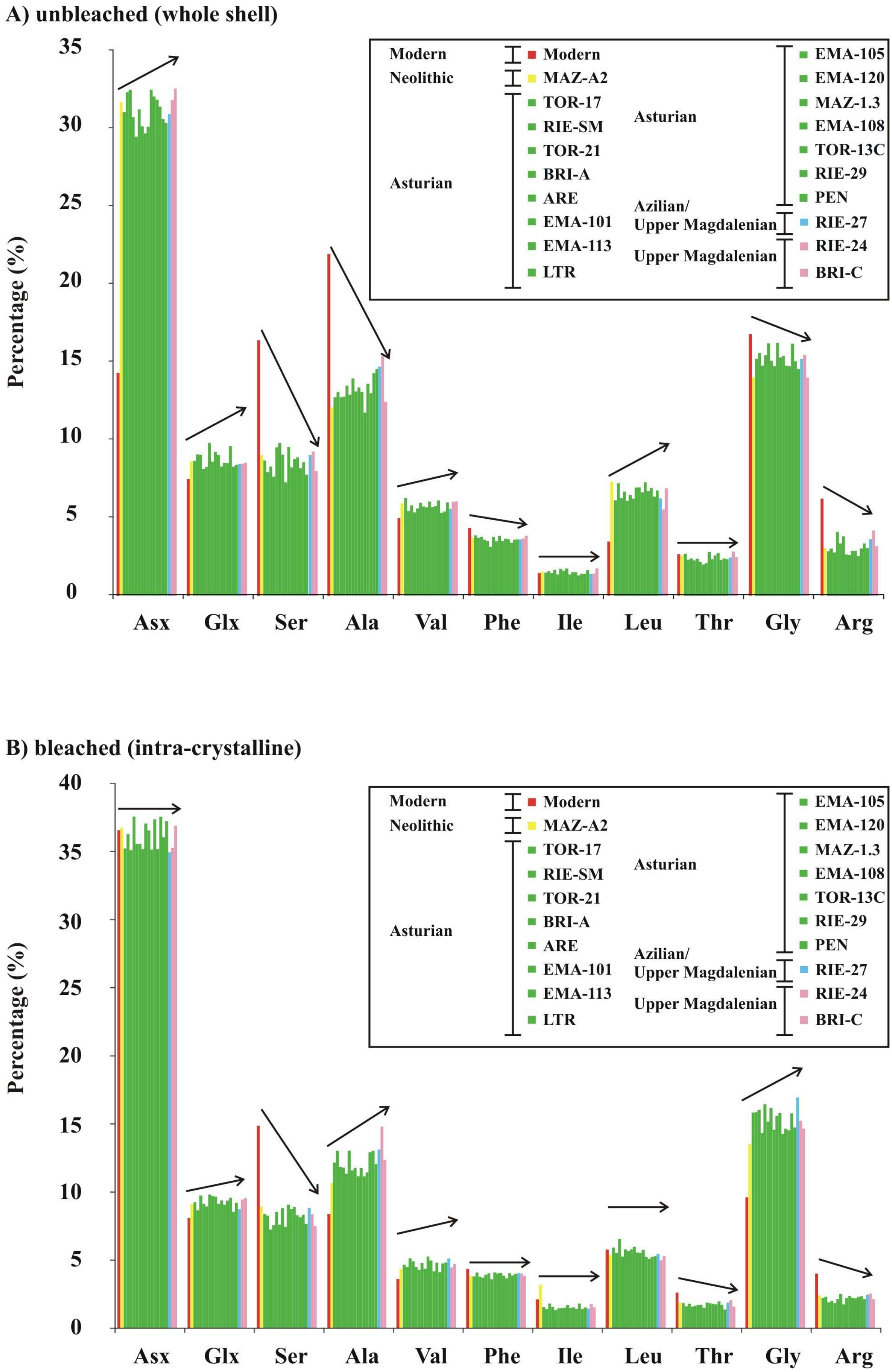
B) bleached (intra-crystalline)

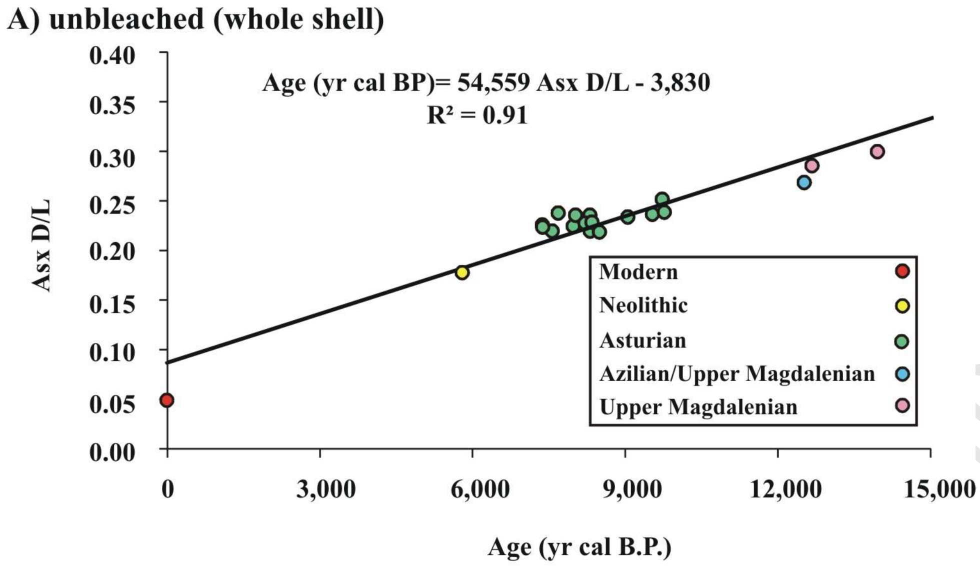


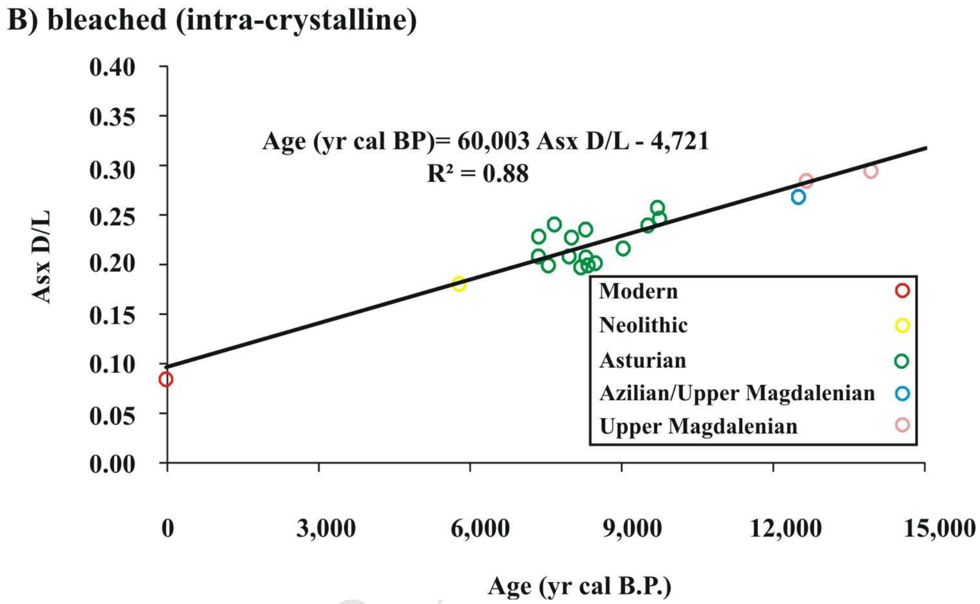


0.5
0.4
0.3
0.2
0.1
0.0
D/L
0
20
40
60
80
100
120
140
160
180
200
220
240
260
Time (h)
Asx (unbleached)
Ala (unbleached)
Glx (unbleached)
Asx (bleached)
Ala (bleached)
Glu (bleached)


500
400
300
200
100
0
Concentration (nmol/mg)
Unbleached
Bleached
Modern
MAZ-A2
TOR-17
RIE-SM
TOR-21
BRI-1
ARE
EMA-101
EMA-113
LTR
EMA-105
EMA-120
MAZ-1.3
EMA-108
TOR-13C
RIE-29
PEN
RIE-27
RIE-24
BRI-C


A) unbleached (whole shell)
Percentage (%)
35
30
25
20
15
10
5
0
Asx
Glx
Ser
Ala
Val
Phe
Ile
Leu
Thr
Gly
Arg
Modern
Neolithic
Asturian
Azilian/
Upper Magdalenian
Upper Magdalenian
Modern
MAZ-A2
TOR-17
RIE-SM
TOR-21
BRI-A
ARE
EMA-101
EMA-113
LTR
EMA-105
EMA-120
MAZ-1.3
EMA-108
TOR-13C
RIE-29
PEN
RIE-27
RIE-24
BRI-C
B) bleached (intra-crystalline)
Percentage (%)
40
35
30
25
20
15
10
5
0


A) unbleached (whole shell)
Age (yr cal BP)= 54,559 Asx D/L - 3,830
R² = 0.91
Asx D/L
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
3,000
6,000
9,000
12,000
15,000
Age (yr cal B.P.)
Modern
Neolithic
Asturian
Azilian/Upper Magdalenian
Upper Magdalenian


B) bleached (intra-crystalline)
Age (yr cal BP)= 60,003 Asx D/L - 4,721
R² = 0.88
Asx D/L
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
3,000
6,000
9,000
12,000
15,000
Age (yr cal B.P.)
Modern
Neolithic
Asturian
Azilian/Upper Magdalenian
Upper Magdalenian