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Research paper

Aspartic acid racemization as a dating tool for dentine: A reality

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ABSTRACT

Given the interest in dating sediments from numerous caves, lakes and fluvial terraces containing fossils and lithic components in Europe, here we provide a complete revision of the amino acid racemization (AAR) (aspartic acid in dentine) dating method in vertebrates. To examine the reliability of this method, which is based on a straightforward sample preparation (previous 3.5 kDa dialysis), we used a range of dental material. We examined human dentine collagen from living donors and remains from historical (16th and 19th centuries) and prehistorical (Neolithic) periods. On the assumption that genus does not affect collagen racemization rates, we also studied Neanderthal material and material from carnivores (cave bear) and several other mammals. To validate our age calculation algorithm, we used a wide series of radiometric datings (ESR and ^{14}C), along with thermoluminescence and AAR dating on invertebrate (ostracode) samples. Our results demonstrate that AAR shows satisfactory correlation between age and the extent of aspartic acid racemization for material from modern humans and for ancient (Pleistocene) mammal remains (cave bears, horses and Neanderthals) and highlight a strong correlation between ages derived from dentine collagen aspartic acid and other dating methods. However, in samples from burial sites (19th and 16th century and Neolithic samples from Syria), it was impossible to establish age at death. We assume that taphonomic processes (time and geochemistry) greatly contribute to the denaturation of the collagen triple helix and higher order structures, thereby allowing the racemization of peptide-bound aspartic acid.

1. Introduction

In many ways the use of amino acid racemization (AAR) as a method to date vertebrate remains for forensic and stratigraphic purposes resembles Jason and the Argonauts' quest for the Golden Fleece. In this regard, our personal challenge has been to place hundreds of bone- and tooth-rich continental deposits (lacustrine, caves and fluvial terraces, among others) in the correct geological time-scale. Many of the problems that we have encountered have arisen from not considering what is referred to as the *site effect*, which includes taphonomy, time-averaging, anthropogenic influence and geological evolution.

The first paper on amino acids in fossilized bone appeared in Ezra and Cook (1957). Since then, the use of collagen amino acid (mostly aspartic acid; Asp) racemization has been widely reported (Arany et al., 2007; Bada et al., 1973, 1974, 1984; Bada and Helfman, 1975; Masters and Bada, 1975; Helfman and Bada, 1975, 1976; Hare, 1980; Bada, 1981, 1985a, 1985b, 1990; Belluomini, 1981; McMenamin et al., 1982; Julg et al., 1987; Blackwell et al., 1990; Marshall, 1990; Belluomini et al., 1991; Elster et al., 1991; Kimber and Hare, 1992; Pfeiffer et al., 1995; van Duin and Collins, 1998; Stone and Stoneking, 1999).

AAR has also been used as a technique to estimate ancient DNA preservation (Poinar et al., 1996; Poinar and Stankiewicz, 1996; Krings et al., 1997, 2000, 1999; Hofreiter et al., 2001, 2004a; Pääbo et al., 2004), although some authors express their reservations about its use for this purpose (Collins et al., 2009; Fernández et al., 2009). In addition, this methodology has been applied to determine the age of modern and historical humans (Helfman and

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Bada, 1975, 1976; Ohtani and Yamamoto, 1991, 1992; Ohtani, 1995; Ohtani et al., 1998, 2002, 2003, 2004), and a complete revision of the sample preparation method has been described in forensic studies (Waite et al., 1999). However, Hare (1980) considered the use of collagen Asp racemization unreliable for dating and criticized the results of Bada et al. (1974), Bada and Deems (1975) and Bada and Helfman (1975) because of the lack of coincidence found between ^{14}C and AAR ages.

At the Biomolecular Stratigraphy Laboratory, we opted to work with dentine and not with bone samples, as the latter material is porous and more susceptible to degradation and contamination by recent amino acids than the more compact dentine. We have extensive experience in the application of Asp racemization as a dating tool (Torres et al., 1998, 1999, 2000a, 2000b). Our laboratory has also studied the racemization kinetics of amino acids in dentine collagen in the presence of water vapor (Llamas et al., 1999; Canoira et al., 2003) (Fig. 1 Supplementary Data). Those results are consistent with the ones reported by Hare (1980), who indicated that the presence of water is a key factor to explain AAR in collagen. However, it was demonstrated that Asp racemization occurs below the temperature of collagen degradation. Canoira et al. (2003) calculated the Arrhenius parameters in thermally-induced racemization of Asp in solid dentine samples from an extant mammal (black bear) and fossil bear remains from three radiometrically dated palaeoentological sites. These authors reported different behavior of Asp racemization in both cases. This observation reinforces the relevance of taphonomy (not controlled experimental conditions), which influences AAR in collagen at low temperatures. The geochemical homogeneity of carbonate-rich cave loams ensures homogeneous "site effects" (Hoyos et al., 1998a; Torres et al., 2003), which is consistent with Hare (1980), as in the cave infills there is little temperature oscillation and a constant moisture content in most cases. In fact, according to Smith et al. (2003), and Sasowsky and Mylroie (2004), humidity in caves is high and not prone to large fluctuations. In fact, deep in caves, temperature may vary only tenths of a degree, and relative humidity changes in fractions of a percent (Toomey, 2009).

In our first collagen analysis performed in bear teeth, we applied the protocol described by Meyer and Goodfriend (1991) for mollusk samples; our results were disappointing because other organic compounds, apart from amino acids, occurred in chromatograms, sometimes interfering in the separation of Asp enantiomers (see section 4.1). Later, using the sample preparation procedure reported by Lafont et al. (1984) and Marzin (1990), we achieved satisfactory results, as organic compounds that interfered in the separation of amino acids were eliminated through a previous dialysis step. We then published the first age calculation algorithm calibrating the Asp D/L values in dentine collagen with Th/U and ^{14}C datings (Torres et al., 2001, 2002).

The present study has two objectives:

1) to attest the all-purpose (forensic science and geological dating) utility of the dentine sample pre-preparation methodology using a dialysis at 3.5 kDa. For this purpose, we discuss the Asp racemization rate in dentine over time using samples from modern humans, from individuals from the 19th and 16th centuries, and from Neolithic and Paleolithic periods of distinct Asian localities, in addition to Upper and Middle Pleistocene mammals (Fig. 1).
2) to report on the reliability of AAR as a dating tool for Pleistocene remains through the improvement of the age calculation algorithm for Asp D/L values in dentine collagen reported by Torres et al. (2002), by using a series of new electron spin resonance (ESR) dates, together with previously used radiocarbon and U/Th ages. We validate the reliability of this equation using samples from archaeological and palaeontological sites (El Sidrón-northern Spain, Ambrona-central Spain, Pinilla del Valle-central Spain, and cave bear sites in Austria and Italy) previously dated by other methods.

2. Material and methods

Fig. 1 and Table 1 show the geographical location of the sites studied, together with some information about the localities, which is completed in Table 2. Most of the sites correspond to inner sediments of the caves (far from the cave entrance). According to our measurements, the sediments show very low annual temperature oscillation (less than 1 °C annual variation, Torres et al., 2003).

2.1. Electron spin resonance (ESR) dating

ESR was carried out on cave bear teeth from several localities in northern Spain (Table 3). Due to the value of the material, we followed semi non-destructive analytical procedures (see Grün, 1995, 2006; Grün et al., 2003). A small piece of tooth enamel was removed in each case. During this process, the enamel was separated from the dentine with a needle and was not cleaned further.

Enamel fragments were mounted in a Bruker ER 218PG1 programmable goniometer and measured at each dose step at 10° angle intervals for 420° (spectra past 360° were used to check for short-term fading effects). ESR measurements were carried out on a Bruker 106 spectrometer with a 15 kG magnet and a rectangular 4102 ST cavity. The samples were recorded with the following measurement parameters routinely applied in the laboratory: accumulation of between 1200 (natural sample) and 200 scans (for the higher dosed samples) with 1.015 Gpp modulation amplitude, 10.24 ms conversion factor, 20.48 ms time constant, 2048 bit spectrum resolution (resulting in a total sweep time of 20.972 s), 120 G sweep width and 2 mW microwave power. The enamel pieces were successively irradiated with the following cumulative doses: 0, 11.9, 23.5, 45.2, 68.4, 113, 152, and 197 Gy. The total measuring time of the machine was 37 days. ESR intensity values were obtained by natural spectrum fitting (see Grün, 2002), dose values by applying a single saturating function with linear conversion, and errors by Monte Carlo simulation (for more details see Grün and Brumby, 1994).

The beta dose rate was determined from large sediment samples from the sites in which the teeth were found. In Amutxate cave, the gamma dose rate was measured *in situ* with a Digidart portable CsI gamma spectrometer.

Uranium (U) in the enamel and dentine was determined with laser ablation (for more details, see Eggins et al., 2003, 2005). The enamel was scanned twice, both scans resulting in similar U distributions. Two models of U uptake (early and linear uptake) were considered for age calculation of the samples.

2.2. AAR

AAR analyses were undertaken on a wide range of samples that can be classified into the following four groups:

- *Modern humans*: samples from age-controlled individuals that were obtained from the Odontological Faculty of the *Universidad Complutense* of Madrid.
- *Historical humans*: samples obtained from the anatomical collection of the Forensic School of the *Universidad Complutense* of Madrid. All these corpses came from dismantled cemeteries from the 16th century (Fortress of La Mota) and 19th century (Monastery of San Francisco). The approximate age of each

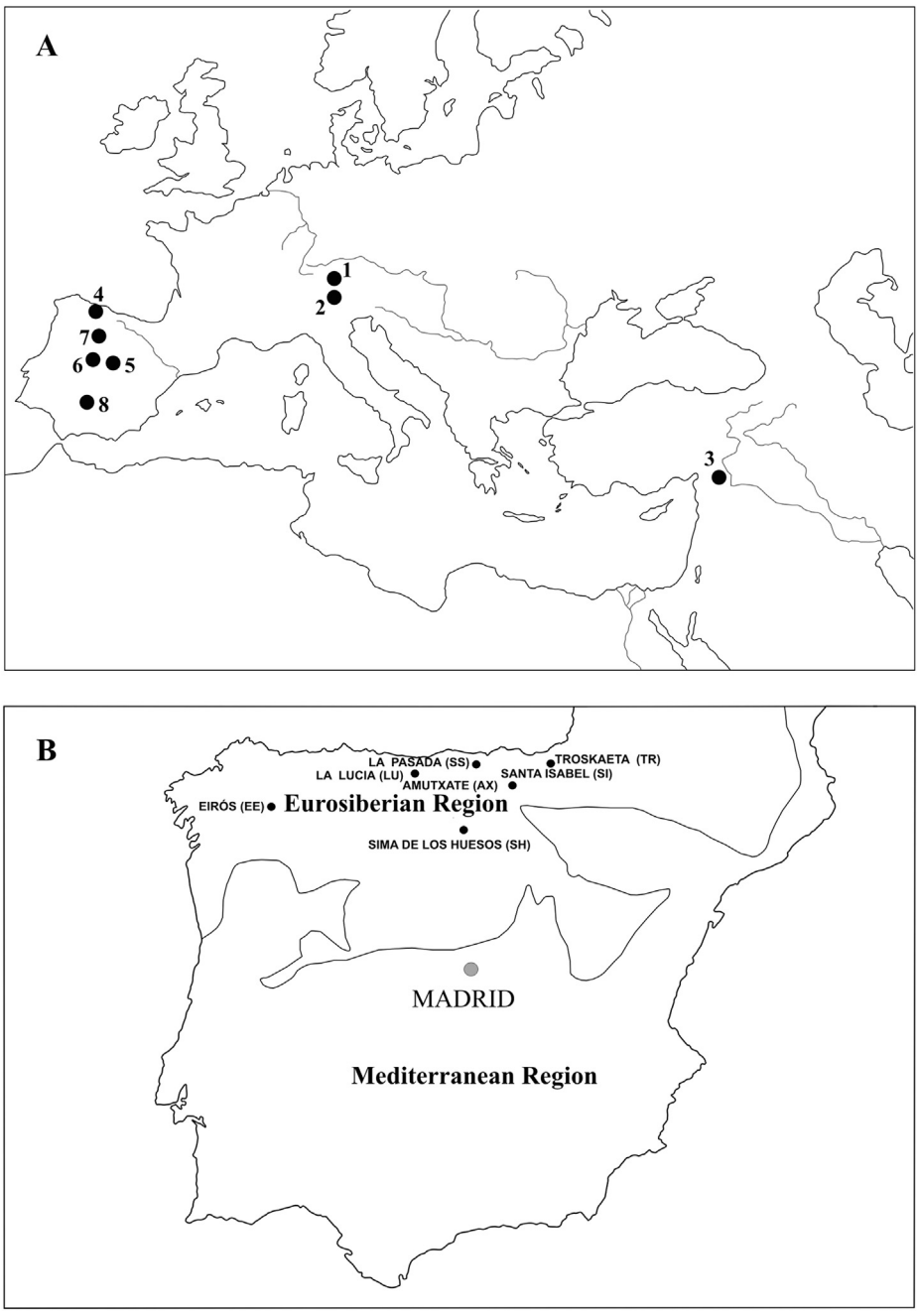

Fig. 1. Geographical location of the sites studied. A) 1: Caves in the Austrian Alps; 2: Conturines cave; 3: Tell Halula (Syria); 4: El Sidrón; 5: Ambrona; 6: Pinilla del Valle karstic system; 7: Monastery of San Francisco cemetery; 8 Fortaleza La Mota cemetery. B) Cave bear localities in the Iberian Peninsula.

individual was estimated after forensic study of the remains (cf. Meindl and Lovejoy, 1985; Ubelaker, 1989; Drusini et al., 1997; Walker et al., 1991). We classified the teeth samples into 5 groups: child II (6–12 years), juvenile (13–20 years), young adult (21–40 years), mature adult (41–60 years), and aged adult (>61 years).

- *Ancient humans*: samples from several phases of the PPNB Neolithic level in Tell Halula (Syria), dated to 8800 ± 60 B.P. by ^{14}C (Molist, 1996). Samples were referred to their archaeological level. The Asp racemization ratios of these samples have been previously reported by Fernández et al. (2009).
- Cave bears (*Ursus spelaeus* and *Ursus deningeri*): a total of 65 of samples were used from Middle and Upper Pleistocene cave bear sites, dated through ^{14}C (Grandal d'Anglade and Vidal Romaní, 1997) and Th/U on a stalactite included in the *U. spelaeus* bed and a flowstone sealing the bone-accumulation in La Lucia cave (Torres et al., 2002), and new ESR ages on tooth enamel.
- To validate the age calculation algorithm established for Asp racemization in cave bear dentine collagen, we used the following samples: 5 teeth from El Sidrón cave (Asturias, Spain; Fig. 1), in which *H. neanderthalensis* remains were dated to *ca.* 45 ka by ^{14}C, ESR and AAR (Torres et al., 2010); *Equus* sp. molars from the karst lacustrine deposits of Ambrona (Soria, Spain), which were dated in this paper to the Middle Pleistocene through AAR analysis of ostracodes; teeth of several *Ursus*

Table 1
Geographical location of the sites studied.

Locality	Latitude	Longitude	Elevation (m a.s.l.)	CMAT (°C)	Location
Monastery of San Francisco	41°52′46″N	5°2′25″E	732	11.0–12.5	Medina de Rioseco (Valladolid, Central Spain)
Fortress of La Mota	37°27′35″N	3°55′46″E	1018	13.0–14.0	Alcalá la Real, Jaén, (S. Spain)
Tell Halula	36°36′0″N	38°3′0″E	400	18–19.5	Middle Euphrates basin, Aleph (Syria)
Eirós	42°15′58″N	7°11′43″W	780	11.0–12.0	Galicia (Triacastela, NW Spain)
La Pasada	44°50′23″N	4°19′16″W	460	10.5–11.5	Guriezo (Cantabria, N. Spain)
La Lucia	44°54′2″N	4°' 33′37″W	510	10.5–11.5	Quintanilla (Cantabria, N. Spain)
Troskaeta	43°0′7″N	2°15′1″W	580	11.0–12.0	Ataun (Guipúzcoa, N. Spain)
Amutxate	42°58′25″N	1°58′1″W	980	11.0–12.0	Aralar mountains (Navarra, N. Spain)
Sima Huesos	42°20′55″	3°0′4.3″W	1022	10.5–11.5	Atapuerca (Burgos, Central Spain)
Santa Isabel	43 15′27″N	3°0′4″W	300	11.0–12.0	Ranero (Vizcaya, N. Spain)
El Sidrón	43°23′7″N	5°19′34″W	167	11.0–12.0	Borines (Asturias, N. Spain)
Ambrona	41°9′39″N	2°29′52″W	1145	10.5–11.5	Ambrona (Central Spain)
Herdengel	47°50′25″N	14°58′38″E	878	6.5–7.5	Lunz am See (Austrian Alps)
Gamssulzen	47°40′56″N	14°17′52″E	1300	6.0–7.0	Totes Gebirge (Austrian Alps)
Ramesch	47°39′0″N	14°15′0″E	1960	6.5–7.5	Totes Gebirge (Austrian Alps)
Conturines	46°33′11″N	11°58′43″E	2750	2.0–3.0	Ladinian Alps (Alta Badia, Italy).
Navalmaillo rockshelter	40°55′28″N	3°48′26″E	1101	10.5–11.5	Pinilla del Valle (Central Spain)
Buena Pinta	40°55′27″N	3°48′25″E	1105	10.5–11.5	Pinilla del Valle (Central Spain)
Camino	40°55′30″N	3°48′29″E	1094	10.5–11.5	Pinilla del Valle (Central Spain)

CMAT: current mean annual air temperature. CMATs of Spanish sites and Tell Halula were in Rivas-Martínez and Rivas-Sáenz (1996–2009), while those of Austrian and Italian sites were in Zentralanstalt für Meteorologie und Geodynamik (2002).

Table 2
Main characteristics of the sites studied.

Locality	Characteristics
Monastery of San Francisco	16th Century cemetery
Fortress of La Mota	Cemetery dated back to 1834
Tell Halula	Man-made hill where different Neolithic occupation episodes accumulate (Molist, 1996). Samples belong to occupational phases VIII-XIII on Pre-pottery Neolithic B levels (7500–7300 cal. B.C.).
Eirós	*U. spelaeus* den, stationally flooded (Torres et al., 1990)
La Pasada	*U. spelaeus* den
La Lucia	Cave bear den with two distinct species (*U. spelaeus* and *U. deningeri*) appeared at different parts of the cavity (the hall-LU-S and the ramp-LU-R, respectively) (cf. Torres et al., 2001, 2002). For the current study, we dated only *U. spelaeus* material from this cave.
Troskaeta	Cave bear den. A subspecies was defined there *U. spelaeus parvilatipedis* (Torres et al., 1991).
Amutxate	*U. spelaeus* den with traces of human presence (Torres et al., 2007).
Sima Huesos	A large cave in which enormous amounts of *U. deningeri* and *H. heidelbergensis* have been recovered from the bottom of a shaft. The site registers several uses: a mixture of a bear trap and human burial site (Aguirre et al., 1988).
Santa Isabel	An *U. deningeri* den affected by tardive vadose reactivations (Torres, 1992).
El Sidrón	High amounts of *H. neanderthalensis*, located in secondary position (Fortea et al., 2003).
Ambrona	Classical archaeological site. Doline-linked lake infill with large amounts of vertebrate remains accumulated in a doline. Though Acheulian artifacts. Formerly considered as result of human exploitation (Howell et al., 1995).
Herdengel	Cave bear den. Occupied by bears for thousands of years (Döppes and Rabeder, 1997; Rabeder, 1999, 2004)
Gamssulzen	Cave bear den
Ramesch	Cave bear den
Conturines	Cave bear den. It is the highest cave bear locality inEurasia (Rabeder, 1992).
Navalmaillo rockshelter	Located in a deeply dismantled karstic system in Pinilla del Valle. The discovery of *Homo neanderthalensis* remains in the 1980s (Alférez, 1985) made it a new landmark of the palaeo-anthropological legacy of the Iberian Peninsula. According to Pérez-González et al. (2010), TL and OSL datings of the deposits place them between MIS6 (upper part)-MIS4).
Buena Pinta	
Camino	

species (formerly *U. spelaeus*) from some Upper Pleistocene localities of the Austrian and Italian Alps, kindly provided by Prof. Rabeder, and dated through ^{14}C and U/Th (Rabeder, 1992, 1999; Döppes and Rabeder, 1997; Hofreiter et al., 2001, 2004a,b); and teeth of various mammal genera (*Equus*, *Cervus*, *Rhinoceros*, *Castor*, *Sus*, Hyena, Bovidae, and Artiodactyla) from the karstic complex in Pinilla del Valle, for which some OSL and TL ages were available (Pérez-González et al., 2010).

Some AAR data on fossil mammal teeth used here (Eirós, La Pasada, Troskaeta, Sima de los Huesos, Santa Isabel, El Sidrón) have already been published (Torres et al., 2000a, 2001), although new localities (Amutxate, Ambrona, caves from the Pinilla del Valle karst system, Herdengel, Ramesch, Gamssulzen, and Conturines caves) were sampled for the first time, and some localities were re-sampled (Sima de los Huesos, Santa Isabel).

Samples of powdered dentine were obtained by drilling a hole 2 mm in diameter and 5–10 mm in depth with a thin diamond-headed device. Samples were taken from the tooth root in carnivores, suidae, and humans, or on the occlusal face of teeth from herbivores. For modern, historical and ancient humans, the teeth were longitudinally sectioned and dentine powder was obtained with a diamond sinter conical drill.

For dentine collagen samples, we followed the dialysis step (3.5 kDa) proposed by Lafont et al. (1984) and Marzin (1990), after de-mineralization of the dentine powder. Ostracode shells from Ambrona site were picked with the aid of a needle, carefully cleaned sonically in distilled deionized (DDI) water, and rinsed with DDI water to remove sediment. Some valves were also cleaned with a small brush under a binocular microscope to eliminate fine debris. In order to remove secondary organic molecules adsorbed to the shells, the valves were then submerged in 3% hydrogen peroxide (H_2O_2) for 2 h following Kaufman (2000) and Hearty et al. (2004). Only translucent specimens were selected for the analysis.

2.2.1. Gas chromatography analysis

The amino acid concentrations and ratios of cave bear remains from Amutxate, Santa Isabel and Sima de los Huesos, and the historical and archaeological human samples from the Fortress of La Mota and Monastery of San Francisco cemeteries and from Tell Halula were measured using a gas chromatograph in the

Table 3
ESR age estimated, including early uptake and linear uptake models, in dentine collagen of ancient bears (*U. spelaeus* and *U. deningeri*) from various localities in the Iberian Peninsula. Some samples were duplicated, with the mean and standard deviation reported. For site locations see Fig. 1 (EE: Eirós, LU: La Lucia; SS: La Pasada; SI: Santa Isabel; TR: Troskaeta; AX: Amutxate).

Sample	Material	ESR sample#	EU age (ka)	LU age (ka)	Mean EU age (ka)	Mean LU age (ka)
EE-523	*U. spelaeus*	1324 A	24.6 ± 1.4	24.9 ± 1.4		
LU-S-109	*U. spelaeus*	1325 A	102.0 ± 10.0	125.0 ± 13.0	108.5 ± 15.6	126.7 ± 19.8
		1325 B	118.0 ± 12.0	129.0 ± 15.0		
LU-S-116	*U. spelaeus*	1326 A	111.0 ± 12.0	112.0 ± 12.0		
LU-S-183	*U. spelaeus*	1327 A	106.0 ± 11.0	114.0 ± 12.0	103.3 ± 14.9	112.5 ± 1
		1327 B	101.0 ± 10.0	111.0 ± 12.0		
SS-1015	*U. spelaeus*	1331 A	24.3 ± 1.5	30.1 ± 1.8		
SS-1112	*U. spelaeus*	1332 A	37.4 ± 3.6	37.8 ± 3.7		
SI-259	*U. deningeri*	1333 B	215.0 ± 17.0	305.0 ± 23.0		
TR-22-3600	*U. spelaeus*	1335 A	48.9 ± 3.7	68.4 ± 4.9	43.0 ± 5.0	61.1 ± 6.7
		1335 B	38.0 ± 3.4	54.7 ± 4.6		
TR-26-4255	*U. spelaeus*	1336 A	78.4 ± 7.2	94.3 ± 8.9	78.4 ± 7.2	94.3 ± 8.9
AX-1192	*U. spelaeus*	1559 A	39.0 ± 2.0	39.0 ± 2.0	39.6 ± 3.6	39.6 ± 3.6
		1559 B	41.0 ± 3.0	41.0 ± 3.0		
AX-0	*U. spelaeus*	1560	44.0 ± 4.0	44.0 ± 4.0		
AX-14	*U. spelaeus*	1561	88.0 ± 6.0	90.0 ± 6.0		
AX-746	*U. spelaeus*	1562	46.0 ± 3.0	48.0 ± 3.0		
AX-103	*U. spelaeus*	1563	85.0 ± 6.0	88.0 ± 6.0		

Biomolecular Stratigraphy Laboratory, following the sample preparation protocol described by Goodfriend (1991) and Goodfriend and Meyer (1991). This procedure involved several steps. Samples were hydrolyzed under a N_2 atmosphere in a mixture of 12 N HCl (2.9 μl/mg) and 6 N hydrochloric acid (100 μl) for 20 h at 100 °C. They were then desalted with hydrofluoric acid, and the supernatant was frozen and dried under vacuum. Amino acids were derivatized in a two-step process, the first involving esterification with 250 μl of 3 M thionyl chloride in isopropanol for 1 h at 100 °C under N_2. The samples were then dried and acylated by reaction with 200 μl of trifluoroacetic acid anhydride (25% in dichloromethane) for 5 min at 100 °C. Excesses of derivative and solvent were evaporated under nitrogen. The sample was taken up in 100 μl of n-hexane, and aliquots of 1–4-μl were injected into a Hewlett–Packard 5890 gas chromatograph. Helium was used as the carrier gas, at a column head pressure of 5.8 psi, with a Chirasil-L-Val-fused silica column (0.39 mm × 0.25 μm × 25 m) from Chrompack. The detector used was an NPD set at 300 °C.

2.2.2. Liquid chromatography analysis

Samples from bears from caves in the Alps, several mammals from the karstic system in Pinilla del Valle, horses and ostracodes from Ambrona, and living human donors were subjected to amino acid analysis using a high performance liquid chromatograph (HPLC) in the Biomolecular Stratigraphy Laboratory, following the sample preparation protocol described by Kaufman and Manley (1998) and Kaufman (2000). This procedure involves hydrolysis, which was performed under N_2 atmosphere in 20 μL/mg 7 M HCl for 20 h at 100 °C in the case of dentine samples (7 μL of 6 M HCl for ostracode valves). The hydrolysates were evaporated to dryness in *vacuo* and then rehydrated in 7 μL 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-1100 HPLC equipped with a fluorescence detector. Excitation and emission wavelengths were programmed at 335 and 445 nm, respectively. A Hypersil BDS C18 reverse-phase column (5 μm; 250 × 4 mm i.d.) was used for the analysis.

Derivatization took place before injection by mixing the sample (2 μL) with the pre-column derivatization reagent (2.2 μL), which comprised 260 mM isobutyryl-L-cysteine (chiral thiol) and 170 mM o-phthaldialdehyde, dissolved in 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 μM 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 25 °C from 1.0 mL/min, from 95% eluent A and 5% eluent B upon injection to 76.6% eluent A, 23% eluent B, and 0.4% eluent C at 31 min.

3. Results

3.1. ESR

For age calculation, we used an updated version of the ESR-DATA program, which uses the Monte Carlo beta attenuation values of Marsh (1999), with an alpha efficiency of 0.13 ± 0.02 (Grün and Katzenberger-Apel, 1994). The resulting ESR age estimates are shown in Table 3 for early U uptake and for linear U uptake (for more details on U-migration and modeling, see Grün and McDermott, 1994). As some samples were analyzed twice, we calculated their mean age. We also determined the average age for each locality. These ages were further used for the establishment of the age calculation algorithm for Asp D/L values measured in dentine collagen.

3.2. AAR

Although recent studies showed that HPLC D/L Asp values are about 5% higher than those obtained by GC (Powell et al., 2013; Wehmiller, 2012, 2013), these differences are very small for the range of Asp D/L values obtained in this study (0.02 for Asp D/L values of 0.40). Moreover, given the similarities between several samples analyzed by GC and HPLC in our laboratory (cf. Ortiz et al., 2009, p.955, see Supplementary Data-Fig. 2), we assume that GC values they can be compared with the new Asp values calculated using HPLC. Here we describe and discuss results from each cluster of samples.

3.2.1. Modern humans

Fig. 2 shows the Asp racemization ratios obtained in odontological samples against patient dental age. Dental ages were calculated by subtracting dental eruption age from individual ages (incisors: 4 years; second premolars: 6 years; canines: 6 years; first premolars: 5 years; first molars: 3 years; second molars: 7 years; third molars: 12 years). A good correspondence between individual age and Asp D/L corrected values was observed, the correlation coefficient being significant and high ($r^2 = 0.94$). Similarly, Helfman

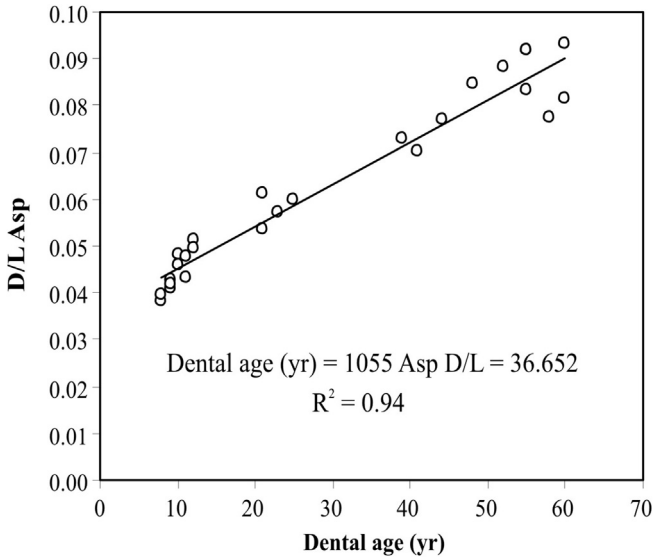

Fig. 2. Aspartic acid racemization in dentine collagen vs. age of modern humans.

et al. (1976) obtained a correlation of $r = 0.979$ between age and Asp D/L values in coronal dentine.

3.2.2. Historical humans From 16th and 19th century cemeteries

No significant relationship was observed between Asp racemization ratios and age-at-death clusters, even when average values inside each age cluster were used (Fig. 3). The samples from the Monastery of San Francisco in Valladolid (7) showed a tendency to have higher D/L values in teeth of older individuals; however, the sample size was very small, and there was a sample from category 5 (aged adult) that showed a low Asp racemization ratio. The samples from the Fortress of La Mota cemetery did not appear to reflect age at death, neither was it possible to establish the burial-age of these samples.

Of note, the racemization ratios in two age distributions were much higher than in samples from modern humans. This observation may indicate the occurrence of post-mortem AAR (Figs. 2 and 3): Asp racemization values in group 1 (child) and 2 (juvenile) ranged between 0.08 and 0.13. These values are similar, or even greater, than those obtained in some Pleistocene samples (Table 4), and could be attributed to different taphonomical conditions. In fact, corpses from the Fortress of La Mota cemetery were buried in an open-air cemetery, specifically in a mass-grave only a few centimeters from the surface and covered with quicklime (CaO) because of an epidemic that caused high mortality in the 16th century. The reaction between quicklime and water can release enough heat to ignite combustible materials, and in the case of human teeth from the Fortress of La Mota cemetery, the exothermic reaction with filtering of meteoric waters through the mass-grave would have produced an increase in their Asp D/L values and could also explain the lack of correspondence between Asp racemization ratios and age-at-death clusters.

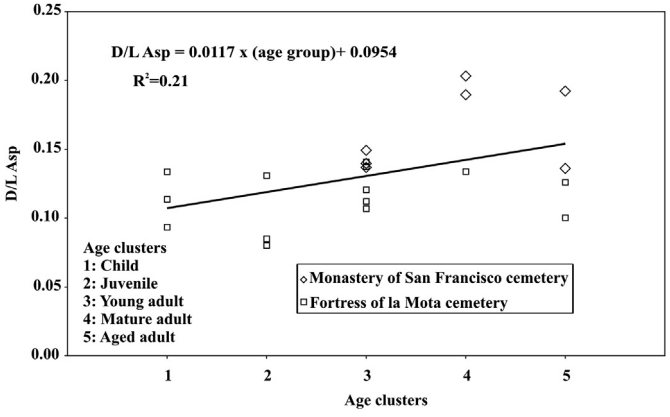

Fig. 3. Aspartic acid racemization in dentine collagen vs. age clusters of historical humans from 16th (Monastery of San Francisco) and 19th century (Fortress of La Mota) cemeteries.

Table 4
Ages calculated using various dating methods (ESR, ^{14}C, U/Th) with their corresponding aspartic acid D/L values used for the determination of the age-calculation algorithm. Amino acid racemization analysis could not be performed on some teeth dated by ESR. Therefore we compared mean ESR ages with mean aspartic acid D/L values for those localities. In Eirós and Sima de los Huesos, we compared the mean age of the sites with the mean aspartic acid D/L values for these localities. Eirós-EE, La Lucia-LU, La Pasada-SS, Santa Isabel-SI, Troskaeta-TR, Amutxate-AX, Sima de los Huesos-SH.

Sample	Age (ka)	Asp D/L
EE	24.9 ± 1.4 (1) 24.1 ± 0.4(2)	0.068 ± 0.009
LU	118.7 ± 26.1 (1)	0.133 ± 0.036
SS	31.5 ± 4.1(1)	0.085 ± 0.024
SI	305.0 ± 23.0(1)	0.399 ± 0.031 (4)
TR	73.1 ± 11.1(1)	0.086 ± 0.014
AX-1192	39.6 ± 3.6(1)	0.053
AX-0	44.0 ± 4.0 (1)	0.048
AX-14	90.0 ± 6.0 (1)	0.069
AX-746	48.0 ± 3.0 (1)	0.050
AX-103	88.0 ± 6.0(1)	0.096
Actual	0.0	0.045
SH	320.0 ± 4.0(3)	0.353 ± 0.036 (4)

1.-ESR (this paper); 2.-^{14}C (Grandal d'Anglade and Vidal Romaní, 1997); 3.-U/Th (Bischoff et al., 1997); 4.- Some more fossil bear teeth from SH (Sima de los Huesos) and SI (Santa Isabel) were sampled here and the mean Asp D/L value changed slightly with respect to that of Torres et al. (2000a, b, 2001).

3.2.3. "Ancient" humans (Neolithic age) from Syria

Fig. 4 shows the Asp racemization ratios for samples found in the Pre-pottery Neolithic B levels (7500–7300 cal. B.C.) (Molist, 1996). Taking into account the short time span between archaeological phases, no age (geological)-Asp D/L correlation was found, and on average the D/L Asp values were similar.

3.2.4. Pleistocene mammals

A very high correlation was achieved in our age-calculation algorithm for Asp racemization ratios when using previously dated

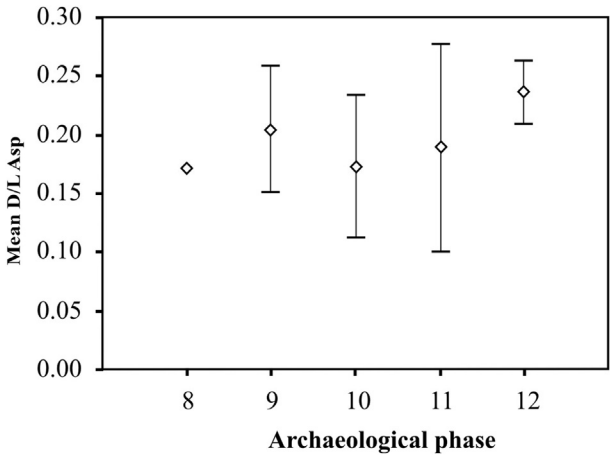

Fig. 4. Aspartic acid racemization in human dentine collagen from burial sites in Tell Halula (Syria).

dentine collagen samples (Fig. 5). Although there was discordance between the age and Asp D/L values of samples AX-14 and EE (Table 4), we kept both to establish the age-calculation algorithm. For this algorithm, we used the racemization values from eight cave localities dated through independent and distinct radiometric methods (Table 4): ^{14}C in bones (Eirós, Galicia; Grandal d'Anglade and Vidal Romaní, 1997), ESR and U series in bear teeth (Sima de los Huesos, Burgos; Bischoff et al., 1997), and ESR data for bear teeth (Amutxate, Troskaeta, Santa Isabel, La Lucia and La Pasada). In the case of ESR dating, no large differences were observed when taking into account early uptake (radionuclide) (EU) or linear uptake (radionuclide) (LU), with the sole exception of the Santa Isabel site (Table 3), in which marked age differences (of 100 ka) were detected. In the former model (Torres et al., 2002), the EU-based ages were selected for the construction of the algorithm; however, after considering the mechanism of "continuous collagen loss" linked to the decrease in Asp content and age (Torres et al., 2000a, 2000b, 2001), we chose to use the LU model, as bones and teeth are geochemically open systems. We used the LU model as there was a very slow and continuous hypodermic water flow that produces an almost regular uptake of U in the fossils.

It must be highlighted that the ESR ages for *U. spelaeus* teeth from La Lucia (between 112.0 ± 12.0 ka and 126.7 ± 19.8 ka, cf. Table 3) were coherent with the U-series dating of a flowstone sealing the cave bear remains (77.231 ± 3.305 ka, cf. Torres et al., 2001). As the ESR ages were obtained directly from teeth, we used these data to establish the algorithm instead of the U/Th age as used in Torres et al. (2001), because the latter age indicated when the cave bear remains were sealed, thus marking the minimum age of the cave bear remains.

A variety of mathematical expressions that describe changes in AAR over time (t) have been used in Quaternary geochronology: the integrated rate equation-FOK (Bada et al., 1970; Wehmiller and Hare, 1971; Bada and Schroeder, 1972), the logarithmic equation (Wehmiller et al., 1988; Goodfriend and Meyer, 1991), parabolic-curve fitting (Mitterer and Kriausakul, 1989; Goodfriend, 1991; Kaufman, 1992; Murray-Wallace and Kimber, 1993; Hearty, 1998; Murray-Wallace et al., 2001), the simple linear equation (Goodfriend, 1987; Goodfriend et al., 1992, 2000; Ellis et al., 1996), the step linear model (Miller et al., 1999; Hearty, 2002, 2003), simple power transformations (Goodfriend et al., 1995, 1996; Kaufman, 2006), and the modification of the integrated rate equation with a power function (Torres et al., 1997; Torres, 1999; Kaufman, 2000; Manley et al., 2000; Ortiz et al., 2002, 2004). In brief, the mathematical expressions for these models differ substantially and none can be applied universally to all amino acids or sample types. Therefore, each equation must be evaluated within the field context of the samples of interest (cf. Goodfriend, 1991; Clarke and Murray-Wallace, 2006; Wehmiller et al, 2012, 2013). In fact, according to Goodfriend (1991), "a model must be chosen empirically for each data set based on the goodness of fit".

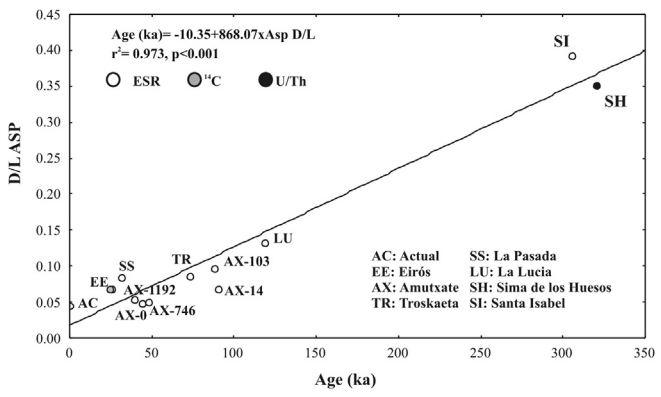

Fig. 5. Age-calculation algorithm for aspartic acid racemization in dentine collagen.

In order to select the best fit for the Asp age-estimation algorithm, we compared the correlation coefficients (r) for various approaches (Table 5). On the basis of the results, we chose to work with the direct linear relationship between Asp D/L values vs. time because it provided the highest correlation coefficient, although the first-order kinetic (FOK) transformation of the Asp D/L values vs. time showed a similar correlation coefficient. The equation (Fig. 5) is as follows:

$$\text{Age(ka)} = -10.4 + 868.1 \times \text{Asp D/L}; r2 = 0.97,\ p < 0.001$$

This algorithm, calculated using new dates, is similar to that established in Torres et al. (2002), although it was calculated using only 3 previously dated sites. This mathematical expression is in accordance with many authors, as a linear racemization rate for Asp and other amino acids can be assumed for racemization ratios up to 0.40 (cf. Masters and Bada, 1977; Mitterer and Kriausakul, 1989; Goodfriend, 1987; Goodfriend et al., 1992, 2000; Clarke and Murray-Wallace, 2006). However, since the rate of AAR usually decreases in late diagenesis, the use of this algorithm is applicable only when it can be demonstrated that the apparent racemization rate is constant. In kinetic experiments on mineralized collagen of bear teeth performed by Llamas et al. (1999) and Canoira et al. (2003), a linear racemization rate was observed until Asp D/L values of 0.45, which were the maximum values measured in the present study. Moreover, Csapò et al. (1998) found a linear relationship between age and racemization ratios, with values up to *ca.* 0.7, for several amino acids (histidine, phenylalanine, aspartic acid, alanine, isoleucine, valine and glutamic acid) in fossil bones. However, Collins et al. (2009) postulate that racemization of Asp eventually changes in the insoluble fraction of collagen, reaching a plateau at D/L values of 0.09.

This linear Asp racemization ratio versus the radiometric age relationship is not unprecedented, as similar results have been published (Julg et al., 1987; Elster et al., 1991; El Mansouri et al., 1996), although a later study analyzing the Asp D/L values in the collagen of the same 10 fossil bones studied by Julg et al. (1987) but using a different derivatizing agent, did not find such a good correlation (Pichet et al., 1989). Bones may be more prone to micro-taphonomic influence than massive enamel-protected dentine (Andrews, 1997).

To check the reliability of our age calculation model, we compared the estimated age using the racemization data from some ^{14}C-dated cave bear sites in Italy and Austria (Rabeder, 1992, 1999; Döppes and Rabeder, 1997; Hofreiter et al., 2001, 2004a,b), a Neanderthal site in Spain (El Sidrón) (Torres et al., 2010), mammal remains from the karstic system of Pinilla del Valle, and a lacustrine locality with associated fossil horses (*Equus* sp.) and freshwater ostracode shells.

The Asp D/L values obtained in *Homo neanderthalensis* remains from El Sidrón cave, in *Equus* sp. teeth from Ambrona, in *Ursus*

Table 5
Correlation coefficients (r) between time and aspartic acid D/L values measured in dentine collagen. All correlations are statistically significant at the level of $p < 0.001$. The highest correlation coefficient is shown in bold.

	D/L Asp	Ln (1 + D/L Asp)/(1-D/L Asp)
Time	**0.973**	0.972
Sqrt time	0.890	0.887

Table 6
Comparison of the ages obtained in different sites using a range of dating methods and those using the age-calculation algorithm for dentine collagen provided in this paper. ^{14}C dates are uncalibrated ages.

Locality	Dating method	Age (ka)	AAR material	Asp D/L	AAR age (ka)
Ambrona	AAR (ostracodes)	171.6 ± 22.8	*Equus* sp.	0.222 ± 0.021	182.4 ± 18.7
Ramesch	^{14}C	*ca.* 64.0–31.0(1)	*U. eremus*	0.085 ± 0.010	63.3 ± 9.0
Gamssulzen	^{14}C	*ca.* 47.0–32.0(1)	*U. ingressus*	0.082 ± 0.011	60.9 ± 9.7
Herdengel	^{14}C	*ca.* 36.2–40.0(1)	*U. eremus*	0.063 ± 0.004	44.1 ± 3.7
Herdengel	^{14}C	*ca.* 58.0–>60.0(1)	*U. ingressus*	0.071 ± 0.005	51.1 ± 5.1
Conturines	^{14}C	*ca.* 41.3–47.5(1)	*U. ladinicus*	0.076 ± 0.007	55.8 ± 6.2
El Sidrón	ESR, OSL, AAR	*ca.*45 (2)	*H. neanderthalensis*	0.068 ± 0.011	48.5 ± 10.1
Navalmaillo (Pinilla del Valle)	TL	Level 1F: 71.6 ± 5.0; 77.2 ± 6.0 (3)	Bovidae, *Rhinoceros, Equus, Castor*	0.274 ± 0.066	234.9 ± 65.3
Buena Pinta (Pinilla del Valle)	TL	Level 3: 63.4 ± 5.5 (3)	*Equus, Cervus*	0.109 ± 0.012(*)	85.5 ± 11.9 (*)
Camino (Pinilla del Valle)	TL	Level 5: 90.9 ± 7.8; 91.6 ± 8.1(3) Upper level: 74.4 ± 6.3 (3)	*Equus, Cervus, Sus,* Hyena, Bovidae, Arctiodactyla	0.119 ± 0.026(*)	94.4 ± 20.6 (*)

(1) Rabeder (1992); Döppes and Rabeder (1997); Rabeder (1999); Hofreiter et al. (2001; 2004a,b); (2) Torres et al. (2010); (3) Pérez-González et al. (2010). (*) some values were discarded for the age calculation due to their high Asp D/L values, falling outside the 2s range of the group following the criteria proposed by Hearty et al. (2004) and Kosnik et al. (2008) (see Supplementary Data), these probably caused by man-mediated heating.

spp. teeth from Alpine caves, and in mammal teeth from the karstic system of Pinilla del Valle were introduced into the age-calculation algorithm established here. The AAR estimated age is the average of the numerical dates obtained for each tooth in each locality (Table 6, Fig. 6), along with the independent age estimates. The AAR age uncertainty is the standard deviation of all the pooled values. The numerical age of the ostracode samples from Ambrona was calculated using the Asp and Glu D/L values (see Supplementary Data-mean Asp D/L = 0.387 ± 0.011; mean Glu D/L = 0.179 ± 0.022), and the age calculation algorithms applied were those established by Ortiz et al. (2004) in the Central and Southern parts of the Iberian Peninsula. The age of the Ambrona site (Table 6) is the average of the numerical dates obtained for each amino acid D/L value measured in ostracodes from this locality. Good correspondence was observed between the two datings of each site (Table 6, Fig. 6), implying that the equation provided here is reliable.

The karstic complex of Pinilla del Valle, which includes the Camino and Buena Pinta caves, showed homogeneous Asp racemization ratios (Table 6), with ages in accordance with TL ages (cf. Pérez-González et al., 2010), although a few samples from each site were discarded because their Asp and Glu D/L values fell outside the 2s range of the group, following the criteria proposed by Hearty et al. (2004) and Kosnik et al. (2008) (see Supplementary Data). However, the AAR age obtained from teeth from the Navalmaillo rockshelter differed greatly to the TL age (Pérez-González et al., 2010).

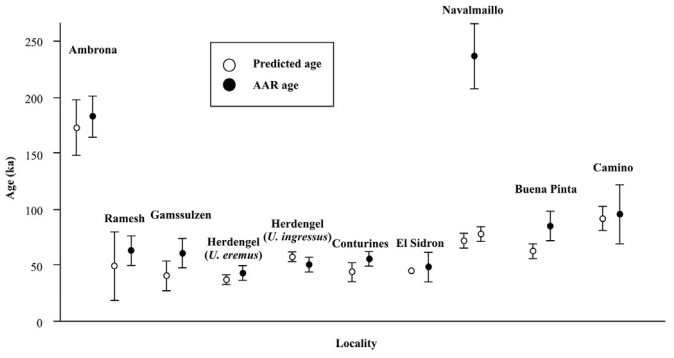

Fig. 6. Comparison of the ages obtained in different sites using a range of dating methods (^{14}C, ESR, OSL, TL, AAR) and those using the age-calculation algorithms for dentine collagen provided in this paper (AAR). Ages appear in Table 6.

4. Discussion

4.1. Dialysis step

In our opinion, the dialysis step proposed by Lafont et al. (1984) and Marzin (1990) is crucial for collagen sample preparation (Fig. 7). In fact, according to Collins and Galley (1998) and Waite et al. (1999), Asp racemization in dentine is significantly influenced by the preparation method. This method (dialysis step) is time-saving compared to that proposed by Ohtani et al. (1998), and Marzin (1990) found a good correlation between ^{14}C and AAR dates using this methodology. El Mansouri et al. (1996) applied a more restrictive dialysis step (10 kDa) and also reported satisfactory ^{14}C-AAR (Asp) racemization; however, their results cannot be directly compared with those obtained in our lab. It is noticeable that none of the regression lines obtained in El Mansouri et al. (1996) for the protein fractions (total extract, and >10,000 Da) and environments (vertebrate remains present together with artifacts within levels made of fine deposits, and those mixed with coarse deposits) intercept the origin. This interception is likely to be explained by the induced racemization linked to the sample preparation protocol (hydrolysis) (Goodfriend, 1987, 1991; Goodfriend and Meyer, 1991), being 0.045 for Asp in dentine collagen (Torres et al., 1999).

4.2. Age at death estimation

The utility of collagen Asp racemization for age estimation in samples from living and recently deceased individuals was demonstrated by Helfman et al. (1976), who obtained a correlation of $r = 0.979$ between age and Asp D/L values in coronal dentine, and by Ohtani and Yamamoto (1991, 1992), Ohtani (1995), and Ohtani et al. (1998, 2002, 2003, 2004). However, according to Waite et al. (1999), Asp and asparagine (Asn) racemization occurs within peptides but is believed to be restricted by the presence of conformational constraints that prevent this process in the collagen triple helix at low temperatures, at least < 37 °C (van Duin and Collins, 1998; Collins et al., 2009). These constraints are linked to the H bonds between chain atoms that stabilize the collagen helicoidal structure and also to the further inhibition of the intermediate cyclic succinimide formation. The size and polarity of the adjacent peptides also affect racemization (Geiger and Clarke, 1987; Fujii et al., 1994). This taphonomic and time-controlled collagen maturation also occurs in kinetic experiments, in which collagen loses its helicoidal structure (cf. Collins et al., 1999).

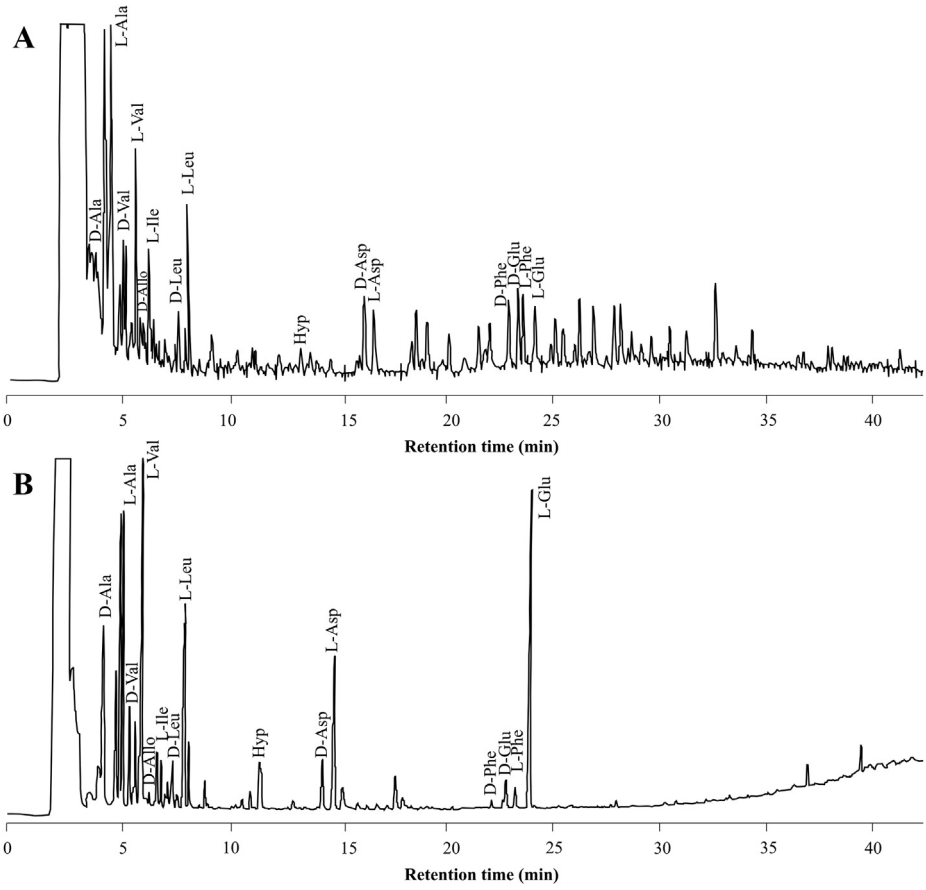

Fig. 7. Chromatograms obtained through the analysis of the same sample (BB1-909). A) total fraction; B) high molecular weight fraction after the dialysis step at 3500 Da.

Here we confirm that Asp racemization is consistent with age (Fig. 2) and that the methodology described here works satisfactorily for forensic studies using a simpler sample preparation protocol.

We also calculated the apparent Asp racemization rate for modern humans (*in vivo*). The racemization reaction of free amino acids is thought to be a first order reversible reaction (Bada et al., 1970; Wehmiller and Hare, 1971; Bada and Schroeder, 1972), which can be expressed as L-amino acid ↔ D-amino acid. k is the reaction rate constant of racemization and its rate equation is expressed as: $\mathrm{Ln}[(1 + \mathrm{D/L})/(1\text{-}\mathrm{D/L})] = 2kt + \text{constant}$, where t is the age. However, Goodfriend (1991) observed that the signal measured as Asp is actually the combined responses of Asp and Asn, with the latter decomposing to the former during acid hydrolysis. Goodfriend (1991) inferred that the pattern of Asp and Asn racemization is due to differences in the rate of succinimide formation from Asn to Asp, and Brinton and Bada (1995) indicated that the effect was due to differences in the rate of racemization and the direct decomposition of Asn to Asp by the hydrolysis of the amide group. Likewise, according to Collins et al. (1999), and Waite and Collins (2000), the initial rapid phase of Asp racemization is caused by a contribution from Asn, and Asp does not follow FOK when peptide-bound. Therefore we chose to follow a linear relationship between D/L Asp values and age at death (Fig. 2).

The apparent Asp racemization rate constant for modern humans obtained in this study was $9.5 \times 10^{-4}\ \mathrm{yr}^{-1}$, which is of the same order of magnitude to that measured in tooth enamel (Helfman and Bada, 1975), dentine (Ohtani, 1995) and enamel and dentine (Ohtani and Yamamoto, 1992). Bada and Brown (1980) also found good correspondence between true age and age determined from Asp racemization in living animals from zoo collections.

4.3. Age estimation in archaeological/palaeoentological sites

Chronological age estimation in human remains from cemeteries (Carolan et al., 1997) and Eskimo remains (Bada and Brown, 1980; Masters, 1984) has been reported. Bada and Brown (1980) described a close correspondence between age at death (calculated from forensic methods) and Asp D/L from remains collected in several medieval burial sites in Czechoslovakia, and in the remains of an Eskimo that died 1600 years ago on St. Lawrence Island. Likewise, after studying the Barrow Eskimo remains, Masters (1984) observed that the extent of Asp racemization in human dentine can be used to assess the chronological age of the individual; however, we were unable to achieve this estimation in the present study, probably because postmortem conditions determine the accuracy of measurements of racemization age at death (Hare, 1980; Masters, 1984). Therefore, it is difficult to assess when post-mortem AAR takes over from the *in vivo* process. Our results suggest that post-mortem AAR might occur after only some centuries; however, on the basis of findings by Bada and Brown (1980) and Masters (1984), it may take place after thousands of years.

Results on Asp racemization in Neolithic samples from Syria showed that there is no burial-age vs. Asp D/L relationship, as the error bars of the mean Asp D/L values of the various samples in all the archaeological levels were similar (Fig 4). These observations thus support a relatively short time interval between stages, within the dating resolution of the technique. This finding confirms that

AAR cannot discriminate between such short intervals (decades and some centuries). The thermal influence on AAR is significant; these samples, ^{14}C-dated at 7500–7300 cal B.C. (Molist, 1996), showed similar Asp D/L values (ca. 0.20) to those of vertebrate remains of the Iberian Peninsula sites dated to ~160 ka (Fig. 5). In open air sites of the Eurosiberian region of the Iberian Peninsula, the current mean annual temperature is 10–12 °C (Rivas-Martínez and Rivas-Sáenz, 1996–2009, Table 1), while in Tell Halula (Syria) it is 18.5–19.5 °C (Rivas-Martínez and Rivas-Sáenz, 1996–2009, Table 1).

These results confirm that the development of a specific age calculation algorithm based on independent ages is required for regions with distinct integrated thermal histories (Hearty et al., 1986; Goodfriend, 1991; Kaufman and Brigham-Grette, 1993; Kaufman, 2006)). It has been recommended that the best model be chosen empirically for each data set on the basis of the goodness of fit for a particular taxon and depositional setting (Clarke and Murray-Wallace, 2006; Kosnik et al., 2008).

The occurrence of cold periods during the Pleistocene, which were much less severe in the Iberian Peninsula than in glaciated areas of Europe, and even less severe in deep cave environments (Torres et al., 2003), did not impair satisfactory correlation between AAR vs. independent datings (^{14}C or ESR). Therefore AAR is considered a suitable dating method for almost the entire Iberian Peninsula, as similar thermal conditions occur (Rivas-Martínez and Rivas-Sáenz, 1996–2009), and, based on the good correspondence with ^{14}C ages, also for areas of Austria and Italy (Table 6). Thus, it is necessary to distinguish between mean annual temperature of the air and the temperature experienced by the sample. In fact, according to Wehmiller (1997), and Miller and Brigham-Grette (1989), diurnal temperature fluctuations are damped out in the first centimeter below the surface in open-air sites, although seasonal oscillation can penetrate at least 1 m into the subsurface. The influence of temperature in sediments from deep areas inside a cave should be negligible, i.e. "for a fossil buried in a cave sediment, any fluctuations around the mean value will be dampened further by the cave sediment" (Smith et al., 2003). In fact, Hoyos et al. (1998b), Torres et al. (2003) and Toomey (2009) quantified that the temperature deep in caves varies less than 1 °C, differences being, in most cases, only tenths of a degree. Thus, although the air temperatures measured near the Austrian and Italian caves were lower than that of caves in the Iberian Peninsula (Table 1), the thermal variations inside the caves, recorded along a complete year using Hobo devices, were very low (lower than 1 °C; Torres et al., 2003) and burial-temperatures are expected to be similar. The use of caves by animals during winter is likely to be due to the milder temperatures found inside the caves.

The high correlation observed between Asp racemization with age in mammal dentine (Fig. 5) and the good correspondence found between AAR ages and those obtained with other methods (Table 6) implies that AAR can be confidently used in dating non-man-mediated cave deposits as well teeth from lacustrine deposits. In this case, the dampening effect of air temperature inside the lacustrine deposits of the karst sinkholes of Ambrona was justified by the high sedimentation rate (Mosquera Martínez, 1995). The apparent racemization rate constant for fossil mammals is 1.2×10^{-6} yr^{-1}, which is similar to those observed by Julg et al. (1987) – 3.0×10^{-6} yr^{-1} and El Mansouri et al. (1996) – 4.4×10^{-6} yr^{-1} in collagen.

In many cases the standard deviation from a single cave appeared to be high, but it is necessary to take into account that cave bear deposits are time-averaged, i.e. the cave remains from the Amutxate cave were accumulated during at least 9 ka (cf. Torres et al., 2007), the average error of each dating being around 10%. Likewise, considerable age variation was also found when large series of ^{14}C datings were made on samples from a single cave bear locality (cf. Hofreiter et al., 2004a,b). The effect of micro-taphonomic differences (Andrews, 1997) may also contribute to this variation, even in the same bone (Collins et al., 2009), although here we analyzed only teeth.

Accurate dating in anthropogenic sites is more difficult. The teeth of herbivores are usually found disarticulated from their alveoli (Toots, 1965; Hill, 1980), thus implying intense human action on the skull. We do not know whether this action was simply mechanical or included heating in a fire in order to extract the marrow, or even the use of bones as fuel. However, the presence of ashes from bonfires points to the possibility of samples having undergone heating, although they were not directly burnt.

Samples from the Navalmaillo rockshelter (and some of Camino and Buena Pinta caves) gave markedly greater ages than those previously obtained through TL and OSL dating methods (Pérez-González et al., 2010). It must be noted that the Navalmaillo rockshelter contains traces of bonfires (ashes). Of great interest is the presence of an old lime-kiln dug in the cave sediments, supposedly of medieval age, although until the 20th century small villages continued to use this kind of kiln to heat limestone chunks to over 900 °C for days. This procedure would have produced a mild heating of bone-bearing beds, thereby accelerating racemization (cf. Masters and Bada, 1977, 1978; Brooks et al., 1990; Hare et al., 1993). This heating would have produced anomalously high D/L values and changes in amino acid composition and concentrations, e.g., decrease in Asp, glycine and alanine with respect to the unheated composition, whereas serine, threonine and arginine were detectable at trace levels or had disappeared completely (Brooks et al., 1990).

We propose that regular, but minor, racemization occurs during the human lifespan. This process may take place in the gap region of collagen (Birk and Tresltad, 1991). The regular process stops around the time of death and there is no correlation between age and racemization thereafter, with taphonomic processes being dominant after only a few centuries or thousands of years after death. El Mansouri et al. (1996) suggested that a period of some thousands of years is required for the start of collagen protein degradation. On the basis of a previous study by our group (Torres et al., 2007), we propose that this maturation consists of collagen swelling, which results in a progressive relaxation of collagen fiber structure (gelatinization), thus allowing a regular Asp racemization rate. A progressive loss of collagen clearly occurs, as reflected in the decrease in total Asp content (Torres et al., 1999, 2001). The buried bone remains in a cave are under favorable taphonomic conditions, in such a way that DNA has been recovered from non-washed Neanderthal bones from a locality included in this paper (El Sidrón), (Briggs et al., 2009).

According to our data (Torres et al., 1999, 2001, 2003), an important loss of collagen was time-related and a marked decrease of Asp occurred. This observation agrees with Hare (1980), who found a progressive decrease of collagen content in heating experiments with water presence but no racemization/epimerization (of isoleucine) was detected. In the case of no water excess, there was no leaching of free amino acids or short-chain peptides derived from collagen gelatinization. These non-desired compounds that racemize at different rates were avoided after the use of the dialysis step. In Abri Pataud, El Mansouri et al. (1996) found a marked influence of the particle size of the bone "sedimentary matrix", the apparent racemization rate of Asp being higher in the most permeable beds, with higher hydraulic transmissivity, and ^{14}C ages, and that the Asp racemization rates correlate much better in the high molecular weight fraction (after dialyzing the sample at 10,000 Da). These authors postulate that racemization and collagen degradation are two coupled phenomena affecting the measurable

racemization. In a similar way, Matsu'ura and Ueta (1980) interpreted that the degree of AAR could be dependent on the dynamics of collagen decomposition.

The selection of dentine was made according to its best preservation in paleontological sites, and in the opinion of Masters (1987, p. 3213) and coinciding with Belluomini and Bada (1985), "dentin analysis may circumvent many of the problems encountered with the analysis of diagenetically altered bone".

Waite et al. (1999) made an extensive review of the use of AAR in forensic science and found that racemization occurs in human dentine, observing that (p. 118) "the insoluble fraction is composed mostly of triple helical collagen and therefore displays the slowest rate of racemization", thus admitting that there is some racemization in intact collagen. Julg et al. (1987) described this process as the spontaneous racemization of the intact collagen, their age-calculation algorithms being applicable (p. 27) "for dating bones as old as 50,000 years, without needing any calibration, whatever their thermal history may be". The aforementioned authors were the first to mention the conformational constraints produced by the triple-helix collagen, which were later confirmed by van Duin and Collins (1998).

Our results on samples from cemeteries and Neolithic settlements in Syria confirm Waite and Collins' (2000) opinion that, for historical samples, the technique may become increasingly inaccurate even when using amino acids from enamel, a presumably more closed system than dentine. Griffin et al. (2009) described different behavior of the enamel system in samples from two medieval cemeteries in England, reporting that this method worked well, and from a post-medieval one in Switzerland, for which a poor relationship between age-at-death and Asp D/L values was found, thus indicating the influence of diagenetic factors.

Judging that there was an absence of a satisfactory mathematical description of Asp racemization kinetics, Collins et al. (1999) proposed an alternative approach based on Asx (Asn + Asp) racemization via Asu formation. This approach successfully predicted racemization rates for collagen between 95 and 140 °C but not at 37 °C.

Samples from historical humans did not reflect age at death or calendar ages and the age distribution queues were much higher than in modern individuals. In our opinion, the decoupling age at death and Asp racemization starts very soon after burial. In some cases burials were in the ground (influenced by atmospheric temperature fluctuations) while in others coffins were piled up or covered with quicklime, as in the Fortress of La Mota cemetery. Thus the "site effect" may have greatly contributed to the distinct racemizaton ratios in coeval remains. In fact, Hare (1974, 1980) demonstrated that epimerization rates change dramatically depending on three scenarios: water absence (negligible), water vapor (high) and water excess (very low). In Pleistocene samples, mainly from deep-cave infills or large carbonate-rich lacustrine sequences, taphonomical conditions are assumed to very similar and therefore would provide similar Asp racemization rates.

5. Conclusions

Here we have demonstrated that the sample preparation and analysis protocol used in this study are effective for the AAR dating of vertebrate remains. Furthermore, they are less time-consuming than other methods (e.g. ^{14}C, ESR, U/Th).

In short, the sample preparation protocol —dialysis at 3.5 kDa— (Lafont et al., 1984; Marzin, 1990) can be confidently used in forensic determinations as well in non-human-mediated sites.

Our data from cemeteries in Spain and an archaeological site in Syria (Tell Halula) do not support this correspondence, although some studies have shown a good chronological age estimation through Asp D/L values in human remains from cemeteries and in Eskimo remains (Bada and Brown, 1980; Masters, 1984; Carolan et al., 1997); however, it should be taken into account that, according to Masters (1984), postmortem conditions are crucial. On the basis of our results, we propose that taphonomical effects can mask the *in vivo* racemization rate.

Asp racemization in collagen can be linked to a denaturation of the triple helix, which decreases the conformational constraints that prevent racemization (van Duin and Collins, 1998), also observed in insoluble collagen (Collins et al., 2009).

It is noticeable that the age-calculation algorithm established here is consistent with the kinetic model observed by Llamas et al. (1999) and Canoira et al. (2003), who found a linear relationship between Asp racemization ratios and time, up to Asp D/L = 0.45 in kinetic experiments on bear teeth collagen. Furthermore, the ages obtained through this model are consistent with those achieved with other dating methods on Pleistocene collagen samples from non-human-mediated accumulations. The almost constant moisture content and little temperature oscillation in deep-cave infills (Hoyos et al., 1998b; Smith et al., 2003; Torres et al., 2003; Sasowsky and Mylroie, 2004; Toomey, 2009), together with the geochemical homogeneity in the sediments, ensure homogeneous "site effects" (cf. Hare, 1980). In human-mediated sites, the heating linked to cooking processes and bonfires may have accelerated racemization; the presence of ashes from bonfires requires careful handling of excavation data and sample position in the site and also cautious interpretation of the data.

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Appendix A. Supplementary data

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