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RESEARCH ARTICLE

To date or not to date? A comparison of different ^{14}C pretreatment methods applied to archeological marine shells from Vale Boi (Portugal)

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Abstract

Mollusk shells are often found in archeological sites, given their great preservation potential and high value as a multipurpose resource, and they can often be the only available materials useful for radiocarbon (^{14}C) dating. However, dates obtained from shells are often regarded as less reliable compared to those from bones, wood, or charcoals due to different factors (e.g., Isotope fractionation, reservoir effects etc.). The standard acid etching pretreatment for mollusk shells is the most used in many ^{14}C laboratories, although another protocol known as CarDS (Carbonate Density Separation) was introduced just over a decade ago. We compare these two methods with two newly proposed methods for intracellular organic matrix extraction. We applied all four methods to samples selected from different archeological layers of the well-known Upper Paleolithic site of Vale Boi, rich in mollusk specimens throughout the stratigraphic sequence. Here we compare our results to previous dates to determine which of these pretreatment methods results in the most reliable ^{14}C dates. Based on the results of this study, all four methods gave inconsistent ages compared to previous dates and the stratigraphic attribution.

Introduction

Mollusk shells are often found in archeological sites, given their great preservation potential and high value as a multipurpose resource. Some mollusk species were used as food, others to make tools such as scrapers or decorative beads for ornamental purposes (Bicho and Haws 2008; Cascalheira et al. 2012; Douka 2011; Tátá et al. 2014). Mollusk shells provide high-resolution records of past environmental conditions and fluctuations, which are reflected in the carbonate structure as variable growth rates and chemical properties (Schöne 2008). Shell stable isotopes are used to estimate and reconstruct paleoenvironmental conditions, namely temperature, precipitation-evaporation patterns, upwelling intensity, and primary productivity (Elliot et al. 2009; Jones and Allmon 1995; Milano et al. 2022; Milano et al. 2020; Sadler et al. 2012; Schöne et al. 2004). Apart from past environmental conditions, shell remains represent a record of the evolutionary history of different human populations, their adaptation strategies, dietary habits, and symbolic thinking (Ramos-Muñoz et al. 2016; Will et al. 2019; Zilhão et al. 2010). Due to the elevated preservation potential of the carbonate crystalline structure, shells can often be the only available materials at archeological sites useful for ^{14}C dating (Brumm et al. 2018; Douka et al. 2013; Ono et al. 2009). However, dating only shells can lead to misinterpretations

due to various potential issues that can arise before and during sample analysis and calibration. Firstly, it is important to perform careful sample selection, both considering the preservation state in terms of diagenesis and time averaging, as well as species selection which could potentially create a difference between the target event and the dated event. Furthermore, there can be issues during sample pretreatment and dating, which can cause insufficient contamination removal or the introduction of exogenous contaminant carbon during analysis and processing. Finally, there are several corrections that are necessary to perform on the obtained radiocarbon date to have a calibrated age (such as isotope fractionation, the marine reservoir effect for marine species and the freshwater effect for riverine species). If all these issues are not considered when performing analysis on shells, it can lead to misinterpretations not only of the archeological context of the site, but also erroneous conclusions about migration events in human evolution. One example is the debate on the site of Ksar Akil in Lebanon which highlights the issue of using only shells when constructing a site chronology (Bosch et al. 2015; Douka et al. 2013). In Ksar Akil, the human remains and any other organic material, were too degraded to perform ^{14}C dating, and the only other available material was mollusk shells (Bosch et al. 2015; Douka et al. 2013). The results from Douka et al. (2013) placed the specimens as roughly contemporaneous to the oldest corresponding remains in Europe, thus casting doubts on the Levant as the point of origin for the dispersal of Upper Paleolithic culture into Europe. On the other hand, Bosch et al. (2015) showed that the human remains from Ksar Akil predate the European Upper Paleolithic as previously thought, confirming the hypothesis of the Levant as the starting dispersal point of Upper Paleolithic culture into Europe (Belfer-Cohen and Goring-Morris 2012; Bosch et al. 2015; Hublin 2012). The two studies used different shell pretreatment methods and sample selection criteria, which might have been part of the reason behind the discrepancy of the dates. The sample preservation state was also different, as one study used ornamental shells, while the other used shells collected for consumption (Bosch et al. 2015; Douka et al. 2013). As demonstrated by this debate, dates obtained only on shells can cause changes in important scenarios that can alter our understanding of events in human evolution. This emphasizes the crucial need to improve the accuracy of the chronometric results and to increase the reliability of shells as suitable dating materials.

Although the ^{14}C dating of shells has been improved over the years (Alves et al. 2018; Douka et al. 2010b; Faivre et al. 2015; Lindauer 2019; Philippsen 2013; Pigati 2002; Reimer and Reimer 2001; Reimer and Reimer 2017; Russell et al. 2011), there are still issues hindering the reliability of the chronometric data. In shells, there is a complex organization of calcium carbonate ($CaCO_3$) units and organic compounds with extraordinary resistance to mechanical and chemical stresses (Barthelat 2010). Yet, over long timescales, diagenesis can still alter their material properties. The primary carbonate phase, often aragonite, can recrystallize into secondary calcite after deposition. During the dissolution and precipitation of secondary calcite, there can be an incorporation of exogenous carbon into the sample. This is one of the processes which can cause a difference in the isotopic composition of the secondary phase compared to the primary one and alter the ^{14}C signal and therefore the resulting age (Douka et al. 2010a). Sand blasting and abrasion were thought to remove surface contamination and altered portions, assumed to also be concentrated mainly on the outer portion of the shells. Furthermore, chemical etching is used in attempts to remove shell portions altered by diagenesis from the ^{14}C dating analysis, which is performed on the entire carbonate fraction. This method has been widely used since the first applications of ^{14}C dating on marine shells, and variations of it are still applied in most laboratories around the world (Brock et al. 2010; Busschers et al. 2014; Chappell and Polach 1972; Dee et al. 2019; Gillespie et al. 1986; Santos et al. 2004). However, the acid etching of the shell surface is not entirely successful in removing altered portions of the shell due to the higher solubility of aragonite compared to calcite and the localized effect of the pretreatment. A novel pretreatment protocol using Carbonate Density Separation (CarDS) was developed by Douka and colleagues in 2010 (Douka et al. 2010a; Russo et al. 2010). This method showed promising results for the removal of secondary calcite from diagenetically altered corals and mollusk shells for ^{14}C dating, representing the most recent innovation in this area. It aims to separate the primary and secondary phase based on their different densities, in order to remove all the potentially contaminated secondary carbonate, not just from the

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surface, thus potentially obtaining a more accurate date for the sample (Douka et al. 2010a; Russo et al. 2010). However, diagenetic processes can often occur without a change in carbonate polymorphs, meaning that aragonite cannot be automatically considered the primary phase and the mere determination of the carbonate polymorph is not indicative of the presence or amount of diagenesis in the sample (Guzmán et al. 2009; Perrin 2004; Toffolo 2021). Although there are studies focusing on the pretreatment of the mineral fraction of the shell, the organic fraction was largely overlooked as it represents a minor portion of the shell (Hadden et al. 2018; Hadden et al. 2019). The main source of carbon in the mineral portion of the shell is the Dissolved Inorganic Carbon (DIC) with a contribution of a small amount of metabolic carbon as well (Gillikin et al. 2006; Hadden et al. 2018; McConnaughey and Gillikin 2008). Carbon of dietary origin is divided between the soft tissues of the mollusk and the shell organic matrix, which was found to be synthetized using mainly carbon from Particulate Organic Carbon (POC; Hadden et al. 2018). A recent study found that the organic intracrystalline matrix plays an important role in the diagenetic mineralogical transformation (Milano and Nehrke 2018). Their results state that the amount and composition of the organic portion of the shell influence the temperature at which the thermally induced transformation from aragonite to calcite occurs. Furthermore, the intracrystalline organic matrix is a closed system that was found to be isolated from the environment (Penkman et al. 2008) making isotopic exchange between the atmosphere and the intracrystalline organic matrix after deposition unlikely. However, attempts to date the organic matrix fraction are scarce and on much younger shells (Hadden et al. 2018; Hadden et al. 2019). Therefore, investigating the ^{14}C signal output of the organic matrix and comparing the results with outputs of the established methods could allow us to potentially improve the accuracy of the chronometric results and increase the reliability of shells as suitable dating materials.

Site under investigation

The archeological site of Vale Boi is a well-known Upper Paleolithic site in southwestern Portugal rich in mollusk shells, making it a great site to use for such methodological experiments. This site is situated between two different environments: the Mediterranean and Atlantic coasts, and it represents the earliest recorded modern human occupation in southwestern Portugal, as attested by the Early Gravettian remains dated to c. 32 ka cal BP (Bicho et al. 2013). It has a long stratigraphic sequence spanning from the early Gravettian, Proto-Solutrean, Solutrean, and Magdalenian (Cascalheira et al. 2012; Manne et al. 2012; Marreiros et al. 2015). Previous studies found evidence of multiple human occupations at this site, proving that it was most likely a seasonal residential camp with a combination of exogenous and regional cultural traits and a diversified use and processing of available resources including mollusk shells (Bicho and Haws 2008; Bicho et al. 2013; Cascalheira et al. 2012; Manne et al. 2012). Furthermore, there was an intense presence of inland and coastal foraging, hunting, and processing of various types and sizes of prey, possible processing of edible plants, as well as the production of various lithic and bone tools, ornaments and art including abundant shell beads (Bicho and Haws 2008; Bicho et al. 2013; Manne 2014; Manne et al. 2012; Marreiros et al. 2015; Pereira et al. 2016; Tátá et al. 2014). The presence of several mollusk species spans the different levels of the site proving the continued use and importance of mollusk shells as a resource throughout the stratigraphic sequence and allowing us to select and pretreat samples of different ages for comparison. Vale Boi has been excavated in three main areas—the Rockshelter, Slope and Terrace areas. Out of these three areas, the Terrace has the longest and most complete archeological sequence (Cascalheira et al. 2012; Manne et al. 2012; Marreiros et al. 2015). In previous studies, numerous ^{14}C dates were available from all areas of the site, including dates on mollusk shells from the Terrace area (Table 1). However, there were some problems with the dates in terms of agreement with the stratigraphic sequence that need further investigation (Cascalheira et al. 2012). Access to previous dates allows us to compare any new experimental results, which might also help clarify the stratigraphic incoherencies.

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Table 1. *Previous dates on samples from the Terrace area of the Vale Boi site*

Archeological attribution	Layer	Material	AMS code	^{14}C age	1σ err	Notes	Ref.
Epipaleolithic	3	Charcoal	AA-63310	8696	54		Tátá et al. 2014
Epipaleolithic	3	Charcoal	Wk-13685	8749	58		Tátá et al. 2014
Epipaleolithic	3	Charcoal	AA-63305	8825	57		Tátá et al. 2014
Epipaleolithic	3	Charcoal	Wk-24761	8886	30		Tátá et al. 2014
Epipaleolithic	3	Olea	Wk-36256	8737	25		Tátá et al. 2014
Epipaleolithic	3	Olea	Wk-36255	8664	25		Tátá et al. 2014
Gravettian	5	Patella	Wk-32144	24381	258	Calcite	Tátá et al. 2014
Gravettian	5	Bone	Wk-31090	24549	165	Min. age – low collagen	Tátá et al. 2014
Gravettian	5	Charcoal	Wk-24762	24769	180		Tátá et al. 2014
Gravettian	5	Patella	Wk-32144.2	23613	240	Aragonite	Tátá et al. 2014
Proto-Solutrean	5	Patella	Wk-50390	20554	75		Tátá et al. 2014
Proto-Solutrean	5	Shell	Wk-42831	20329	90		Cascalheira et al. 2017
Proto-Solutrean	5	Charcoal	Wk-42830	20818	107		Cascalheira et al. 2017
Gravettian	5	Littorina littorea	Wk-44416	22358	80		Belmiro et al. 2021
Gravettian	5	Bone	Wk-31089	24183	161	Min. age – low collagen	Tátá et al. 2014
Gravettian	5	Patella	OxA-25710	25050	100	Calcite	Tátá et al. 2014
Gravettian	5	Pecten	Wk-32145	25181	293	Min. age – burnt	Tátá et al. 2014
Gravettian	5	Patella	Wk-30677	25196	103	Calcite	Tátá et al. 2014
Gravettian	5	Patella	Wk-30679	25317	99	Calcite	Tátá et al. 2014
Gravettian	5	Charcoal	Wk-26801	27720	370		Tátá et al. 2014
Gravettian	5	Patella	Wk-30677.2	22235	173	Aragonite	Tátá et al. 2014
Gravettian	5	Patella	Wk-30679.2	25390	255	Aragonite	Tátá et al. 2014
Early Gravettian	6	Patella	Wk-30678	25579	98	Calcite	Tátá et al. 2014
Early Gravettian	6	Pecten	Wk-35712	26026	114		Tátá et al. 2014
Early Gravettian	6	Pecten	Wk-35713	25930	122	Aragonite	Tátá et al. 2014
Early Gravettian	6	Charcoal	Wk-35717	28012	192	Arbutus	Tátá et al. 2014
Early Gravettian	6	Pecten	Wk-32146	28321	422	Calcite	Tátá et al. 2014
Early Gravettian	6	Pecten	Wk-50396	41384	998		Tátá et al. 2014
Early Gravettian	7	Acanthocardia	Wk-32147	27141	365	Aragonite	Tátá et al. 2014
Early Gravettian	7	Nassarius	Wk-35714	25964	110	Calcite	Tátá et al. 2014
Early Gravettian	7	Patella	Wk-30676	24318	90	Calcite	Tátá et al. 2014
Early Gravettian	7	Patella	Wk-30676.2	26353	284	Aragonite	Tátá et al. 2014
Early Gravettian	7	Pecten	Wk-50394	26403	149		Tátá et al. 2014
Early Gravettian	8	Pecten	Wk-50393	27349	172		Unpublished

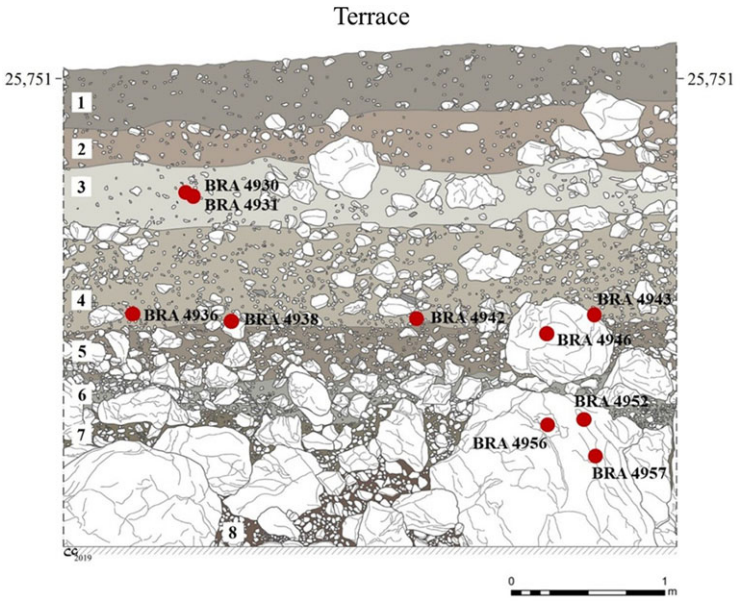

Figure 1. *The samples used in this study shown as red dots in their sampling positions within each archeological layer in the Terrace area of the Vale Boi site.*

Materials and methods

We performed analysis and ^{14}C dating on a total of 10 archeological shell samples, collected from Layers 3–7 at the Terrace area of Vale Boi. Some of the samples were divided into several pieces to perform the different pretreatment methods. In addition to the shell samples, two bone samples from Layer 6 in the Terrace area of Vale Boi were dated.

The bone samples were pretreated following the standard acid-base-acid method followed by ultrafiltration (Talamo et al. 2021; Talamo and Richards 2011) at the Department of Human Evolution at the Max Planck Institute for Evolutionary Anthropology (Leipzig, Germany). The Accelerator Mass Spectrometry (AMS) dates were obtained at the at the Klaus-Tschira AMS laboratory of the Curt-Engelhom-Centre for Archaeometry (CEZA; Mannheim, Germany).

All selected shell samples were given the unique identifying BRAVHO (Bologna Radiocarbon Laboratory Devoted to Human Evolution) lab number (BRA n°) and their stratigraphic position is shown in Figure 1.

Shell pretreatment methods

Here, we introduce two methods aiming to extract the intracellular organic matrix of the shell and compare them to the two previously mentioned methods from literature (Dee et al. 2019; Douka et al. 2010a; Russo et al. 2010). These four methods are summarized in Figure 2 as Methods A, B, C and D. Method A was based on a protocol routinely used in organic matrix extraction from modern coral and mollusk samples (Falini et al. 2013; Reggi et al. 2014). The advantage of this method is the use of a dialysis membrane, which allows to extract both the soluble and the insoluble organic matrix from the shell and minimizes the loss of organic material. Method B is quite similar, following mostly the same steps, although it does not include the use of a dialysis membrane. It was developed based on the routine collagen extraction protocol used in the BRAVHO ^{14}C laboratory (Talamo et al. 2021; Talamo and Richards 2011). Method C corresponds to the CarDS protocol (Douka et al. 2010a; Russo et al. 2010) and Method D to the standard acid etching pretreatment used in most laboratories across the world (Dee et al. 2019). Before applying these methods to archeological specimens, they were tested on modern

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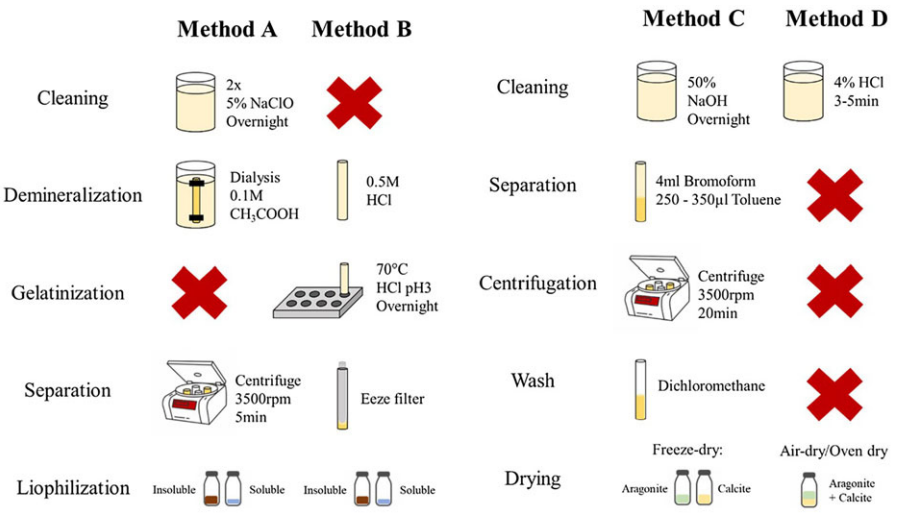

Figure 2. *Summary and comparison of the four methods tested, with red crosses for steps not performed.*

specimens. These tests, as well as further clarifications on the protocols used are presented in the Supplementary information file (SI). Comparing the ^{14}C dates obtained from all four methods we could determine the best method to routinely apply on mollusk shells, which would exclude pretreatment as an issue when considering the use of shells for radiocarbon dating. This will be one step towards achieving more reliable results and helping obtain trustworthy chronologies for sites where shells are the only available material for dating.

Method A—Organic matrix extraction using a dialysis process: The shell samples were first cleaned with a mechanical drill to remove any sediment adhered to the shell surface, they were further cleaned by leaving them in a 5 vol.% sodium hypochlorite solution overnight to remove external organic matter, as it can be contaminating (Penkman et al. 2008). Subsequently, the shells were rinsed in MilliQ water several times to wash off the sodium hypochlorite and any loose debris and left to air-dry before hand-crushing them to powder in an agate mortar. Then the crushed shells were further crushed in an automatic mill to obtain a finer powder, which was sieved with a 150 µm mesh stainless steel sieve. An aliquot of 2.5 g of each sample was put into a labelled glass tube and once again left in 5 vol.% sodium hypochlorite solution overnight for a thorough removal of non-intracrystalline organic material. It was then rinsed three times with MilliQ water and oven-dried for two days at 60°C. The powder was then transferred into SpectraPor 3 regenerated cellulose membranes for dialysis (MWCO = 3.5 kDa, flat width 45 mm, no glycerol humectant) and dispersed with 5 mL of deionized water. The sealed membranes were then put into 1 L of a 0.1 M CH_3COOH solution under stirring. The solution was changed every five days until the samples were decalcified, subsequently it was replaced by MilliQ water to reach a pH value of around 6. The obtained dispersion containing organic matter was centrifuged at 3500 rpm (2301 × g) for 5 min to separate the soluble (liquid) and insoluble (solid) fractions, which were then lyophilized and weighed.

Method B—Organic matrix extraction (as for collagen extraction): After an initial mechanical cleaning of the shell surface, the samples were ground and sieved as for Method A. The powder was then inserted into labelled test tubes with a defined volume of a 0.5 M HCl solution. The solution was periodically changed until the powder stopped producing effervescence. Then, the samples were rinsed in MilliQ water three times before proceeding with another 15 min HCl (0.5 M) step followed by other three rinses. Then the samples were put in a pH 3 (0.001 M) HCl solution in a heater block at 70°C overnight. After letting them cool down, each sample was filtered using an Elkey labs Eeze filter, to tentatively separate the soluble and insoluble fractions and transferred into glass vials to be frozen and lyophilized. Alternatively, centrifugation can also be used to separate the two fractions, as described for

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Method A. However, as opposed to Method A, the soluble fraction is mostly lost during pretreatment in this case as it is not protected by the dialysis membrane. This method was based on (Talamo et al. 2021), with modifications to account for the different material types.

Method C—Carbonate Density Separation (CarDS): After the initial mechanical cleaning steps, the shells were soaked in a 50% NaOH solution overnight to remove the external organic protein fraction. The samples were then ground and sieved as for the previous methods. An X–ray Diffraction (XRD) analysis was performed on the powder to identify the proportions of aragonite and calcite before proceeding with the separation protocol. A portion of 100–800 mg of powder was then inserted into a 10 mL test tube where 4 mL of 99% bromoform solution and 250–350 µL of toluene were added. The mixture was then centrifuged for 20 min at 3500 rpm to separate the two polymorphs. The supernatant (presumable calcite) fraction was carefully pipetted into another glass tube, and both fractions were washed in dichloromethane and deionized water before freezing and lyophilization. This method was based on previous work by different authors (Douka et al. 2010a; Russo et al. 2010).

Method D—Acid etching: This pretreatment consisted of mechanical surface cleaning followed by an acid etching of the shell surface in HCl (4%) to remove the outermost layer. For shells used in these experiments, the etching lasted from 3 to 5 minutes. The shells were then ground and sieved as for the other methods. This method followed the protocol described in Dee et al. (2019).

The four methods were applied to specimens from the Terrace area of the archeological site of Vale Boi. The selected samples in this context were *Crassostrea* sp. and *Pecten* sp., as these were the only two species present in different archeological layers with enough material to be used for four different pretreatment methods. These specimens were selected based on their weight to perform all methods on the same specimen when possible. In case enough material from the same specimen was not available, we selected a specimen from the same layer and same species to be able to compare the resulting ^{14}C dates.

X-ray diffraction analyses

X-ray powder Diffraction (XRD) was performed using a PANalytical X-ray Diffractometer at the Department of Chemistry "Giacomo Ciamician" of the University of Bologna. The raw XRD pattern files were processed using the Profex 4.3.2a software (Döbelin and Kleeberg 2015) to determine the phases present in the powders (i.e., presence of aragonite/calcite) and their relative quantity. All the details on the analysis and resulting XRD patterns on modern and archeological shells are included in the SI.

Graphitization and CO_2 AMS dates

All shell samples were sent to the ETH laboratory in Zurich, Switzerland for AMS dating in the ETH MICADAS (Mini Radiocarbon Dating System (Wacker et al. 2010a). Samples with a low weight yield (<10 mg) from method A and B were measured with the gas ion source (Ruff et al. 2007). The organic samples were combusted with an EA (Ruff et al. 2010). The resulting CO_2 was isolated with a versatile gas interface (Wacker et al. 2013), from where it was introduced into the gas ion source of the MICADAS (Fahrni et al. 2013) for radiocarbon analysis as a standard procedure. The organic matter extracted with methods A and B, which had a sufficient weight yield (>10mg), was processed as other organic samples (i.e., collagen and cellulose), it was graphitized at the BRAVHO laboratory of the University of Bologna, using the coupled EA-AGE III graphitization system (Wacker et al. 2010b) and pressed into AMS targets as described by Tassoni et al. (2023). During processing, all samples were accompanied by weight-matched standards and blanks (Oxalic and phthalic acid). To convert CO_2 to graphite in the AGE graphitization system, we used iron as catalyst (Němec et al. 2010; Wacker et al. 2010b), which results in a mixture of graphite and iron (more iron than graphite) in the target. In the ion source of the AMS, a cesium beam is directed to the target creating carbon ions, and this process is diluted by the iron present in the target. The more iron is present, the lower the ion yield and thus the

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current will be. In the AGE, graphitization requires at least 3 mg of Fe for 1 mg of C. If the sample contains less than 1mg of C, the ratio of Fe:C will increase, so the current will decrease. Thus, we performed graphitization tests on the samples to define the carbon content of the material. The test revealed that the carbon content was very low (1–5% C). Therefore, for four of the samples of insoluble organic matter, with lower carbon yields (BRA 4957 – Method A, BRA 4952 – Method B, BRA 4938 – Method B and BRA 4930 – Method A), only 2 mg of Fe was used during graphitization to allow for sufficient AMS ion current. Furthermore, samples of oxalic acid were graphitized with all the shell samples with the according iron amounts, as standards. Finally, for the carbonate fractions treated with Methods C and D the samples were sent directly to the ETH in gas chromatography (GC) vials. Phosphoric acid was used to release CO_2 from the carbonates, subsequently following the aforementioned standard procedure used by ETH. For all samples dated, phthalic acid blanks graphitized in the BRAVHO laboratory were included to correct the resulting ages.

Pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS)

Analytical pyrolysis is a powerful technique capable of providing chemical information about organic matter at a molecular level. The use of Py-GC-MS to characterize the chemical composition of natural organic matter has been reported in studies related to differences in ^{14}C ages (Ferro-Vázquez et al. 2019). Furthermore, Py-GC-MS was used to identify possible changes in the composition of fresh mussels (*M. galloprovincialis*) after exposure to cyanotoxins (Diez-Quijada et al. 2020). Analytical pyrolysis has also been applied to the characterization of the intra-skeletal organic matrix in hard corals (Adamiano et al. 2014).

In this study, analyses by Py-GC-MS were performed to determine if the insoluble materials extracted with Methods A and B were consistent to the chemical characteristics of the organic matrix of the shell and checking for the occurrence of any external material in the samples. Py-GC-MS analysis was performed using an EGA/PY-3030D micro-furnace pyrolyser (Frontier Laboratories Ltd., Japan) coupled with a 7890 Agilent HP gas chromatograph (GC) connected to a 5977 Agilent HP quadrupole mass spectrometer (MS) (Agilent Technologies, USA). A small crucible capsule containing weighed shell sample (2–4 mg) was introduced into the furnace and then pyrolyzed at 600°C for 100 s using helium as carrier gas (1 mL min^{-1}) and an interface temperature of 280°C. The evolved gases were then directly injected into the GC-MS for analysis. The GC injector was operated in split mode with a 10:1 ratio at 280°C. Pyrolysis products were separated by a HP-5MS fused silica capillary column (stationary phase poly[5% diphenyl/95% dimethyl]siloxane, 30 m × 0.25 mm i.d., 0.25 µm film thickness, Agilent Technologies, USA) with the following temperature program: 50°C to 300°C at 10°C min^{-1}, then hold for 10 min at 300°C, using helium as carrier gas (1 mL min^{-1}). The MS was operated in EI positive mode (70eV, scanning 45–500 *m/z*) with transfer line temperature 250°C, ion source temperature 230°C and quadrupole temperature 150°C. Tentative identification of the pyrolysis products was performed by comparison with MS library and published studies.

Results

In terms of complexity, time and equipment required for performing the methods, the simplest and most rapid of the methods performed was Method D. Methods A and B required the longest time to complete, however the procedures were relatively simple. Method C on the other hand, was the most complicated to perform successfully and it required working with bromoform and toluene, both highly hazardous substances.

Weight and carbon yields

The final weight of all archeological samples is shown in Table 2. All four methods lead to the reduction of the initial sample weight, resulting in 0.1 wt.% to 81.3 wt.% of the initial sample weight (Table 2).

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Table 2. *Information on the Archeological samples used in the experiments. The end weight and radiocarbon age are shown as the Soluble/Insoluble fraction for Methods A and B, as Aragonite/calcite for Method C, and for Method D as the whole carbonate fraction*

BRAVHO lab code	Species	Method	Layer	Yield (wt.% of initial weight)	Start weight (mg)	End weight (mg) *C content (ugC)*		^{14}C Age BP ± 1σ Error	
						Soluble/Aragonite	Insoluble/Calcite	Soluble/Aragonite	Insoluble/Calcite
BRA 4930	*Crassostrea sp.*	A	3	0.3	6609	14.1 *191*	4.2 *104*	5260 ± 30	3309 ± 72
		B		0.8	6385	47.8 *755*	1.7 *11*	5409 ± 22	N/A
BRA 4931	*Crassostrea sp.*	C	3	75.2	761	572.4 *981*	0	4596 ± 23	N/A
		D		18.5	724	134 *998*		5522 ± 24	
BRA 4942	*P. maximus*	A	4	0.8	7430	56.4 *532*	2.6 *34*	8318 ± 25	7704 ± 93
BRA 4938	*P. maximus*	B	4	0.6	5577	31.2 *555*	2 *7*	8275 ± 23	N/A
BRA 4943	*P. maximus*	C	4	66.2	886	454.2 *993*	131.9 *995*	22266 ± 70	25102 ± 95
BRA 4936	*P. maximus*	D	4	48.4	2285	1107 *980*		22499 ± 74	
BRA 4946	*P. maximus*	A	5	0.3	1582	3.7 *50*	1.6 *2*	6781 ± 87	N/A
		B		0.2	1546	2 *60*	0.6	5054 ± 77	N/A
		C		50.1	262	99.3 *979*	32 *996*	18927 ± 54	19153 ± 54
		D		53	294	155.7 *986*		20562 ± 61	
BRA 4952	*P. maximus*	A	6	0.2	4527	6.7 *217*	1.9 *61*	12975 ± 123	15617 ± 153
		B		0.5	4514	20.6 *437*	1.9 *9*	8609 ± 23	N/A
		C		79.7	431	0	343.8 *999*	N/A	27374 ± 121
		D		28.9	415	119.9 *984*		33416 ± 240	
BRA 4957	*P. maximus*	A	7	0.8	2861	20.8 *597*	2.3 *2*	5428 ± 21	N/A
		C		60.1	235	97.3 *989*	43.8 *994*	20632 ± 61	20839 ± 62
		D		12.2	205	25.1 *993*		25091 ± 96	
BRA 4956	*P. maximus*	B	7	1.5	2413	33.9 *523*	1.8 *21*	4555 ± 21	858 ± 83

Methods A and B caused the most substantial loss of material, as only the organic matter was extracted, resulting in 0.1 wt.% to 2.0 wt.% of the initial shell weight. The mean values of 0.5 wt.% for Method A and 0.7 wt.% for Method B align with the expected organic matter content in mollusk shells of 0.01–5% of the shell weight (Berger et al. 1964; Hadden et al. 2018; Hadden et al. 2019; Marin et al. 2012), considering an additional loss of material during the pretreatment steps. Furthermore, the low yields for archeological shells could be expected due to degradation, even though the organic matrix in the shell is considered to remain well preserved (Berger et al. 1964). The difference in yield between the two methods could be due to a better dissolution in Method A of the carbonate fraction. A higher proportion of soluble organic matter is maintained in the sample due to the use of the dialysis technique as opposed to direct dissolution used in Method B, which causes most of the soluble fraction to be lost during pretreatment. For methods C and D, the carbonate fraction is used, thus resulting in a higher weight yield after pretreatment (from 12.2 wt.% to 81.3 wt.% of the initial weight). The mean weight yield for Method C was 66.3 wt.%, while for Method D the mean was 32.2 wt.%. In Method C, the material is lost mostly due to the various pretreatment steps, as this method doesn't imply the use of acid to dissolve the shell. This explains the yield difference between Methods C and D since the latter uses an acid to dissolve the outer portion of the shell. As the authors of Method C (Douka et al. 2010a) also stated, the loss of material using acid etching usually varies from 30 to 80% of the initial weight. See the weight yield comparison with modern specimens in the SI (Section 3). The carbon yield of all pretreated samples is shown in Table 2. Samples with weight yields under 10 mg, which were not graphitized, had very low carbon yields (2–217 µg of carbon). Samples with less than 20 µg of carbon were considered unreliable and excluded from the study. Graphitized samples yielded 191–755 µg of carbon, with all but two samples providing what is considered as sufficient carbon for routine AMS dating (500 µg; Butkus et al. 2022). Carbonate samples had the highest carbon yields (979–999 µg), which are values considered sufficient to obtain reliable radiocarbon determinations.

XRD analysis

For Methods A and B, the XRD analysis was performed after pretreatment on both the soluble and insoluble fractions. The results obtained were in line with the expectations, as the soluble organic matrix fraction did not contain any carbonates, while the insoluble one had traces of carbonates in some cases where the dissolution was not complete, as well as traces of quartz in some other cases (Figure S7, Section 4 in the SI). Presumably, the traces of quartz derive from contamination in the seashells, however since there is no carbon in quartz this should not affect the radiocarbon age.

All the samples used for Method C were mostly calcite with only a small fraction of aragonite (Table 3 and S3, Figures S10–S13, Section 4 in the SI). In case of both *Pecten* sp. and *Crassostrea* sp., this was expected, as they are primarily calcite (Carriker and Palmer 1979; Stenzel 1963; Turekian and Armstrong 1960), except for muscle binding areas which were found to consist of aragonite in various species of oysters (Carriker and Palmer 1979). The only high aragonite sample was BRA 4952 (>90% aragonite; Table 3). All other samples were over 99% calcite, thus we attempted a single separation to separate high Mg calcite (HMC, higher density, >4% $MgCO_3$) from low Mg calcite (LMC, lower density, <4% $MgCO_3$), as stated possible by the authors of the method (Douka et al. 2010a). HMC and aragonite are considered the metastable polymorphs, which under the influence of different factors (such as the pH and Mg:Ca ratio values, various kinetic, biological and hydrographic factors) are expected to recrystallize into the more stable LMC after the death of the organism (Douka et al. 2010a) and references therein. After pretreatment, sample BRA 4952 resulted in a slightly higher aragonite proportion (Table 3), while the calcite samples in some cases showed traces of aragonite that were previously not detected. Furthermore, no differences in average Mg content were detected in the calcite samples before and after separation, given the similar lattice parameters in the XRD patterns (Figure 3 and S10–S13, Section 4 in the SI). This method would be more effective in cases with a high proportion of both polymorphs and high variations in Mg content in the shell. Many marine organisms incorporate

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Table 3. *Percentages of calcite and aragonite before and after the application of Method C to archeological shells. Whole—percentages before separation; Aragonite/calcite—the two resulting fractions which presumably are either aragonite or calcite. The two columns showing the weight percentage of calcite/aragonite are the results of XRD pattern analysis, showing actual percentages obtained in the separated fractions*

Method C			
BRAVHO lab code	Fraction (presumable)	Calcite (wt.%)	Aragonite (wt.%)
BRA 4931	Whole	99.06	0.00
BRA 4931	Calcite	99.74	0.15
BRA 4943	Whole	100.00	0.00
BRA 4943	Calcite	99.47	0.40
BRA 4943	Aragonite	99.83	0.17
BRA 4946	Whole	99.98	0.00
BRA 4946	Calcite	100.00	0.00
BRA 4946	Aragonite	99.95	0.04
BRA 4952	Whole	7.24	91.15
BRA 4952	Aragonite	6.48	92.55
BRA 4957	Whole	99.29	0.20
BRA 4957	Calcite	99.11	0.00
BRA 4957	Aragonite	99.77	0.00

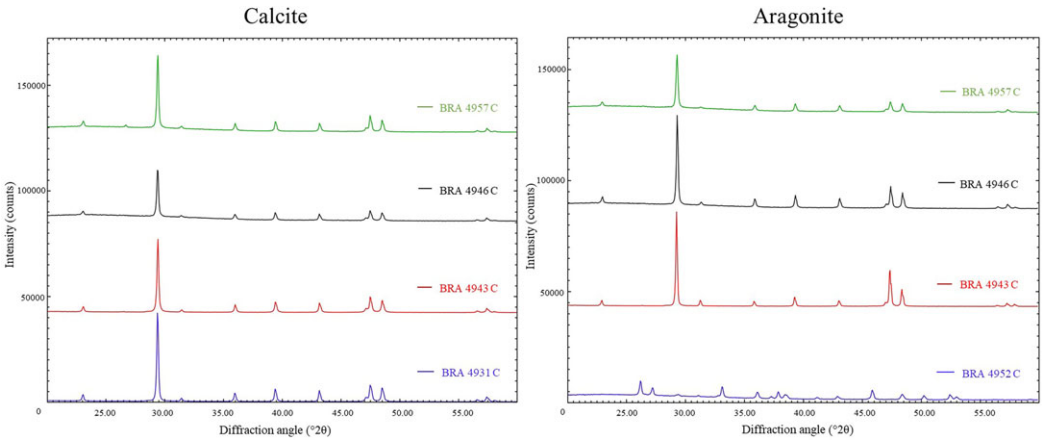

Figure 3. *XRD patterns of the "calcite" (left) and "aragonite" (right) fractions after separation using Method C. The samples are positioned along the vertical axis to differentiate them from one another.*

both HMC and LMC into their skeletons to increase its resistance and hardness (Bianco-Stein et al. 2022; Ma et al. 2008, 2009; Polishchuk et al. 2017). Therefore, the presence of these two calcite polymorphs may not indicate diagenetic recrystallization.

Archeological samples treated with Method D showed similar proportions of calcite and aragonite before and after applying the protocol. A slight decrease in aragonite percentage could have been expected given the preferential dissolution of aragonite during the acid etching of shells (Vita-Finzi and Roberts 1984). Interestingly, archeological sample BRA 4952 contained a higher amount of aragonite in the portion pretreated with Method D compared to the portion pretreated with Method C.

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Furthermore, the results obtained from the XRD analysis revealed little to no change in the mineral phase composition in the carbonate samples pretreated for this study, which could indicate that the samples most likely have not been subjected to heavy recrystallization. However, to truly assess the preservation state of the shells, a simple polymorph determination is not enough, and should be combined with other tests such as microscopic examination of the crystalline structure, or the examination of the organic matter preservation (Guzmán et al. 2009; Perrin 2004; Toffolo 2021). Some of the differences in the XRD results could also be explained by differences in original polymorph proportions which can vary due to shell ontogeny and thickness, as well as due to the climatic and environmental conditions in which the mollusks secreted their exoskeletons. Moreover, the portions analyzed for each method were different fragments taken from the same shells, which may contain variable proportions of the two polymorphs. To avoid this in future experiments, the shells could be ground to powder whole and only then separated for pretreatment with the different methods. Alternatively, Feigl's solution could be applied to the samples to determine the presence of calcite and aragonite prior to analysis. This is a common practice useful to determine areas of the shell to select for radiocarbon dating, and it allows for the identification of the two polymorphs prior to the use of any type of wet chemical pretreatment (Checa et al. 2007; Gray and Smith 2004; Schöne et al. 2017). However, in this study it was important to have all the material available for pretreatment in order to perform multiple pretreatment methods on the same sample. Therefore, this type of selection was not performed for this study.

Radiocarbon AMS dates

The radiocarbon ages of all the samples are shown in Table 2. All results are reported as uncalibrated dates and expressed in BP (years before 1950). Results from Method A and B were expected to overlap and mostly did so in Layers 3 and 4, while in Layers 5, 6 and 7 Method B resulted in even younger ages compared to Method A. In Layer 3 the carbonate fractions from Methods C and D overlap with the dates on the organic fraction from both Method A and B. However, for all the remaining layers, the carbonate fractions resulted in much older ages.

Method A—The shells treated with Method A gave ages ranging from 15,617 ± 153 to 3,309 ± 72 BP. Both the insoluble and soluble fractions from Method A gave very young dates with no clear trend. Only three of the extracted soluble fractions resulted in enough material to obtain reliable radiocarbon measurements. In two cases, for samples BRA 4930 and BRA 4942, the soluble fraction was significantly younger than the insoluble fraction extracted from the same shell. On the other hand, the soluble fraction from sample BRA 4952 resulted significantly older than the insoluble fraction from the same shell.

Method B—Method B gave similar results to those from Method A, giving even younger ages ranging from 8,609 ± 23 to 858 ± 83 BP. This method resulted only in insoluble fractions, with one exception, as the soluble fractions were mostly lost during pretreatment. The only sample resulting in enough soluble organic matrix to be dated was BRA 4956 from Layer 7, and it was an outlier with an extremely young age of 858±83 BP.

Method C—The radiocarbon ages resulting from shells treated with Method C ranged from 27,374 ± 121 to 4,596 ± 23 BP. The aragonite fractions were consistently older compared to the calcite fractions resulting from the same shell even though the differences were not substantial in two out of three cases resulting in 226- and 207-years difference in Layers 5 and 7, BRA 4946 and BRA 4957 respectively. The only major difference between the calcite and aragonite fractions was 2836 years in sample BRA 4943 from Layer 4.

Method D—Method D resulted in an age range from 33,416 ± 240 to 5,522 ± 24 BP. The age obtained for sample BRA 4931 from Layer 3 is similar to the results for this Layer obtained by the other methods. In Layer 4, the age obtained for sample BRA 4936 was close to the age obtained from Method C for the calcite fraction of sample BRA 4943 from the same Layer, resulting significantly younger than

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the aragonite fraction from the same shell. However, in this case it is important to underline that Methods C and D were performed on two different samples. Therefore, a direct comparison might not be reliable. The ages obtained for shells from Layers 5, 6 and 7 were older than those resulting from Method C both from aragonite and calcite fractions.

Comparison with previous results—Based on previous results on charcoal samples (Tátá et al. 2014) the expected ^{14}C age for samples from Layer 3 was ~8660 to ~8880 BP (Table 1). However, the shells used in this study yielded younger ages across all four methods, even though a direct comparison of different materials and species can cause errors in interpretation. Although ages obtained with Method C and D for Layer 4 matched the expected range for Layer 5 (~20,300–25,400 BP, Table 1), they were older than ages obtained by the same methods for Layer 5 (~19–20,000 BP). For Layer 5, Methods A and B produced ages significantly younger than expected (6781 ± 87 and 5054 ± 77 BP, respectively for the same sample), the sample pretreated with Method D aligned with previous dates, while results from Method C were slightly younger. Layer 6 exhibited high variability, with previous ^{14}C results ranging from ~26,000 to ~41,000 BP (Table 1). The carbonate fractions from the same sample collected in this layer resulted in ages of 27,374 ± 121 BP for Method C, and 33,416±240 BP for Method D. The two dates on bones from Layer 6 of the Terrace area resulted in two considerably different dates of 27,600 ± 140 for sample MAMS-19366 and 20,260 ± 80 BP for sample MAMS-19367. Layer 6 aragonite fraction mostly agreed with previous shell results, though dates varied significantly in this layer. For Layer 7, the expected ^{14}C age range was ~24,300–27,100 BP (Table 1). The sample from this layer pretreated with Method D matched this range (~25,000 BP), while Method C resulted in younger ages for the same sample (~21,000 BP). In Layers 6 and 7, Method D produced dates ~5000 years older than Method C's for the same shells, though some previous shell dates were even older (Table 1). When considering only dates obtained on *Pecten* sp. for layers 5–7, our dates from Method C are considerably younger, with Method D showing closer, yet still younger ages.

Pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS)

In this study, only the insoluble fractions of the extracted organic matrix were analyzed as the soluble fractions were insufficient to perform Py-GC-MS. The pyrograms of the archaeological samples (Figure 4) were characterized by a variety of pyrolysis products that included (1) aliphatic hydrocarbons, mainly *n*-alkanes from *n*-decane to *n*-tetratiacontane, (2) aromatic hydrocarbons principally diphenyl, monocyclic (from benzene to C_4-benzenes) and polyaromatic hydrocarbons (PAHs, indenes, naphthalenes, phenanthrene and alkylated forms), (3) nitrogen-containing compounds (pyrroles, pyridines, aromatic and long chain aliphatic nitriles), (4) aliphatic (fatty acids, palmitic and oleic acids) and aromatic oxygenated compounds (furaldehydes, phenols, benzofurans).

Discussion

Weight and carbon yields

The weight yields obtained for Methods A and B were in line with the expectations based on literature and previous results (Berger et al. 1964; Hadden et al. 2018, 2019; Marin et al. 2012). It is important to acknowledge the large amount of material needed to perform Methods A and B compared to Methods C and D, considering both the low weight and carbon yields. This makes it difficult to choose Method A or B instead of Method C or D, since the amount of material available for pretreatment can often be scarce. Furthermore, having a lower yield makes the extracted material more susceptible to contamination, the effect of which, if present, will be much greater than in a higher yield sample. For example, the same quantity of modern carbon introduced in a sample of 1mg would have a much more significant effect on the radiocarbon age than in a sample of 100 mg. In previous work it was demonstrated that even samples with low carbon yields (<100 µg C) resulted in reliable radiocarbon

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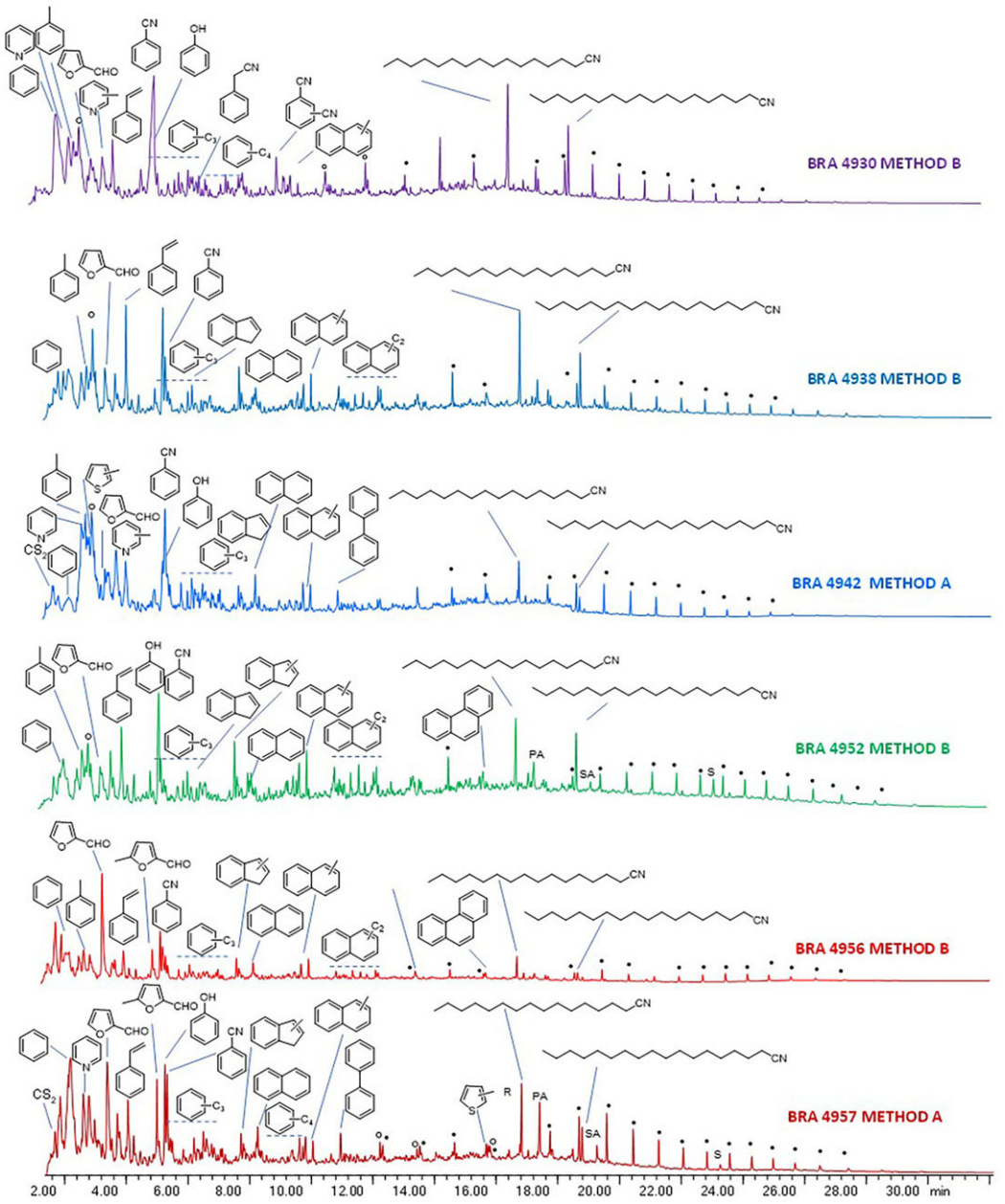

Figure 4. *MS-pyrograms from Py-GC-MS of the insoluble fraction of archaeological samples. The molecular structures were reported for some of the most intense peaks. CS2: carbon disulphide; PA: palmitic acid; SA: stearic acid; S: squalene; (o): alkenes; (■): alkanes.*

dates when using the direct CO_2 measurement method, used for the organic matter samples in this study with less than 10 mg of material (Fewlass et al. 2017; Fewlass et al. 2019). AMS dates obtained from samples which resulted in carbon content below 20 µg were not considered reliable and were excluded from this study. Furthermore, for sample graphitization, a carbon content of 500 µg is considered sufficient for routine AMS dating (Butkus et al. 2022). Samples with carbon content from 50–300 µg of carbon were successfully graphitized using the AGE III graphitization system with the routine method

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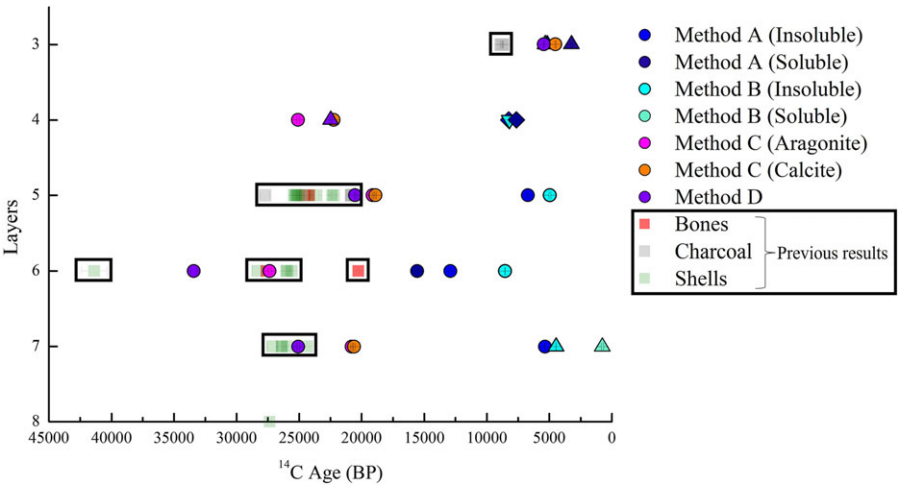

Figure 5. *A graph showing the radiocarbon age (X-axis) resulting from the four different methods applied on shells with different shapes indicating different shell samples used (Layer 3: BRA 4930 triangle; BRA 4931 circle; Layer 4: BRA 4942 diamond; BRA 4938 inverted triangle; BRA 4936 triangle; BRA 4943 circle; Layer 5: BRA 4946 circle; Layer 6: BRA 4952 circle; Layer 7: BRA 4956 triangle; BRA 4957 circle) and previous results from charcoal, bones and shells (squares) from five different archaeological layers (Y-axis). The error bars (±1σ) are shown within the symbols for most samples, given the large scale of the graph, and the tightness of the error range.*

(Solís et al. 2015), and a dilution method was also developed to bring the carbon content values up to 500 µg for aerosol measurements (Butkus et al. 2022). In this study, the carbon content of graphitized samples was below the 500 µg threshold only in case of two samples which had 437 and 191 µg of carbon. Therefore, the carbon yield of the samples in this study, although very low in some cases, was sufficient for reliable AMS measurements to be performed. However, the low carbon yield, as previously mentioned, could render the effect of any present contamination in the samples much more pronounced.

Evaluation of diagenesis and contamination

The soluble fractions were expected to give the most reliable date, as this fraction was isolated from external influences and did not contain carbonate. However, the soluble fractions from Method A resulted younger than the insoluble fractions in most cases and the only soluble fraction resulting from Method B was an outlier and extremely young for an early Gravettian layer (Figure 5) possibly indicating that the soluble fraction is more susceptible to modern contamination. However, this could also be due to the very small amount of the soluble fraction and the low carbon content that was extracted, which would be more affected by any potential contamination. Furthermore, the results obtained for both Method A and Method B are extremely young considering previous dates, and dates from this study obtained using the carbonate fraction. The lack of overlap between the carbonate fractions with the organic matrix in all layers except Layer 3 could be due to the use of a different species, since for Layer 3 *Crassostrea* sp. was used and for all the other layers *Pecten* sp. was used. The radiocarbon age of mollusk shells can be influenced by a variety of factors, including habitat (Lindauer et al. 2021), mineralogy (Douka et al. 2010b) and feeding ecology (England et al. 2013). The two species used in this study are both filter feeders (Santhanam 2018a). However, *Crassostrea* sp. is an intertidal to subtidal species, while *Pecten* sp. is a subtidal species, which might have an influence on the radiocarbon content of the samples. In previous studies, some of the *Pecten* sp. samples collected were

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heavily abraded and since they live at depths of 10–110 m, they were almost certainly collected from the beach, thus possibly giving older ages than the age of their use at the site (Manne et al. 2012).

The differences between the dates on *Pecten* sp. from previous studies compared to the results presented here could be partly explained by "shelf life" in beach deposits, as well as variable preservation and taphonomy. However, this does not explain the differences among methods applied to the same shell (Figure 5). For Layers 5 and 7 the differences in age between aragonite and calcite, separated with Method C, were not substantial, which was expected since both fractions contained similar percentages of calcite and aragonite. Given the differences in age between the calcite and aragonite fractions in Layer 4, it is possible that there was some diagenetic alteration of the calcium carbonate. This could have occurred with potential incorporation of exogenous carbon into the sample and no change in the crystalline structure given the prevalence of one of the two polymorphs in all samples. Furthermore, intrashell variation in diagenetic effects was hypothesized in a previous study after the application of both XRD and Scanning electron microscopy which did not always show consistent results (Barton 2012). Additionally, some of the samples which were determined as well-preserved gave radiocarbon determinations which despite that were statistical outliers (Barton 2012). This confirms the hypothesis that diagenetic effects cannot always be detected merely by examining the carbonate polymorphs present in the sample. Therefore, it is necessary to perform multiple lines of analysis to confirm the preservation state of shell samples prior to radiocarbon dating.

Comparison among methods and with expected ages

The ages obtained from Methods A and B were considerably younger than the expected ages for the archeological Layers 3–7 in the Terrace area of the Vale Boi site (Figure 5). To properly compare the results of the four pretreatment methods, we will focus on the only two samples on which all four methods were performed. Samples BRA 4946 and 4952 show that the age obtained using the organic matrix presents a shift towards younger ages by over ten to fifteen thousand years. A similar discrepancy is observed in sample BRA 4957 pretreated with three of the methods. Therefore, despite the theoretical potential the organic matrix has, the results of this experiment demonstrate that it is challenging to obtain reliable results using this fraction. Given the consistency of the results obtained for both methods, the systematic error in dates is likely related to the process of organic matrix extraction irrespective of the specific methods used, or possible contamination during pretreatment. Considering the ages of the samples pretreated with Methods A and B, in case modern contamination was present, half or more of each sample would have to be modern to obtain such results. Given the low weight yield of the organic matrix extracted and its low carbon content, even a small amount of modern contamination could have affected the results in such a way. Since the ages obtained from Methods C and D resulted in much older ages for all layers except Layer 3, it is possible that the contamination is specific to the organic matrix fractions and possibly species specific. In further research, the effect of the species used should be examined to determine if the organic matrix of different species from the same layers gives variable results, given the natural differences among species in terms of habitat, diet and even polymorph proportions. The overlap with the ages from the carbonate fractions in Layer 3 could also be explained by the smaller effect of modern contamination on younger samples (Talamo et al. 2021). However, given the effect certain habitats, diets and species-specific mineralogy on the radiocarbon ages obtained on mollusk shells, it should be common practice to use only a determined species for radiocarbon dating. Moreover, fungi and/or microorganisms may introduce younger contamination into the organic fractions of a sample during storage and preparation (Wohlfarth 1998). Furthermore, another way to introduce younger contamination after shell deposition is the development of inorganic intracrystalline carbonate cement within a shell structure (Douka et al. 2010a; Webb et al. 2007). This fraction would be removed in Methods A and B, together with the rest of the carbonate fraction. Nonetheless, secondary carbonate precipitates can be formed by microorganisms which also leave fatty acid signatures in the intracrystalline structure of the shells (Busschers et al. 2014). This type of non-carbonate contamination

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would be maintained in the sample after pretreatment. Given the differences in age between the organic matrix and the carbonate fractions from our shells, microbially-induced recrystallization could explain these results. This type of recrystallization could have occurred with no change in the polymorphic state of the carbonates as seen in the XRD results for the samples used in this study. Even though the intracrystalline organic matrix has been shown to be a closed system (Penkman et al. 2008), what this entails in terms of radiocarbon analysis is yet to be understood. Since the carbonate fractions were unaffected, or significantly less affected by the contamination, this would mean that the organic matrix might be more susceptible to it. However, at the present time and with such a small amount of material, it is impossible to consider the organic matrix extraction a feasible method for dating archeological mollusk shells.

It is important to note that the shell dates were not corrected for the marine reservoir effect, thus a direct comparison with bone dates from Layer 6, and among the different species will only be reliable once the correction of the marine reservoir effect is applied. Nonetheless, based on the ^{14}C ages, all the results in Layer 6 are inconsistent and the differences are larger than this correction (Figure 5). Previous results from Layer 6 indicated two sets of human occupation around 3000 years apart from one another (Bicho et al. 2013), which could correspond to the dates we obtained. Furthermore, the bone pretreatment method is well-established, proven to eliminate exogenous contaminant carbon from the sample (Talamo et al. 2021). Thus, the differences in Layer 6 might instead be due to vertical movement and mixing within and between the layers, which were also identified in previous work (Bicho et al. 2013). Vertical movement of samples between layers could also explain why we obtained younger ages for Layer 5 compared to Layer 4, and those from Layer 7, compared to Layer 6. However, it does not explain the difference between the dates obtained for Layer 6 from the same shell (BRA 4952, Figure 5). Furthermore, when considering only the carbonates from the samples BRA 4946 and 4952 pretreated with all four methods, and sample BRA 4957 pretreated with three of the methods, Method D is constantly showing older ages compared to Method C. This is also observed in BRA 4931 which was pretreated both with Method C and D. The age differences range from around a thousand years in BRA 4946 to around six thousand years in BRA 4952. The dates obtained with Method D overlap better with previous results. However, the samples from literature were also pretreated using acid etching, similarly to Method D. Based on this data alone, it is difficult to determine with certainty which of these two methods is showing the correct ages. In previous work, heavy recrystallization was detected in some of the shell samples (Bicho et al. 2013). Even though our XRD results do not show such differences in terms of aragonite to calcite transformations, there could have been some diagenetic alterations without a change in crystalline structure which were not detected. Analysis of the shell crystalline structure would help identify possible diagenetic alterations, which did not result in change in the carbonate polymorph to improve the sample selection in cases where shells are used to construct site chronologies. The pyrolysis products we obtained for the insoluble organic matrix of the archeological samples pretreated with Method A and B are normally detected upon Py-GC-MS of diagenetically degraded natural organic matter (Brown et al. 2000). A similar suite of pyrolysis products was reported for the sedimentary matrix of samples of the Upper Palaeolithic sequence of Abri Pataud that were attributed to charred organic matter mixed to other organic materials (Braadbaart et al. 2020). We performed a comparison of the pyrolytic patterns obtained on archeological shells with patterns from modern samples (See SI, Section 4). This comparison evidenced changes indicative of degradative processes related to diagenesis (a more detailed discussion is available in the SI, Section 4). It is worth noting that the samples obtained from method A (BRA 4930, BRA 4942, BRA 4957) generated Py-GC-MS traces featured by a suite of peaks tentatively identified as sulphur-containing compounds. In particular, carbon disulphide, alkyl tiophenes, benzotiophenes and a broad peak at the central part of the chromatogram attributed to S_8. The presence of tiophenes was typically encountered in the pyrograms of natural organic matter degraded under anoxic conditions (Çoban-Yıldız et al. 2006). The samples used in this study could have spent a significant amount of time in this type of conditions in marine sediments, given the fact that they were most likely collected from the beach. However, tiophenes could be formed as artefacts during pyrolysis by the reaction of elemental sulfur with fatty acids (Saiz-Jimenez

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1995). Besides, the presence of sulfur compounds was also observed in the pyrogram of the modern samples (See SI, Section 4) and the sample BRA 4930 treated with method A suggesting differences in the pyrolytic pattern caused by sample treatment. This would support the hypothesis that the extraction protocol might affect the obtained organic matrix.

All four methods gave ages inconsistent with the stratigraphical attribution of the samples and the expected age for the respective archeological layers given the previous results (Figure 5). Samples from Layer 6 gave the oldest ages for all four methods, and the samples from Layer 4 were consistently older than those from Layer 5, for all four methods. Most of the previous results on shells come from dates on *Patella* sp. which is a grazing species feeding on algae by scraping them off the rocky substrate (Santhanam 2018b). This might cause age distortions by the introduction of older carbon from the limestone rocks present in the Vale Boi area (Bicho et al. 2013; Tátá et al. 2014). It is worth noting that the use of different mollusk species might cause large discrepancies in the results (For example, see England et al. 2013). On the one hand, due to the influence of variable diets, climatic and environmental conditions of each species which influences their initial biochemical composition, and on the other hand due to their different uses at the site. A previous study found differences among shell dates of up to 2000 years which they attributed mainly to heavy recrystallization, but also mentioned vertical sample movement as a potential issue (Bicho et al. 2013). This underlines the need of securing the stratigraphical attribution of the samples and further studying the potential disturbances and bioturbation in the stratigraphical units of the site. Furthermore, it is necessary to perform experiments using more samples to evaluate differences among the results when using different species from the same layer in a secure stratigraphical context.

Conclusions

The extraction of the organic matrix of the shells in theory could be a potentially useful method for radiocarbon dating, given that the intracrystalline organic matrix of the shell is protected from the surrounding environment in a closed system. However, after the application, the weight and carbon yields were very low for both method A and B, and the resulting ages were significantly younger than those resulting from carbonate fractions of the same shell specimens. This means that further research is needed to explore ways to perform the organic matter extraction without risk of contamination. A potential way to decrease the effect of contamination is to obtain samples with a higher weight and, subsequently higher carbon yields. Moreover, it is important to take into consideration the large amount of material needed to perform the organic matrix extraction (Method A and B) compared to the amount needed for the carbonate pretreatment (Method C and D). Therefore, to be able to apply this approach to more archeological specimens, which are often extremely valuable, further investigation needs to be done. Method C is a useful method for aragonite and calcite separation in shells presenting both calcium carbonate polymorphs in significant amounts. However, in this study most of the samples consisted of one of the two polymorphs, and the traces of the other polymorph were difficult to remove. Therefore, this method did not have enough of an effect to justify the effort of performing the pretreatment. Furthermore, there could be diagenetic effects that occur without aragonite to calcite transformations, which would not be detected by XRD nor removed by Method C. As demonstrated by the pyrolysis results from samples pretreated with Methods A and B, degradation can occur without an apparent effect on the crystalline structure of the samples. Therefore, performing a more detailed analysis of the crystalline structure of shells before radiocarbon dating could reveal alterations that were not identified in this study and that might influence the resulting age estimates. Finally, Method D is the quickest and simplest method performed, and it gave results closer to the expected ages compared to the other methods, in most cases. When we consider sites where only shells are available for radiocarbon dating, it is crucial to carefully select the species and samples, as they must be in alignment with the method employed. These choices should be made in close collaboration between radiocarbon specialists and site archeologists as they can greatly affect the accuracy of the age determination and interpretation.

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Overall, all the results were inconsistent both for shells and for the two bones analyzed in the Terrace area of Vale Boi in comparison with the previous results and with the archeological attribution. Given the complexities of the Vale Boi archeological site and the potential mixing in the stratigraphical sequence, it is likely that some of the inconsistencies in ages obtained from the different layers are due to factors not directly linked to dating shells. However, dates obtained from the same individuals using different methods showed significant differences, highlighting the importance of using efficient pretreatment protocols. The results of this study underline the difficulty of obtaining reliable radiocarbon dates on marine mollusk shells, and the need for additional method improvements for their pretreatment and contamination removal.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/RDC.2024.80

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