Research Article

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An Experimental and Methodological Approach of Plant Fibres in Dental Calculus: The Case Study of the Early Neolithic Site of Cova del Pasteral (Girona, Spain)

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Abstract: Perishable organic raw materials such as plant fibres have been widely used since the time of the earliest human groups, although their poor preservation limits our knowledge of their use. Filling this gap requires a focused search for plant fibre evidence in non-perishable materials. Since teeth are sometimes used in fibre processing, evidence of fibres can be sought in mineralised bacterial plaque, and therefore, its analysis can potentially be useful in the study of plant–human relationships. Experimental protocols have been tested to improve the recovery of microremains in dental calculus, although these are typically focused on microremains other than plant fibres. This article investigates the possibility that plant fibres are identified less often in dental calculus because the corrosive agents used cause their disappearance. Ethylenediaminetetraacetic acid (EDTA) and hydrochloric acid agents at 0.2 and 0.5 M were applied for 5, 30, 90, and 170 h to six different modern plant fibres considering their use in archaeological craft activities and showed that none of the procedures affected their characteristics. After testing the same approach on medieval control dental calculus samples, a selected methodology (0.5 M EDTA) was applied to ancient dental calculus from the Neolithic site of Cova del Pasteral (La Cellera de Ter, NE Spain) as a case study to identify the plant fibres present in it.

Keywords: plant fibres, dental calculus, decalcification, methodology

1 Introduction

1.1 Plant Fibres Record in Archaeology

Perishable organic raw materials such as wood, plant fibres, and skins have been widely used since the time of the earliest human groups. They are not commonly preserved in archaeological contexts and consequently have not usually been studied in detail in archaeology, even though they are key to understanding human

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adaptations, past technological skills, and ecological knowledge (Aranguren et al., 2018; Conard et al., 2020; Gleba & Harris, 2019; Hardy et al., 2020; Hurcombe, 2014; Rios-Garaizar et al., 2018). The poor preservation of perishable material has limited our knowledge of past plant crafts, such as those made from plant fibres (Hurcombe, 2014). Botanically, they are described as sclerenchyma cell bundles associated with vascular plant support and nutrient and water transport functions (Evert, 2006). However, in fibre crafts, the complete stem or leaves can also be used. Their composition is based on lignin and cellulose in varying concentrations, which gives durability, flexibility, and strength to the fibres (Evert, 2006).

Although direct archaeological evidence is scarce (Hurcombe, 2014), the use of this material has been well documented since Upper Pleistocene chronologies (Barber, 1994; Hardy, 2008; Kvavadze et al., 2010; Soffer, 2004). Other indirect evidence can be obtained from the analysis of use-wear preserved on lithic and bone tools that attest work with plant materials. Current information about their use is usually provided by the analysis of fibre impressions on durable materials such as clay or pottery (Guerra-Doce et al., 2024; Romero-Brugués et al., 2022; Soffer et al., 2000; Wigforss, 2014). Direct evidence of plant fibre-based crafts becomes more frequent in early and mid-Holocene contexts such as the examples found in southern Iberian sites as three charred braid cords from Coves de Santa Maira site (12,900–10,200 cal BC) (Aura Tortosa et al., 2019), the dehydrated cords, footwear/sandals and baskets from Cueva de los Murciélagos (7986–3740 cal BC) (Martínez-Sevilla et al., 2023), the waterlogged and charred cordage and basketry from La Draga (5324–4977 cal BC) (Herrero-Otal et al., 2021, 2023; Herrero-Otal, 2024; Romero-Brugués, 2022; Romero-Brugués et al., 2021), the charred and dehydrated baskets and a cord fragment from Coves del Fem (4991–4545 cal BC) (Herrero-Otal et al., 2021; Romero-Brugués et al., 2021), and basketry from Cova de les Cendres (Bernabeu Aubán & Fumanal García, 2009), among others. The preservation of organic materials is limited to exceptional environmental conditions, such as persistent waterlogging, extreme aridity, or processes like carbonisation or mineralisation.

The limited direct evidence for plant fibre-based materials in archaeology hinders our understanding of past craft activities, including variability in raw materials, technology, functionality, and distribution (Hurcombe, 2014), and also their role in past societies, where they preceded other material cultures, such as pottery, but survived the introduction of new materials and are still part of a living material culture all around the world (Kuoni, 1981). Their study is therefore crucial in providing a different perspective on aspects of human evolution. Filling this gap requires a focused search for evidence of the use of plant fibre in other non-perishable materials, like the tools used in plant fibre processing. In this sense, the use of teeth as a third hand in plant craft processing, which has been recorded in ethnographic and archaeological contexts (Bocquentin et al., 2005; Lozano et al., 2008; Milner & Larsen, 1991; Romero et al., 2024; Schulz, 1977; Scott & Jolie, 2008), opens an opportunity to approach the fibres that can be potentially preserved in durable or mineralised materials such as bacterial plaque adhered to teeth as this research is focused on. Therefore, the recovery of plant fibres from this mineralised material may involve disaggregation of the matrix using decalcifiers, which may prove to be an effective method of obtaining information in the absence of direct remnants of fibre-based materials.

Although the analysis of dental calculus microdebris is mainly focused on the presence of starch and phytoliths to explain dietary and medicinal practices (Fiorin et al., 2019; Hardy et al., 2012, 2016; Henry et al., 2012; Horrocks et al., 2014; Power et al., 2015, 2018; Radini & Nikita, 2023; Radini et al., 2016, 2019; Tromp & Dudgeon, 2015), plant fibres are also found. Debris of bast fibres – extracted from the inner bark or secondary phloem of dicotyledonous plants – such as hemp (*Cannabis* sp.), flax (*Linum usitatissimum*), and nettle (*Urtica dioica*), as well as cotton (*Gossypium* sp.) have been recorded by several researchers (Blatt et al., 2011; Cristiani et al., 2016; MacKenzie et al., 2022; Radini et al., 2016; Romero et al., 2024).

The presence of plant fibres in archaeological context can result from craft activities such as the production of textiles and baskets (Blatt et al., 2011; Radini et al., 2017), as well as from building or repair materials (Norström et al., 2019). Linking the presence of fibre debris in dental calculus to craft activities requires the consideration of other anthropological parameters, such as dental wear. This connection can be established when there is a significant concentration of debris and a large enough sample size of individuals for statistical analysis (Radini et al., 2019). This presence is justified by the use of dentition in extra-masticatory activities, which has been documented from an ethnographic and archaeological perspective. Table 1 exemplifiers some

archaeological and ethnographic examples of the use of teeth as a third hand from different chronological context and cultures. In this regard, the preservation of plant fibres in dental calculus samples demonstrates the necessity to be explored and enriched by biological, anthropological, and archaeological approaches to better understand the use of teeth as a third hand. As mentioned, dental calculus analyses can be potentially useful for studying plant–human relationships although limitations in interpretations should be considered (Hardy et al., 2018). It has been discussed how representative the results are of the role plants played in human activities and environment description (Radini & Nikita, 2023) in both quality and quantity (Hardy et al., 2018; Power et al., 2021). Methodological issues may also be considered regarding the results of debris immersed in dental tartar deposits. Some chemical compounds usually used to disaggregate the matrix can be damaging to organic remains like plant debris (Le Moyne & Crowther, 2021; Tromp et al., 2017). More research is now being carried out to improve methodologies for the extraction of remains (Hardy et al., 2018; Power et al., 2021; Radini et al., 2017).

The aim of this research is to explore the presence, recovery, and identification of plant fibres in dental calculus samples. The objective is to propose a methodological procedure for their recovery when embedded in a calcified dental calculus matrix, as they are often overlooked in this type of analysis. In order to determine whether this oversight is due to limitations in the recovery method, difficulties in identification, both factors or other reasons, this research combines experimental, methodological, and descriptive work by experimenting with the degradation of plant fibres using different decalcifiers and control medieval samples, as well as applying a selected methodology to Neolithic samples as a case study.

Table 1: Archaeological and ethnographic examples of the use of teeth as a third hand from different chronological context and cultures

Site	Chronology	Dental modification evidence	Dental calculus analysis	Reference
Sima de los Huesos (Spain)	Middle Pleistocene	Vestibular striations Enamel flakes	No	Lozano et al. (2008)
Mallaha site (Palestina)	13000–9500 AC	Abraded grooves	No	Bocquentin et al. (2005)
Taforalt (Morocco)	11900–10800 BP	Chipping Notching Interproximal grooves	No	Bonfiglioli et al. (2004)
Ajvide (Sweden)	Middle Neolithic	Occlusal facets, labial, interproximal striae Chipping	No	Molnar (2008)
Gricignano d'Aversa (Italy)	2500–1800 BC	Occlusal and para-occlusal grooves	Yes	Sperduti et al. (2018)
Castellón Alto (Spain)	2141–1703 cal BC	Interproximal, labial, and occlusal grooves Labial notching Enamel chippings Polished enamel	No	Lozano et al. (2021)
Laderas del Castillo (Spain)	*ca.* 1900 cal BC	Rounded U-notches Chipping Abraded surfaces	Yes	Romero et al. (2024)
Thjodhild's Church (Greenland)	1000–1150 AD	Notches	No	Scott and Jolie (2008)
Stone Lake site (California)	100 BC–1200 AD	Abraded grooves	No	Schulz (1977)
Oxyrhynchus (Egypt)	Byzantine period	Lingual grooves	No	Agustí-Farjas (2017)
Australian Aborigine	Ethnographic	Vestibular striations Enamel flakes	No	Lozano et al. (2008)
Greenland Eskimos	Ethnographic	Grooves	No	Pedersen (1947)

2 Materials

2.1 Plant Fibres for a Methodological Proposal

The methodology involved the selection of different plants that have been documented for craft purposes at different times in prehistory and history, as well as those that are still being used. This selection aimed to be representative of the different types of plant fibres that are and have been traditionally used in/for basketry and textile production. Differences in the way they are normally physically processed were also taken into account (Kuoni, 1981).

From this methodological perspective, three monocotyledonous species were selected: esparto grass (*Stipa tenacissima*), bulrush (*Scirpus holoschoenus*), and cattail (*Typha* sp.) (Table 2). Esparto grass is an endemic dry plant found in the central and southern parts of the Iberian Peninsula. Its use is documented from Mesolithic chronologies (Aura Tortosa et al., 2019; Martínez-Sevilla et al., 2023) to the present, and it is traditionally known as one of the most used plant fibres in Iberia. In addition, bulrush and cattails, both monocotyledonous hydroponic plants, were selected. Both have been traditionally used in basketry in different parts of the Iberian Peninsula, such as the northeast, since the establishment of the first farmers and herders in the Neolithic (Herrero-Otal et al., 2021; Herrero-Otal, 2024; Romero-Brugués et al., 2021). In order to study dicotyledonous, two different herbaceous plants were used: nettle (*Urtica dioica*) and flax (*Linum usitatissimum*), and the bast from an arboreal plant, the lime tree (*Tilia* sp.) (Table 2). In the first two cases, they are bast fibres, and in the case of the lime tree, the fibres are extracted from the secondary xylem. The use of these families of dicotyledonous has been very widespread since the Neolithic from northern to southern Europe (Altorfer & Médard, 2000; Bender Jørgensen, 1992; Gramsch, 1992; Herrero-Otal et al., 2023; Herrero-Otal, 2024; Médard, 2003; Miettinen et al., 2008; Mineo et al. 2023; Piqué et al., 2018; Wigforss, 2014). The state of processing refers to the treatment the fibres need to be submitted to in order to make them malleable and usable for crafting. Although there is a wide diversity of ways to process this plant material, the most common ones are physical crushing or retting in water. In the present research, the processing of the plant fibres was chosen according to how they are normally used in traditional crafts. Thus, for the monocotyledonous families, esparto grass was used both unprocessed and processed, and cattail and bulrush unprocessed. The three dicotyledonous (flax, stinging nettle, and lime tree) were used in their processed state.

2.2 Dental Calculus Samples

2.2.1 Control Sample: The Castell de Besora Site (Santa Maria de Besora, Spain)

The Monumental Complex of the Castell de Besora is located on top of a hill, 1 km from the village of Santa Maria de Besora (Osona). On this summit, which is at 1025 m.a.s.l., lie the remains of the Castell de Besora, which was built in the ninth century, and the church of Santa Maria, constructed in the eleventh century

Table 2: Classification of the plant fibre species used in the methodological approach

Division	Class	Order	Family	Genus	Specie	Plant part	Processing state
Angiospermae	Monocotyledoneae	Poales	Poaceae	*Stipa*	*S. tenacissima*	Leaf	Unprocessed Processed
Angiospermae	Monocotyledoneae	Poales	Cyperaceae	*Scirpus*	*S. holoschoenus*	Stem	Unprocessed
Angiospermae	Monocotyledoneae	Poales	Typhaceae	*Typha*	*Typha* sp.	Leaf	Unprocessed
Angiospermae	Dicotyledoneae	Malpighiales	Linaceae	*Linum*	*L. usitatissimum*	Stem bast	Processed
Angiospermae	Dicotyledoneae	Rosales	Urticaceae	*Urtica*	*U. dioica*	Stem bast	Processed
Angiospermae	Dicotyledoneae	Malvales	Malvaceae	*Tilia*	*Tilia* sp.	Bast	Processed

(Busquets & Fàbregas, 2008). Besora was an important power centre during the medieval period until the castle's decline in the fourteenth century. The church of Santa Maria remained active until the eighteenth century when a new church was built on the village plain. The land surrounding the church building was the necropolis for the local population, and a significant number of skeletons have been excavated.

Burial CB21-187 corresponds to an adult female individual buried in the parish necropolis surrounding the church, near the atrium arches. The anthropological field information records an adult female with a large amount of tartar deposits; these were sampled for the methodological approach of the article to test different procedures instead of using more ancient and valuable samples in order to prevent their loss. Dental calculus was collected from teeth 31, 32, and 33 (FDI System).

2.2.2 Neolithic Case Study: The Cova del Pasteral Site (La Cellera de Ter, Spain)

The archaeological site of Cova del Pasteral refers to a set of naturally communicated caves nearly 300 m in length located in El Pasteral (La Cellera de Ter), in the northeast of the Iberian Peninsula 20 km west of Girona (Figure 1). The caves emerge in an outcrop of limestone on the northern side of a hill called Muntanya de Canet or Puig de Gria. This burial cave with Neolithic archaeological materials was found at the end of the twentieth century when the area was exploited as a marble quarry.

The site was visited numerous times by residents in the area who recovered archaeological materials from the site. Riuró (1942) published the first description of the cave, and the materials recovered are currently stored in the Museu d'Arqueologia de Catalunya (MAC). In the 1980s, speleologists made some interesting new finds on the cave, and the first archaeological analysis of the materials was published. Bosch Lloret (1985) carried out a study of the materials recovered from the site and dated them in a wide chronocultural period: from the Early to the Late Neolithic/Chalcolithic. Furthermore, the anthropological analysis of the human remains identified at least 23 individuals (9 from the early phase of the site and 14 from the latest) (Campillo & Vives, 1985). Since the discovery of a possible natural entrance to the cave (Entrance-1) in the 2020 archaeological field season (García, 2020), the annual fieldwork from 2020 confirmed the existence of this access as

Figure 1: Location of Cova del Pasteral and Entrance-1 between the marble outcrops.

well as its richness in prehistoric remains. The pottery record from the excavations has confirmed the chronocultural adscriptions made by Bosch Lloret (1985). Together with the first radiocarbon results, this dates the use of Cova del Pasteral in *ca.* 5000–3000 BC.

The material from the site studied in this research was recovered in the Early Epicardial Neolithic layer (UE1008). It is characterised by the high concentration of pottery fragments chronologically ascribed to this chronological phase and dispersed human remains (Rosillo et al., 2022). Anthropological studies have found that at least 12 individuals from all age groups were buried in the cave during this period. In addition, the analysis of the bone remains has identified degenerative osteoarthritis in vertebrae, calcification of the anterior ligament of the joints, and infectious pathologies in the mouth, such as caries (Agustí-Farjas, 2022).

Dental calculus analysed in the article was collected from two isolated teeth from the Epicardial Neolithic layer. Both individuals were adults with different wear patterns and no pathologies. Supragingival tartar deposits from buccal surfaces were extracted from a maxillary right first premolar (Tooth 14) and from a maxillary lateral incisor (Tooth 22) (Figure 2).

3 Methods

Decalcification is used in various medical, research, and industrial processes and is essential for studying the organic components of materials. Decalcification can be carried out using different methods such as heat, electricity, and chemical agents. The latter are divided into strong (inorganic) acids such as nitric acid (HNO_3) and hydrochloric acid (HCl), weak (organic) acids such as acetic acid (AcOH), and chelating agents such as ethylenediaminetetraacetic acid (EDTA) (Gupta et al., 2014). The action of these compounds is based on the chemical reaction with the calcium present in the material in order to dissolve it into salts forming compounds. Factors such as concentration, exposure time, temperature, and agitation also play an important role in the action of these agents (Gupta et al., 2014; Sheehan & Hrapchak, 1980). The aggressiveness of strong inorganic acids like HCl can damage soft-tissue structures and negatively affect cellular integrity. However, they usually take less time to complete decalcification procedures, making them suitable for highly mineralised specimens. Conversely, weak organic acids take longer to complete decalcification and are used for hard materials such as dense cortical or large bones. Chelating agents, like EDTA, do not behave like inorganic or organic acids, but bind to metal ions, predominantly calcium and magnesium and are therefore more respectful of organic matter, and do not damage tissue or affect their stainability (Gismondi et al., 2018; Hendy et al., 2018).

A bibliographic review of dental calculus analysis was carried out in order to select different solvents, concentrations, and reaction times to test their effect on plant fibres. The most common decalcification methodology used in dental calculus decalcification is based on the use of hydrochloric acid (HCl) (Fiorin

Figure 2: *In situ* tartar deposits from the Cova del Pasteral site: isolated Tooth 14 (left) and Tooth 22 (right).

et al. 2019; Gismondi et al., 2018; Mackie et al., 2017; Tromp et al., 2017; Warinner et al., 2014a; among others) and EDTA (Fagernäs et al., 2020; Juhöla et al., 2019; Mackie et al., 2017; Modi et al., 2020; Soto et al., 2019; Tromp et al., 2017; among others), or even the non-chemical disintegration of calculus (Bucchi et al., 2019). Some authors recently recommended the use of EDTA instead of HCl based on the higher rates of microremain recovery (Tromp et al., 2017) and the null or minimal modification of the starch granules (Modi et al., 2020; Soto et al., 2019). Nonetheless, HCl is more commonly used in concentrations ranging from 0.05 to 9.5 M. This is an aspect to be considered because of the important loss of information due to the chemical processes the material is submitted to. Decalcification processes including chemical concentrations and solvent exposure times may have a degradation effect on organic microremains, such as starch grains, pollen, or even plant fibres (Le Moyne & Crowther, 2021).

Experimental protocols to test the effectiveness of different concentrations of EDTA and HCl agents have also been performed, and the use of EDTA has been proposed as a gentler decalcifier that increases the amount of microremains recovered from dental calculus samples (Modi et al., 2020; Tromp et al., 2017). Although other researchers documented that EDTA could cause alterations in starch granules (Soto et al., 2019), the latest experimental analysis proposed by Le Moyne and Crowther (2021) concluded that the use of low concentrations of HCl or EDTA is appropriate for dental calculus decalcification and recovery of starch grains.

Traditional focus on dental calculus research has been the analysis of starch granules preserved within dental calculus from early human diets (Hardy et al., 2012, 2016; Henry et al., 2012; Horrocks et al., 2014; Power et al., 2015, 2018; Tromp & Dudgeon, 2015). However, more research should be performed to focus on plant fibres. The analysis of plant fibres immersed in a dental calculus matrix owing to fibre crafting is a promising line of research, but it has been underexplored in terms of both the methodological approach and the debris identification. To analyse this, in this article, EDTA and HCl solvents were selected and applied at 0.2 and 0.5 M for 5, 30, 90, and 170 h to both the plant fibres and the dental calculus samples, as explained below.

3.1 Selection of Vegetal Species and Testing Protocol

The samples of plant species were washed to remove any residual sediment using a Bransonic® 5510EDTH ultrasound machine in the Anthropology Unit of the Department of Animal Biology, Plant Biology and Ecology (BABVE) at the Universitat Autònoma de Barcelona. The washing procedure consisted of immersing the fibres in ultrapure Mili-Q water in the ultrasonic machine for 15 min. This process was repeated three times, changing the ultrapure water between each bath. The samples were then dried in a laboratory oven for approximately 48 h or until completely dry.

Two different decalcification procedures were used in the current methodological approach, taking into account previous analyses of dental calculus samples. Following this, EDTA and HCl were used in two different concentrations (0.2 and 0.5 M) for four different reaction times (5, 30, 90, and 170 h). The procedure consisted of immersing the fibres in the different solvents during different periods. The samples were then transferred into 1.5 ml Eppendorf tubs and washed with ultrapure Mili-Q water. Washing involved shaking the samples manually and with a vortex and centrifuging them using a microcentrifuge machine for 5 min at 3,000 rpm. This procedure was repeated three times to remove any residual solvent. The samples were then mounted for optical microscopy using Euromex PB5265 Entellan permanent mounting medium.

In the case of unprocessed monocotyledonous, both transverse (cross section) and epidermal sections were prepared, whereas, in the case of processed monocotyledonous and dicotyledonous, the fibres were mounted directly, corresponding to longitudinal sections. The samples were examined under 50–500× magnification using an Olympus BX43 optical microscope with a polarising filter coupled with an Olympus DP26 camera linked to Olympus cellSens software. The observation was performed in the Archaeobotany Laboratory of the Prehistory Department at the Universitat Autònoma de Barcelona.

3.2 Dental Calculus Analysis

The same methodological approach carried out on the modern plant fibres was then performed on control samples from the Castell de Besora site to test the decalcification process and to demonstrate the effect of the solvents used on plant fibres immersed in dental calculus samples prior to the analysis of Neolithic dental calculus. Finally, the tested methodology was applied to ancient dental calculus material from the Neolithic site of Cova del Pasteral as a case study to identify the fibres in the calculus.

3.2.1 Sampling and Decontamination

A published protocol for the extraction of microremains from archaeological human dental calculus was followed for the initial steps of selection, documentation, removal, storage, weighing, and decontamination (Fiorin & Cristiani, 2024), although some steps were adapted to our samples, such as the speed and time of centrifugation to obtain pellets. Following this protocol, dental calculus samples were selected according to their size and origin, quantity, colour, and texture. The teeth were photographed before and after removing the tartar. The whole process was carried out in the Anthropology Unit of the BABVE at the Universitat Autònoma de Barcelona.

The teeth were then cleaned mechanically with distilled water using a new soft toothbrush. Tartar was removed from each side of the tooth using a dental scaler. In addition, tartar was extracted separately according to the part of the tooth from which it originated (lingual, buccal, distal, medial, or occlusal). The samples were weighed and transferred into 1.5 ml Eppendorf plastic tubes.

Decontamination of the samples consisted of removing any residual soil adhering to their outer surface. Contaminants were extracted from the sediments using ultrapure water and shaken manually several times at minimum speed in a vortex. The deposits were then cleaned manually under a stereomicroscope using a nylon brush soaked in drops of 0.5 M HCl. At this point, the samples were rinsed with ultrapure water and centrifuged up to three times to remove any residual sediment or HCl. The samples were then transferred to new sterile 1.5 ml Eppendorf tubes and dried until processed for calculus disaggregation.

Sample processing was performed under strict contamination-free conditions. Extraction and handling were performed in a laminar flow hood cleaned with detergents, 5% sodium hypochlorite, and 90% ethanol. The work surface was additionally shielded with aluminium. The researchers used starch-free gloves, lab coats, and face masks, while all tools were either cleaned or replaced between samples. To further ensure the reliability of the process, environmental control slides were placed in the laminar flow hood, as were fibres from the lab coats and face masks. This decontamination protocol was adapted from several established and published guidelines (Cristiani et al., 2018; Cummings et al., 2018; Gismondi et al., 2018; Hardy et al., 2016; Romero et al., 2024; Weyrich et al., 2017).

3.2.2 Disaggregation, Microscopical Observation, and Identification

With regard to the tartar used in the methodological approach, from the Castell de Besora site, the same solvents and times as in the plant fibre methodological approach were used. Thus, EDTA and HCl at 0.2 and 0.5 M were used for 5, 30, 90, and 170 h. Dental calculus samples were transferred to 1.5 ml Eppendorf tubes and washed with ultrapure Mili-Q water. Washing consisted of manual and vortex shaking and centrifugation in a microcentrifuge for 5 min at 5,000 rpm. The samples were then immersed in the different solvents and mixed manually and by vortexing several times during the different decalcification times. When the samples were disaggregated, they were centrifuged at 5,000 rpm for 5 min, and the supernatant was removed. The pellet was rinsed with ultrapure Mili-Q water and centrifuged again to remove any residual acid. It should be noted that in some cases, the time and speed of centrifugation were modified to achieve pellet deposition. This procedure was repeated three times for each sample to remove any residual solvent.

For the case study samples, from the Cova del Pasteral site, following the results obtained from the methodological approach with the modern plant fibres and the test with control medieval dental calculus, 0.5 M EDTA was chosen to disaggregate them. In this case, the exposure time was until the sample was completely disaggregated, but in no case more than 170 h. The rest of the procedure was the same as for the control samples.

Dental calculus samples were then mounted on optical microscope slides with pure glycerine and sealed with transparent nail polish. The samples were examined with the same optical microscope as used for the plant fibres in the same laboratory facilities. Finally, the microremains were photographed, measured, described, and compared with modern reference materials. It should be noted that greater attention was paid to microremains found embedded in the dental calculus matrix and those situated in the centre of the samples, which supports the absence of contamination.

4 Results

4.1 Methodological Approach

4.1.1 Plant Fibre Materials

Each of the plant fibres was observed in detail in order to identify any changes in their appearance due to the use of 0.2 and 0.5 M EDTA and HCl. In the case of the unprocessed fibres, both the transverse section and the epidermis were observed. In the case of processed fibres, it is difficult to observe both the transversal section and the epidermis (because processing usually involves physical damage), so in this case, the longitudinal characteristics were considered.

- *S. tenacissima* (unprocessed). Unprocessed esparto grass leaves are naturally flat, but in arid environments, they convolute to reduce their size and limit water transpiration. This folding is clearly visible in the transverse section views as protuberances on the abaxial surface containing isolated vascular bundles surrounded by a thick sclerenchyma tissue. Adaxial and abaxial epidermis are different in appearance as the former is smooth with short and long cells alternation, while abaxial epidermis is characterised by a high concentration of silica hairs and stomata in the inner part of the protuberances (Figures S1–S4).
- *S. tenacissima* (processed). The previous characteristics of unprocessed esparto grass are mostly lost when it is physically processed. The transversal section becomes difficult to observe, although, in both adaxial and abaxial surfaces, some of the cells are visible in some scattered parts. Otherwise, inner structures like parenchyma and sclerenchyma tissue are visible longitudinally. In both cases, they correspond to histological structures very similar to other plant families and species and they are not characteristic enough for raw material determination (Figures S5–S8).
- *S. holoschoenus* (unprocessed). Bulrush stems are characterised by a rounded or oval cross section in which the inner part can be full of parenchyma/aerenchyma tissue, depending on the species and the age of the stem. In the case of *S. holoschoenus,* this medulla part is empty. Even so, the isolated vascular bundles are arranged in different rows along the tissue, with the large ones in the outer part surrounded by sclerenchyma tissue. Collenchyma is also clearly visible in the transversal section. As it is an aquatic plant, the epidermis is characterised by a high number of stomatal cells arranged in longitudinal lines along the tissue and separated by two or three lines of epidermal cells (Figures S9–S12).
- *Typha* sp. (unprocessed). Cattail leaves differ in the appearance of their vascular bundles from the monocotyledonous species described above. Small ones are near the epidermis, and large ones are in the columns of the parenchyma along the aerenchyma chambers. The epidermis, both adaxial and abaxial, displays a high number of stomata dispersed along the surfaces (Figures S13–S16).
- *L. usitatissimum* (processed). Flax fibres are similar to nettle fibres and also correspond to sclerenchyma fibres. The surface of flax fibres appears smooth and has characteristic knots or cross-marks at regular

intervals. In cross-section, flax fibres are polygonal or irregular in shape although they are not visible in our samples as only longitudinal views are present (Figures S17–S20).
- *U. dioica* (processed). Stinging nettle fibres are visible when the stem of the plant is processed and retted in water and correspond to the sclerenchyma tissue of the plant, which surrounds the vascular system of the secondary phloem. They have a central lumen running the length of the fibre. The surface appears slightly rough or striated due to the orientation of the cellulose microfibrils that provide structural support and are used in determination (Figures S21–S24).
- *Tilia* sp. (processed). What is microscopically visible in the cross section of the lime tree bast is the secondary phloem. It derives from the vascular cambium. In this research what is visible in the images is the longitudinal view where rays appear empty as they were previously retted in water (Figures S25–S28).

These characteristics explained for each of the plant families are the ones used in their identification. Moreover, in all the cases, they are visible in all the solvent concentrations and timings used in this study, and no major alterations were observed.

4.1.2 Plant Fibres in the Dental Calculus Control Sample: The Castell de Besora Site

Quantitatively, several plant fibres were found in the dental calculus of individual CB21-187 from the Castell de Besora site (Table 3). Methodologically, no qualitative differences were found between the fibres recovered from this individual when treated with 0.2 and 0.5 M EDTA for 5, 30, 90, and 170 h (Figure 3). Although EDTA is a chelating agent known for its decalcifying properties, enabling it to dissolve calcium carbonate deposits and other forms of precipitated calcium, its use in dental calculus does not appear to affect the identification characteristics of plant fibres at these concentrations for periods up to 170 h (Figure 3a–h). Very similar results were obtained using HCl at different concentrations (0.2 and 0.5 M) and timings. Even with these concentrations and times of HCl, no qualitative changes were observed in the current study protocol (Figure 3i–p). It should be noted that the tartar in the material studied was not completely decalcified, as seen in Figure 3, although microremains were visible with this protocol. From a quantitative point of view and in a general overview, more fibres have been recovered in the samples treated with EDTA than the ones decalcified with HCl (Table 3). The samples were obtained from three different teeth (Teeth 31, 32, and 33).

The samples from the Castell de Besora site were prepared as microscope slides where the cross sections were not visible. However, some of the documented fibres appear to have a rounded section with frayed edges. In longitudinal sections, the fibres have an internal striated texture and are colourless. Under polarised light, many of these fibres have a brownish hue, with visible transverse lines corresponding to cross-sectional markings. These characteristics are commonly described in bast fibre identification studies (e.g. Bergfjord & Holst, 2010; Haugan & Holst, 2013, 2014; Lukešová et al., 2017, 2019) and have been proposed as one of the most reliable methods for identifying bast fibres when combined with complementary techniques (Haugan & Holst, 2013).

Although the cross section of these fibres is not visible, their diameters are consistent with flax, hemp, and nettle plant fibres, which were most commonly used in textile production during this time period (Andersson Strand, 2012; Bergfjord & Holst, 2010; Haugan & Holst, 2013, 2014; Sperduti et al., 2018). In addition to the

Table 3: Number of plant fibres recovered in each protocol of the medieval control samples from Castell de Besora

			5 h	30 h	90 h	170 h
EDTA	Tooth 31	0.2 M	10	7	5	13
EDTA	Tooth 31	0.5 M	1	12	7	16
HCl	Tooth 32	0.2 M	1	4	6	9
HCl	Tooth 33	0.5 M	2	2	1	2

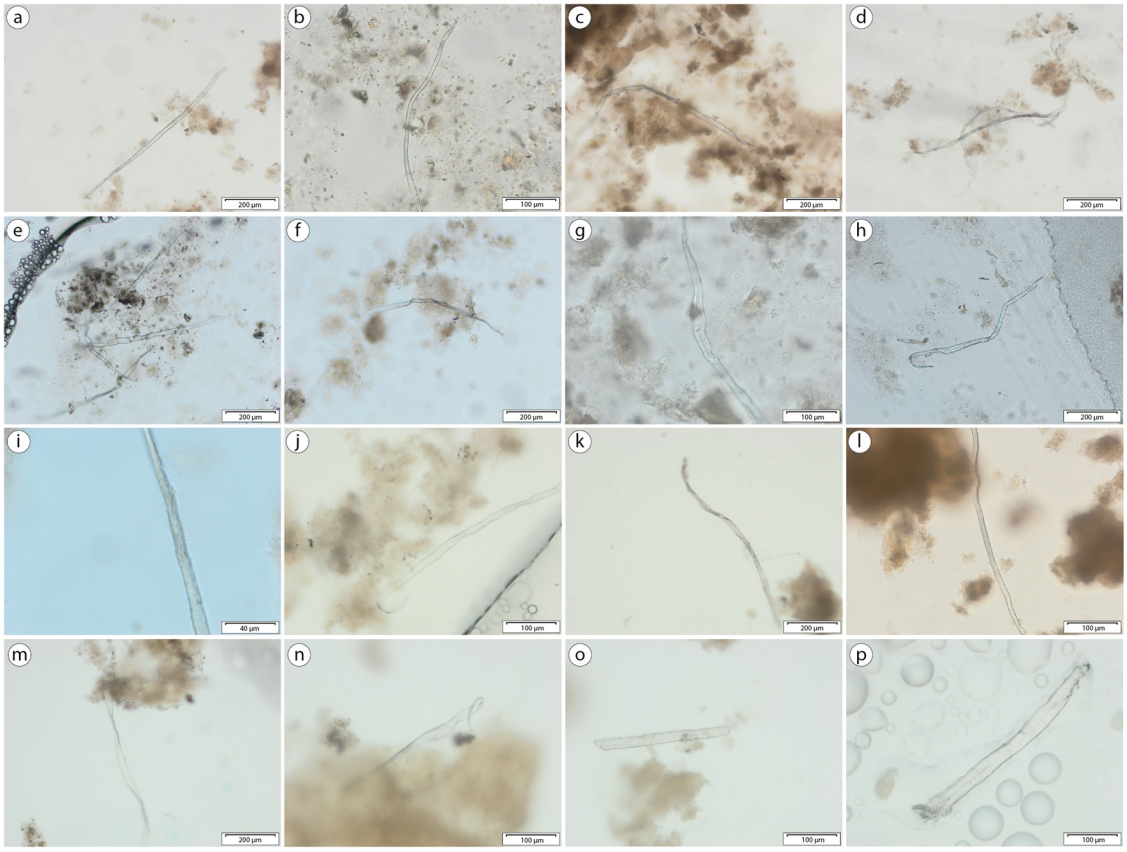

Figure 3: Plant fibres recovered from dental calculus from the Castell de Besora Site: (a) 0.2 M EDTA-5 h, (b) 0.2 M EDTA-30 h, (c) 0.2 M EDTA-90 h, (d) 0.2 M EDTA-170 h, (e) 0.5 M EDTA-5 h, (f) 0.5 M EDTA-30 h, (g) 0.5 M EDTA-90 h, (h) 0.5 M EDTA-170 h, (i) 0.2 M HCl-5 h, (j) 0.2 M HCl-30 h, (k) 0.2 M HCl-90 h, (l) 0.2 M HCl-170 h, (m) 0.5 M HCl-5 h, (n) 0.5 M HCl-30 h, (o) 0.5 M HCl-90 h, and (p) 0.5 M HCl-170 h.

identification of plant fibres, other microremains such as starch grains, phytoliths, fungal spores and indeterminate residues have also been documented.

4.2 Microremains in the Neolithic Case Study: The Cova del Pasteral Site

The results obtained from the decalcification of Neolithic dental calculus indicate the recovery of such microremains as plant fibres, multicellular plant tissues, starch grains, fungi, animal hair, and unidentified materials and minerals. Although this research is focused on the identification of plant fibres, attempts have also been made to identify the plant tissue remains.

The plant fibres found in the dental calculus samples from the Cova del Pasteral site generally had a round cross-section with a smooth surface (Figure 4a–c). However, these characteristics are not sufficiently reliable for the precise identification of plant species, as they are consistent with cellulosic fibres derived from a variety of plant sources, including bark, wood, leaves, or stems of both monocotyledonous and dicotyledonous plants. Furthermore, although it remains a challenge to determine the exact part of the plant from which the processed fibres originate, it is plausible that they correspond to the sclerenchyma tissue typically used for fibre extraction in dicotyledonous plants as seen under polarised light (Figure 4a–c).

Vegetal tissue remains were considered to be multicellular structures. Although it was not possible to identify all of them, two showed clearly recognisable features. The first could correspond to a fragment of a Poaceae/Gramineae, as long dendritic cells are visible (Figure 4d), even though the part of the plant could not be identified. In two more multicellular tissue remains, parenchyma cells consistent with non-defined monocotyledonous are visible (Figure 4e–f), although the genus, or even the family, could not be identified.

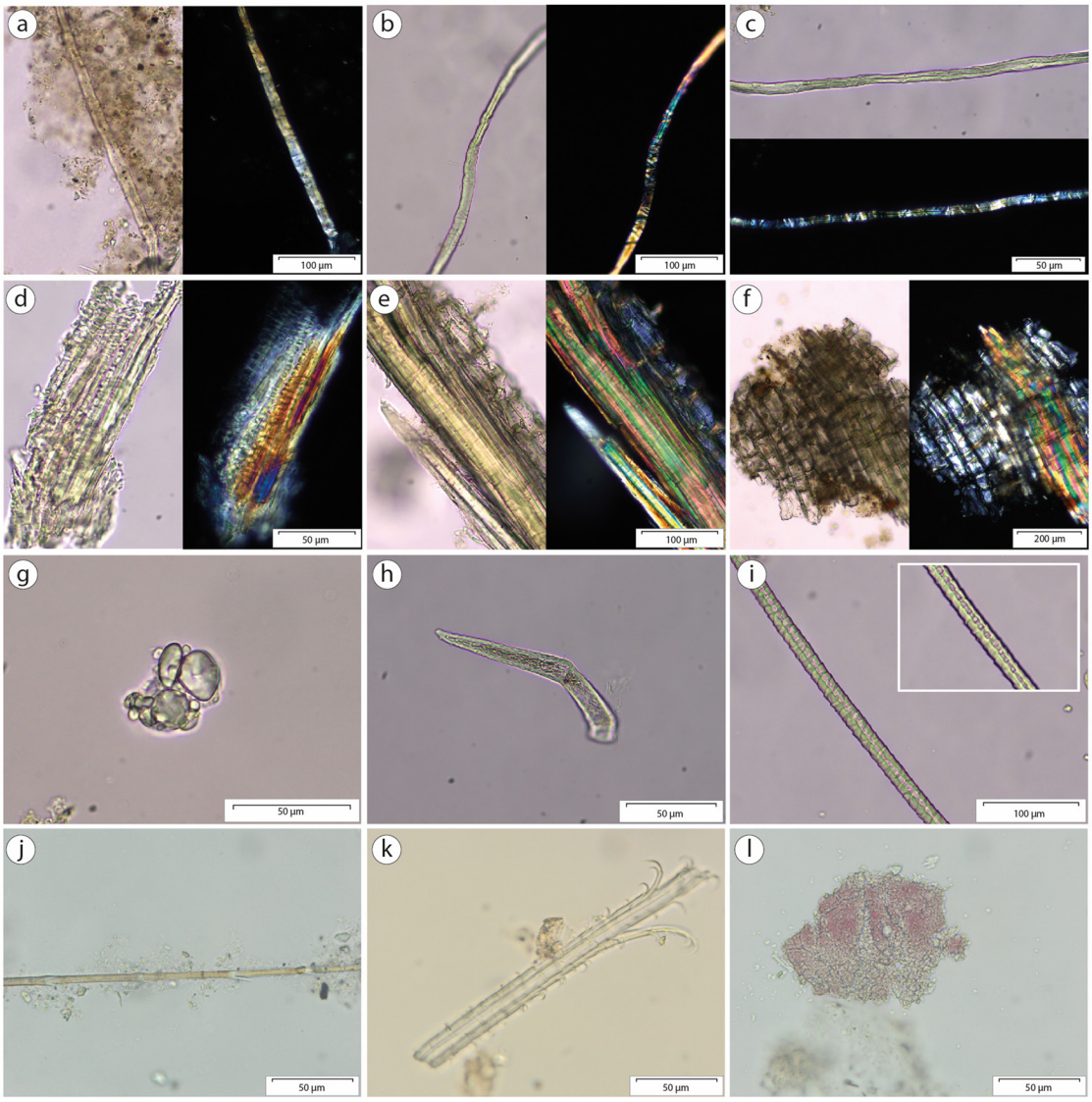

Figure 4: Optical microscopy images of microremains found in dental calculus from the Neolithic case study samples from the Cova del Pasteral site: (a)–(c) plant fibres; (d) Poaceae/Gramineae tissue; (e) and (f) non-identified monocotyledonous plant tissues; (g) starch grains; (h) possible fungal spore; (i) animal hair; (j) and (k) unidentified microremains, and (l) unidentified coloured mineral.

Several microremains corresponding to other categories were also identified in the dental calculus from the case study in this article, although from a quantitative perspective, the most common microremain typology found in the Neolithic dental calculus samples are plant fibres followed by the indeterminate plant tissue (Table 4). Some of the other recognised categories correspond to starch grains (Figure 4g), possible fungal spores (Figure 4h), animal hair (Figure 4i), unidentified microremains (Figure 4j and k), and coloured minerals (Figure 4l).

Table 4: Microremains found in the Neolithic case study samples from Cova del Pasteral

	Plant fibres	Plant tissue	Starch grains	Animal hairs	Non-identified microremains	Non-identified coloured minerals
Tooth 14	18	12	5	1	7	1
Tooth 22	13	15	0	0	4	2

5 Discussion

Dental calculus is the naturally mineralised bacterial plaque that adheres to the teeth and becomes calcified if not removed when it is soft. Dental calculus contains an organic fraction (amino acids, peptides, carbohydrates, and lipids) and an inorganic fraction composed mainly of calcium phosphate salts (Blatt et al., 2011; Lieverse, 1999; Middleton, 1994). It is associated with saliva production, which is a guarantee against post-mortem inclusions. During its formation, particles of diverse nature can be trapped, including starch granules, phytoliths, fungal spores, plant fibres, animal hairs, and other lithological compounds such as pigments (Blondiaux & Charlier, 2008; Power et al., 2018; Radini et al., 2019).

New insights into the palaeodiet and subsistence patterns of ancient populations have been provided by the identification of microremains entrapped in dental calculus matrix, demonstrating the consumption of inhaled or ingested materials during daily activities or the use of teeth as a third hand for extra-masticatory work (Buckley et al., 2014; Cristiani et al., 2018; Hardy et al., 2016; Mickleburgh & Pagán-Jiménez, 2012; Musaubach, 2012; Radini, 2016; Sperduti et al., 2018; among others). Molecular analyses have also been developed in relation to dental calculus to detect proteins, isotopes, DNA, and associated metabolites (Adler et al., 2013; De La Fuente et al., 2013; Hendy et al., 2018; Preus et al., 2011; Warinner et al., 2014b; Wright et al., 2021). In addition, particles within the dental calculus matrix provide an opportunity to infer past human life by combining physical anthropology, biomolecular analysis, and archaeobotany.

5.1 The Use of EDTA and HCl in Modern Plant Fibres and Dental Calculus Control Samples

Some authors suggest that the selection of decalcifiers depends on such variables as the urgency of the study, the degree of mineralisation of the samples, and the objective of the research. Numerous studies have been carried out to develop new decalcifiers and improve existing ones to meet the criteria for the most efficient decalcifier. The current research has sought the best decalcifying agent for archaeological dental calculus samples in order to prevent the loss of the organic record inside, such as plant fibres. The ideal agent removes calcium completely without damaging tissue morphology. The results of the methodological approach of this research on different vegetal species used for the extraction of plant fibres showed that the use of chelating agents such as EDTA or strong acids such as HCl do not affect the histological structure of plant fibres at 0.2 and 0.5 M, at least for the first 170 h. Their subsequent use of control samples of dental calculus from individual CB21-187 showed that they completely decalcify without affecting the structural characteristics of plant fibres.

Therefore, in the current study, we focused on the characteristics of the microremains, specifically the plant fibres, and not on their quantitative presence. However, the fact that more plant fibres were recovered in the samples treated with EDTA in the protocol sample should be mentioned. It should also be noted that the presence of microremains in dental calculus samples is not homogeneous, making quantitative results difficult to interpret as the samples also came from three different teeth (although they are anatomically close to one another, and the components should be very similar). We cannot discard that the recurrent lower presence of plant fibres in the samples treated with HCl (both 0.2 and 0.5 M) could be related to a greater impact of HCl, but the fact that no other evidence of degradation has been observed on the surface of the fibres suggests that there is no relation.

Although there is no standardisation of the methods used to decalcify dental calculus, the literature has grown in recent years. Authors have tried different methods such as no chemical treatment (Power et al., 2018), while other chemical methods using different solvents such as EDTA or HCl have been tested (Boyadjian, 2018; D'Agostino et al., 2019; Hardy et al., 2016; Tromp et al., 2017). The choice of decalcification method is very important, as it may affect the number and identification of microremains (Bucchi et al., 2019).

Similar studies have been carried out on the recovery of other organic materials from dental calculus. Le Moyne and Crowther (2021) analysed the recovery of starch granules and suggested that the use of 0.5 M HCl

for prolonged periods did not cause significant morphological changes or complete dissolution of different starch granules. However, they suggest that chemical interactions between HCl and the calcium phosphate of the dental calculus matrix may affect starch recovery during decalcification as the reaction between HCl and the calcium phosphate is exothermic, and this increase in heat may cause starch granule degradation. Other authors suggest that phosphated starch in dental calculus may be more susceptible to disruption by calcium ions released during HCl decalcification (Mercader et al., 2018; Tromp et al., 2017). Conversely, Tromp et al. (2017) argued that the use of EDTA reduces the calcium ions they sequester, reducing their reactivity and potentiality. An advantage of the use of EDTA is related to DNA and protein analysis, providing greater opportunities to study the materials, considering that this type of analysis is destructive. Although some researchers reported no morphological changes on starch granules, others found morphological changes on the surface of starch granules (Le Moyne & Crowther, 2021; Soto et al., 2019). Le Moyne and Crowther (2021) conclude that the minor alterations they report are negligible and do not affect starch recovery and identification, although they recommend further research. They, like Tromp et al. (2017), also state that 0.5 M EDTA (pH: 8) is a more suitable decalcifying agent than corrosive HCl for the recovery of microremains from dental calculus.

5.2 Plant Fibres From the Cova del Pasteral Site

Although our results showed that the use of EDTA and HCl for the demineralisation of archaeological dental calculus for plant fibre recovery at 0.2 and 0.5 M for a maximum of 170 h did not affect the appearance of plant fibres, we prioritised the use of the EDTA chelating agent due to its chemical properties of sequestering calcium ions in the Neolithic samples. Its use also allows more opportunities to study the materials, if we bear in mind that this type of analysis is destructive as regards DNA and protein analysis, as other researchers have suggested (Le Moyne & Crowther, 2021; Soto et al., 2019; Tromp et al., 2017). We also observed a higher number or plant fibres recovery in the control samples treated with EDTA (although this might be related to a non-homogeneous presence of microremains in different dental tartar). The concentration was chosen to accelerate the process so that the time to complete calculus disaggregation was 70 h.

The identification of plant fibres through the study of indirect evidence is particularly challenging due to the low resolution of some of the documented diagnostic plant features and the lack of comprehensive reference materials. This makes accurate identification and analysis difficult for researchers. In addition, similarities between different plants often prevent specific differentiation.

Dental calculus can be studied because it is a mineralised material, and the presence of plant fibres can be explained by the use of teeth for extramasticatory activities or even their inhalation from the environment. Although linking the presence of fibre debris on dental calculus to craft activities requires the consideration of additional anthropological parameters, such as dental wear, this link is feasible when there is a high concentration of debris and a sufficient number of individuals to allow for robust statistical analysis (Radini et al., 2019).

This method is particularly effective when the sample size is large enough to provide meaningful data, allowing researchers to draw more accurate conclusions about past human activity and behaviour. The identified microremains revealed the presence of plants in the analysed dental calculus in the form of both plant fibres and plant tissues. Although the plant fibres could not be determined due to the absence of diagnostic features, they are very similar to cellulose fibres. These fibres appear in a wide range of sizes and appearances, as cellulose is present in all plants, both monocotyledonous and dicotyledonous (Vanderghem et al., 2012). As regards the presence of cellulose fibres, this cannot be a definitive sign that this is due to the use of plant fibres, since they are present in a great variety of structures and elements of plants. Thus, their origin might be inferred from the consumption of plant leaves and stems, but also from seeds and fruits for nutritional purposes. More hypotheses could be made if more contextual information about the individual, the site, and the environment could be obtained through anthropological and archaeobotanical studies, but in the case study of this research, this information is not available, so no further information can be extracted. The same conclusion can be drawn from the presence of plant tissues, which will be discussed below.

In the case of plant tissues, different cell types have been identified, such as those from the epidermis, sclerenchyma, and parenchyma tissues of stems and leaves of monocotyledonous. However, the characteristics did not allow the identification of specific families or genera, except for an indeterminate Poaceae/Gramineae. The presence of monocotyledonous in general and cereals in particular in the Early Neolithic in the study area is chronologically proven by the appearance of agricultural practices in the area. Moreover, several archaeobotanical studies from relatively proximate archaeological sites, such as the settlement of La Draga (5292–4713 cal BC; Banyoles, Girona), have revealed the presence of different species of monocotyledonous, such as *Triticum durum/turgidum* type, *Hordeum distichum, Triticum dicoccum, Triticum monococcum,* and *Triticum timopheevi* (Antolín, 2013; Piqué et al., 2021). Other monocotyledonous identified at the site include *Cladium mariscus, Cyperus fuscus, Phragmites australis, Typha angustifolia,* and *Typha latifolia* (Antolín & Buxó, 2011) through carpological analysis, and a *Cyperus* sp. as an underground storage organ (Berihuete-Azorín et al., 2018). The use of monocotyledonous families in the production of handicrafts such as plant fibre cords and baskets at the site must also be considered, as three different Poaceae/Gramineae, one Cyperaceae, and one Typhaceae family were used (Herrero-Otal et al., 2021; Herrero-Otal, 2024). The use of Cyperaceae is also recorded in other archaeological sites in northeastern Iberia, such as the Coves del Fem site (*ca.* 6065–4545 cal BC; Ulldemolins, Tarragona), where fragments of baskets and a single small piece of cord made from sedges and Juncaceae were found (Herrero-Otal et al., 2021; Romero-Brugués, 2022; Romero-Brugués et al., 2021).

The presence of various plant tissues such as plant fibres and other histological parts in the samples of this study cannot be directly related to the use of teeth as a third hand or tool because no contextual knowledge is available such as individual biological information about the individuals, oral pathologies and/or characteristics and other osteological details or even the associated burial goods. However, this study demonstrates the possibilities of fibre survival in dental calculus, which can be a powerful tool to complement other approaches such as the use of wear traces or Activity Induced Dental Modification, as it has been called by some authors (Lozano et al., 2021). It should also be noted that the use of teeth as a tool or as a third hand can imply a wide variety of actions, such as the processing of plant fibres, such as softening or spinning, but also the processing of seeds with a more alimentary objective. The identification of specific parts of plants – both monocotyledonous and dicotyledonous – could help to determine the origin of the presence of plant fibres in dental calculus, depending on whether the identified parts of certain species were consumable for humans. More detailed experimental or anthropological work is recommended in order to identify differences in the microwear patterns produced by different types of processing of plants associated with different activities (Emperaire, 2002).

6 Conclusions

The present study aimed to find a non-aggressive decalcification method for the extraction of plant fibres that can become embedded in the dental calculus matrix. To this end, seven different plant fibres, traditionally used in handicrafts, were exposed to two different concentrations of EDTA and HCl, the most commonly used decalcifiers in dental calculus analysis, for different periods of time up to 170 h. The results of this methodological approach showed that none of the treatments affected the quality appearance of the plant fibres, preserving their identifying characteristics. These steps were then repeated on control samples of tartar, with the same results, although quantitatively more plant fibres were recovered in the samples treated with EDTA. Based on these results, other references, and the chelating properties of EDTA, it was decided to use that decalcifier for the disaggregation of Early Neolithic samples from the Cova del Pasteral site. The identified microremains revealed the presence of plants in the analysed dental calculus in the form of both plant fibres and plant tissues, in agreement with previous archaeobotanical studies for other archaeological sites in the surroundings. However, a direct relationship between the use of teeth and the treatment of plant fibres cannot be reported due to the lack of further anthropological information on the individuals from whom the samples were taken. We conclude, as have other authors, that future studies of archaeological dental

calculus should use EDTA for decalcification, as this chelating agent reduces the reactivity of metal ions, it is gentler on microbotanical remains, and it also allows other analytical procedures such as DNA or protein extraction to be carried out. In sum, this study has successfully demonstrated the survival of plant fibres in dental calculus samples, employing a methodology that can be explored and complemented with other approaches – biological, anthropological, and archaeological – to infer the use of teeth as a third hand for multiple purposes.

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