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Enhanced functional data retrieval from Palaeolithic stone tools by lipid analysis

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ABSTRACT

Despite the great potential of lipid biomarkers in archaeological science, their analysis in stone tools has been overlooked. The lipid retention capacity of Palaeolithic stone tools, along with the potential utility of the biomarkers they may harbour as a functional proxy, remains largely unknown. Here, we extracted lipid biomarkers from flint flakes and limestone pebbles from the Middle Palaeolithic site of El Salt (SE Spain) and analysed them using gas chromatography-mass spectrometry (GC-MS) and gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). Adjacent sediments were also analysed for comparison. We provide evidence that Palaeolithic stone tools preserve a diverse array of lipid biomarkers including fatty acids, *n*-alkanols, sterols and terpenoids, the analysis of which allowed us to determine whether the tools were used and/or hafted. The isotopic characterization of individual fatty acids preserved on tools' working edges enabled us to identify lithic residues as fats resulting from the processing of ruminant animal species, or as lipids from non-ruminant animal and/or plant taxa. This introduces into functional studies a novel approach that adds taxonomic resolution and complements current techniques such as use-wear and micro-residue analyses. Our findings highlight the remarkable preservation potential of biomolecular remains within the Palaeolithic record and underscore the importance of exploring them in different kinds of materials and contexts.

1. Introduction

Biological markers or biomarkers play a pivotal role in archaeological science (Evershed, 2008a). This is because they are molecular fossils likely to preserve after burial and are diagnostic of specific biota (Eglinton and Calvin, 1967; Gaines et al., 2009; Peters et al., 2004). Compared to other biomarkers such as proteins, carbohydrates or DNA, lipids are the most resistant to decay and translocation due to their hydrophobic properties (Evershed, 1993, 2008a).

Since the 1970s, gas chromatography-mass spectrometry (GC-MS) analysis of lipid biomarkers preserved in archaeological materials has yielded valuable data about past human societies (Condamin et al., 1976; Evershed, 2008a; Thornton et al., 1970). Most studies have focused on lipid residues in pottery, but also in ecofacts such as human and faunal remains, resin and tar, and coprolites, providing valuable information about past diet, technology and natural resource exploitations (Charters et al., 1993; Colonese et al., 2015; Condamin et al., 1976; Craig et al., 2013; Degano et al., 2019; Dudd and Evershed, 1998; Evershed et al., 1995; Lin et al., 1978; Lucquin et al., 2016; Niekus et al., 2019). Analysis of lipids preserved in archaeological soils and sediments has also been useful for palaeoenvironmental reconstruction, identification of human occupation surfaces, and characterization of pyrotechnologies (Brittingham et al., 2019; Bull et al., 1999; Choy et al., 2016; Collins et al., 2017; Connolly et al., 2019; Leierer et al., 2019).

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The incorporation of gas chromatography coupled to isotope-ratio mass spectrometry (GC-IRMS) into these investigations has been crucial as it has allowed us to narrow down biomarker sources by determining the carbon and hydrogen isotope composition of individual compounds. In this regard, the $\delta^{13}C$ values of $C_{16:0}$ and $C_{18:0}$ fatty acids have been extensively used to distinguish different organic products (e.g., from ruminant, non-ruminant, marine and freshwater animals, and plants) preserved in archaeological materials such as pottery and sediments by comparison with present-day reference fats and oils (Buonasera et al., 2023; Craig et al., 2013; Dudd and Evershed, 1998; Lucquin et al., 2016; Tomé et al., 2022).

Despite the great potential of lipid biomarkers, lithics –the most ubiquitous objects of Pleistocene archaeological sites– have not yet been extensively studied through these analytical techniques. Residue analysis on lithics has advanced considerably over the past decades, employing approaches ranging from optical microscopy for the visual characterization of residues to more recent techniques such as Raman microscopy (Bordes et al., 2017, 2018) and FTIR microspectroscopy for organic compound identification (Monnier et al., 2018; Solodenko et al., 2015; Venditti et al., 2019), SEM-EDS for elemental analysis and microstratigraphic examination (Hayes and Rots, 2019; Jahren et al., 1997), and integrated multi-analytical protocols (Monnier et al., 2013). Within this expanding analytical toolkit, lipid biomarker analysis through gas chromatography-mass spectrometry can provide additional molecular-level, high-resolution information for the detection and identification of archaeological residues.

GC-MS has been widely employed to identify macroscopic residues such as tar, resin and bitumen (Boëda et al., 1996; Cârciumaru et al., 2012; Charrié-Duhaut et al., 2013; Croft et al., 2018; Degano et al., 2019; Hauck et al., 2013; Mazza et al., 2006; Niekus et al., 2019; Perrault et al., 2016; Sauter et al., 2000; Villa et al., 2015), which have been reported to occur on stone tools in a few Paleolithic sites. However, very few studies have analysed lithic tools without visible residues in search of biomarkers, which may remain undetectable at both macroscopic and microscopic scales (Buonasera, 2005, 2007; Luong et al., 2017, 2018, 2019; Mazzia and Flegenheimer, 2015; Quigg et al., 2001). While these pioneering investigations have provided promising preliminary data, several key challenges and questions remain largely unexplored, limiting a comprehensive understanding of the potential of lipid biomarkers in lithic studies.

First, the lipid retention potential of the type of rocks on which lithic artifacts are made, such as flint or limestone, is not well known. To date, no research has explored the molecular preservation of lipid biomarkers on Middle Palaeolithic stone tools without visible residues. Second, although previous research applying chromatography techniques to lithics has provided clues about tool use, the application of lipid biomarkers in functional studies still faces several challenges and knowledge gaps that need to be addressed. On the one hand, establishing whether lipid molecular residues preserved in lithics are use-related or derived from postdepositional natural processes (i.e., absorbed from the surrounding sedimentary environment) is not straightforward and is still an unresolved issue (Buonasera, 2005, 2007; Luong et al., 2017, 2018, 2019). This has been central in the field of micro-residue studies, motivating extensive experimental work showing that analysing the spatial distribution of residues and comparing them with surrounding sediments is key to distinguishing use-related residues from environmental or incidental deposits (Langejans, 2011; Lombard and Wadley, 2007; Wadley and Lombard, 2007). This is because non-use-related residues tend to be randomly distributed across a tool's surface, whereas use-related residues typically exhibit a consistent spatial pattern, being more abundant around the working edge (Langejans, 2011; Langejans and Lombard, 2015; Lombard and Wadley, 2007; Wadley and Lombard, 2007). However, for lipids, limited work on these matters exists. In this context, several studies have assumed that lipid residues contained in archaeological heated rocks derive from cooking activity without contemplating potential postdepositional sources (Quigg et al., 2001). Although some papers included data from adjacent sediment samples (Buonasera, 2007; Luong et al., 2018; Mazzia and Flegenheimer, 2015), off-site rocks (Buonasera, 2005), and/or by analyzing different areas of stone tools (e.g., active edges/areas vs. non-active surfaces) (Buonasera, 2007; Luong et al., 2017, 2018, 2019) to control for lipids absorbed from depositional environment, these studies are very few, or the sediments considered were contextually problematic. Another approach has been to assume that lithic remains not showing any use-wear traces potentially preserve only environmental lipid residues, and could thus be used as a control for such residues (Luong et al., 2019), although the presence of use-related residues cannot be ruled out on lithics without use-wear traces (Crombé et al., 2001; Langejans, 2011). On the other hand, compound-specific isotope analysis (CSIA), which may hold potential to identify the biotic sources of fatty acids preserved and involved in tool use, has never been tested and implemented in lithic functional studies.

Our paper aims to shed light on these issues through a lipid biomarker study of anthropogenic flint flakes and limestone pebbles and their adjacent sediment from El Salt Middle Palaeolithic Site (Alcoy, Spain). GC-MS analysis was conducted in combination with compound-specific $\delta^{13}C$ analysis of individual fatty acids by GC-C-IRMS with the objectives of (i) exploring the preservation potential of lipid biomarkers in Middle Palaeolithic flint and limestone objects, and (ii) assessing the viability of utilizing lipid residues and their isotopes on lithic tools as functional proxies in Middle Palaeolithic research.

2. Materials and methods

2.1. Samples

25 flint flakes and 3 limestone pebbles (Table 1, Table S1) from the Middle Palaeolithic site of El Salt (Alcoy, SE Spain) (Fig. 1, Fig. S1, SI Appendix) were sampled for analysis. Several sediment control samples ($n = 8$) from the same stratigraphic context were also collected for analysis (Table 1, SI Appendix). All the samples belong to Stratigraphic Units (SUs) XI and Xb, dated 60.7 ± 8.9 and 52.3 ± 4.6 ka BP, respectively (Galván et al., 2014) (Fig. 1, Table 1). All the archaeological samples were excavated and recovered using solvent-washed metal tools and wearing nitrile gloves, packed in previously combusted (550 °C, 10 h) Al foil, and stored at −20 °C until further processing.

The archaeological flint samples generally consist of cortical and full-production flakes, obtained through a recurrent centripetal Levallois knapping modality, from flint nodules of the Serreta, Mariola, and Beniaia types (Table S1, SI Appendix). Some of the flakes present simple retouching on one (sidescrapers) or two (retouched points) of their edges (Fig. 2, Table S1). The limestone pebbles (Fig. 2) are three naturally rounded pebbles, most likely deriving from the Oligocene conglomerates that are located around El Salt. None of the pebbles were technologically modified, but S4 shows macroscopic use-wear traces on one of its poles, including abrasion, scattered pitting and linear scars attributable to activities involving percussion, pressure, and short, irregular dragging movements (Fig. S2).

A non-archaeological flint nodule from each flint type present in our assemblage ($n = 3$; Mariola, Serreta and Beniaia) was also collected in the Serpis valley fluvial deposits for analysis (Table S2). While sediments can serve as controls for potential environmental lipids associated with lithics, non-archaeological flint nodules provide a baseline biomarker signal of the rock, which may include organic matter trapped during its formation and/or the burial history of the host rock.

2.2. Lipid extraction

2.2.1. Flint flakes

Flint flakes were first rinsed with ultrapure water (Milli-Q®) to remove any sediment adhering to their surfaces. Lipids from some flakes were extracted by submerging the objects in a mixture of

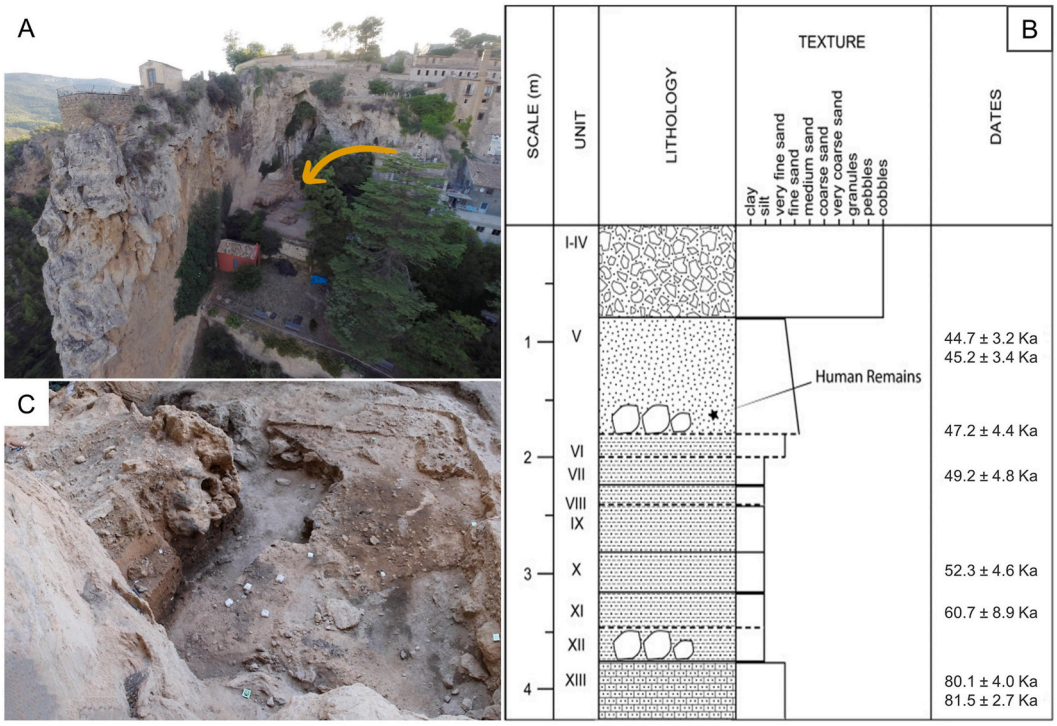

Fig. 1. The archaeological site of El Salt. (A) Site overview. (B) Stratigraphic units and absolute dates (Galván et al., 2014). (C) Excavation surface of SU Xb.

Table 1
Sample information. See SI Appendix for detailed descriptions of the sedimentary facies. CSEM: *conventional solvent extraction method*; TLE sap: *TLE saponification method*. NA: not applicable. †These samples were subjected to two separate extractions (i.e., from "*a*" and "*b*" areas).

Lithic sample	Element	SU	Associated sedimentary sample	Length (cm)	Width (cm)	Thickness (cm)	Weight (g)	Flint type	Extraction method
S4	Limestone pebble	Xb	Sed-La12	6.0	5.0	3.5	148.5	NA	*CSEM*
S4DD*a*	Limestone drill dust	Xb	Sed-La12	NA	NA	NA	1.0	NA	*CSEM*
S4DD*b*	Limestone drill dust	Xb	Sed-La12	NA	NA	NA	1.0	NA	*CSEM*
S20	Limestone pebble	Xb	Sed-La12	5.0	5.0	3.0	71.6	NA	*CSEM*
S20DD	Limestone drill dust	Xb	Sed-La12	NA	NA	NA	1.5	NA	*CSEM*
S35	Limestone pebble	Xb	Sed-La12	5.7	4.2	3.5	134.1	NA	*CSEM*
S35DD	Limestone drill dust	Xb	Sed-La12	NA	NA	NA	1.5	NA	*CSEM*
S3	Sidescraper	Xb	Sed-La12	6.0	4.0	0.7	12.5	Serreta	*CSEM*
S24	Unretouched flint flake	Xb	Sed-La12	3.5	2.5	0.5	4.3	Mariola	*CSEM*
S25	Unretouched flint flake	Xb	Sed-La12	3.9	3.4	0.3	1.9	Mariola	*CSEM*
S1	Unretouched flint flake	Xb	Sed-La13	5.9	4.3	1.2	22.5	Beniaia	*CSEM*
S2	Unretouched flint flake	Xb	Sed-La13	2.3	3.6	0.3	3.0	Beniaia	*CSEM*
S8	Unretouched flint flake	Xb	Sed-La13	3.2	2	0.4	2.1	Mariola	*CSEM*
S13	Unretouched flint flake	Xb	Sed-La13	3.5	2.5	0.8	3.5	Serreta	*CSEM*
S23	Unretouched flint flake	Xb	Sed-Lg14	3.6	3.5	0.7	4.8	Mariola	*CSEM*
S22†	Retouched point	Xb	Sed-Lg14	5.6	2.9	0.8	12.2	Beniaia	*CSEM*
S6†	Unretouched flint core-flake	Xb	Sed-Lg14	3.4	3.0	1.1	8.9	Mariola	*CSEM*
S5	Unretouched flint flake	Xb	Sed-La14	2.8	3.7	0.8	5.0	Beniaia	*CSEM*
S14	Unretouched flint flake	Xb	Sed-La14	3.0	2.2	0.7	2.8	Beniaia	*CSEM*
S15†	Unretouched flint flake	Xb	Sed-La14	4.9	3.4	1.4	13.5	Serreta	*CSEM*
S26†	Sidescraper	Xb	Sed-La14*	6.3	3.6	1.2	29.1	Beniaia	*TLE sap*
S28†	Sidescraper	Xb	Sed-La14*	5.0	3.6	1.3	19.3	Mariola	*TLE sap*
S19	Unretouched flint flake	Xb	Sed-Lg15	5.0	3.9	1.2	14.5	Beniaia	*CSEM*
S7	Unretouched flint flake	XI	Sed-Lm1	2.7	2.8	0.7	3.4	Serreta	*CSEM*
S9	Unretouched flint flake	XI	Sed-Lm1	2.9	2.0	0.7	3.4	Serreta	*CSEM*
S10	Sidescraper	XI	Sed-Lm1	3.6	2.4	0.7	5.5	Beniaia	*CSEM*
S11	Unretouched flint flake	XI	Sed-Lm1	4.7	2.5	0.9	10.7	Beniaia	*CSEM*
S16†	Sidescraper	XI	Sed-Lm1	4.1	2.7	1.9	21.3	Serreta	*CSEM*
S17†	Unretouched flint flake	XI	Sed-Lm1	4.8	4.5	1.3	26.1	Mariola	*CSEM*
S18†	Sidescraper	XI	Sed-Lm1	4.9	2.6	0.7	10.8	Serreta	*CSEM*
S21†	Sidescraper	XI	Sed-Lm1*	5.9	3.2	1.0	19.0	Beniaia	*TLE sap*
S27†	Retouched point	XI	Sed-Lm1*	5.0	3.3	0.9	14.3	Beniaia	*TLE sap*

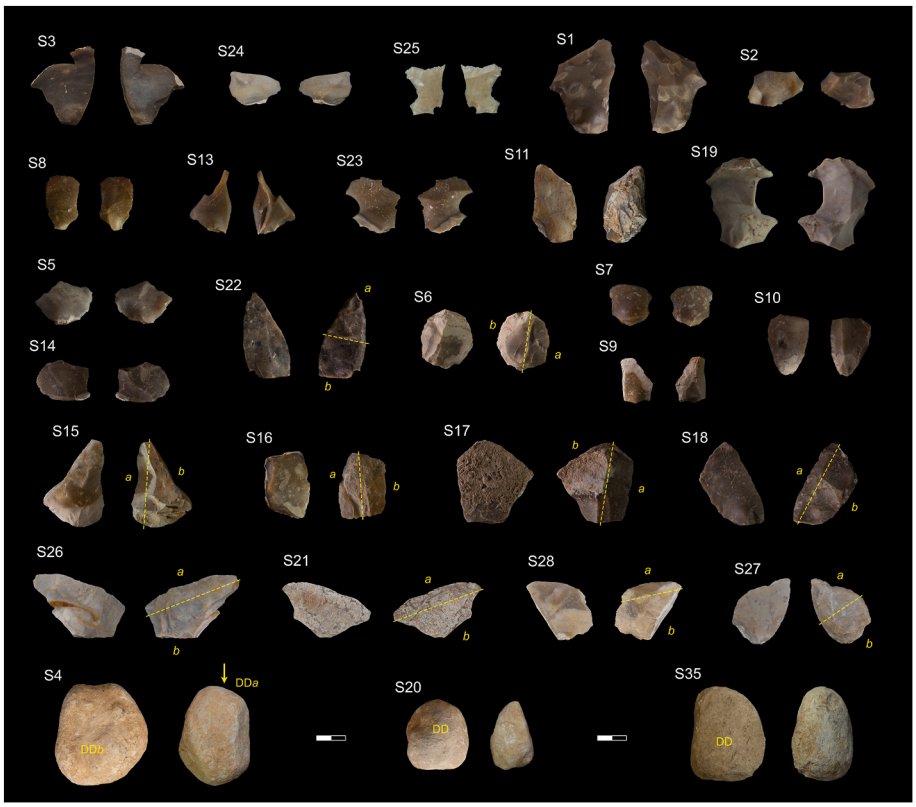

Fig. 2. Flint flakes and limestone pebbles from El Salt analysed in this paper. The yellow dashed lines indicate the two areas extracted (yellow "*a*" and "*b*") in selected flakes. "DD" indicates where drill dust samples were obtained from the pebbles. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

dichloromethane and methanol (DCM:MeOH, 3:1, v/v, 40–60 mL) contained in a clean beaker and applying sonication (see SI Appendix) to first assess the preservation of lipids on the lithic remains from El Salt. For flakes showing a potential working edge and a potential prehensile area, two separate lipid extractions were performed (Fig. 2, Table 1) with the aim of identifying possible use-related residues through the study of their spatial distribution (Langejans, 2011). First, the potential working edge (or the distal end in the case of retouched points) was selectively extracted by immersing only that part of the artifact in the solvent mixture with the help of clean metal tweezers while the rest of the object was covered with combusted (550 °C, 3h) Al foil, and sonication was applied (see SI Appendix). Subsequently, the entire artifact was submerged in the minimum necessary volume of the solvent mixture and sonication was also applied. The resulting sample extracts —corresponding to the potential working edge and the rest of the piece— were labelled with the suffixes "*a*" and "*b*" (e.g., S21*a* and S21*b*) (Fig. 2, Table 1).

2.2.2. *Limestone pebbles*

Limestone pebbles were first rinsed with ultrapure water (Milli-Q®) to remove any sediment adhering to their surfaces. For each pebble, two independent lipid extraction procedures were applied: (1) a drilling method and (2) a submersion method.

(1) For the drilling method, 1 g of limestone was obtained by pulverizing the surface of the pebble (<1 cm depth) using a solvent-cleaned diamond drill bit. These limestone drill dust samples were labelled with the suffix "DD" (e.g., S35DD). In the case of pebble S4, an additional drill dust sample (S4DD*a*) was collected from one of the poles that showed use-wear macro-traces (Fig. 2, Fig. S2, Table 1), with the aim of identifying a potential functional lipid signal. The drilled sample with use-wear traces was labelled by appending the suffix "*a*", in contrast to the sample taken from an area without use traces ("*b*") (i.e., S4DD*a* and S4DD*b*). Lipids from the resulting limestone drill dust were extracted using a 40 mL mixture of DCM:MeOH (3:1, v/v) via ultrasound-assisted extraction (SI Appendix).

(2) For the submersion method, the remaining portions of the limestone pebbles after removing the drill dust samples, were fully immersed in a mixture of DCM and MeOH (3:1, v/v, 40–60 mL) contained in a clean beaker and applying sonication (SI Appendix).

2.2.3. *Sediments*

Lipids from sediments (2–5 g) were extracted with a mixture of DCM and MeOH (3:1, v/v) via ultrasound-assisted extraction (see SI Appendix).

2.2.4. *Non-archaeological flint nodules*

Non-archaeological flint nodules were first rinsed with MeOH and DCM to minimise contamination from handling. Then, the samples were shattered/pulverized by hammering and <2 cm chips in size/powder were collected for analysis. Lipids from flint samples (Table S2) were extracted using a 40 mL mixture of DCM:MeOH (3:1, v/v) via ultrasound-assisted extraction (see SI Appendix).

2.3. *Lipid fractionation and analysis*

The total lipid extract (TLE) from most of the samples (Table 1) was fractionated into four different polarity fractions (F1: hydrocarbons; F2: ketones; F3: *n*-alkanols and sterols; and F4: fatty acids) through a SiO_2 column using several mobile phases (*conventional solvent extraction*

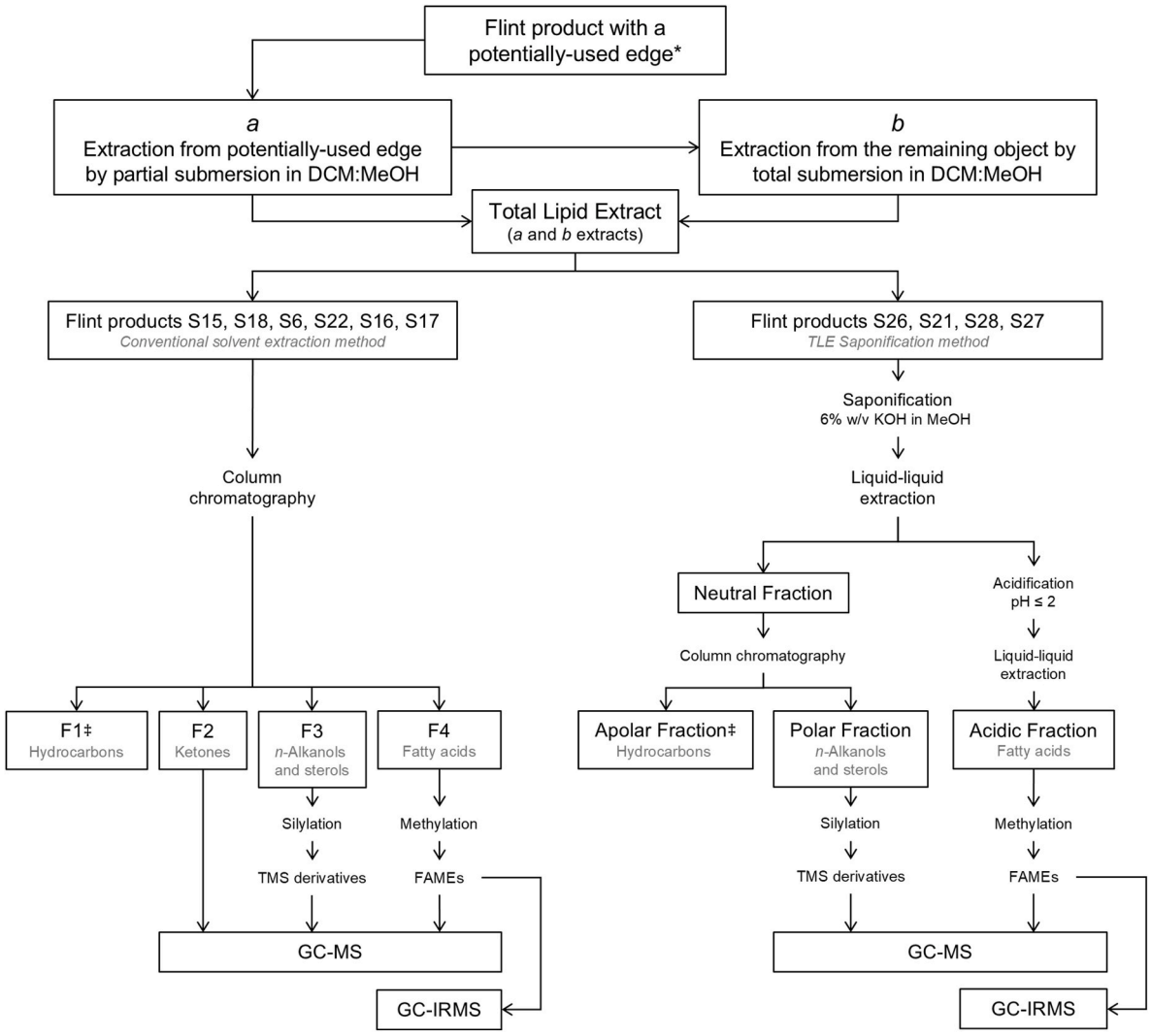

Fig. 3. Flowchart of the analytical workflow for the two methods (*conventional solvent extraction method* and *TLE saponification method*) used for lipid biomarker analysis of flint products from El Salt. The method used for each of the samples subjected to two separate extractions from different areas is indicated. *Potential working edges were determined based on technological and morpho-potential features (although use-wear traces can also be used for this purpose, as recommended by Luong et al., 2019; provided modern lipid contamination is excluded). ‡These lipid fractions were not included in this work.

method, Fig. 3, see SI Appendix).

With the aim of evaluating the efficiency of different analytical approaches, the total lipid extracts from some flint flakes and adjacent sediments (Table 1) were saponified with 1.5 mL 6 % w/v KOH in MeOH and allowed overnight at room temperature and subsequently separated into neutral (hydrocarbons, *n*-alkanols and sterols) and acidic (fatty acids) fractions through liquid-liquid extraction (*TLE saponification method*, Fig. 3, see SI Appendix). Further separation of the neutral fraction into apolar (hydrocarbons) and polar (*n*-alkanols and sterols) fractions was carried out using an Al_2O_3 column (Fig. 3, see SI Appendix).

Here, we present the results for F2, F3 and F4 from the *conventional solvent extraction method*, and the neutral-polar and acidic fractions from the *TLE saponification method* (Fig. 3). These extracts were analysed by gas chromatography-mass spectrometry (GC-MS) (Fig. 3, see SI Appendix). Subsequently, the compound-specific $\delta^{13}C$ values of $C_{16:0}$ and $C_{18:0}$ fatty acids from the selected samples subjected to two extractions (i.e., from the "*a*" and "*b*" area extracts; Table 1) and their adjacent sediments were measured by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) (Fig. 3, see SI Appendix).

2.4. Lipid identification and quantification

Lipid compounds were identified by comparison of their retention times and mass spectra with those of reference compounds (37 component FAME mix C_4–C_{24}, 200–600 mg/L in DCM; and fatty acids C_{26}, C_{28}, and C_{30}) and using the NIST mass spectra library (v. 2.2). Concentrations were estimated by comparing the peak areas of the analytes to those of known quantities of the internal standards.

Lipid concentration in the sediments, limestone drill dust samples obtained from the pebbles, and chips/powder obtained from non-archaeological flint nodules were normalized based on the sample weight and expressed in ng/g sample. Conversely, given that potentially extracted lipids from the flakes and the pebbles (via submersion) would likely be preserved on the lithic surface it is not adequate to quantify them by sample weight; instead, they were quantified in total ng per object or per extracted area (i.e., "*a*" and "*b*"). Although comparing lipid concentrations between sediments and lithic artifacts is challenging in this manner, this approach is intended to provide a general idea of the lipid concentration present in the artifacts and allow us to compare it to a specific amount of adjacent sediment.

3. Results

3.1. Flint flakes

All flint flakes yielded even-numbered saturated fatty acids, with $C_{16:0}$ and $C_{18:0}$ being the most abundant (Fig. 5, Table S4). Some flakes also exhibited other organic compounds such as unsaturated fatty acids, *n*-alkanols, sterols, ketones and terpenoids (Fig. S3, Table S3, Table S4).

Quantitative and qualitative differences in lipid composition were observed between different areas of some flakes (i.e., "*a*" and "*b*" extracts) (Table 2, Fig. 5). The potential working edge ("*a*" extracts) often contained substantially higher amounts of fatty acids compared to the rest of the tool ("*b*" extracts) (Table 2, Fig. 5). This pattern was particularly pronounced in most sidescrapers (S26, S21, S28, S18), where fatty acid concentrations at the working edge were 2.5–6.5-fold higher than in other areas of the tool. Comparable differences were observed in one retouched point (S27, 3.6-fold higher at the active distal part) and one unretouched flake (S15, 8.3-fold higher at the working edge). Moreover, the edges of sidescrapers S21 and S26 showed 29.0- and 18.8-fold more unsaturated fatty acids ($C_{16:1}$ and $C_{18:1}$), respectively, than the rest of the piece, while the retouched point S27 exhibited 4.9-fold higher levels at the distal active part. Other lithic elements showed only minor differences in fatty acid content between the working edge and the rest of the object, or even lower amounts at the edge (samples S6, S22, S16, and S17).

Regarding qualitative differences, sidescraper S21 displayed long-chain, even-numbered saturated fatty acids and *n*-alkanols, as well as cholesterol, exclusively at the potential working edge (Table 2). In contrast, flake S15 contained 7-oxodehydroabietic acid solely in the potential prehensile area ("*b*" extract) (Table 2). Samples S6 and S17 also showed slight qualitative differences between the "*a*" and "*b*" extracts (Table 2).

The carbon isotopic composition of $C_{16:0}$ and $C_{18:0}$ fatty acids was measured in samples where lipids were extracted from two distinct areas. The isotopic values of fatty acids from lithic extracts "*a*" and "*b*" differed to varying degrees, with no consistent pattern observed across the samples. $\delta^{13}C_{16:0}$ values ranged from −32.3 ‰ to −23.9 ‰, and $\delta^{13}C_{18:0}$ values from −29.9 ‰ to −25.8 ‰ (Fig. 7, Table S5).

3.2. Limestone pebbles and drill dust samples

All limestone samples, including both submerged pebbles and their respective drill dust samples, yielded even-numbered saturated fatty acids, primarily $C_{16:0}$ and $C_{18:0}$ (Fig. 5, Table S6). Other organic compounds such as *n*-alkanols, and cholesterol were also detected in some samples (Table S6).

In pebble S4, where two drilled samples were collected, the region showing use-wear traces (S4DD*a* sample) contained over fourfold higher fatty acid concentrations and more than twice the cholesterol levels compared to the area without traces (S4DD*b*) (Table 2, Fig. 5). Additionally, C_{16} *n*-alkanol was detected exclusively in the "*a*" extract. Isotopic analysis revealed a $\delta^{13}C_{16:0}$ value of −25.7 ‰ and a $\delta^{13}C_{18:0}$ value of −29.5 ‰ for S4DD*a*, whereas S4DD*b* exhibited a $\delta^{13}C_{16:0}$ value of −28.1 ‰ and a $\delta^{13}C_{18:0}$ value of −29.5 ‰ (Fig. 7, Table S5).

3.3. Sediments

Sediments were characterised by the prevalence of even-numbered saturated and unsaturated fatty acids, and even-numbered *n*-alkanols (Fig. S4, Fig. S5, Table S7, Table S8). Other organic compounds including ketones (C_{29}, C_{31}), terpenoids (dehydroabietic acid, friedelin), sterols (campesterol, stigmasterol, β-sitosterol, cholesterol) and stanols (stigmastanol, cholestanol, coprostanol, epicoprostanol) were also identified in some of the samples (Table S7, Table S8). The carbon isotopic values of sedimentary fatty acids were consistently higher than those preserved on the lithic samples, with $\delta^{13}C_{16:0}$ values ranging from −26.5 ‰ to −25.8 ‰, and $\delta^{13}C_{18:0}$ values from −27.1 ‰ to −24.8 ‰ (Fig. 7, Table S5).

3.4. Non-archaeological flint nodules

Few organic compounds, including even-numbered *n*-alkanols and the fatty acids $C_{16:0}$ and $C_{18:0}$ were detected in the non-archaeological nodules at low concentrations (Table S9).

4. Discussion

4.1. Middle Palaeolithic stone tools as lipid archives

Our study provides novel evidence showing that Middle Palaeolithic lithic tools preserve a diverse array of lipid biomarkers including fatty acids, *n*-alkanols, sterols and terpenoids. The absence of macroscopic residues on our samples implies that lipids are only preserved at molecular scale. This highlights the potential of molecular-based approaches in lithic residue investigations and suggests the possibility of targeting any archaeological stone tool for lipid analysis, even when residues are not visible.

Unlike pottery, lithics present a challenge in organic residue analysis due to the inability to remove an external surface layer that may have been exposed to contaminants beforehand. This makes contamination of

Table 2
Fold differences in fatty acids between "*a*" and "*b*" extracts on lithic samples. Fold differences are calculated as the ratio of fatty acid amounts in the "*a*" extract to those in the "*b*" extract. NA: not applicable - unsaturated fatty acids were not detected. Other qualitative differences in lipid data between *a* and *b* extracts are also noted.

Sample	Element	Fold difference of total fatty acids (saturated + unsaturated) between *a* and *b* extracts	Fold difference of saturated fatty acids between *a* and *b* extracts	Fold difference of unsaturated fatty acids between *a* and *b* extracts	Other qualitative differences between *a* and *b* extracts
S26	Sidescraper	2.5-fold	2.3-fold	18.8-fold	
S21	Sidescraper	6.4-fold	6.0-fold	29.0-fold	Long-chain, even-numbered saturated fatty acids and *n*-alkanols, and cholesterol, in S21*a*
S28	Sidescraper	3.9-fold	4.0-fold	0.8-fold	
S27	Retouched point	3.6-fold	3.5-fold	4.9-fold	
S15	Unretouched flake	8.3-fold	8.3-fold	NA	7-Oxodehydroabietic acid in S15*b*
S18	Sidescraper	4.8-fold	4.8-fold	NA	
S6	Unretouched flake-core	1.5-fold	1.5-fold	NA	C_{18} *n*-alkanol in S6*a*
S22	Retouched point	0.3-fold	0.3-fold	NA	
S16	Sidescraper	0.6-fold	0.6-fold	NA	
S17	Unretouched flake	1.5-fold	1.5-fold	NA	C_{18} *n*-alkanol in S17*a*
S4	Pebble	4.1-fold	4.1-fold	NA	C_{16} *n*-alkanol in S4DD*a*

the samples with modern lipids a significant concern. Here, the archaeological origin of the lipids is inferred for the following reasons: (1) rigorous precautions were taken throughout the entire analytical process, including the use of nitrile gloves and solvent-cleaned metal tools for excavation, sampling, and handling, as well as the packaging of the samples in combusted Al foil; (2) lipids commonly found in human skin, such as squalene and cholesterol (Girod et al., 2012; Whelton et al., 2021), were either not detected in our samples or were present at trace amounts; (3) the lithic samples yielded notably low amounts of lipids, consistent with an ancient origin and in line with what is typically found in aged archaeological materials (Evershed, 2008b); (4) artifacts from which two extractions were performed exhibited variable lipid compound types, quantities, and isotopic compositions depending on the extracted area.

These findings validate the reliability of our methodology, thereby establishing the potential for its application in diverse archaeological contexts, and across various types of lithic remains. To our knowledge, our samples are the oldest archaeological artifacts without any macroscopic residue from which lipid biomarkers have been successfully extracted, identified, and isotopically characterised.

4.2. Lipid preservation

Studying the degree of lipid preservation in lithic remains presents a challenge. As biomarkers extracted from the stone tools were likely preserved on the lithic surface (see discussion below), lipid concentrations were expressed in ng/object or ng/area (see 2.4. section). This complicates direct comparisons between lipids extracted from lithic artifacts and those from sediments, which are quantified as ng of lipids per gram of sediment. However, our quantitative data provides a glimpse of the lipid content present on the artifacts and enables comparison with a specific amount of sediment (e.g., 1 g), as shown in Fig. 4, offering a general idea of the degree of lipid preservation on the lithic remains compared to their adjacent sediments.

Pebbles and their respective drilled dust samples yielded higher lipid concentrations compared to both 1 g of adjacent sediment and flint flakes processed using the same procedure (Fig. 4). This suggests that the high porosity of limestone could enhance the absorption, trapping, and preservation of lipid residues, thereby shielding them from decay and microbial activity. This makes limestone more efficient as a reservoir of archaeological biomarkers than flint. This interpretation is consistent with observations from micro-residue experimental studies, which indicate that the porous and irregular surfaces of rocks can promote better residue retention than glass-like ones (Langejans, 2010), where lipid residues are more exposed and accessible to microbial activity (Monnier and May 2019).

Flakes generally showed higher lipid content than 1 g of adjacent sediment when processed using the free lipid extraction method, whereas the *TLE saponification method* revealed higher lipid amounts in sediments (1 g) than in flakes (Fig. 4). The latter procedure also proved significantly more effective than the conventional solvent extraction method, enabling the detection of a greater diversity and higher quantities of lipids from both lithics and sediments. This difference does not reflect a greater extraction *per se*, but rather that saponification cleaves the ester bonds in esterified lipids (e.g., acylglycerols) present in the TLE, releasing free fatty acid and other moieties that are more amenable to conventional GC-MS analysis. The pronounced differences in lipid yields between saponified and non-saponified samples likely indicate that lipids at El Salt predominantly occur in esterified forms. This may be attributed to the good preservation state of organic matter in the El Salt sedimentary deposit (Leierer et al., 2019; Mallol et al., 2013; Rampelli et al., 2021), which would diminish lipid hydrolysis, thereby limiting the release of free fatty acids and other compounds (Craig et al., 2004; Dudd et al., 1998; Regert et al., 1998). Polar lipids may also have undergone post-depositional reactions, potentially forming stable insoluble complexes via polymerization and interaction with components of

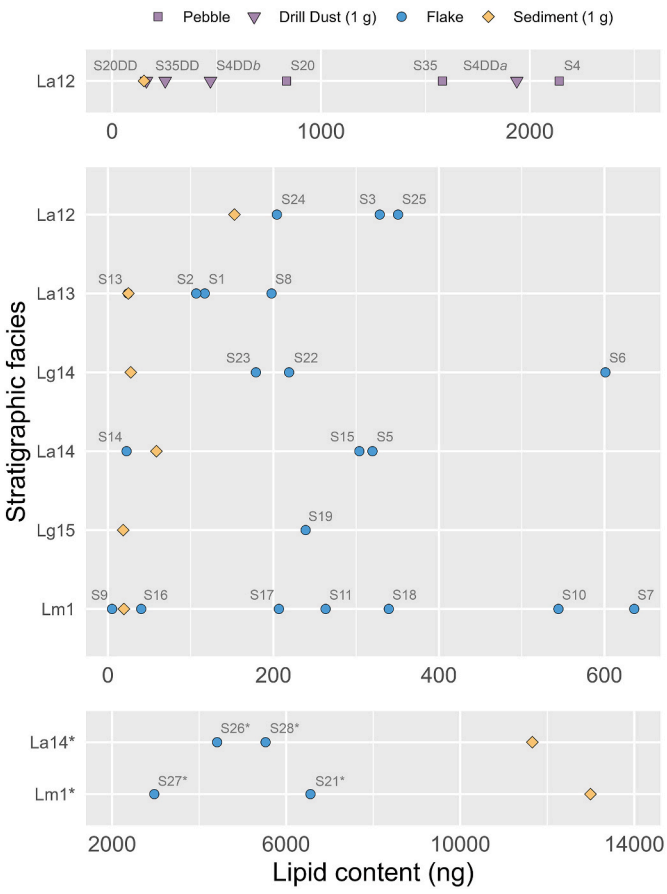

Fig. 4. Lipid content recovered from the entire pebbles, drill dust samples (1 g) obtained from the pebbles, and flakes in comparison to 1 g of sediment of the sedimentary facies in which they were found. For samples subjected to two separate extractions, i.e., from "*a*" and "*b*" areas, lipid content from both extracts were added. *These samples were processed using the *TLE saponification method.*

the organic and/or mineral matrix of sediments and flint, which would require alternative analytical approaches such as direct strong base or acid extraction for analysis (Correa-Ascencio and Evershed, 2014; Craig et al., 2004; Regert et al., 1998; Stern et al., 2000).

While our method does not enable us to pinpoint the specific preservation mechanisms of lipids on flint flakes, the identified biomarkers might be preserved either as molecular residues or as part of micro-residues adhering/adsorbing to the surfaces, trapped within microcracks, depressions, crevices, cortex, and mineralized matrix of polishes, or as a thin film smeared on the surfaces (Anderson, 1980; Bordes et al., 2017, 2018; Croft, 2021; Evans and Donahue, 2005; Langejans and Lombard, 2015; Luong et al., 2019; Shanks et al., 2001; Smith et al., 1970; Wadley et al., 2004). Fatty acids are well represented in our samples, in line with numerous studies that have reported fat residues on Palaeolithic stone tools (Bordes et al., 2018; Lombard, 2008; Luong et al., 2019; Solodenko et al., 2015; Venditti et al., 2019). Despite their high susceptibility to degradation, unsaturated fatty acids have also been identified in some of our flakes, particularly along retouched edges. Similar findings were reported by Bordes et al. (2018), who identified unsaturated fatty acid residues on Middle Palaeolithic stone tools from Denisova Cave using Raman spectroscopy. Their preservation on our samples may reflect the role of surface irregularities and microcracks formed during retouch in trapping lipids and reducing their exposure to oxidation and bacterial activity (Croft, 2021; Evershed, 1993; Mazzia and Flegenheimer, 2015; Shanks et al., 2001), although tool use likely also contributed to higher lipid amounts in these areas, as discussed in

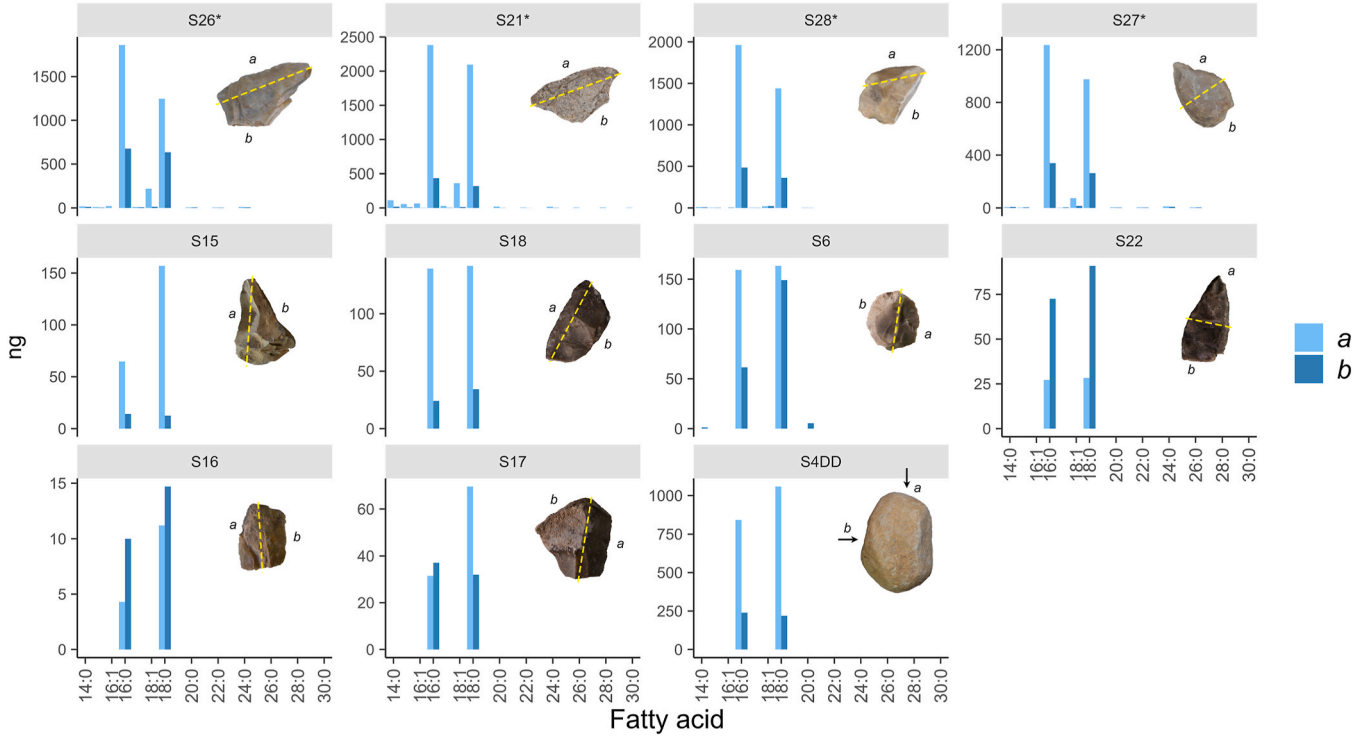

Fig. 5. Bar charts showing the abundances of saturated and unsaturated fatty acids extracted from flake areas "*a*" and "*b*", and from the samples drilled from pebble S4, i.e., S4DD*a* and S4DD*b*. *These samples were processed using the *TLE saponification method*.

section 4.3. Further research combining both microscopic and biomarker methodologies in residue analysis could yield valuable insights into the mechanisms governing lipid preservation in archaeological lithic remains. Employing techniques such as mass spectrometry imaging (Hammann et al., 2020) or Raman microscopy (Bordes et al., 2017, 2018) for direct visualization of lipids could also provide additional clarity on this matter. On the other hand, the extent to which lipid residues on stone tools could have been affected by post-depositional processes, such as microbial activity, phosphatisation, mineral precipitation or decalcification, remains unclear and requires dedicated experimental investigation, similar to approaches applied in micro-residue research (Cnuts and Rots, 2024; Croft et al., 2016; Langejans, 2010; Monnier and May 2019).

4.3. Enhanced functional data retrieval

Distinguishing between use-related and environmental residues in lithics poses a significant challenge due to the potential introduction of environmental residues from sediment onto lithics. This issue can be addressed by analyzing sediment control samples for shared residues and comparing residues on the active areas of the tools to those on the rest of the artifact (Barton et al., 1998; Langejans, 2011; Langejans and Lombard, 2015; Lombard and Wadley, 2007; Luong et al., 2019; Wadley and Lombard, 2007).

The lipids preserved in most of our lithic samples mirror those identified in the sedimentary matrix and, in some cases, also in non-archaeological flint nodules to a lesser extent. In the latter case, the amounts and types of lipids detected are very low, and therefore any potential contribution of lipids naturally present in the flint to the extracts from archaeological flakes is expected to be minimal (Table S9, see SI Appendix for extended discussion). In contrast, the sediments at El Salt are much richer in lipids, and their similarity to those found on the lithics complicates the differentiation of use-related lipids from environmental ones, particularly in cases where the entire artifact was extracted as a single unit. Nevertheless, the availability of spatially distributed lipid data in selected samples (n = 11, Fig. 5, Table 1, Table 2) enables us to address the challenges of identifying use-related lipids on the lithics and their distinction from environmental ones, as discussed below.

Analysis of one of the pebbles revealed that the area exhibiting abrasion, scattered pitting and linear scars (S4DD*a* sample, Fig. S2) contained over fourfold fatty acids and more than twofold the cholesterol levels compared to the area without use-wear traces (Sample S4DD*b*, Fig. 5, Table 2). This strongly suggests the functional significance of the lipids from the S4DD*a* sample and their association with the activity that produced the use-wear traces.

For the flakes, the potential working edge ("*a*" extracts) often yielded substantially higher lipid content compared to the rest of the tool ("*b*" extracts) (Fig. 5, Table 2). This pattern is especially marked in most sidescrapers (S26, S21, S28, S18), where fat residues on the working edge were between 2.5- and 6.5-fold higher than on the rest of the tool (Fig. 5, Table 2). Comparable differences were found on one retouched point (S27, with 3.6-fold more fatty acids at the active distal part) and one unretouched flake (S15, with 8.3-fold more fatty acids at the working edge) (Fig. 5, Table 2). These results suggest that these flakes, in which fatty acid levels on the active areas were at least twice those of the rest of the piece, were likely used in the processing of organic materials (Table 3), while the identification of diagnostic biomarkers in some cases provides further insights into worked materials, as discussed below.

Use-related plant residues were identified on the working edge of sidescraper S21 based on the exclusive presence on this area (S21*a* extract) of diagnostic higher plant-derived long-chain, even-numbered saturated fatty acids and *n*-alkanols (Fig. 6, Table 2, Table 3) (Eglinton and Hamilton, 1967; Ficken et al., 2002). The significantly higher concentration of $C_{16:1}$ and $C_{18:1}$ on this area (29-fold greater in S21*a* compared to S21*b*; Figs. 6 and 5, Table 2), both unsaturated fatty acids that are characteristic constituents of plant oils (Spangenberg and Ogrinc, 2001; Steele et al., 2010), also support this interpretation, aligning with previous use-wear evidence of woodworking at El Salt

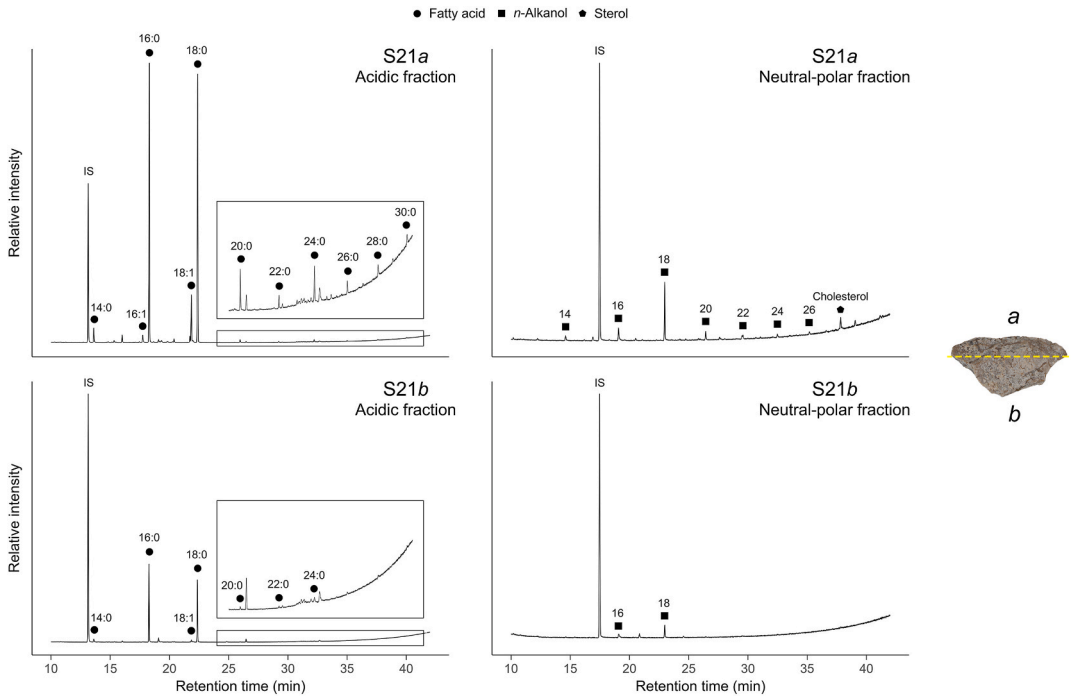

Fig. 6. Partial total ion chromatograms of acid and neutral-polar lipid fractions of "*a*" (above) and "*b*" (below) extracts from sidescraper S21 from El Salt. IS: internal standard.

Table 3
Functional information retrieved from selected lithic samples (i.e., subjected to two separate extractions from different areas) using lipid biomarker and compound-specific isotope analysis. NA: not applicable.

Sample	Lithic element	Use based on lipid data	Functional information retrieved based on lipid compound and/or amount data	Functional information retrieved based on isotopic data
S26	Sidescraper	Yes	No diagnostic data	Plant or ruminant/ non ruminant animal material processing
S21	Sidescraper	Yes	Higher plant material processing	NA
S28	Sidescraper	Yes	No diagnostic data	Plant or ruminant/ non ruminant animal material processing
S27	Retouched point	Yes	No diagnostic data	Plant or ruminant/ non ruminant animal material processing and possible hafting
S15	Unretouched flake	Yes	Possible conifer resin-based hafting	Plant or ruminant/ non ruminant animal material processing
S18	Sidescraper	Yes	No diagnostic data	Plant or ruminant/ non ruminant animal material processing
S6	Unretouched flake-core	No	NA	NA
S22	Retouched point	No	NA	NA
S16	Sidescraper	No	NA	NA
S17	Unretouched flake	No	NA	NA
S4DD*a*	Pebble	Yes	No diagnostic data	Ruminant animal material processing

(Bencomo et al., 2023). The detection of cholesterol—associated with both plant and animal tissues (Behrman and Gopalan, 2005; Hartmann, 1998)—exclusively on S21*a* further underscores the functional significance of these lipids, reinforcing the interpretation of this flake's use in plant processing.

A diagnostic conifer resin degradation marker, 7-oxo-dehydroabietic acid (Colombini and Modugno, 2009), was exclusively detected on the potential prehensile area of S15 (i.e., S15*b*, Table 2), suggesting the possible use of a conifer resin-based hafting, in line with evidence of Pinaceae resin use in Middle Palaeolithic contexts (Degano et al., 2019) and the abundance of pine in the region during Pleistocene (Vidal-Matutano, 2017; Vidal-Matutano et al., 2017). This interpretation is further supported by the technological features of the flake: on the ventral face, the bulb was removed through multiple flat removals, while on the dorsal face, the basal area was thinned (Fig. 2), both modifications being consistent with intentional hafting preparation. Nonetheless, alternative explanations for the presence of 7-oxodehydroabietic acid on S15*b* must be considered. One possibility is post-depositional deposition of residues from the surrounding sediment. However, no conifer-derived compounds were detected in the sediment from which the flake was recovered (Sed-La14), and environmental conifer remains such as pine needles are not a predominant component in the sedimentary matrix of El Salt SU Xb (Leierer et al., 2019). Another possibility is incidental deposition from hearths (which are predominantly pine-fueled at El Salt (Vidal-Matutano, 2017; Vidal-Matutano et al., 2018)), either through contact with embers or the settling of charcoal particles. Yet, no charcoal residues were observed on the flake. A third scenario involves residues resulting from woodworking activities; however, this is unlikely given that the S15*b* area lacks a functional edge.

So far, we have shown that most side-scrapers (S26, S21, S28, S18), one retouched point (S27), one unretouched flake (S15), and one pebble (S4) were likely used to process organic materials, as evidenced by the comparison of lipid content on their working areas with that of the rest of the tool (Table 3). Among these, S21 was likely used to process plant materials, as discussed above (Table 3). For the remaining tools interpreted as used, subsequent comparison of carbon isotopic compositions

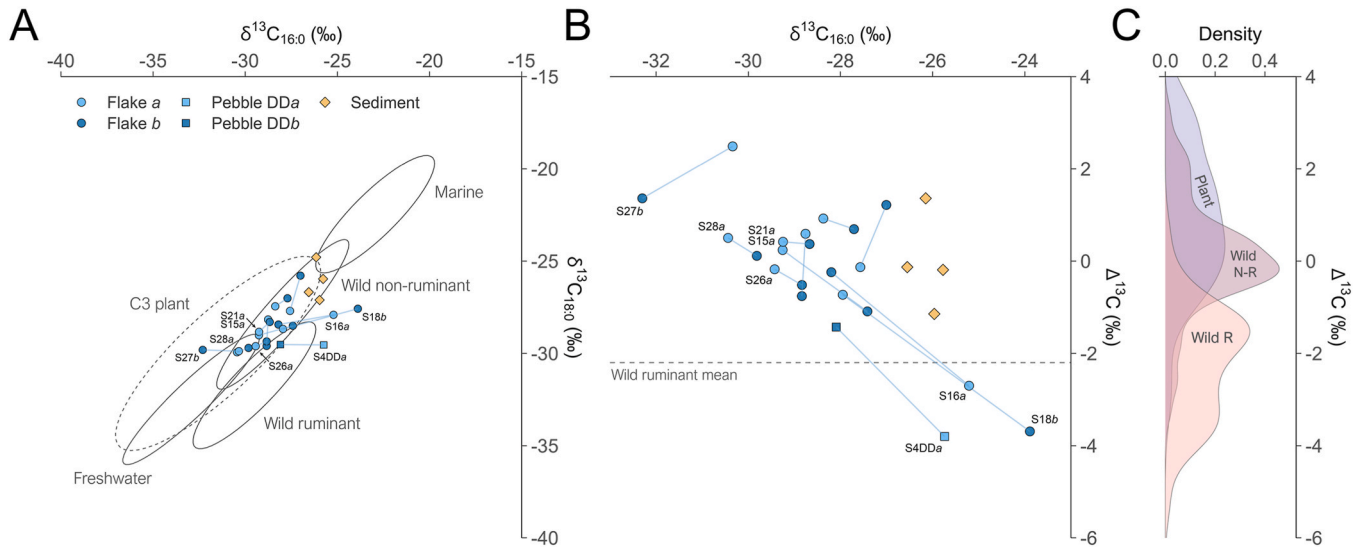

Fig. 7. **A**: Scatter plot of $\delta^{13}C_{16:0}$ values against $\delta^{13}C_{18:0}$ values for lipids extracted from two areas ("*a*" and "*b*") of individual flakes, drill dust samples from pebble S4 (S4DD*a* and S4DD*b*), and their adjacent sediments (i.e., Sed-La12, Sed-Lg14, Sed-La14, Sed-Lm1) from El Salt. Each flake's areas "*a*" and "*b*" isotopic data are connected by blue lines. Statistical ellipses (1σ) indicate the distribution of $\delta^{13}C$ values for reference fatty acids from 397 modern wild animals and plant samples (Dataset S1, SI Appendix), providing a comparative framework for evaluating potential sources. **B**: Scatter plot of $\delta^{13}C_{16:0}$ values against $\Delta^{13}C$ values ($\delta^{13}C_{18:0} - \delta^{13}C_{16:0}$) for lipids extracted from the areas "*a*" and "*b*" of the flakes, drill dust samples collected from pebble S4 (S4DD*a* and S4DD*b*), and sediments from El Salt. $\Delta^{13}C$ values highlight differences between $C_{16:0}$ and $C_{18:0}$ isotopic compositions, which help distinguish ruminant versus non-ruminant and plant lipid sources. $\Delta^{13}C$ reference values are shown as density plots (Wild N-R: wild non-ruminant; Wild R: wild ruminant) from Dataset S1, allowing visual comparison with archaeological samples (**C**). The mean $\Delta^{13}C$ value for wild ruminant fats (−2.2 ‰) is indicated. Samples discussed in the text are labelled. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

of $C_{16:0}$ and $C_{18:0}$ fatty acids of lithics with modern authentic reference fats and oils (Dataset S1, SI Appendix) led us to further delve into the materials worked with the tools, as discussed below.

The sediment samples generally exhibit higher $\delta^{13}C_{16:0}$ and $\delta^{13}C_{18:0}$ values compared to lithics (Fig. 7A), likely indicating a different source of lipids. All flint products determined as used by lipid data (see above, Table 3) plot within the wild non-ruminant and plant ranges (Fig. 7A), suggesting the preservation of either one of these fats or a mixture of both on the lithics. If we consider the zooarchaeological data from El Salt SU X (Pérez, 2023), potential non-ruminant animals butchered with these tools are horse (*Equus ferus*) and wild boar (*Sus scrofa*). The possibility that the fats on the lithics originated from Neanderthals handling tools cannot be entirely ruled out (Luong et al., 2017). Among the plant species that may account for the residues, there are many possibilities, as the anthracological, carpological and micromorphological records of SU X show a presence of pine (*Pinus nigra-sylvestris*), hackberry (*Celtis* sp.), and species within the genus *Acer*, *Quercus*, *Juniperus*, *Buxus*, *Salix-Populus*, *Ephedra*, *Pistacia* and the Fabaceae family, among others (Leierer et al., 2019; Mallol et al., 2013; Vidal-Matutano et al., 2018). Use-related lipids identified on the working edges of samples S26, S28, S18, S27 and S15 are therefore suggested to result from the processing of these plant and/or animal taxa (Table 3).

The isotopic values of the fatty acids recovered from the proximal part of the retouched point S27 ("*b*" extract) plot exclusively within the C3 plant range (Fig. 7A). This may suggest a potential association with plant-based hafting materials or residues from contact with a wooden haft, given the significant isotopic difference between the sample and its adjacent sediment, as well as the tool's pointed morphology and the post-retouching proximal thinning flake extractions (Fig. 2).

Three lithic samples showed presence of ruminant fats based on their low $\Delta^{13}C$ values ($\delta^{13}C_{18:0} - \delta^{13}C_{16:0}$), applying a stringent lower threshold of −2.2 ‰ ($\Delta^{13}C$ mean for wild ruminants) (Fig. 7B, C). One of these samples is the pebble area with use-wear macro-traces (S4DD*a*), where a functional lipid signal was identified (see above, Table 3). The detection of ruminant fats in these samples indicates that the tool was likely used for processing ruminant animals, most likely red deer (*Cervus elaphus*), Iberian ibex (*Capra pyrenaica*), chamois (*Rupicapra rupicapra*) and/or aurochs (*Bos primigenius*), given their dominance among ruminant animals in the faunal record of El Salt SU X (Pérez, 2023). The use of the pebble on these taxa could involve butchering tasks such as marrow extraction, as well as other processing activities like meat tenderizing or hide working, which may explain the formation of the use-wear traces; in the former case, it also aligns with diagnostic anthropogenic alterations (e.g., percussion notches) observed in the faunal record of the site (Pérez, 2023; Pérez et al., 2019).

The other two samples with $\Delta^{13}C$ values compatible with ruminant fats are S18*b* and S16*a* (Fig. 7B). S18*b* corresponds to a potential prehensile area lacking an edge (Fig. 2), making it unlikely to have been used as an active working surface. The low lipid content recovered from this area further supports this interpretation (Fig. 5, Table 3). S16*a* is a potential working edge (Fig. 2); however, the quantitative and qualitative lipid data do not allow us to distinguish whether the lipid residues in this sample are functional or environmental in origin (Fig. 5, Table 3). The presence of ruminant fats in these samples requires further investigation, but one plausible explanation is the leaching of lipids from neighboring faunal remains via syn- or post-depositional processes (Luong et al., 2017, 2019).

While our interpretations are strongly supported by the current data, further validation through use-wear analysis would provide additional confidence (Luong et al., 2019). More importantly, experimentation, as has been demonstrated in the study of micro-residues (Cnuts and Rots, 2024; Croft et al., 2016; Langejans, 2010; Monnier and May 2019), will be crucial to understanding the extent, mechanisms, and modes of lipid residue preservation and migration patterns on stone tools across different diagenetic environments and processes, as well as to better distinguish use-related lipids from environmental ones, ultimately aiding in the validation and establishment of this research line. Comparing Palaeolithic fats with modern authentic reference isotopic data also requires careful consideration, given the still-developing isotopic dataset for wild animals and plants and their potential overlap in biological source ranges (Fig. 7A). Expanding this isotopic reference collection, particularly using modern wild animals and plants from environments

similar to the study site or archaeological faunal and plant remains, would significantly enhance the precision of this approach. Future work could also benefit from analyzing a broader set of lithic tools and testing alternative lipid extraction methods (Correa-Ascencio and Evershed, 2014; Regert et al., 1998; Stern et al., 2000; Zhang et al., 2022) or advanced analytical techniques such as HTGC-MS and HPLC-MS to refine the methodology further and explore additional dimensions of the data.

Our study pioneers the application of compound-specific isotope analysis (CSIA) to organic residues on lithics, introducing a groundbreaking approach to investigating the functionality of archaeological stone tools. The exploration of this method stands as a pivotal step towards establishing a research line with potential far-reaching implications, introducing into functional studies a novel approach that adds a level of taxonomic resolution. Importantly, it could be pivotal when addressing tools that may not have retained micro-wear after use. The method's non-destructive nature allows for its integration with other analytical techniques on the same stone tool, making a multi-proxy approach the most effective way to analyze the lithic record from a functional perspective. To fully test the potential of this approach, we recommend the following sequential analytical methodology: 1) targeted sampling for organic residue analysis (refer to Material and Methods for details); 2) micro-residue analysis (provided modern lipid contamination is excluded); 3) lipid biomarker and CSIA of individual fatty acids from different lithic areas (ensuring the minimum required quantity of fatty acids for CSIA; the more areas analysed, the better); 4) use-wear analysis.

CRediT authorship contribution statement

Javier Davara: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization. **Cristo M. Hernández:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Daniel Carrizo:** Writing – review & editing, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation. **Antonio V. Herrera-Herrera:** Writing – review & editing, Validation, Methodology, Investigation, Data curation. **Eneko Iriarte:** Writing – review & editing, Investigation. **Carolina Mallol:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jas.2025.106427.

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