Heritage Science

RESEARCH

Open Access

Novel nannofossils extraction methods from paintings, coupled with GC–MS for provenance determination and binder analysis

Victory Armida Janine Jaques[1*], Jakub Trubač[2], Jiří Rathouský[3], Tomáš Cajthaml[4] and Katarína Holcová[1]

Abstract

The use of calcareous nannofossils for provenance analysis is a new-old topic for cultural heritage. Several studies have already mentioned it for ceramic, but less for paintings. Preparatory layers of the paintings are often made with chalk, which is composed of microfossils. To extract a calcareous nannofossils assemblage from a painting layer, we need to disaggregate it. The method is to plunge the micro-samples into water and heat it if water alone does not work. The disaggregation process takes a long time and is not efficient in terms of quantitative results. In this work, we aimed to develop a disaggregation method that increases the number of determinable nannofossils extracted from a painting micro-sample. As these samples are valuable and unique, we decided that a combination of analyses on the disaggregated micro-sample should be tried to extract the most information from it. We studied the possibility of binder determination by gas chromatography–mass spectrometer after the nannofossils assemblage extraction on the residual liquid from the disaggregation. The method we are presenting is easy to apply, has a high disaggregation rate for most paintings, and a low impact on binders fatty acids for their determination.

Keywords: Cultural heritage, Painting, Calcareous nannofossils, Extraction, Provenance, Binder, FAME, GC–MS

Introduction

Cultural heritage objects from their findings are considered mysterious, and many scientists are working to unveil their secrets. Most artefacts are rare and unique, which implies that non-invasive and non-destructive methods are preferred. For the conservation and restoration of artworks, the material must be carefully selected, and the provenance of the material source is particularly important [1–4]. Conservators and restorers are the main actors in the use of provenance information by their works related to the gain of information on an object for the conservators and the restoration with the correct material for the restorers.

Provenance and analyses

The regional provenance of a material can give two pieces of information about an object: where it was made (closest material is used for convenience and/or economic reasons) or if there was a commercial relationship between the artwork creation place and the material provenance [5]. Specific materials like pigments can come from specific places for their unique properties, which is a trace of valuable and commercially interesting roads [6].

Several methods of provenance analysis can be used on painted artefacts. Trace elements, mineralogy, wood essence, palynology and organic matter analyses are

*Correspondence: victory.jaques@gmail.com

[1] Institute of Geology and Palaeontology, Faculty of Science, Charles University, Albertov 6, 128 43 Prague 2, Czech Republic
Full list of author information is available at the end of the article

some common ones [7, 8]. Mineralogical analysis can be done with on-site non-invasive spectroscopic techniques like Fourier Transformed Infra-Red (FTIR) [9, 10], Raman and X-ray fluorescence spectroscopy (XRF) [11, 12] or UV Visible Reflectance (UV–VIS) [13, 14], but only the surface and bulk analysis can be carried out this way. Stratigraphy, particle distribution in the layer or grain identification is poorly or not available this way. Overpaintings, dirt, varnishes and other things can be misleading for the interpretation of the results [15, 16].

Micro-sampling allows strongly reliable results for the restoration of the artefacts. Stratigraphy, component determination and distribution, crystallography and morphology are significant for the understanding of how an artwork is constructed. The bones and flesh of it can be discovered in all their beauty and complexity for a better understanding of the artist himself by micro-sampling. For these reasons, micro-sampling is important and often used for semi-destructive analysing techniques like cross-sections for light microscopy (LM) and scanning electron microscopy (SEM).

Painting stratigraphy

There are several artwork types and painting techniques that have been evolving throughout history [8, 17–19]. The ground of a painting is made of one or more preparatory layers applied on canvas, wood panel or coarse wall, meant to flatten, smooth and homogenised before the pictorial layer. The preparatory layers are mostly whitish, but can also be coloured with clays or earth pigments, to increase the visual painting depth [20, 21]. The ground layers can be as complex as the pictorial layers, from the application technique to the materials used. Natural chalk is one of the materials used for the preparatory layers of painting [22, 23], and has been known since ancient Egypt. In Medieval and new-age European workshops, several types of chalk were used from historical quarries (Rügen, Germany; Champagne, France; Mons, Belgium; Bologna, Italy). Indeed, transporting the chalk was not economically advantageous, as there was a market for chalk in the region where it was extracted [22]. While Bologna chalk is significantly different from being built of gypsum (Messinian), the natural chalk from northwest Europe (Upper Cretaceous) is composed of microorganisms' tests with more or fewer impurities.

Microfossils

The assemblages of microorganisms are one of the keys to the provenance identification of material through the determination of their geological age deposition. Microorganisms refer, in this study, to calcareous nannofossils [24], foraminifera [25], diatoms [26, 27] and palynomorphs [28–30]. They are common in source rock materials like clay and chalk. Combined with other methods for refinement or verification, the provenance determination of cultural heritage objects through micropalaeontology is encouraging [31]. We focus this study on the extraction of calcareous nannofossils from historical paintings and polychrome statues with clay or chalk preparatory layers [8, 32]. Minute calcareous biomineralized bodies up to 10 µm represent calcareous nannoplankton formed mainly by unicellular algae. They commonly occur in marine sediments from Triassic to Recent and may be rock-forming. Many of these rocks (typically chalk) were used for art. Calcareous nannofossils were already used for ceramic provenance analysis [33–37], but are less analysed for painted artworks [21, 38]. In the geological field, calcareous nannofossils have been used since the last century sixties and for provenance analysis since the nineteenth century [39, 40]. These are significant biostratigraphic markers, due to their quick speciation in time and space [24], determining the age of the rock deposition (geological age of the source rock material) relation between rock layers and assemblage evolution of the calcareous nannofossils. In many cases, the paleobiogeographic origin of the source rock material can be established [41, 42]. The micrometre size and abundance of calcareous nannofossils in the rock make them appropriate for millimetric art samples [43] as an artwork sample does not exceed 1 mm most of the time and has a submicrogram volume.

Sample binders

In natural samples, the particle binding is mainly an inorganic process (mineral cementation), and the organic matter rarely participates in the binding and hardening process of rock. The disaggregation of such samples by the chemical process of water or mechanically by the freeze–thaw method is effective. On the contrary, artworks are man-made mixtures of natural and synthetic components, creating specific chemical and morphological interactions not always occurring in nature. A binding medium is chosen according to its purpose (functional, aesthetic, protective) [44]. The components and their interactions determine the hardness of the layer and its chemical decomposition/evolution [45, 46]. In this case, the binding medium plays a critical role. It can be a pure or a mixture of processed organic matter, like rabbit skin glue, tree resin, oil or inorganic cement (fresco). The clay and/or chalk are mixed with a binder and applied to the support. The binder disperses the pigments and permits their adhesion and cohesion on a support after the solvent (mostly water) evaporation. The binder hardens over time. There can be interactions between some pigments and the binders that can change their conformation with ageing [47]. The restoration of a painting and

its long-term conservation relies on binder identification [48–50]. Even with the constant improvement of resolution and sensitivity of non-destructive methods [47], they are often inconclusive on the specific type of binder used due to the complexity of the mixtures [51–53]. Gas-chromatography mass–spectrometry (GC–MS) is a destructively widely used and well-known method for precise organic matter determination [54–58], which is sensitive and works with a small amount of material [59, 60]. Casoli [61] explains the procedure of derivatization of fatty acids for GC–MS analysis, and it has already been extensively studied in the literature [62]. Fatty acid methyl esters (FAME) identified by GC–MS can be related to specific materials, and their interpretation is rich in information about the technique, the school, and the period of production. The concentration of fatty acids (FA) in a sample indicates the type of organic components present (plant or animal-based, species); some FA markers [63] and their ratio can inform about the drying process (age) and the presence of other chemicals, such as degradation products, can clarify some interpretations [51, 63–66]. As White explains, a good analyst can identify the type of binder according to the FA pattern. Mills [65] also showed that the ratio of palmitic/stearic acid is stable over time and can be used for oil identification. Later, several studies confirmed it [63, 67, 68], except for Tammekivi et al. [51] and some others, who emphasized using other criteria for confirmation. Still, according to Tammekivi et al. [51], the concentration of the particles (pigments) has a substantial impact on the fatty acid correlated to drying oils.

Calcareous nannofossils determination and preparation

The determination of calcareous nannofossil is based on a combination of different morphological features (shape, size, the morphology of the central area, for detailed information see [116]), which vary for each species. Their usual determination is done with an optical microscope at its highest magnification (optic limits). SEM cross-section analyses, common for artwork stratigraphy observation, offer high-resolution images of the layers and their components, including microfossils present in a painting micro-sample. However, calcareous nannofossil assemblages cannot be determined from a cross-section. The nannofossils are mainly hidden inside the matrix, and the orientation of the ones at the surface does not always allow their identification. Counting of the specimens is also excluded, which suppresses any quantification possibility with this method. The usual nannofossils identification and quantification method is the preparation of a smear or spray slide (nannofossils in solution, then dried and mounted on a glass plate) observed under a polarised microscope with a minimum of 1000× magnification. It is the easiest and most cost-effective method for the determination and quantification of nannofossils [69]. To prepare the slides, the nannofossils should be released from the matrix and cleaned from the other materials (matrix, organic binders, varnish) that could hide their morphology [70–72]. The standard nannofossils decantation and extraction method was developed for natural rocks and great material quantities [70, 73, 74]. Strong acids and mechanical destruction can be used depending on the rock [75]. For painted artworks, the common methods are not always efficient because of the binders and the small volume of material available.

Cultural heritage samples are precious, unique micrometric, and non-reproducible. For those reasons, specific rules are to be applied when dealing with artwork micro-samples related to contamination, loss of material or information. When the necessity to disaggregate a sample occurs, like for the extraction of calcareous nannofossils or precise binder determination, we think that combining the analyses with single sample destruction is important to the community. This study focuses on the development of a disaggregation procedure of painted micro-samples to extract calcareous nannofossils for material provenance analysis coupled with binder determination. Both analyses provide significant information for restorers, conservators, art historians and museums for better artwork comprehension, storage of environmental parameters and more accurate restorations.

Materials and methods

Specimens

The raw materials used for the preparation of the model samples (MS) were champagne chalk (CC; Kremer 58000) mixed with cold-pressed linseed oil (LO; Umton Barvy 3212) or rabbit skin glue (RSG; Kremer 63025) for the preparatory layers [45, 47, 76]. Champagne chalk is known for its historical use in paintings, and its broad literature on calcareous nannofossils and geological settings [77–79], as well as the binders [80].

Three model samples (MS) were prepared with different component ratios (Table 1).

The RSG was soaked in hot water for 2 h and regularly stirred. The glue was made from 10 g of granules for 125 mL of water (according to the recipe from Slánský

Table 1 Ratio of components composing the preparatory layer for each model sample

[%]	Chalk (CC)	Rabbit skin glue (RSG)	Linseed oil (LO)	Pigment layer
MS1	50	50	0	0
MS2	50	0	50	0
MS3	50	50	0	Casein

[81]), mixed with CC and left for drying. MS2 with LO was dried at room temperature for 2 months. MS3 is a historically accurate sample prepared by the restorer Quentin Arguillère. On top of the preparatory layers, he applied yellow earth mixed with casein to remind of a pictorial layer. All samples used for this experiment were smaller than 3 mm for a maximum thickness of 2 mm.

Chemicals

The tested solvents were distilled water (H_2O), which has a very low effect on carbonates and organic binders, hydrogen peroxide 7% (H_2O_2) that oxidised organic matter in an effective and quick way [69, 71, 82], ethanol 96% (E) that will dissolve oil or glue components, acetone (A) 99.6% that can dry organic matter and dissolve varnishes, Hydrochloric acid 5% (HCl) that is corrosive for inorganic and organic matter, xylene 99% (X) that dissolve organic components [83] and finally Petrol medical R 95% (PB) [71, 84]. These solvents are common in most laboratories, whether for cleaning or use in more complex chemical procedures. We also investigated two micro-emulsions (ME) primarily prepared for the surface cleaning of paintings that differ in their active component (quaternary base) and their concentration. Their alkaline nature was thought not to attack the calcareous particles, and the presence of surfactants should weaken the adhesion of organic matter to an inorganic surface. The emulsions consisted of an aqueous solution of non-ionic triblock copolymer poly (propylene glycol-block-ethylene glycol-block-propylene glycol) at a concentration above its critical micellae concentration, 1-pentanol serving as a co-surfactant, and a quaternary base at 9.1% or 4.6% concentration. The preparation of the microemulsions was based on the procedure according to the patent CZ 305527 [85]. The high efficiency of these emulsions is achieved by a combination of chemical and physical actions. The chemical action lies in the cleavage of the covalent bonds of the proteins (chemical action) and the facilitated removal of fission products from the surface of the treated material by changing the surface tension. The physical function is enabled by the concerted action of the surfactant and co-surfactant.

Solvents, temperature, ultrasonic testing

Pure chalk (CC) was mixed with each chemical to evaluate their impact on the nannofossils. The same procedure was applied to the linseed oil (LO) and the rabbit skin glue (RSG) [86] (Fig. 1). The outcome was that the chemicals damaging the calcareous nannofossils (CC) were discarded, as well as the chemicals without effect on the binders (RSG, LO). Hydrogen peroxide was kept because it could have a lower impact on the nannofossils when embedded in a binder.

The efficiency of the chosen chemicals (H_2O, H_2O_2, ME1, ME2) was tested on the model samples (MS1, MS2, MS3) at room temperature and at 70 °C [87, 88]. 70 °C was chosen for being slightly higher than the temporary protein denaturation temperature, which is around 65 °C [87]. The denaturation of the molecules weakens their binding properties [89, 90].

For this procedure, the micro-sample and the chemical in the open assay tube are fixed in a water bath, allowing for evaporation. The heat is evenly distributed in the water bath, which improves its management, controlled with an external thermometer.

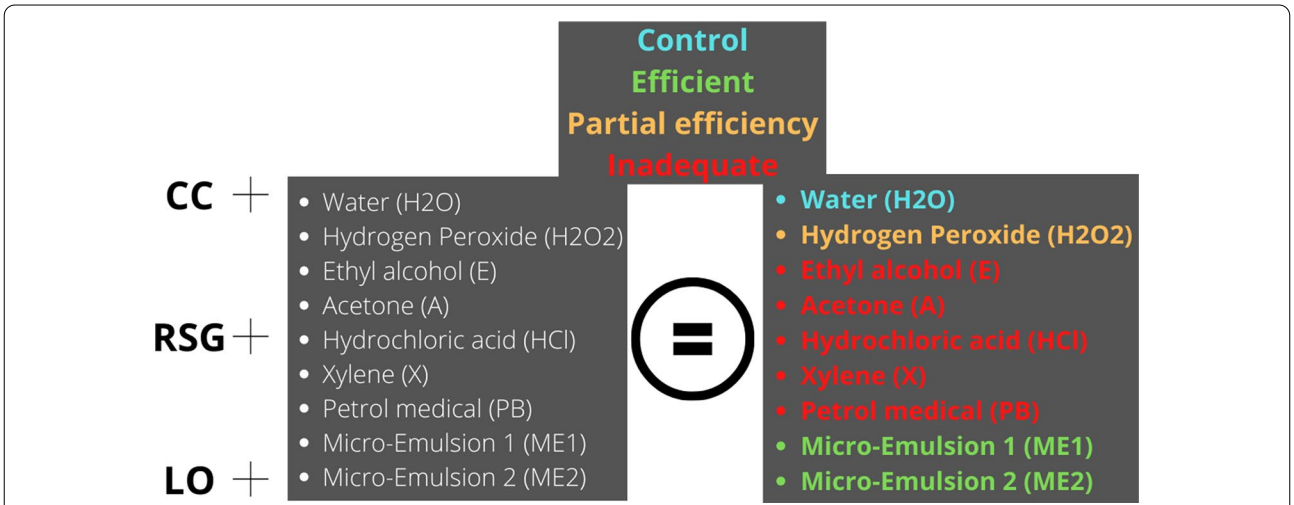

Fig. 1 Impact of the chemicals on chalk, linseed oil and rabbit skin glue, and outcome of the test with the chemicals kept for the disaggregation methods

The examination of the ultrasonic bath, Elmasonic S 10 H of 37 kHz ultrasonic from impulsion waves with time and temperature control, on calcareous nannofossils was carried out with chalk in distilled water and a timer at 0 s, 10 s, 30 s, 60 s, and 120 s. The three model samples were also tested in the same manner to observe the mechanical disaggregation [91].

Disaggregation of micro-specimens

The first disaggregation method (M1) is based on protein denaturation and liquefaction processes by temperature, and mechanical disaggregation with the ultrasonic bath coupled to a solvent.

Each micro-sample was inserted into an assay tube, filled with one of the solvents. The tubes were fixed open in a water bath at 70 °C. After the evaporation of the solvent, the tubes were refilled with the same solvent, and the ultrasonic was run for 0 s, 10 s, 30 s, 60 s or 120 s. When half of the solvent evaporated, ethanol was added to the solution as the last step to clean the particles. The assay tube was shaken and vertically left to settle for 10 to 30 s before preparing the smear slides.

Method 2 (M2) consists of the chemical disaggregation of the micro-samples with patented micro-emulsions. Each micro-sample was in an assay tube filled with one of the micro-emulsions. The impact of each micro-emulsion on the micro-samples was observed after 5 min, 30 min and 1 h. The smear slides were then prepared.

Method 3 (M3) is a combination of the first two methods M1 and M2 to increase the reliability and efficiency of the disaggregation on a more global scale. Each micro-sample was in a specific assay tube filled with micro-emulsion. The open assay tube was fixed in a water bath at 70 °C for 30 min to 2 h, depending on the disaggregation of the sample. The micro-emulsion evaporated during the process and had to be refilled several times according to the time of evaporation. When the sample was disaggregated, the assay tube was filled with ethanol and shaken. The solution was finally set for 10 s in the ultrasonic bath. This part of the procedure was added to mechanically disaggregate the last clumps, and the ethanol was meant to clean the particles from the micro-emulsion. The smear slide was then prepared.

Smear slide preparation

Each solution from the disaggregated samples was prepared as two smear slides at least, based on Blaj and Henderiks [92], with slight additions as follows: A part of Canada balsam was gently heated (≤ 60 °C) over a hot plate along with the glass slides (1×7 cm). Distilled water was added to the assay tube if some liquid was missing, and then left to settle vertically for 30 s. A single-use pipette, to avoid contamination, was used for each disaggregated micro-sample to pour some drops over the glass slide. The solution was taken 2 mm over the bottom of the assay tube to its top. After complete evaporation of the liquid, a drop of Canada balsam was applied with a thin glass rod on the glass slide. A coverslip of #0 thickness was set on top of the hot preparation until the Canada balsam was uniformly distributed. Bubbles were removed by gently pushing the thin glass from the middle to the corners with a large glass rod. The smear slide was left to cool down, and then cleaned from the Canada balsam excess with xylene and ethanol.

The smear slides were analysed under a Nikon Eclipse N500 microscope bright-field and cross-polarized light, in transmission mode from the Institute of Geology and Palaeontology, Charles University, and a Reichert microscope from CEITEC CT Laboratory in Brno.

Calcareous nannofossils counting

The quantification of calcareous nannofossil was done from 10 frames dispatched in"L" or a"T" shape (Fig. 2A) at 560× magnification (Fig. 2B). It follows the rules used by Backmann et al. [93] who chose a specific number of frames to be counted at a specific magnification. Each frame contains at least 1 nannofossil to be considered (Fig. 2B). If no nannofossils were counted, it means the whole slide was observed at 560× magnification (Fig. 2B). Less than 50 specimens counted (whether whole or broken) were considered inconclusive results. The determination of the nannofossil species was done at 1000× magnification (Fig. 2C).

Analysis of fatty acid methyl esters by GC–MS

The analysis consists of the esterification of lipids, then injection, separation, identification, and quantitation of the fatty acid methyl esters (FAME) [53]. Gas chromatography coupled with an ion trap mass spectrometer (GC–MS; Varian 450 GC, Varian 240 MS) was used to analyse the FAME. The detection limit was 0.02 µg/g. Briefly, the mixture of a 50 mM phosphate buffer, methanol, and chloroform was used for the extraction, followed by alkaline methanolysis and analysis by GC–MS. Each material and solvent was analysed separately (linseed oil, rabbit skin glue, chalk, micro-emulsions) for the determination of chemical fingerprints. Different ratios of materials were mixed (50%/30%/10%), and their FA patterns were compared to the ones from the reference analyses. MS1, MS2 and MS3 were analysed as untouched samples and already disaggregated by micro-emulsions. The samples had dry masses between 0.1 and 0.5 mg or were already dissolved in 1.5 mL

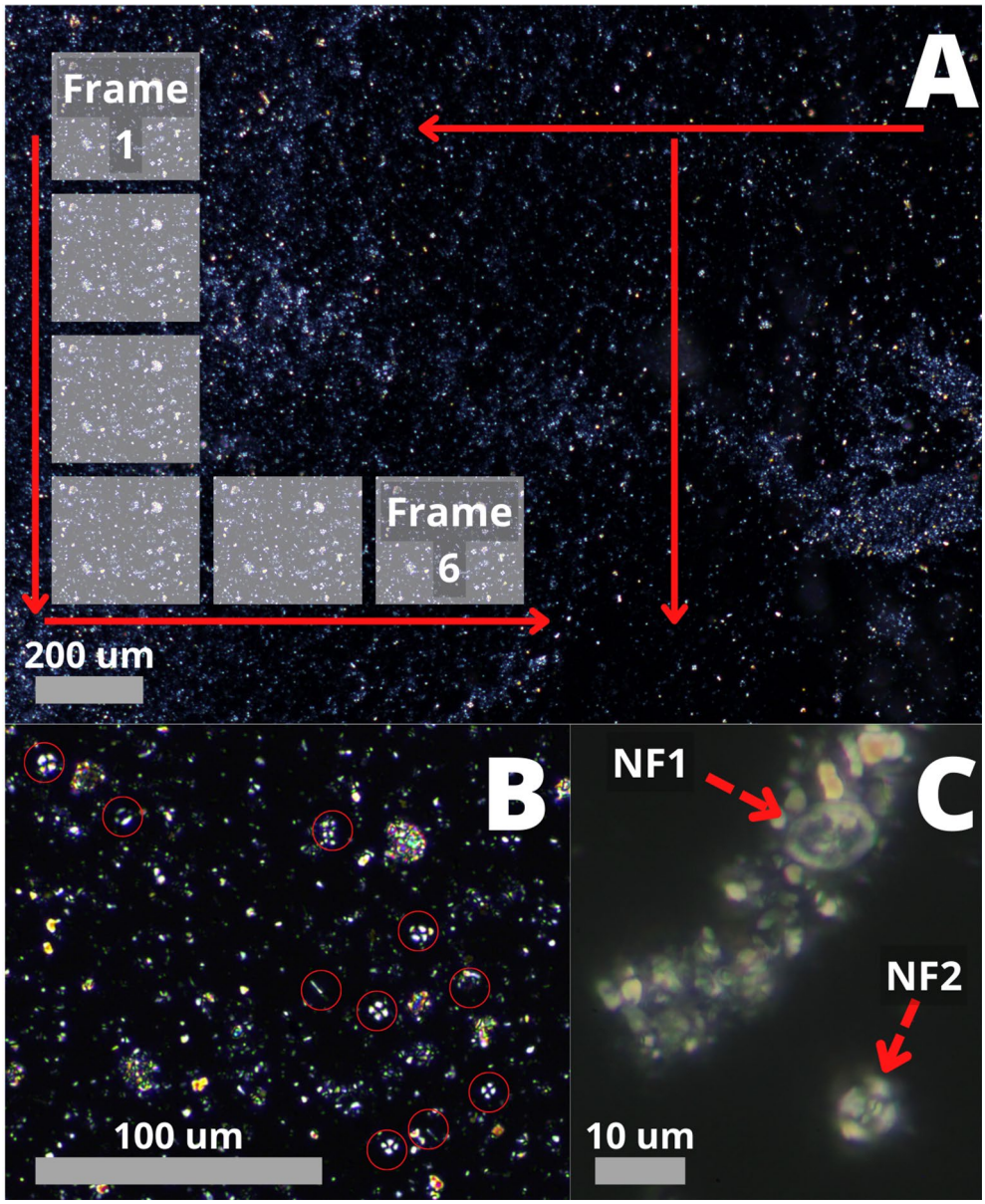

Fig. 2 Cross-polarized light microscope images of chalk smear slides. **A** "L" or "T" shape used for the direction in which the frames (1 to 6 here; frames are not at scale) at 560× magnification were observed. **B** 560× magnification at which the counting of the nannofossils was done. Some of the most visible nannofossils in the picture are highlighted (red circle). **C** The determination of the nannofossils was done at 1000× magnification. NF1 is in an aggregate and cannot be properly determined, while NF2 is nicely visible

assay tubes with the solvents used for the disaggregation methods 1, 2 and 3.

Results and discussion

Although several nannofossils' extraction procedures already exist [73, 75, 94], we considered them not adapted to the organic binders and the small volume of material available.

The results describe the reaction of the micro-samples during their disaggregation according to each method, and rely on the nannofossil quantification.

The counting of nannofossils was done under an optical microscope, even though it may lead to taxonomical problems [95, 96]. The determination of the nannofossil species was not the focus of this study, and is not discussed here. The average number of counted nannofossils

for the 3 model samples (MS1, MS2, MS3) is relatively different from the reference quantification done on pure chalk with water. For MS1 prepared with rabbit skin glue, the average value counted was 307 nannofossils. MS2 (linseed oil binder) contained 112 nannofossils, while MS3 (historical model sample) had 101 nannofossils. The reference quantification count was 300 nannofossils from 10 frames. The commonly accepted total of counted nannofossils for a "good" quantification should be between 300 and 400 nannofossils per slide [97]. The counted nannofossils difference can be due to the distribution of them onto the smear slide. All slides were first observed at 100× magnification, and the most well-distributed site was taken for the counting (low number of aggregates, separation of the nannofossils). We suggest that smear slides could also be prepared, according to Gallagher [75], for a combined determination through scanning electron and optical microscopes, as also developed in other works [98–101]. This will ensure proper identification of the different species present.

Interaction solvents—Chalk

The ratio between whole and broken nannofossils was used to define the impact of each solvent on the nannofossils. Water was used as a reference, as it is the most commonly used solvent for rock disaggregation in micropalaeontology. The other solvents that had non-/low interference with the nannofossils were ethanol (Eth), acetone (Ace), xylene (Xyl), distilled water (H_2O) and the two micro-emulsions (ME1, ME2) (Fig. 1). We observed that the ratio complete/broken is very similar for all of them (Fig. 3). Except for hydrochloric acid (HCl), which disaggregated all chalk particles, and peroxide (H_2O_2), where a higher number of broken nannofossils were counted compared to water. Although several extraction methods use peroxide to remove the natural organic parts of a sample [102–104], it can impact the nannofossils that contain some magnesium and induce more breakage, as it has an impact on calcium carbonate [105].

Interaction temperature—Chalk

The temperature increase induces the entropy of the molecules, reducing their binding ability, which supports the dissolution of the oily and proteinaceous binders from the inorganic components. The temperature with which we are working had no visible impact on the nannofossils, partially corroborating the work of Chairopoulou [106] even though the study of Coto [107] showed an increasing solubility under certain conditions already from 20 °C on the contrary. This procedure alone does not work, but helps the solvent mix with the organic particles, breaks the bonds of the molecules, and reduces the time of disaggregation. Older samples are less sensitive to temperature rise. We observed that the effect of temperature on the sample disaggregation is related to the solvent used and depends on the type of binder. Rabbit skin glue is more prone to react to heat, while linseed oil is less. The

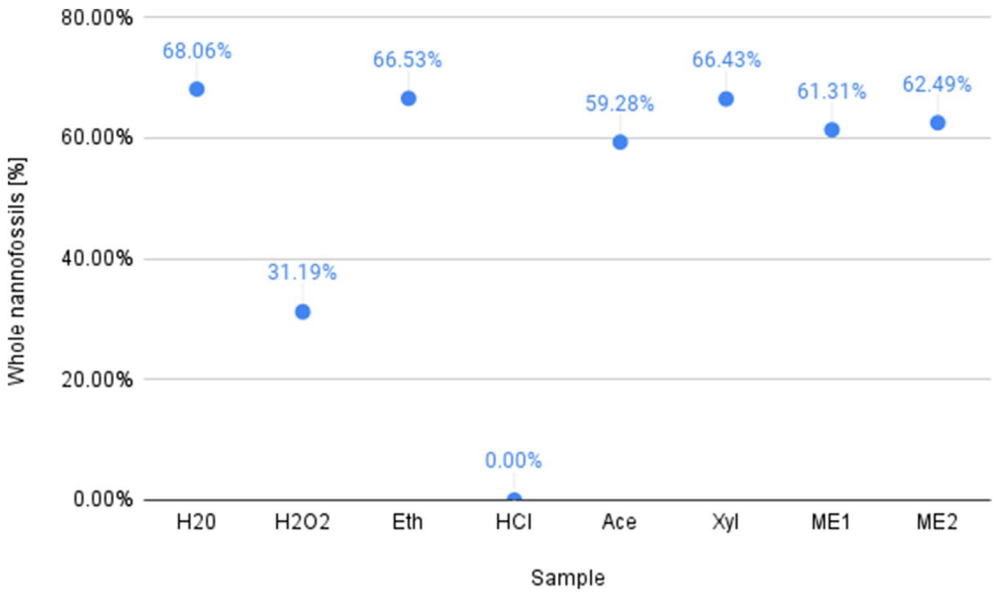

Fig. 3 Evaluation of the interaction between the solvent and the percentage of whole/broken calcareous nannofossils in the champagne chalk. The CC was only mixed with distilled water (H_2O) for control. Peroxide (H_2O_2), ethanol (Eth), hydrochloric acid (HCl), xylene (Xyl) and the two chosen micro-emulsions (ME1, ME2) were tested as disaggregation solvents

best effect on the model samples was 70 °C, even without other procedures. The pH level of the solution is related to solvent concentration and temperature, and can have an impact on the disaggregation process. This could be interesting to assess in a follow-up study.

Interaction ultrasonic—Chalk

The ultrasonic bath can partially break inorganic cement and facilitate the removal of residual binders from particles [91, 108]. Particle separation is also possible through centrifugation [109], but we did not consider it a common or easy access device for most laboratories. Several works studying micro- and nannofossils used an ultrasonic bath apparently without impact. In Clark's studies [91], he showed that the disaggregation of nannofossils in a typical 60-W lab sonicator should be limited to less than 5 min for cleaning 3 µm nannofossils in volumes of water around 25 mL. Smaller volumes will therefore take less time. We used 1.5 mL in our study. The presence of nannofossils over 5 µm was reduced (Fig. 4). 8% can be negligible over a large material volume full of nannofossils, but when working with small volumes of material and scarce nannofossils, the impact is significant on nannofossils assemblage determination and data interpretation. The ultrasonic bath was useful at the end of the disaggregation procedure to break the last aggregates and clean the remaining binder from the inorganic particles. 10 s of ultrasonic time in a 60-W ultrasonic bath is suggested for this final cleaning step.

Disaggregation methods

During the M1 disaggregation procedure, the model sample MS01 (rabbit skin glue; Fig. 5) was dissolved with water, peroxide and both micro-emulsions. Peroxide was kept for testing, even though it had a proven direct impact on the nannofossils in pure chalk (Fig. 3). The hypothesis was that the binder could "protect" part of the nannofossils from the solvent. The number of whole nannofossils from MS01 increased by 2.5× compared with pure chalk in peroxide, while the number of broken nannofossils decreased by 1/4. The reaction between rabbit skin glue and peroxide allows fewer molecules to react primarily with chalk particles. This preserves the chalk particles as long as the binder is around. The disaggregation was very low with model samples MS04 (linseed oil; Fig. 6) and MS16 (historical; Fig. 7), which showed a high variation in their results. The peroxide was discarded because its result could not be predicted if the samples were of unknown composition.

Water disaggregated MS01 (Fig. 5), but not MS04 (Fig. 6), and neither did MS16, also with rabbit skin glue like MS01 (Fig. 7). Water can facilitate disaggregation by partially rehydrating the binder, but it is not efficient.

The M2 disaggregation procedure showed an effective disaggregation of MS01 with both micro-emulsions ME1 and ME2. ME2 showed a very good dissolution (no aggregates to very small ones) on all micro-samples and seemed more effective on MS04 than ME1, even though ME1 is more concentrated in the quaternary base. With both micro-emulsions, the solution turned orange when

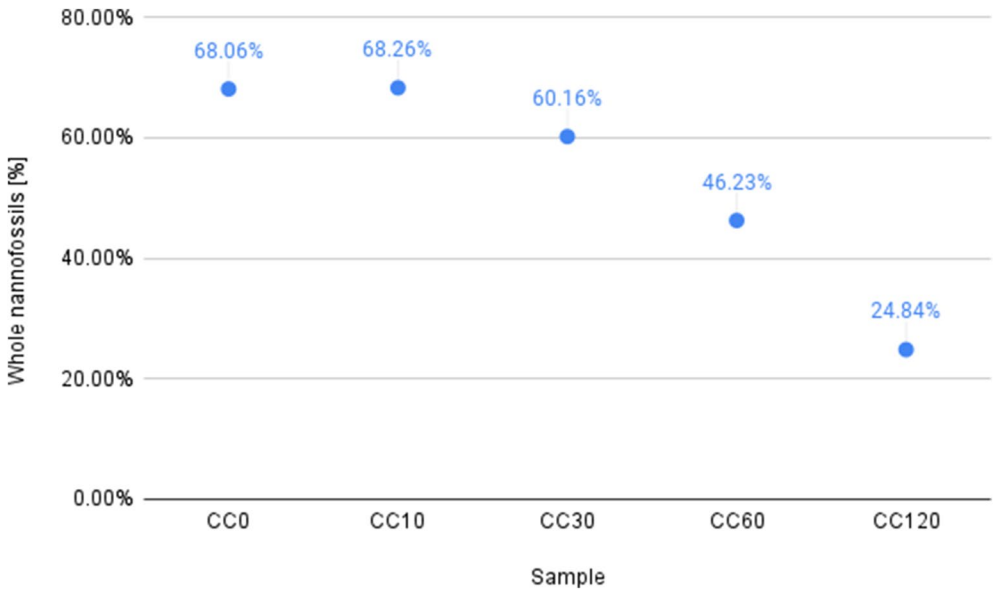

Fig. 4 Evaluation of the interaction between the ultrasonic time and the number of calcareous nannofossils after the treatment. Champagne chalk (CC) was soaked in distilled water during the ultrasonic procedure. The ultrasonic was set for 0', 10', 30', 60' and 120's (CC0 to CC120)

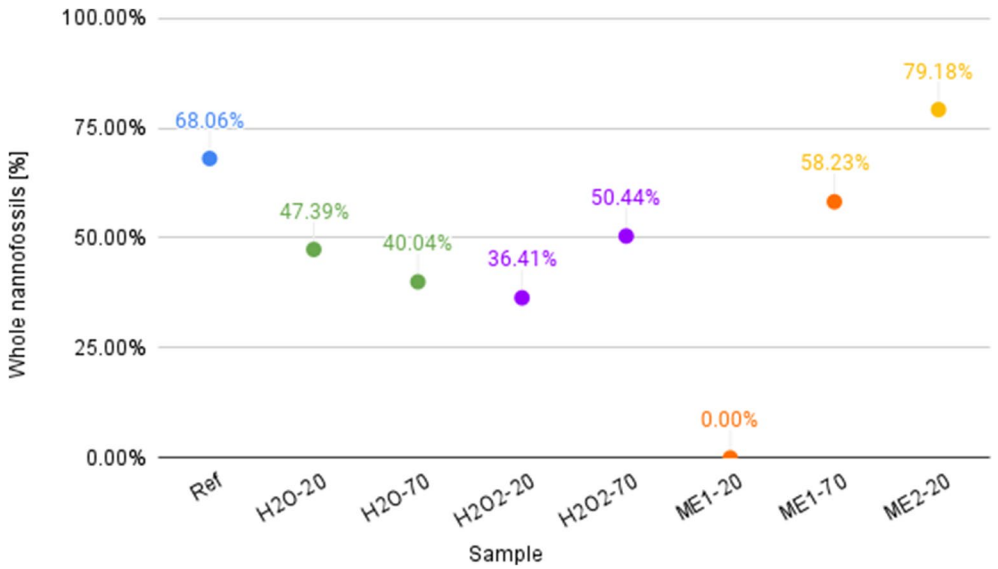

Fig. 5 Quantification of calcareous nannofossils from the micro-sample MS01 disaggregated by various solvents. Dissolution with water (H_2O) was compared with peroxide (H_2O_2) and the two micro-emulsions (ME1, ME2). The impact of the temperatures of 20 °C and 70 °C is also shown ("H_2O-20" = water at 20 °C or "H_2O-70" = water at 70 °C). The ratio of whole and broken nannofossils in the micro-samples is expressed as the percentage of whole nannofossils. Ref is the value of this ratio in the original rock material

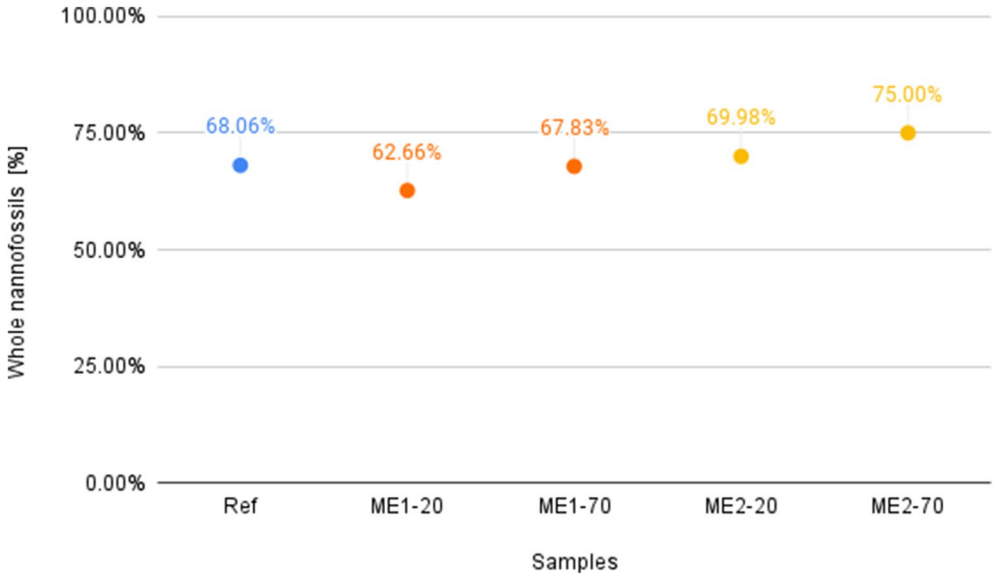

Fig. 6 Quantification of calcareous nannofossils from the micro-sample MS04 disaggregated by the two micro-emulsions ME1 and ME2 at 20 °C or at 70 °C ("ME1-20" = Micro-emulsion 1 at 20 °C or "H_2O-70" = Micro-emulsion 1 at 70 °C). MS04 disaggregated with water showed 0% of counted nannofossil and is therefore not shown in the graphic. The ratio of whole and broken nannofossils in the sample is expressed as the percentage of whole nannofossils. Ref is the value of this ratio in the original rock material

in contact with the linseed oil only (Fig. 8). This can be due to (1) the formation of degradation products of proteins due to the action of the strong alkaline quaternary base, resulting in the formation of an insoluble anion–cation complex between the quaternary ammonium ions and the proteins at an alkaline pH [110] or (2) the destruction of the triple helix conformation of collagen. The absence of such a reaction with rabbit collagen can be due to the character of the protein [111]. The separation of the material is excellent, the solution does not turn

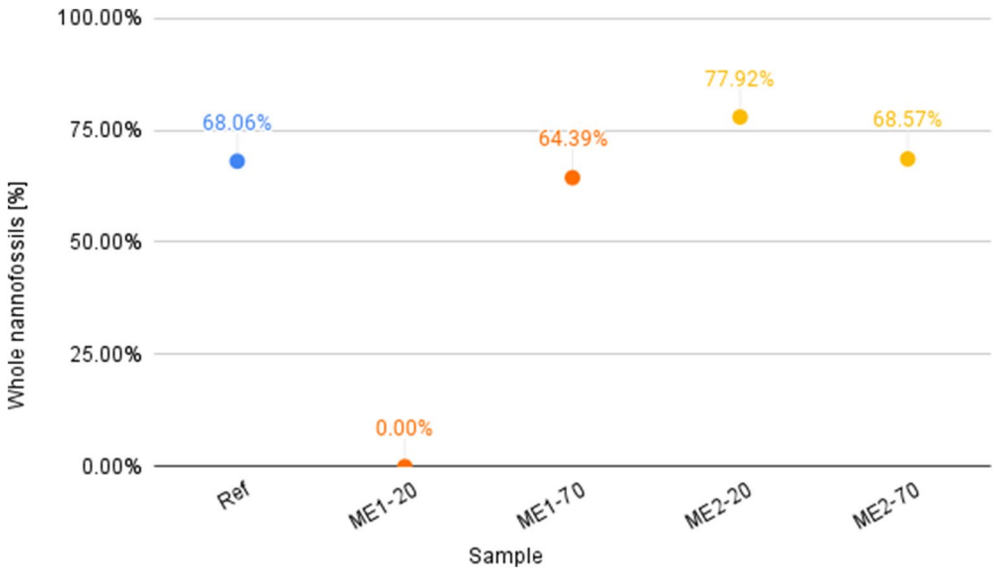

Fig. 7 Quantification of calcareous nannofossils from the micro-sample MS16 disaggregated by the two micro-emulsions ME1 and ME2 at 20 °C or at 70 °C ("ME1-20" = Micro-emulsion 1 at 20 °C or "H_2O-70" = Micro-emulsion 1 at 70 °C). A problem was encountered with ME1-20 smear slides, where 0 nannofossils were counted. We interpreted it as a preparation issue and not related to nannofossils dissolution according to the other results. The disaggregation of MS16 with water showed 0% of nannofossil, and is therefore not shown in the graphic. The ratio of whole and broken nannofossils in the sample is expressed as the percentage of whole nannofossils. Ref is the value of this ratio in the original rock material

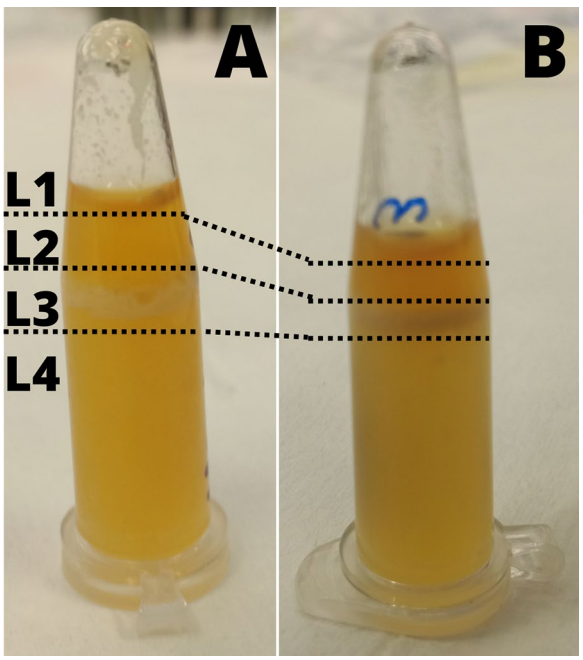

Fig. 8 The solution becomes orange when **A** ME1 and **B** ME2 are in contact with linseed oil. After some time, a density separation (L1 low density to L4 higher density) of the materials is visible with colour variations (L1: dark orange, L2: lighter orange, L3: whitish, L4: orange pale; all four are opaque solutions)

into a gel, and there is a very low dissolution of the nannofossils with the micro-emulsions, as shown in Fig. 3. Both micro-emulsions ME1 and ME2 dissolved rabbit skin glue. During the optical microscopic observation, a layer on top of some particles was visible and could be related to one of the components in the micro-emulsions. This can hinder the determination of nannofossils.

The M3 disaggregation procedure gave similar results to M1 on MS04 with linseed oil (Fig. 6) micro-sample, while M2 showed a better disaggregation of the micro-sample MS01 with rabbit skin glue (Fig. 5). MS16 micro-sample with rabbit skin glue and casein was quicker and better disaggregated with this disaggregation procedure M3 than with the first two disaggregation procedures M1 and M2. The optical microscopic observations of the M3 disaggregation procedure showed no material segregation compared to the M2 procedure (Fig. 8), and showed generally fewer aggregates (Fig. 2) than M1 and M2. We therefore had a higher rate of whole nannofossils counted.

Binder analysis

In our study, we analysed single materials and solvents (RSG100, LO100, ME1, ME2), and mixtures

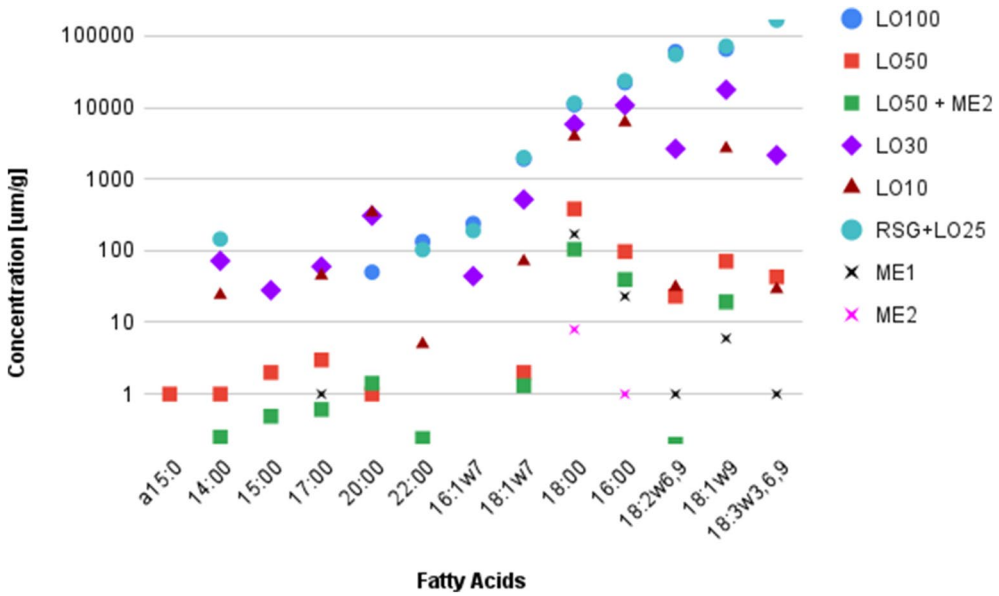

Fig. 9 Linseed oil fatty acids (FA) concentration on a logarithmic scale. Palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic (18:3) FA are typical markers for linseed oil. Chromatograms are in the Additional file 1

(RSG50/30/10, LO50/30/10—for example 50% RSG for 50% chalk (CC) = RSG50).

9 different fatty acids were detected in pure linseed oil (LO100) (Fig. 9; Additional file 1: Table S1) for 49.2% lipids (m/m). Some typical FA markers for linseed oil are palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic (18:3), which we found in all samples containing LO. The ratio of palmitic/stearic acid points out the oil type and linseed oil, and is mostly reported to be between 1.2 and 2.4. All samples containing linseed oil had a P/S ratio between 1.58 and 2.05, except for LO50 (P/S 0.25). We cannot explain it, as it appears comparable to LO50 mixed with ME2 (P/S 0.38; Additional file 1: Table S1 + Fig. S7). We observed that the P/S ratio decreased when the chalk concentration was increased (Additional file 1: Table S1), which corroborates the observations of Tammekivi et al. [51] about the impact of different pigments and their concentration on the binder and the FA ratios. This confirms the question about the FA ratios validity to interpret the oil type, which needs to be further studied.

The drying stage of the oil can be evaluated by the presence of Linoleic (fresh oil), Linolenic, Oleic (old oil) acids, as well as Azelaic, Suberic and Sebacic acids, which are degradation products typical in drying oils [61, 112] (Additional file 1: Fig. S3). These dicarboxylic acids were not detected in our samples, which can be due to our methodology (inefficient transesterification reaction in the experimental conditions), the "freshness" of the oil (lack of polymerization due to insufficiently aged oil), the drying conditions and/or the amount of chalk mixed with the binder [112–114]. This absence needs to be clarified in further study. The presence of Hypogeic acid (16:1ω7) is interesting, as it can be related to the order of the Malpighiales, which contains the common flax seed (Linum linseed oil) according to PlantFAdb [115], but was only detected in LO100, LO30 and RSG + LO25 (Fig. 9; Additional file 1: Figs. S3 + S5).

In LO50, LO30 and LO10, additional FAs such as myristic acid (14:0), 12-methyltetradecanoic acid (a15:0), pentadecyclic acid (15:0) and margaric acid (17:0) were found, partially due to external contamination (finger fat, preparation surface, tools), as they also appear in rabbit skin glue (Additional file 1: Figs. S1 + S4). Rabbit skin glue is composed of 98% of collagen and water. There is a possibility that depending on how well the rabbit skin glue was refined, FA traces can appear, but in our case, it is probably unrelated.

Concerning the micro-emulsions, ME1 had a similar pattern of FA to rabbit skin glue, which tends to confirm external contamination (Fig. 9; Additional file 1: Fig. S6). ME2 only had palmitic and stearic acids detected in very low concentrations (< 10 µm/g; Additional file 1: Table S1) for a P/S ratio of 0.13, and ME1 had a P/S ratio of 0.14. FAs detected in the micro-emulsions are considered external contaminations. The FA patterns in LO50 and LO50 + ME2 are similar (Additional file 1: Figs. S6 + S7), except for the presence

of behenic acid (22:00) in LO50 + ME2 and a strong decrease of linoleic acid (18:2ω6,9) compared to LO50. The impact of micro-emulsions on linseed oil FA pattern identification can be considered negligible (Additional file 1: Table S1).

Conclusion

Three disaggregation procedures of calcareous nannofossil extraction were designed and tested on three types of model micro-samples based on painting materials to enable the determination of the geological material source. Calcareous nannofossils can be used for provenance determination of the material or artwork when combined with other data. The disaggregation methods M1, M2, and M3 were developed to avoid the dissolution of carbonates by testing different (1) solvents to dissolve common organic binders used in paintings, (2) two different temperatures to temporarily denature proteins and weaken the binder bond, and (3) ultrasonic cleaning timing for the mechanical destruction of the aggregates and particles cleaning. The best combination was obtained with the third protocol, M3, by the combination of the micro-emulsion ME2 at 70 °C and ultrasonic cleaning of 10 s at the end of the whole procedure. This protocol disaggregated the three types of micro-samples and showed a low amount of aggregates in the assay tube before the preparation of the smear slide. It also had the highest calcareous nannofossil content and was in close agreement with the reference sample nannofossil quantification.

Even though provenance analysis is key information, the destruction of a cultural heritage micro-sample is always undesirable. This led us to combine the nannofossil extraction with binder analyses, crucial for the restoration and conservation of artwork. Binders interact with the inorganic components, and the environment can have a strong impact on them. Their precise determination ameliorates the conservation of the artefact.

Gas-chromatography analyses can precisely determine organic binders even with very small volumes of material. But primarily, the effect of the solvents on the organic binder had to be determined. Therefore, the raw materials (solvents and micro-samples) were measured, and then the mixtures. Both micro-emulsions, ME1 and ME2, had a low influence on the fatty acid content. We want to emphasize the need for further study of different derivative procedures for GC–MS, tests on real samples, and analyses of protein analysis. The designed and tested calcareous nannofossils extraction methods can easily be applied in any laboratory with a good disaggregation rate on various types of samples. The information gained by this disaggregation procedure is valuable for conservation, restoration, and historical investigation of provenance and techniques.

Abbreviations

FAME: Fatty acid methyl esters; GC–MS: Gas chromatography–mass spectrometer; FTIR: Fourier Transform InfraRed spectroscopy; UV–VIS: UltraViolet Visible Reflectance; XRF: X-ray fluorescence spectroscopy; SEM: Scanning electron microscopy; LM: Light microscopy; MS: Model sample; ME: Micro-emulsion; CC: Champagne chalk; LO: Linseed oil; RSG: Rabbit skin glue; Xyl: Xylene; Eth: Ethanol; H_2O_2: Peroxide; H_2O: Distilled water; Ace: Acetone; HCl: Hydrochloric acid.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40494-022-00773-8.

Additional file 1: List of compounds identified by GC–MS with the chromatograms for each sample, as well as for linseed oil, rabbit skin glue and micro-emulsions.

Acknowledgements

Thanks go to the ALMA laboratory from Prague/CZ for the introduction to the painted samples preparation topic. The Archaeological Institute from the Czech Academy of Science and the Central European Institute of Technology in Brno/CZ were of great help concerning the microscopes. We also want to acknowledge Jaroslav Semeráde for his invaluable help with the GC–MS analysis. Special acknowledgement goes to Quentin Arguillère, who prepared the historically accurate model samples. The raw materials were bought from Výtvarné potřeby Zlatá Lod´, Národní 416/37—Platýz, 110 00 Praha 1, which we thank for their kind advice.

Author contributions

JR prepared the micro-emulsions. JT conducted the GC–MS and FAME analysis with the participation of TC. KH participated in micropaleontological data elaboration and interpretation. VJ elaborated the concept, developed the disaggregation methods, interpreted the data, and wrote the main parts of the manuscript. All authors read and approved the final manuscript.

Funding

The study was supported by the institutional funding provided by the Centre for Geosphere Dynamics (UNCE/SCI/006) and by the GAUK project number 956120, COOPERATIO.

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests

The authors declare no conflict of interest regarding the publication of this article, and that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author details

[1]Institute of Geology and Palaeontology, Faculty of Science, Charles University, Albertov 6, 128 43 Prague 2, Czech Republic. [2]Institute of Geochemistry, Mineralogy and Mineral Resources, Stable and Radiogenic Isotope Research Laboratory, Faculty of Science, Charles University, Albertov 6, 128 43 Prague 2, Czech Republic. [3]J. Heyrovský Institute of Physical Chemistry of the CAS, Dolejškova 3, 18223 Prague, Czech Republic. [4]Institute for Environmental Studies, Faculty of Science, Charles University, Benátská 2, 128 01 Prague 2, Czech Republic.

Received: 11 March 2022 Accepted: 14 August 2022
Published online: 30 August 2022

References

1. Turk J, Mauko Pranjić A, Hursthouse A, Turner R, Hughes JJ. Decision support criteria and the development of a decision support tool for the selection of conservation materials for the built cultural heritage. J Cult Herit. 2018;37:44–53.
2. Pernet B, Silverman ER, Valentich SP. The seashells of an iconic public artwork: diversity and provenance of the mollusks of the watts towers. JCMS. 2019;17:1.
3. Draganov D, Hunziker J, Heller K, Gutkowski K, Marte F. High-resolution ultrasonic imaging of artworks with seismic interferometry for their conservation and restoration. Stud Conserv. 2018;63:1–15.
4. Radis M, Iacomussi P, Aghemo C. Optical characteristics and visual appearance for artwork materials. In: Pezzati L, Targowski P, editors. Optics for arts, architecture, and archaeology V. Bellingham: SPIE; 2015. p. 95270J.
5. Tasker A, Wilkinson IP, Williams M, Morris M, Cooper NJ, Fulford MG. Provenance of chalk tesserae from a roman town-house in Vine Street, Leicester. Britannia. 2013;44:219–46.
6. Nöller R, Hahn O. Illuminated manuscripts from turfan tracing silk road glamour by analyzing pigments. Sci Technol Archaeol Res. 2015;1:50–9.
7. Zagora J. SEM-EDX pigment analysis and multi-analytical study of the ground and paint layers of Francesco Fedrigazzi's painting from Kostanje. ceroart. 2013; EGG 3.
8. Antunes V, Candeias A, Coroado J, Serrão V, Cachão M, Carvalho ML. A multidisciplinary approach to the study of the brightening effects of white chalk ground layers in 15th and 16th century paintings. Anal Methods. 2016;8:4785–97.
9. Gruchow F, Machill S, Thiele S, Herm C. Imaging FTIR spectroscopic investigations of wood: paint interface of aged polychrome art objects. In: Schreiner M, editor. e-Preservation Science: morana-rtd.com; 2009. p. 145–50.
10. Schulz H, Kropp B. Micro spectroscopy FTIR reflectance examination of paint binders on ground chalk. Fresenius J Anal Chem. 1993;346:114–22.
11. Antunes V, Candeias A, Carvalho ML, Oliveira MJ, Manso M, Seruya AI, et al. GREGÓRIO LOPES painting workshop: characterization by X-ray based techniques. Analysis by EDXRF, μ-XRD and SEM-EDS. J Inst. 2014;9:C05006–C05006.
12. Coccato A. Application of Raman and X-ray fluorescence spectroscopies to Cultural Heritage materials -he non-destructive examination of paintings, pigments, and their degradation. Doctoral dissertation. Faculty of Arts and Philosophy, Ghent University; 2017.
13. Picollo M, Aceto M, Vitorino T. UV-Vis spectroscopy. Physical Sci Rev. 2019. https://doi.org/10.1515/psr-2018-0008.
14. Kuroda R, Sugawa S. UV/VIS/NIR imaging technologies: challenges and opportunities. In: Dhar NK, Dutta AK, editors. Image sensing technologies: materials, devices, systems, and applications II. Bellingham: SPIE; 2015. p. 8.
15. Shackley MS. Is there reliability and validity in portable X-ray fluorescence spectrometry (PXRF)? SAA Archaeol Rec. 2010;10:17–20.
16. Liritzis I, Zacharias N. Portable XRF of archaeological artifacts: current research, potentials and limitations. In: Shackley MS, editor. X-ray fluorescence spectrometry (XRF) in geoarchaeology. New York, NY: Springer; 2011. p. 109–42.
17. Antunes V, Candeias A, Oliveira MJ, Lorena M, Seruya AI, Carvalho ML, et al. Calcium sulfate fillers and binders in Portuguese 15th and 16th centuries: ground layers from a family painting workshop—study by multianalytical spectroscopic techniques. Microchem J. 2016;125:290–8.
18. Antunes V, Valadas S, Serrão V, Carvalho ML, Candeias A, Mirão J, et al. Josefa d'Óbidos workshop from panel to canvasMultianalytical approach to materials and technical evolution of the most significant Portuguese painting workshop of the 17th century. J Mol Struct. 2019;1188:31–41.
19. Hradil D, Hradilová J, Holcová K, Bezdička P. The use of pottery clay for canvas priming in Italian Baroque—an example of technology transfer. Appl Clay Sci. 2018;165:135–47.
20. Zagora J. Historical development of coloured grounds in italian painting from the 15th to the mid-18th century—present insights and open questions. Croat Conserv Inst Yearb. 2017;8:73–94.
21. Hradil D, Hradilová J, Lanterna G, Galeotti M, Holcová K, Jaques V, et al. Clay and alunite-rich materials in painting grounds of prominent Italian masters—Caravaggio and Mattia Preti. Appl Clay Sci. 2020;185:105412.
22. Geyssant J. Features and characteristics of calcium carbonate. In: Tegethoff FW, editor. Calcium carbonate. Basel: Birkhäuser; 2001. p. 2–15.
23. Mayhew TD, Ellis M, Seraphin S. Steatite and calcite natural white chalks in traditional old master drawings. J Am Inst Conserv. 2012;51:175–98.
24. Bown PR, Lees JA, Young JR. Calcareous nannoplankton evolution and diversity through time. In: Thierstein HR, Young JR, editors. Coccolithophores. Berlin: Springer; 2004. p. 481–508.
25. Haynes JR. Foraminifera. 1st ed. London: Palgrave Macmillan; 1981.
26. Theriot E, Herbarium D, Round FE, Crawford RM, Mann DG. The diatoms. Biology and morphology of the genera. Syst Biol. 1992;41:125–6.
27. Parr JF, Taffs KH, Lane CM. A microwave digestion technique for the extraction of fossil diatoms from coastal lake and swamp sediments. J Paleolimnol. 2004;31:383–90.
28. Riding JB, Kyffin-Hughes JE. A direct comparison of three palynological preparation techniques. Rev Palaeobot Palynol. 2011;167:212–21.
29. McLean D, Chisholm JI. Reworked palynomorphs as provenance indicators in the Yeadonian of the Pennine Basin. Proc Yorks Geol Soc. 1996;51:141–51.
30. Pound MJ, O'Keefe JMK, Marret F. An overview of techniques applied to the extraction of non-pollen palynomorphs, their known taphonomic issues and recommendations to maximize recovery. Geol Soc Lond Spec Publ. 2021;511:63–76.
31. Fiorentino A. The potential of nannofossil analysis applied to archaeological studies: the case of the Riace's Bronzes. J Nannoplantkon Res. 1998;20:101–3.
32. Hradil D, Hradilová J, Bezdička P, Švarcová S. Differentiation between anonymous paintings of the 17th and the early 18th century by composition of clay-based grounds. Appl Clay Sci. 2015;118:8–20.
33. Wilkinson I, Quinn P, Williams M, Taylor J, Whitbread I. Ceramic micropalaeontology. In: Hunt A, editor. The oxford handbook of archaeological ceramic analysis. Oxford: Oxford University Press; 2016. p. 265–87.
34. Quinn PS, Day PM. Calcareous microfossils in bronze age aegean ceramics: illuminating technology and provenance. Archaeometry. 2007;49:775–93.
35. Quinn PS. Calcareous nannofossils as a tool for the provenance determination of archaeological ceramics, building materials and related artefacts. In: Williams M, Hill T, Boomer I, Wilkinson IP, editors. The archaeological and forensic applications of microfossils: a deeper understanding of human history. London: The Geological Society on behalf of The Micropalaeontological Society; 2017. p. 159–75.
36. Quinn PS, Day PM. Ceramic micropalaeontology: the analysis of microfossils in ancient ceramics. J Micropaleontol. 2007;26:159–68.
37. Quinn PS. The occurrence and research potential of microfossils in inorganic archaeological materials. Geoarchaeology. 2008;23:275–91.
38. Kędzierski M, Kruk MP. Similarity and provenance of underpainting chalk grounds based on their nannofossil assemblages cluster analysis. J Cult Herit. 2018;34:13–22.
39. Švábenická L, Reháková D, Svobodová A. Calpionellid and nannofossil correlation across the Jurassic–Cretaceous boundary interval, Kurovice Quarry, Outer Western Carpathians. In: Berichte der Geologischen Bundesanstalt. Wien: 10th Int. Symp. Cretaceous; 2017. p. 252.
40. Švábenická L. Coccoliths in the chalk material of high Gothic paintings (14th and 15th centuries, Bohemia). Vestn Cesk Geol Ust. 1994;69:47–51.
41. McIntyre A, Bé AWH. Modern coccolithophoridae of the atlantic ocean—I. Placoliths and cyrtoliths. Deep Sea Res Oceanogr Abstr. 1967;14:561–97.
42. Okada H, Honjo S. The distribution of oceanic coccolithophorids in the Pacific. Deep Sea Res Oceanogr Abstr. 1973;20:355–74.
43. Gibbs SJ, Shackleton NJ, Young JR. Identification of dissolution patterns in nannofossil assemblages: a high-resolution comparison of synchronous records from Ceara Rise, ODP Leg 154. Paleoceanogr Paleoclimatol. 2004;19.
44. Caruso F, Orecchio S, Cicero MG, Di Stefano C. Gas chromatography–mass spectrometry characterization of the varnish and glue of an ancient 18th century double bass. J Chromatogr A. 2007;1147:206–12.
45. Elert K, Herrera A, Cardell C. Pigment-binder interactions in calcium-based tempera paints. Dyes Pigm. 2018;148:236–48.

46. Karakaş F, Çelik MS. Stabilization mechanism of main paint pigments. Progr Org Coat. 2018;123:292–8.
47. Pellegrini D, Duce C, Bonaduce I, Biagi S, Ghezzi L, Colombini MP, et al. Fourier transform infrared spectroscopic study of rabbit glue/inorganic pigments mixtures in fresh and aged reference paint reconstructions. Microchem J. 2016;124:31–5.
48. Bower NW, Blanchet CJK, Epstein MS. Nondestructive determination of the age of 20th-century oil-binder ink prints using attenuated total reflection fourier transform infrared spectroscopy (ATR FT-IR): a case study with postage stamps from the Łódź Ghetto. Appl Spectrosc. 2016;70:162–73.
49. van der Werf ID, Calvano CD, Laviano R, Simonetti A, Sabbatini L. Multitechnique chemical characterisation of a 12–13th-century painted Crucifix. Microchem J. 2013;106:87–94.
50. Colombini MP, Andreotti A, Bonaduce I, Modugno F, Ribechini E. Analytical strategies for characterizing organic paint media using gas chromatography/mass spectrometry. Acc Chem Res. 2010;43:715–27.
51. Tammekivi E, Vahur S, Vilbaste M, Leito I. Quantitative GC-MS analysis of artificially aged paints with variable pigment and linseed oil ratios. Molecules. 2021;26:2218.
52. Andreotti A, Bonaduce I, Colombini MP, Gautier G, Modugno F, Ribechini E. Combined GC/MS analytical procedure for the characterization of glycerolipid, waxy, resinous, and proteinaceous materials in a unique paint microsample. Anal Chem. 2006;78:4490–500.
53. Colombini MP, Modugno F. Characterisation of proteinaceous binders in artistic paintings by chromatographic techniques. J Sep Sci. 2004;27:147–60.
54. Bonaduce I, Cito M, Colombini MP. The development of a gas chromatographic-mass spectrometric analytical procedure for the determination of lipids, proteins and resins in the same paint microsample avoiding interferences from inorganic media. J Chromatogr A. 2009;1216:5931–9.
55. Lluveras A, Bonaduce I, Andreotti A, Colombini MP. GC/MS analytical procedure for the characterization of glycerolipids, natural waxes, terpenoid resins, proteinaceous and polysaccharide materials in the same paint microsample avoiding interferences from inorganic media. Anal Chem. 2010;82:376–86.
56. Quehenberger O, Armando AM, Dennis EA. High sensitivity quantitative lipidomics analysis of fatty acids in biological samples by gas chromatography-mass spectrometry. Biochim Biophys Acta. 2011;1811:648–56.
57. Dettmer-Wilde K, Engewald W, editors. Practical gas chromatography—a comprehensive reference. Berlin: Springer; 2014.
58. Hübschmann H-J. Handbook of GC-MS: fundamentals and applications. 3rd ed. Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA; 2015.
59. Calvano CD, van der Werf ID, Palmisano F, Sabbatini L. Revealing the composition of organic materials in polychrome works of art: the role of mass spectrometry-based techniques. Anal Bioanal Chem. 2016;408:6957–81.
60. Smoluch M, Sobczyk J, Szewczyk I, Karaszkiewicz P, Silberring J. Mass spectrometry in art conservation—with focus on paintings. Mass Spectrom Rev. 2021. https://doi.org/10.1002/mas.21767.
61. Casoli A, Musini PC, Palla G. Gas chromatographic-mass spectrometric approach to the problem of characterizing binding media in paintings. J Chromatogr A. 1996;731:237–46.
62. Dron J, Linke R, Rosenberg E, Schreiner M. Trimethylsulfonium hydroxide as derivatization reagent for the chemical investigation of drying oils in works of art by gas chromatography. J Chromatogr A. 2004;1047:111–6.
63. Mateo-Castro R, Gimeno-Adelantado JV, Bosch-Reig F, Doménech-Carbó A, Casas-Catalán MJ, Osete-Cortina L, et al. Identification by GC-FID and GC-MS of amino acids, fatty and bile acids in binding media used in works of art. Fresenius J Anal Chem. 2001;369:642–6.
64. Hermans JJ, Keune K, van Loon A, Iedema PD. The crystallization of metal soaps and fatty acids in oil paint model systems. Phys Chem. 2016;18:10896–905.
65. Mills JS. The gas chromatographic examination of paint media. Part I. Fatty acid composition and identification of dried oil films. Stud Conserv. 1966;11:92–107.
66. Manzano E, Rodriguez-Simón LR, Navas N, Checa-Moreno R, Romero-Gámez M, Capitan-Vallvey LF. Study of the GC-MS determination of the palmitic-stearic acid ratio for the characterisation of drying oil in painting: La Encarnación by Alonso Cano as a case study. Talanta. 2011;84:1148–54.
67. Gelzo M, Corso G, Pecce R, Arcari O, Piccioli C, Dello Russo A, et al. An enhanced procedure for the analysis of organic binders in Pompeian's wall paintings from Insula Occidentalis. Herit Sci. 2019;7:12.
68. van den Berg JDJ, van den Berg KJ, Boon JJ. Determination of the degree of hydrolysis of oil paint samples using a two-step derivatisation method and on-column GC/MS. Progr Org Coat. 2001;41:143–55.
69. Hünnig Bom M, do MonteGuerra R, Concheyro A, Fauth G. Methodologies for recovering calcareous nannofossils from bituminous claystone. Micropalaeontology. 2015;61:165–70.
70. Green OR. Extraction techniques for calcareous nannofossils. In: Green OR, editor. A manual of practical laboratory and field techniques in palaeobiology. Dordrecht: Springer; 2001. p. 366–74.
71. Kennedy AE, Coe AL. Development of the freeze–thaw processing technique for disaggregation of indurated mudrocks and enhanced recovery of calcareous microfossils. J Micropaleontol. 2014;33:193–203.
72. Hauser LM. An integrated microwave technique for releasing microfossils from an indurated bone bed. J Micropaleontol. 2015;35:2015–2005.
73. Bolli HM, Saunders JB, Perch-Nielsen K, editors. Plankton Stratigraphy: Planktic Foraminifera, Calcareous Nannofossils and Calpionellids. 2nd ed. Cambridge: Cambridge University Press; 1989.
74. Švábenická L. Calcareous nannofossils of the upper karpatian and lower badenian deposits in the carpathian foredeep, moravia (Czech Republic). Geol Carpath. 2002;53:197–210.
75. Gallagher LT. Tertiary calcareous nannofossils from the central and southern North Sea Basins, and their biostratigraphical application. Doctoral dissertation. University of London; 1988.
76. Ghezzi L, Duce C, Bernazzani L, Bramanti E, Colombini MP, Tiné MR, et al. Interactions between inorganic pigments and rabbit skin glue in reference paint reconstructions. J Therm Anal Calorim. 2015;122:315–22.
77. Stover LE. Cretaceous Coccoliths and Associated Nannofossils from France and the Netherlands. Micropaleontology. 1966;12:133–67.
78. Gennaro M, Wonham JP, Gawthorpe R, Sælen G. Seismic stratigraphy of the Chalk Group in the Norwegian Central Graben, North Sea. Mar Petrol Geol. 2013;45:236–66.
79. Dessenne JL, Duffaut P. Les propriétés rhéologiques de la craie et leur influence sur le percement de galeries. La Houille Blanche. 1970;56:477–88.
80. Mills J, White R. Organic chemistry of museum objects. 2nd ed. London: Routledge; 1987.
81. Slánský B. Technika malby Díl I., Malířský a konservační materiál. 2nd ed. Praha: Paseka; 2003.
82. Anantha Padmanabha Setty MG. Preparation and method of study of fossil diatoms. Micropaleontology. 1966;12:511–4.
83. Michalski S. A physical model of the cleaning of oil paint. Stud Conserv. 1990;35 sup1. Preprints of the Contributions to the Brussels Congress, 3–7 September 1990: Cleaning, Retouching and Coating:85–92.
84. Pingitore NE, Fretzdorff SB, Seitz BP, Estrada LY, Borrego PM, Crawford GM, et al. Dissolution kinetics of $CaCO_3$ in common laboratory solvents. SEPM JSR. 1993;63:641–5.
85. Rathouský J, Herman V. Čisticí směs pro odstraňování hydrofobizačních ochranných povlaků na bázi organokřemičitých polymerů (Cleaning mixture for the removal of hydrophobization protective coatings). 2015.
86. Stoye D, Freitag W, editors. Paints, coatings and solvents. 2nd ed. Hoboken: Wiley; 1998.
87. Fields M, Spencer N, Dudhia J, McMillan PF. Structural changes in cartilage and collagen studied by high temperature Raman spectroscopy. Biopolymers. 2017;107:e23017.
88. Veis A, Cohen J. The degradation of collagen. II. The solubilization process in the Acid pH range. J Am Chem Soc. 1955;77:2364–8.
89. Privalov PL. Thermodynamic problems of protein structure. Annu Rev Biophys Biophys Chem. 1989;18:47–69.
90. Lee B. Isoenthalpic and isoentropic temperatures and the thermodynamics of protein denaturation. Proc Natl Acad Sci USA. 1991;88:5154–8.
91. Clark DF. Effects of ultrasonic pressure on calcareous nannofossils. Geology. 1973;1:61–2.
92. Blaj T, Henderiks J. Smear and spray preparation techniques put to the test (II): reproducibility and accuracy of calcareous nannofossil assemblage counts. J Nannoplankton Res. 2007;29:92–100.

93. Backman J, Shackleton NJ. Quantitative biochronology of Pliocene and early Pleistocene calcareous nannofossils from the Atlantic, Indian and Pacific oceans. Mar Micropaleontol. 1983;8:141–70.
94. Lord AR. A stratigraphical index of calcareous nannofossils. Chichester: Ellis Horwood Ltd.; 1982.
95. Andruleit H. A filtration technique for quantitative studies of coccoliths. Micropaleontol. 1996;42:403–6.
96. Saavedra-Pellitero M, Flores Villarejo JA, Baumann KH, Boeckel B, Sierro Sánchez FJ. Comparison of different preparation and analysis techniques for quantitative coccolith studies focusing on biogeographic patterns of species. Micropaleontology. 2011;57:139–61.
97. Negri A, Giunta S, Hilgen F, Krijgsman W, Vai GB. Calcareous nannofossil biostratigraphy of the M. del Casino section (northern Apennines, Italy) and paleoceanographic conditions at times of Late Miocene sapropel formation. Mar Micropaleontol. 1999;36:13–30.
98. Perch-Nielsen K. Eine Preparationstechnik zur Untersuchung von Nanno-plankton im Lichtmikroskop und im Elektronenmikroskop. Medd fra Dansk Geol Forening. 1967;17:129–31.
99. Moshkovitz S. A new method for observing the same nannofossil specimens both by light microscope and scanning electron microscope and preservation of types. Israel J Earth Sci. 1974;23:145–7.
100. Sherwood RW, Levin HL. Scanning electron and optical microscope procedure for viewing of individual Coccoliths. Tulane Stud Geol Paleontol. 1973;10:103–6.
101. Moshkovitz S. New types of cover-slip and mounting-slide with a graticule for examination of the same small object both in light microscope and in scanning electron microscope. Micr Acta. 1978;80:161–6.
102. Stoll HM, Ruiz Encinar J, Ignacio Garcia Alonso J, Rosenthal Y, Probert I, Klaas C. A first look at paleotemperature prospects from Mg in coccolith carbonate: cleaning techniques and culture measurements. Geochem Geophys Geosyst. 2001;2.
103. Koch C, Young JR. A simple weighing and dilution technique for determining absolute abundances of coccoliths from sediment samples. J Nannoplankton Res. 2007;29:67–9.
104. Bairbakhish AN, Bollmann J, Sprengel C, Thierstein HR. Disintegration of aggregates and coccospheres in sediment trap samples. Mar Micropaleontol. 1999;37:219–23.
105. Faimon J, Stelcl J, Kubesová S, Zimák J. Environmentally acceptable effect of hydrogen peroxide on cave "lamp-flora", calcite speleothems and limestones. Environ Pollut. 2003;122:417–22.
106. Chairopoulou MA, Kratzer F, Gross R, Herrmann M, Teipel U. Influence of the temperature on coccolith-containing systems from *Emiliania huxleyi* cultivations. Chem Eng Technol. 2020;43:904–12.
107. Coto B, Martos C, Peña JL, Rodríguez R, Pastor G. Effects in the solubility of $CaCO_3$: experimental study and model description. Fluid Phase Equilib. 2012;324:1–7.
108. Geisen M, Bollmann J, Herrle JO, Mutterlose J, Young JR, Bollmann J, et al. Calibration of the random settling technique for calculation of absolute abundances of calcareous nannoplankton. Micropaleontology. 1999;45:437–42.
109. Katz BJ. Preparation of calcareous nannofossil assemblages for chemical examination. J Paleontol. 1978;52:497–500.
110. Chinard FP. Interactions of quaternary ammonium compounds and proteins. J Biol Chem. 1948;176:1439–47.
111. Cheng H, Chen M, Liao L, Zhiqiang L. Chemical and physical behaviour of collagen fibre in alkaline solutions. J Soc Leath Technol Chem. 2009;93:140–4.
112. Pitthard V, Stanek S, Griesser M, Muxeneder T. Gas chromatography–mass spectrometry of binding media from early 20th century paint samples from Arnold Schönberg's Palette. Chromatographia. 2005;62:175–82.
113. Shilling MR, Carson DM, Khanjian HP. Evaporation of fatty acids and the formation of ghost images by framed oil paintings. Western Association for Art Conservation (WAAC)—Newsletter. 1998;21.
114. Surowiec I, Kaml I, Kenndler E. Analysis of drying oils used as binding media for objects of art by capillary electrophoresis with indirect UV and conductivity detection. J Chromatogr A. 2004;1024:245–54.
115. Ohlrogge J, Thrower N, Mhaske V, Stymne S, Baxter M, Yang W, et al. PlantFAdb: a resource for exploring hundreds of plant fatty acid structures synthesized by thousands of plants and their phylogenetic relationships. Plant J. 2018;96:1299–308.
116 Young JR, Bown PR, Lees JA. Nannotax3 website. Int Nannoplankton Ass. 2022. https://www.mikrotax.org/Nannotax3. Accessed 21Apr 2022.

Publisher's Note