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Integrated Protocol for Phytolith and Diatom Extraction from Carbonised Food Crusts via Ashing V.1

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We use this protocol and it's working

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Abstract

Carbonised food crusts on pottery or other receptacles are valuable contexts for the study of ancient meals as they represent in-situ remnants of ancient meals and other preparations. Yet they represent one of the lesser studied contexts for extracting microremains in archaeological research. A variety of approaches have been published for the extraction of microbotanical remains from food crusts (García-Granero et al. 2018; Lusheck and Thompson 2007; Pigford and Zutter 2015; Saul et al. 2013). Some published protocols for the extraction of microbotanical remains from archaeological carbonised food crusts in attempt to co-extract starches and phytoliths simultaneously from a single food crust, often relying on disaggregation to minimise the impact of sampling (García-Granero, et al. 2018; Saul et al. 2013). These methods often rely on hydrogen peroxide (H₂O₂) to freely liberate food remains from the carbonised food crust matrix. However food crust can be highly durable and resistant to disaggregation, and even if disaggregated, the resulting supernatant will be rich in carbonised particles. If microremain density is low, it is extremely laborious if even possible, to reliably detect microremains in the clutter of carbon. Counting must be aided by removing as much carbon from a sample as practical. Other approaches focus on nitric acid extraction, although effective in isolating phytoliths and producing 'clean' samples, it poses some risks to user health. In this method, we highlight combustion with hydrochloric acid ash removal as a simple, fast, and safe method to produce easy to count silica-based microremains in samples with an optional clay removal step based on the rapid phytolith extraction method (Katz et al. 2010).

Materials

Equipment

Precision scale (at least 0.0001g)
High-speed centrifuge (6000 rpm) for 1.5 mL tubes
Sonicator
Tube racks (1.5 ml)
Fume hood
Vortex mixer
Laboratory drying oven (30–60°C)
Laboratory muffle oven (600°C)
20–50 ml crucibles
Lab coat
Dental cures
Safety glasses
Fine point marker
Pencil

Consumables

3 ml plastic pipette
Microscopy slides
Microscopy cover slips
Non-powdered gloves
Surgical face mask
Recording sheet
Wooden cocktail sticks

Optional

Aluminium foil
Weigh boats
100–1000 µl mechanical pipette

Reagents and mounting media

Entellan® New
Glycerine
Hydrochloric acid, HCl @ 5–12% v/v
Hydrogen peroxide, H₂O₂ @ 30% v/v
Sodium polytungstate, 3Na₂WO₄ · 9WO₃, (SPT) @ 2.35g/cm³



Before start

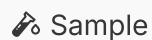
As phytoliths and diatoms are microscopic, risks of unnoticed cross-contamination must be accounted for. Although phytoliths and diatoms are not frequently found as a common laboratory contaminant, there is a risk of cross-contamination in facilities where phytoliths are extracted from reference plants. Working surfaces should be exhaustively cleaned with hot water and soap. While sodium hydroxide-based cleaning will be required if the facility is used for studying ancient starch. Cleaning should be done regularly to minimise risks, particularly in areas where dry sediment or plant extracts are prepared, as well as worktops and around balances.

For the study of starch or other non-silica microremains, subsampling of food crusts is recommended.



Sampling

1



Before commencement of sampling, extant food crusts should be documented in-situ with photography. Carbonised food crusts can be dislodged, ideally in chunks, from pottery or stone cooking vessel surfaces using cleaned dental curesettes onto creased weighing paper. This should ideally be underlain with a layer of smoothened tinfoil laid over the working surface which may catch spilled particles. Collected food crust can be transferred from the creased paper into prelabelled and weighed 1.5 ml plastic centrifuge tubes. The required mass of food crust varies enormously depending on the richness, but with high-carbon situations 50–70 mg is ideal. However this protocol is suitable down to 5 mg. Low mass samples present difficulties such as phytolith or diatom counts may be too low statistically and thus, samples below 10 mg are impractical. In addition, If organic residue analysis is desired with some of the sample, storage in glass, or foil and plastic is preferred.

Ashing

6h 30m

2 The sample can then be transferred into 20–50 ml crucibles labelled with a pencil burned at 500°C for 5 hours in a muffle oven. Higher temperatures increase the risk of damage to phytoliths and diatoms (Arasuna and Okuno 2018; Piperno 2006). If preparing a very large sample, for example, > 1 g, care should be taken to use a larger crucible to avoid creating anaerobic conditions that would prevent the elimination of carbon from the sample.

5h



3 After combustion, the samples can be allowed to cool for about 60 minutes. They can be transferred into new 1.5 ml prelabelled and preweighed centrifuge tubes. If it is rich in clay particles, it may become apparent at this stage through the appearance of a brown to orange colour rather than the expected white/grey colouration. If the sample is rich in clays, an additional step can be taken to reduce them. This is not essential, but it may be helpful. The filled tubes should then be weighed to record the soluble and insoluble fraction weight.

1h 30m



Food crusts from archaeological pottery prior to combustion treatment



Food crusts from archaeological pottery after combustion treatment

Decalcification

2d 1h 5m

- 4 Add ~0.4–0.8 ml Hydrochloric acid (HCl) @ 5–12 % v/v. Saturation of the ashes may be impaired by debris and the process can be aided with tapping the sides of the tube. After the reactions have ceased, which usually takes a few minutes, the tubes can then be diluted with water until full. 25m
- 5 Centrifuge the samples at 2000 × g for 10 minutes. 10m
- 6 The supernatant can be removed with a 3 ml plastic pipette, and water can be added once again to a sample to further remove acid residue. This process should be completed thrice to clean samples of residual acid fully. 30m
- 7 The sample tubes with their Acid Insoluble Fraction (AIF) can now be dried in a drying oven set at 50°C for 48 hours and then they are ready for mounting. 2d



Clay removal (optional)

32m 6s

- 8 In samples where clay removal is required, this can be done after drying using a variant of the Rapid Phytolith Extraction method (Katz et al. 2010). A weighed amount of extract between 20 and 50 mg should be taken in a 0.5 ml conical plastic centrifuge tube. 1m *
- 9 Add 500 μ l of sodium polytungstate solution (SPT, $\text{Na}_6(\text{H}_2\text{W}_{12}\text{O}_{40}) \cdot \text{H}_2\text{O}$) with a specific gravity of 2.35 g/cm³ with a 100–1000 μ l mechanical pipette. 2.35 g/cm³ specific gravity is selected to allow phytoliths and diatoms to float, while low enough to allow quartz, feldspar and other minerals to sink. 1m
- 10 The tube is vortexed for approximately 3 seconds. 3s
- 11 It is then sonicated for about 10 minutes. 10m
- 12 The vortexing is repeated. 3s
- 13 The tube is then centrifuged for 10 minutes at 5000 rpm. 10m
- 14 An aliquot of 50 μ l of the supernatant is pipetted and placed on a glass microscope slide covered with a 24 mm $\times$ 24 mm cover slip. The microremains on the slide can now be counted.
- 15 Alternatively, the SPT can immediately be removed. This involves dilution of the tube with water until full and then centrifuging at 2000 $\times$ g for 10 minutes. Ideally, the waste supernatant can be collected for recycling through particle filtration and evaporation. After drying, the sample is ready for mounting and storage of the remaining sample. 10m

Remove any remaining char products (optional)

3d 0h 1m

- 16 In some samples substantial amounts of char products may remain. In severe cases char can be readily seen as a flecked black component in the sample. These can be reduced through the addition of an oxidation step through hydrogen peroxide (H_2O_2) while wearing safety gloves. *
- 17 Add ~1 ml of 30% hydrogen peroxide to the dry sample in the 1.5 ml tube. Allow gases to escape from the tube. 1m



- 18 Reaction can be encouraged by leaving the tube in a drying oven at 50°C. As the hydrogen peroxide decomposes into water and evaporates, more hydrogen peroxide can be added to further clean the samples if required. Degradation of charcoal can be a lengthy process, taking several days. However, this is an aggressively prolonged treatment and may result in fewer articulated phytoliths (Parr et al. 2001). Remaining hydrogen peroxide can be removed through centrifugation, or gravity sedimentation.

3d

Mounting permanent microscopy slides

2w 0d 0h 6m

- 19 Add ~1mg of the extracted phytoliths and diatoms to a slide. Prepare the weighed amount in a sample boat or add the extract to a slide zeroed on a balance.
- 20 Add two drops of the Entellan New [®] mounting medium with a new sterile disposable pipette. The drops are let run from the pipette shaft rather than released from the pipette bulb.
- 21 Mix the Entellan New [®] and extract powder on the slide with a disposable wooden cocktail stick to evenly distribute the phytoliths/ and diatoms across the slide if a quantitative approach is required.
- 22 Add a clean cover slip on the sample, (coverslip size depends on the volume of the sample and mounting media). 22 mm x 22 mm is often a convenient size.
- 23 Allow the slide to begin curing in a flat position in a fume hood to allow ventilation of toxic fumes. After about a day, an initial examination of the slide can be conducted provided the mounting medium has set at the edges of the coverslip. Failure to do this may result in damage to microscope objectives. Complete drying may take two weeks.

2m

1m

1m

2m

2w

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