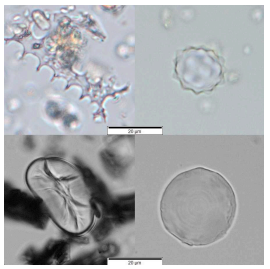
Jan 25, 2023

Protocol for the double extraction of starch grains and phytoliths from sediments, V.1



DOI
https://dx.doi.org/10.17504/protocols.io.5jyl8jdq8g2w/v1

Carlos Santiago-Marrero[1], Jaime R. Pagán-Jiménez[2,3]

[1]Universitat Pompeu Fabra;
[2]Cultural Heritage & Plantscape Research, Bargelaan 200, 2333CW, Leiden, Netherlands;
[3]Royal Netherlands Institute of Southeast Asian and Caribbean Studies, Reuvensplaats 2, 2311BE Leiden, Netherlands.

Carlos G. Santiago-Marrero
Universitat Pompeu Fabra

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.5jyl8jdq8g2w/v1

Protocol Citation: Carlos Santiago-Marrero, Jaime R. Pagán-Jiménez 2023. Protocol for the double extraction of starch grains and phytoliths from sediments, V.1. **protocols.io** https://dx.doi.org/10.17504/protocols.io.5jyl8jdq8g2w/v1

License: This is an open access protocol distributed under the terms of the **Creative Commons Attribution License**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working
We use this protocol and it's working

Created: December 16, 2022

Last Modified: January 25, 2023

Protocol Integer ID: 74104

Keywords: Archaeobotany, Starch grains, Microbotanical remains, Phytoliths, starch grains extraction, full degradation of starch grain, starch grain, gravimetric extraction of lighter starch grain, separation of microbotanical remain, protocols for phytolith extraction, phytoliths from sediment, sodium polytungstate protocol of phytoliths extraction, sediment, removing organic matter, phytoliths extraction, lighter starch grain, sediment sample, archaeological sediments in tandem, utilised chemical reagent, carbonates from sediment, archaeological sediment, extraction, chemical reagent, aggressive chemical reagents for the elimination, microbotanical remain, protocol for the double extraction, aggressive chemical reagent, reagent, heavier phytolith, phytolith, double extraction, organic matter, removing undesired particle, amount of residue, universitat pompeu fabra laboratory of environmental archaeology

Disclaimer

DISCLAIMER – FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK

The protocol content here is for informational purposes only and does not constitute legal, medical, clinical, or safety advice, or otherwise; content added to **protocols.io** is not peer reviewed and may not have undergone a formal approval of any kind. Information presented in this protocol should not substitute for independent professional judgment, advice, diagnosis, or treatment. Any action you take or refrain from taking using or relying upon the information presented here is strictly at your own risk. You agree that neither the Company nor any of the authors, contributors, administrators, or anyone else associated with **protocols.io**, can be held responsible for your use of the information contained in or linked to this protocol or any of our Sites/Apps and Services.

Abstract

Most of the protocols employed for the separation of microbotanical remains from sediments follow the same principles: (1) preparing the sediment samples to be analysed (e.g., weighing, labelling, etc.); (2) deflocculation or particulate dispersal; (3) removing undesired particles (organic matter, carbonates, clay); (4) and extraction of desired remains (e.g., phytoliths, starch grains, fibres or pollen) (Torrence 2006). However, a major concern when applying a combined protocol is the adverse effect that the utilised chemical reagents could have on the proxies intended to be recovered (Pearsall *et al.* 2004, Torrence 2006, Henry *et al.* 2016). This is particularly relevant when targeting microbotanical remains with quite different physicochemical properties. For example, protocols for phytolith extraction from sediments typically employ aggressive chemical reagents for the elimination of organic matter, causing severe damage or the full degradation of starch grains (Coil *et al.* 2003, Piperno 2006, Henry *et al*. 2016, Le Moyne and Crowther 2021).

Many researchers have addressed the possible ways to extract both microbotanical remains from archaeological sediments in tandem (Pearsall *et al.* 2004, Torrence 2006, García-Granero *et al.* 2016, 2018, Henry *et al.* 2016, Pearsall 2016). One of the most common approaches is the “piggy-back” procedure developed by Chandler-Ezell and Pearsall (2003). This method is based on the gravimetric extraction of lighter starch grains with a density that ranges up to 1.60g/cm³ (Henry *et al.* 2016) and heavier phytoliths ranging in density up to 2.3g/cm³ afterwards (Pearsall *et al.* 2004, Piperno 2006). This way, starch grains are not exposed to the reagents employed for removing organic matter and carbonates from sediments. The protocol here presented is a modified version of the protocol for phytoliths and starch grains extraction described by Pearsall 2016 (363 - 364), and the Sodium Polytungstate protocol of phytoliths extraction developed by Madella and colleagues (1998), used at the Universitat Pompeu Fabra Laboratory of Environmental Archaeology. The whole process takes around 7 to 14 days to be completed, although time may vary depending on the amount of residue or sediment to be analysed.

Image Attribution

Phytolith and starch images by Carlos G. Santiago-Marrero.

Guidelines

Recycling of reagents

Instructions for recycling SPT are available on the Sometu website under the “How to use” section of the product description. Link: http://www.sometu.de/howtouse.html.

Disposal of used reagents after use

Used reagents must be discarded following the parameters established by the regulatory entities and university or research regulations of each country.

Materials

Equipment

Precision scale (at least 0.0001g)
Sonicator (ultrasonic and heated bath)
High-speed centrifuge (6000 rpm) for tubes of 5 mL
High-speed centrifuge (4000 rpm) for tubes of 50 mL
Vortex mixer
Tube racks (2 ml and 50 ml)
Fume hood
Laboratory drying oven (30-60ºC)
Lab coat
Safety glasses
Safety earmuff
Fine point marker
Pencil

Consumables

Sterile microcentrifuge tubes (2 mL)
Sterile centrifuge tubes (50 mL)
Sterile pipette tips (100 - 1000µl)
Sterile disposable pipette
Sterile glass vials (10 mL)
Microscopy slides
Microscopy cover slips
Non-powdered gloves
Hairnet (recommendable)
Surgical face mask
Aluminium foil
Adhesive labels
Recording sheet

Reagents and mounting media

Distilled water, H_2O
Entellan® New
Ethanol, C_2H_6O @ 10% v/v
Glycerine
Hydrochloric acid, HCl @ 5% v/v
Hydrogen peroxide, H_2O_2 @ 30% v/v and 8% v/v
Sodium hexametaphosphate, $(NaPO_3)_6$ (Calgon) @ 5% v/v
Sodium hypochlorite, NaOCl (Bleach) @ 5% v/v
Sodium polytungstate, $3Na_2WO_4 \cdot 9WO_3$, (SPT) @ 1.8g/cm3 and 2.30g/cm3

Safety warnings

Description of the chemicals and safety guidelines

Entellan® New
(CAS-No.) 1330-20-7
(EC-No.) 1272/2008
H226 Flammable liquid and vapour
H312 + H332 Harmful in contact with skin or if inhaled
H315 Causes skin irritation

P210 Keep away from heat.
P302 + P352 IF ON SKIN: wash with plenty of soap and water
P304 + P340 IF INHALED: remove victim to fresh air and keep at rest in a position comfortable for breathing.

Glycerine
(CAS-No.) 56-81-5
(EC-No.) 200-289-5
GHS Classification of substance in accordance to Regulation (EC) No. 1272/2008 (CLP/GHS): Not a hazardous substance or mixture.

Potential health effects
Can be irritating to the eyes
Can be harmful if ingested
Can be harmful if inhaled. Avoid breathing mist.
Can be irritating to the skin.

Most important symptoms
After inhalation: ON HEATING: irritation of respiratory tract.
After eye contact: redness, lacrimation.
After ingestion: nausea, vomiting, diarrhoea.

First Aid Measures
IF INHALED: remove the victim into fresh air. If symptoms continue, get medical attention.
ON SKIN contact: Wash immediately with soap (15 min).
IN EYE contact: Rinse immediately with water for 15 min. Get medical attention if symptoms persist.
IF INGESTION: DO NOT induce vomiting. If vomiting occurs, have the victim lean forward to prevent aspiration. Seek medical attention.

Special hazard

DIRECT FIRE: combustible, keep away from flames.
INDIRECT FIRE: temperature above flashpoint: higher fire/explosion hazard.

Hydrochloric acid
(CAS-No.) 7647-01-0 (EC-No.) 231-595-7;231-596-7
(EC Index-No.) 017-002-01-X
(REACH-no) 01-2119484862-27.

H314 - Causes severe skin burns and eye damage.
H335 - May cause respiratory irritation.

P301+P330+P331 - IF SWALLOWED: rinse mouth. Do NOT induce vomiting.
P303+P361+P353 - IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water/shower. P304+P340 - IF INHALED: Remove person to fresh air and keep comfortable for breathing.
P305+P351+P338 - IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present, and easy to do. Continue rinsing.
P310 - Immediately call a POISON CENTER/doctor.

Hydrogen peroxide
(CAS-No.) 7722-84-1 (EC-No.) 231-765-0 (EC Index-No.) 008-003-00-9
(REACH-no) 01-2119485845-22.

H271 - May cause fire or explosion; strong oxidiser.
H332 - Harmful if inhaled.
H302 - Harmful if swallowed.
H314 - Causes severe skin burns and eye damage.

P210 - Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking. P280 - Wear protective gloves/protective clothing/eye protection/face protection.
P301+P330+P331 - IF SWALLOWED: rinse mouth. Do NOT induce vomiting.
P304+P340 - IF INHALED: Remove person to fresh air and keep comfortable for breathing.
P310 - Immediately call a POISON CENTER/doctor.

Sodium hexametaphosphate
(CAS-No.) 68915-31-1 (EC-No.) 272-808-3
(EC Index-No.) not applicable.
(REACH-no) 05-2118406971-42.

IF INHALED: fresh air.
IF ON SKIN: Take off immediately all contaminated clothing. Rinse skin with water/ shower.
IF IN EYES: rinse out with plenty of water. Remove contact lenses.

IF SWALLOWED: after swallowing: make the victim drink water (two glasses at most). Consult a doctor if feeling
unwell.

Sodium hypochlorite
(CAS-No.) 7681-52-9 (EC-No.) 231-668-3
(EC Index-No.) 017-011-00-1
(REACH-no) not applicable.
H290 May be corrosive to metals.
H314 Causes severe skin burns and eye damage.
H410 Very toxic to aquatic life with long-lasting effects.

P301+P330+P331 IF SWALLOWED: rinse mouth. Do NOT induce vomiting.
P303+P361+P353 IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water [or shower].
P305+P351+P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P310 - Immediately call a POISON CENTER/doctor.

Sodium polytungstate
(CAS-No.) 12141-67-2 (EC-No.) 412-770-9
(EC Index-No.) 074-001-00-X
(REACH-no) 01-2120061128-61-xxxx.

H302 - Harmful IF SWALLOWED.
H318 - IF ON EYES: Causes serious eye damage.
H412 - Harmful to aquatic life with long-lasting effects.

P305+P351+P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P310 - Immediately call a POISON CENTER/doctor.

Before start

Lab environment contamination, even cross-contamination of archaeological samples in laboratories, is a constant concern in microbotanical research (Crowther *et al*. 2014, Henry 2020, Pagán-Jiménez *et al.* 2016). This is particularly important for starch due to the industrial use of wheat, potato and maize starch in everyday products that may cause false positives (modern starch contaminant) (Fullagar 2006, Crowther *et al*. 2014, Henry 2020). Once modern starch grains (and phytoliths to a certain extent) are mixed with archaeological sediment samples, it becomes impossible to differentiate between them (Henry 2020). For that reason, it is advisable to follow protocols for avoiding contamination starting in the field, once the archaeological feature or artefact to be analysed has been exposed (Mercader *et al.* 2017).

All surfaces and materials in the laboratory should be exhaustively cleaned in advance using a 5% v/v solution of Sodium hypochlorite (Bleach) which hydrolyses and destroys starch grains that may be present in the working area and on equipment (modern contaminants) (Ragheb *et al.* 1995, Crowther *et al.* 2014). It is advisable to submerge all equipment (e.g., tubes, vials, spatulas, pipette tips, etc.) in boiling water after cleaning with bleach in order to completely eradicate any potential starch grain contaminant that may have survived the chemical exposure. The ventilation in the laboratory must be turned off, and only one person should be present during the extractions to avoid airborne starch or the air movement and suspension of particles. Hairnets and face masks should be worn all the time, and no gloves should be used during starch grains procedures (even non-powdered globes can contain starch grains). Hands should be constantly washed with a starch-free soapy solution at intervals between sample preparation to avoid any other source of contamination.

Before starting the extraction procedure: label a new sterile pipette tip (100-1000µl) for each sample and place the pipette tip box in a new clean plastic bag. This tip will be used throughout the whole extraction process, leaving at least 4 empty spaces between each tip to avoid cross-contamination. Label a new 50 mL and 2 mL centrifuge tube, and a 10 mL glass vial for each sample, weigh after labelling (with the cap on) and record the weight of each recipient (Figure 1).

Note

- This procedure is designed for 50 mL centrifuge tubes and a standard amount of 4g of sediment sample. When analysing smaller amounts of sediments (e.g., sediment from grinding stones, lithic tools or pottery) adjust the quantities accordingly.
- The precision pipette and tips can be substituted with individually wrapped disposable sterile pipettes (Figure 2). Make sure to label the pipette and its wrap, 1 for each sample to be analysed.

Figure 1. Labelled 50 mL centrifuge tube, pipette tip (100-1000µl), Eppendorf® tube and a glass vial: on the side (left) and on the top (right).

Figure 2. Labelled wrapped 3 mL plastic sterile disposable pipette.

Prepare in advance a sample track sheet to record every step in the extraction procedures (Table 1). The format of the sheet may change based on the researcher's preference, but in general, it should contain five main items related to the protocol's general steps: (1) Clays deflocculation; (2) Starch grains; (3) Carbonates and organic matter elimination; (4) Phytoliths (5) Microscopy slide mounting (Figure 3).

Combined Starch and Phytolith Protocol Recording Sheet														
Sample	Defluctuating				Starch			Phytolith						
Lab ID	Analysed sample (g)	Tube weight (g)	S in tube (g)	Samples (g) after Calgon	Vial weight (g)	Vial sample Dry	Extracted starch	After Digestion	(AIF)	Vial weight (g)	Vile (g) (w/sample)	Extracted Phy	Slide Weight	Extract on slide g

Table 1. Recording sheet example.

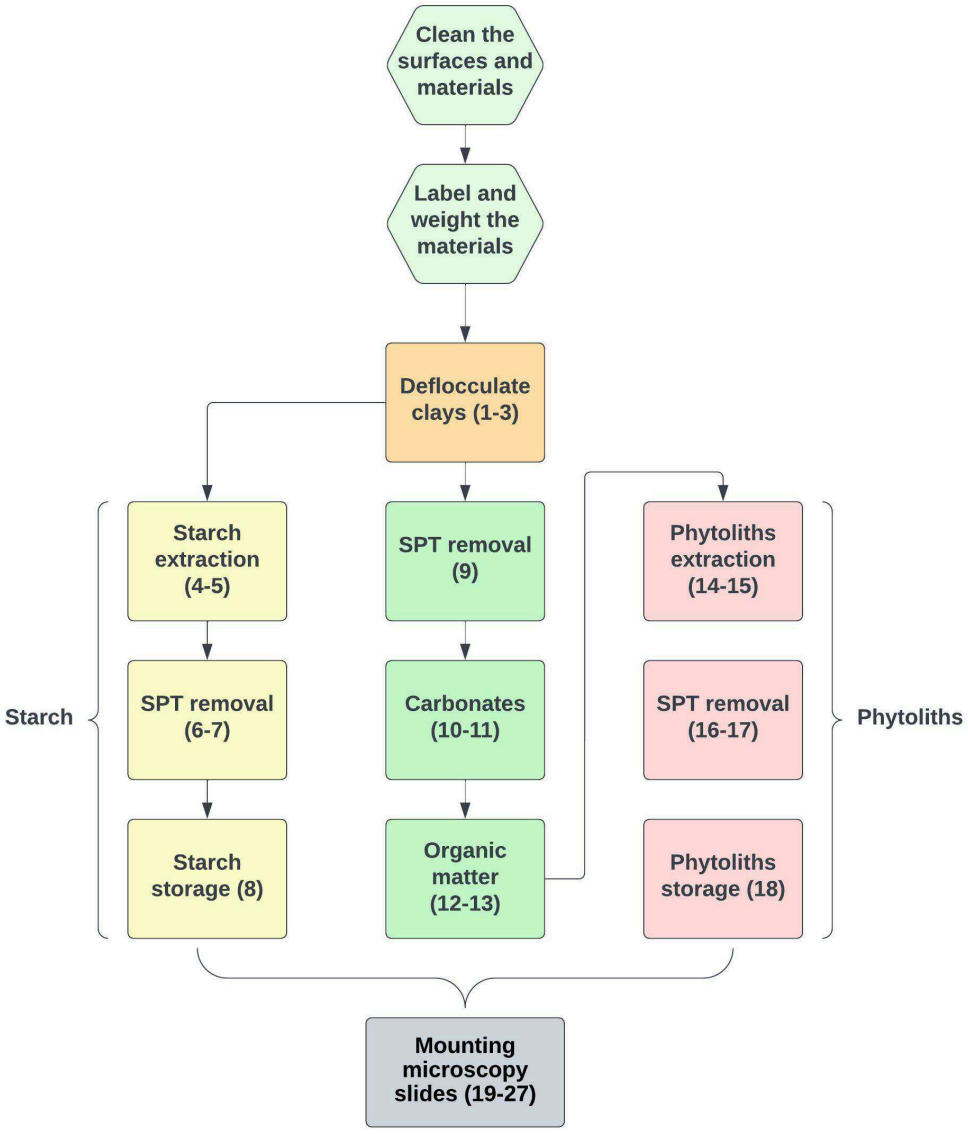


Figure 3. Summary of the steps followed for the double extraction protocol of starch grains and phytoliths.

References

-Chandler-Ezell, K. and Pearsall, D.M., 2003. Piggyback microfossil processing: Joint starch and phytolith sampling from stone tools. *Phytolitharian Newsletter*, 15 (3), 2–8.
-Coil, J., Alejandra Korstanje, M., Archer, S., and Hastorf, C.A., 2003. Laboratory goals and considerations for multiple microfossil extraction in archaeology. *Journal of Archaeological Science*, 30 (8), 991–1008.
-Crowther, A., Haslam, M., Oakden, N., Walde, D., and Mercader, J., 2014. Documenting contamination in ancient starch laboratories. *Journal of Archaeological Science*, 49, 90–104.
-Fullagar, R., 2006. Starch on Artefacts. *In*: R. Torrence and H. Barton, eds. *Ancient Starch Research*. Walnut Creek, California: Left Coast Press., 177–204.

-García-Granero, J.J., Lancelotti, C., and Madella, M., 2016. A methodological approach to the study of microbotanical remains from grinding stones: a case study in northern Gujarat (India). *Vegetation History and Archaeobotany*, 26 (1), 43–57.
-García-Granero, J.J., Urem-Kotsou, D., Bogaard, A., and Kotsos, S., 2018. Cooking plant foods in the northern Aegean: Microbotanical evidence from Neolithic Stavroupoli (Thessaloniki, Greece). *Quaternary International*, 496, 140–151.
-Henry, A.G., ed., 2020. *Handbook for the Analysis of Micro-Particles in Archaeological Samples*. Cham: Springer International Publishing.
-Henry, A.G., Debono Spiteri, C., Büdel, T., Hutschenreuther, A., Schmidt, S., and Watzke, J., 2016. Methods to isolate and quantify damaged and gelatinized starch grains. *Journal of Archaeological Science: Reports*, 10, 142–146.
-Le Moyne, C. and Crowther, A., 2021. Effects of chemical pre-treatments on modified starch granules: Recommendations for dental calculus decalcification for ancient starch research. *Journal of Archaeological Science: Reports*, 35, 102762.
-Madella, M., Powers-Jones, A.H., and Jones, M.K., 1998. A Simple Method of Extraction of Opal Phytoliths from Sediments Using a Non-Toxic Heavy Liquid. *Journal of Archaeological Science*, 25 (8), 801–803.
-Mercader, J., Abtosway, M., Baquedano, E., Bird, R.W., Díez-Martín, F., Domínguez-Rodrigo, M., Favreau, J., Itambu, M., Lee, P., Mabulla, A., Patalano, R., Pérez-González, A., Santonja, M., Tucker, L., and Walde, D., 2017. Starch contamination landscapes in field archaeology: Olduvai Gorge, Tanzania. *Boreas*, 46 (4), 918–934.
-Pagán-Jiménez, J.R., Guachamín-Tello, A.M., Romero-Bastidas, M.E., and Constantine-Castro, A.R., 2016. Late ninth millennium B.P. use of Zea mays L. at Cubilán area, highland Ecuador, revealed by ancient starches. *Quaternary International*, 404, 137–155.
-Pearsall, D.M., 2016. *Paleoethnobotany: A Handbook of Procedures*. Third. London & New York: Routledge.
-Pearsall, D.M., Chandler-Ezell, K., and Zeidler, J.A., 2004. Maize in ancient Ecuador: results of residue analysis of stone tools from the Real Alto site. *Journal of Archaeological Science*, 31 (4), 423–442.
-Piperno, D.R., 2006. *Phytoliths: A Comprehensive Guide for Archaeologists and Paleoecologists*. AltaMira Press.
-Ragheb, A., Abdel-Thalouth, I., and Tawfik, S., 1995. Gelatinization of Starch in Aqueous Alkaline Solutions. *Starch - Stärke*, 47, 338–345.
-Torrence, R., 2006. Starch in Sediments. *In*: R. Torrence and H. Barton, eds. *Ancient Starch Research*. Walnut Creek, California: Left Coast Press., 145–176.

Clays deflocculation

3m

1 Take a labelled and previously weighed new 50 mL sterile tube and carefully add the corresponding sample to be processed. Cover the mouth of the tube with aluminium foil and poke small holes (Figure 4). This will allow the samples to dry and minimise the chance for airborne contaminants to enter the sample by accident. Put the tube with the sample into the drying oven at a maximum temperature of 32°C to avoid starch gelatinisation. Weigh the tube until it stops losing weight, then the sample will be completely dry. Weigh the tube again with the cap on, and add (up to 45 mL) a 5% v/v solution of Sodium hexametaphosphate (Calgon), close the tube, shake and leave Overnight .

Note

- Shake the samples by hand or with a vortex mixer.
- Use an ultrasonic bath in case sediments are too compacted or clay aggregates are present (until sediment is loosened).

Safety information

- Wear a lab coat and safety glasses.
- Chemical handling must be done inside the fume hood.
- Wear a safety earmuff when using the ultrasonic bath.

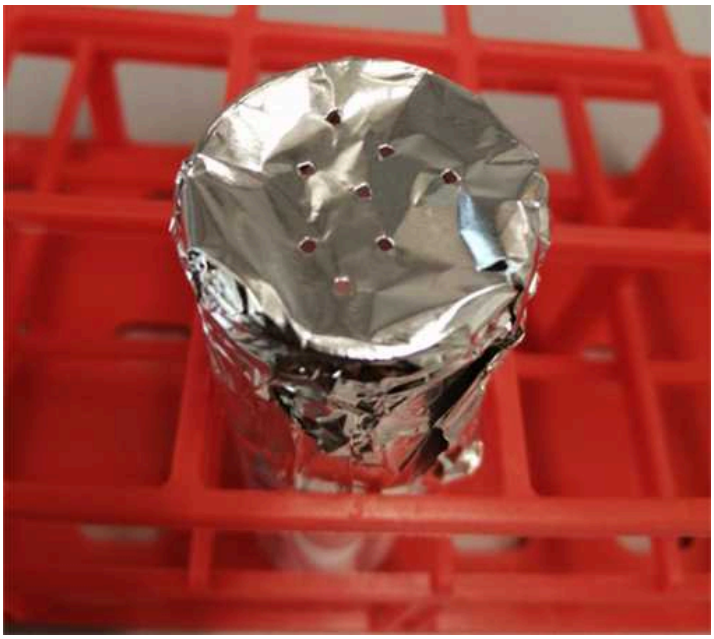


Figure 4. Example of how to cover with aluminium foil.

2 After 24:00:00, shake the samples with Calgon (Figure 5), centrifuge for 00:03:00 at 1500 rpm and discard the supernatant always leaving around 3 to 5 mL of supernatant above the sediment in the tube. Top up with distilled H_2O up to the 45 mL mark and centrifuge again for 00:03:00 at 1500 rpm and discard the supernatant one more time. Repeat this step at least 4 more times, or until you are able to clearly see through the supernatant in the tube. 1d 0h 6m

Note

- Make sure that no sediment remains attached to the internal tubes' side walls when using a centrifuge with a rotor accommodating tubes at an angle.
- Use a new sterilised and labelled plastic pipette (one pipette for each sample) to remove the supernatant.

Figure 5. Sediment samples after 24 h in Calgon.

3 Discard the supernatant once more, leaving around 2 to 3 mL of supernatant in the tube, cover the mouth of the tube with aluminium foil and poke small holes. Repeat the drying process described in step 1, at no more than 32°C. Once dried, weigh the tube with the sample and the cap on, and subtract the original weight of the empty tube to calculate the amount of removed clays.

Starch grains extraction

3m

4 Add 5 mL of Sodium polytungstate (SPT) with a specific gravity of 1.8g/cm3. Add more if necessary, it should double the amount of sediment in the tube. Shake gently and centrifuge for 00:03:00 at 1500 rpm. 3m

5 Recover all the floating fraction with the corresponding sterile pipette tip and transfer it to the labelled and weighed new 2 mL tube (Figure 6). Do not top up the tube above half its capacity (no more than 1 mL).

Note

- This step can be repeated to make sure to recover all the starch grains in the sediment sample and the material that could remain attached to the tube walls.
- If the floating fraction is too large for the 2 mL tube, transfer the recovered portion to a new labelled 50 mL tube and continue with the SPT removal as described in steps 16 and 17. Transfer it back to the corresponding tubes afterwards.

Figure 6. Floating fraction containing starch grains and other particles with the same gravimetry.

6 **SPT removal** 3m

Add distilled H_2O to the tube with the recovered floating fraction up to the 2 mL mark and centrifuge for 00:03:00 at 7000 rpm.

7 3m

Pipette out the supernatant with the corresponding labelled pipette tip until the 1.5 mL mark, and save the supernatant to recycle the SPT. Top up with distilled H_2O, shake gently and centrifuge for 00:03:00 at 7000 rpm two more times (3 in total). Be mindful that at this point, the density remains high, and starch grains could still be suspended in the middle part of the solution (Figure 7A). Repeat the process two more times until the 1 mL mark, and then two more times until reaching the 0.5 mL mark. Afterwards, do not top up the tube anymore. At this point, the sample should have been washed 7 times in total (Figure 7B).

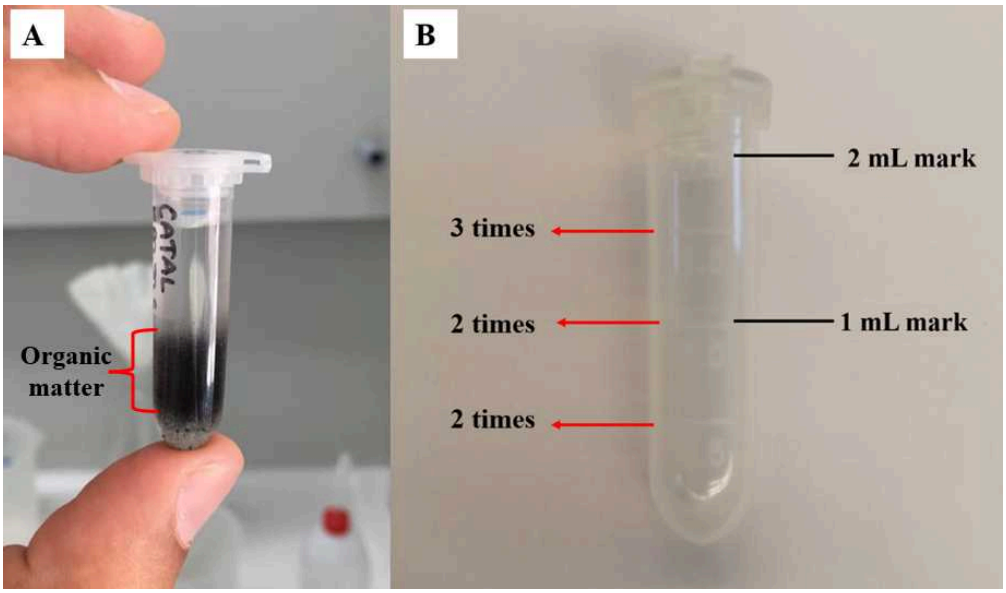


Figure 7. Example of the SPT removal process. **A.** Floating fraction after first centrifuge round. Notice the organic matter suspended between (or from) the bottom and/or to the middle of the tube; **B.** Guide for the washing procedure.

8 **Starch residue storage**

Cover the mouth of the tube with aluminium foil paper and poke small holes (Figure 8). Repeat the drying process described in step 1, at no more than 32°C to avoid starch gelatinisation by heat. Weigh the tube with the dry sample and subtract the original weight of the empty tube to know the amount of the recovered starch sample. Starch samples are ready to be mounted or stored.

Note

The recovered sample could contain high levels of organic matter (e.g., micro-charcoal). In those cases, the recovered fraction can be subjected to mild oxidation using hydrogen peroxide at **8% v/v** following the instructions described in steps 12 and 13.

Figure 8. Example of a 2 mL tube containing the washed recovered floating fraction ready to dry.

Carbonates and organic matter elimination

9m

9 **SPT removal from remaining sediments** 3m

Add distilled H_2O up to the 45 mL mark to the original 50 mL tube containing the sediment sample, and centrifuge for 00:03:00 at 1500 rpm. Remove the supernatant, always leaving around 3 to 5 mL of supernatant above the sediment in the tube, save the supernatant to recycle the SPT. Top up with distilled H_2O and repeat this step three more times (more if the supernatant is still not clear).

Safety information

Turn on the ventilation, and wear safety glasses and gloves from now on.

10 **Carbonates elimination**

Discard the supernatant leaving around 2 to 3 mL in the tube and add 15 mL of Hydrochloric acid (HCl) at 5% v/v to the 50 mL tubes. This step should be done

progressively with a disposable pipette to avoid aggressive reactions. Place the tubes with the samples in a water bath at approximately 40°C until the reaction stops (e.g., no more bubbling occurs when adding HCl). Do not seal the tubes to allow gas to be released.

Note

Stop adding HCl if the reaction is too strong and starts to overflow. Wait until it stops reacting, or add distilled water to the tube if the reaction is too violent.

11 **HCl removal** 3m

Top up the 50 mL tube with distilled H_2O up to the 45 mL mark, shake gently and centrifuge for 00:03:00 at 1500 rpm and discard the supernatant always leaving around 3 to 5 mL of supernatant above the sediment in the tube. Repeat this step at least three more times (more if the supernatant is still not clear).

12 **Organic matter elimination**

Discard the supernatant leaving around 2 to 3 mL of supernatant above the sediment in the tube and add 15 mL of Hydrogen peroxide (H_2O_2) at 33% v/v. Place the tube with the sample in a water bath at approximately 40°C until the reaction stops (e.g., no more bubbling occurs when adding a drop of H_2O_2). Do not seal the tube to allow gas to be released.

13 **H_2O_2 removal** 3m

Top up the 50 mL tube with distilled H_2O up to the 45 mL mark, shake gently and centrifuge for 00:03:00 at 1500 rpm and discard the supernatant leaving around 3 to 5 mL of supernatant above the sediment in the tube. Repeat this step at least three more times (more if the supernatant is not clear).

14 Discard the supernatant leaving around 2 to 3 mL of supernatant above the sediment in the tube, cover the mouth of the tube with aluminium foil and poke small holes. Dry in a drying oven at 60-70°C following the drying process described in step 1. Weigh the tube with the dry sample again with the cap on and subtract the original weight of the empty tube to obtain the weight of the remaining sediment sample after the carbonates and organic matter elimination, also known as the Acid Insoluble Fraction (AIF).

Phytoliths extraction

9m

15 3m

Add 15 mL of SPT with a specific gravity of 2.35g/cm3. Adjust the amount of added SPT if necessary, it should double the amount of sediment in the tube. Shake gently and centrifuge for 00:03:00 at 1500 rpm.

16 Recover the floating fraction (residues of interest or silicates) with the corresponding labelled pipette tip and transfer it to a new labelled 50 mL tube. The entire floating fraction has to be recovered.

Note

This step can be repeated one more time to make sure to recover all the silicates in the sediment sample.

17 3m

SPT removal

Top up the tube with the recovered floating fraction with distilled H_2O until the 45 mL mark and centrifuge for 00:03:00 at 2000 rpm. Discard the supernatant until the 20 mL mark, leaving the residue at the bottom. Save the supernatant and recycle the SPT.

18 3m

Top up with distilled H_2O until the 45 mL mark, shake gently and centrifuge for 00:03:00 at 2000 rpm three more times and discard the supernatant until the 5 mL mark, leaving the residue at the bottom. The supernatant of the last two washes can be discarded.

19 **Phytoliths (silicates) storage**

Use the corresponding pipette tip to transfer the remaining H_2O and residues at the bottom of the tube to the corresponding labelled and weighed 10 mL glass vial and let it dry in the drying oven at 60-70°C following the drying process described in step 1. After drying, weigh the vial with the residues (silicates) and subtract the original weight of the vial to know the amount of extracted silicates. The phytolith sample is ready to be mounted on a microscopy slide or stored.

Mounting microscopy slides - starch grains

20 Wash a new microscopy slide and coverslip with a 5% v/v solution of bleach. Rinse first with distilled H_2O, and then with ethanol. Let it dry and label the microscopy slide.

21 10m

With a new sterilised pipette, add a few drops of the mounting medium (a solution of 50% glycerine and 50% distilled H_2O) to the 2 mL tube containing the dried extracted

starch sample, mix and let it hydrate for 00:10:00. Always use a new sterilised pipette per sample.

Note

The amount of mounting medium to be added may vary depending on the amount of sample and the presence of other particles such as micro-charcoal. Usually, 2 drops are enough.

22 Add a few drops of the solution from the 2 mL tube to the corresponding clean and labelled microscopy slide and cover with a clean cover slip. The slide is ready to be studied under the microscope.

Note

The amount of starch sample to be added to the slide may vary depending on the size of the cover slip. Around 2 to 3 drops should be added when using a 24 mm x 24 mm cover slip.

23 Once the scanning of the slide is done, store it in a flat position in a microscopy slide folder or microscopy slide storage box.

Note

Optional: use clear nail polish to fix the cover slip to the slide. This step should be done at the very end of the study since nail polish can cause cross-contamination from one slide to another.

Mounting microscopy slides - phytoliths

24 Wash with ethanol, label and weigh a new microscopy slide.

25 Place around 1 mg of the corresponding extracted silicates on the slide and weigh the slide with the silicates to know the exact weight of the sample on the slide.

26 Add a few drops of the mounting medium (in this case, Entellan New®) with a new sterile disposable pipette. Mix the Entellan New® and silicates on the slide with a

stainless-steel micro spatula. Wash the spatula with ethanol very well before and after mounting a new slide.

Note

The amount of mounting medium to be added may vary depending on the size of the cover slip. Around 4 drops should be enough when using a 24 mm x 24 mm cover slip.

27 Place a clean cover slip on the sample and leave the slide in a flat position in the fume hood to let the sample sit and eliminate noxious fumes. After a few hours, the slide is ready to be studied under the microscope.

28 After finishing with the slide scanning, leave the slide for two weeks in the fume hood to let the Entellan New® dry. Then, store the slide in a microscopy slide folder or microscopy slide storage box.