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Acid-free protocol for extracting fossil pollen from Quaternary sediments

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Abstract

Quaternary palynology commonly requires the use of acids such as hydrogen fluoride (HF), sulfuric acid (H₂SO₄), hydrochloric acid (HCl) to extract pollen grains and spores from sediments. In South America, the restricted access of these acids made the development of fossil pollen studies challenging. Here we propose an alternative protocol for pollen processing exempted of the use of acids. This protocol was developed to enable broader, safer, and less expensive access to palynological research since the barriers imposed by reagent controls are withdrawn. Moreover, our protocol responds to environmental protection policies. Special attention was paid to the quality of the pollen grains and spores obtained at the end of processing with similar or even better preservation than conventional protocols. Also, our protocol was tested on three different sediment environments (peat, lake, and swamp). Our successful and satisfactory results provide new research opportunities in Quaternary palynology for those countries where the reagents are not available or too expensive.

Keywords: pollen extraction; sediment; hydrofluoric acid; clay

1. Introduction

Pollen grains are part of the plant reproductive process and are found in different types of sediments due to a range of dispersion modes (Heusser & Stock 1984; Erdtman 1986; Traverse 1988; Faegri & Iversen 1989; Moore, Webb, et al. 1991; Colinvaux et al. 1999; Traverse 2007). The extraction of the pollen grains from sediment requires the use of acids such as Hydrofluoric acid (HF), Hydrochloric acid (HCl), and Sulfuric acid (H₂SO₄) to eliminate the mineral and organic components of the sediment (Assarsson & Granlund 1924; Lennie 1968; Heusser & Stock 1984; Faegri & Iversen 1989; Moore, Weeb, et al. 1991; Colinvaux et al. 1999; Bennett & Willis 2001; Traverse 2007). The pollen grain external structure is very resistant to those physical and chemical treatments. However, in many countries, most of these acids have their usage and access controlled by rigorous regulations since they are categorized as highly hazardous chemicals to human health, physical structures, drug preparation, and destructive for the environment. This constraint drastically prevented the development of this field of research in South America or in Africa.

As most existing protocols were designed for organic matter-rich sediment (Bates 1978), the main recurring concern among Quaternary palynologists regarding an acid-free procedure lies in the preparation of samples enriched in clay. Indeed, for these samples, the large amount of clay particles prevents the proper observation of the pollen grains and spore as they become too diluted or masked by silica particles, hindering the observations. However, in some protocols (Bates 1978; Heusser & Stock 1984), a deflocculating clay step was added to solve this problem. The authors added a sodium pyrophosphate solution to the samples in an early step of the

procedure as a clay deflocculant, using sieves to separate and discard the clay and applying the traditional acidic chemical protocol to the samples.

Here we propose an alternative acid-free palynological processing protocol to facilitate palynological research in places where these acids are not accessible. Our protocol uses sodium metaphosphate (NaPO_3) as a clay deflocculant, potassium hydroxide (KOH) as a detergent, and zinc chloride (ZnCl_2) as a precursor for the flotation of the pollen grains. The proposed protocol was tested on three different sediment depositions, peat, lacustrine, and swamp. Finally, our results were compared to equivalent samples treated by the conventional acidic protocols.

2. A brief review of some main acid-free techniques

In palynology, traditional laboratory techniques for palynomorph extraction use acid digestion in sedimentary rocks or unconsolidated sediments. These chemical treatments are at environmental and health risks and have high costs. Some techniques without acids have been developed to reduce risks and costs but maintain the same analytical pattern as traditional methods described below.

One of the first protocols developed without the use of acids was the method of separating microfossils by deflocculation was Knox (1942), using bromoform (CHBr_3) at 2.3 g.cm^{-3} and acetone ($\text{C}_3\text{H}_6\text{O}$). According to the author, this method is very efficient for samples with a low microfossil concentration. Arms (1960) analysed material from southwestern USA and Mexico and concentrated the palynomorphs once verifying the formation of insoluble colloids after the HF step. The technique consists of separating palynomorphs based on a gas separation: the solution prepared with 5% of water-soluble depressant, 5% of pine oil in distilled water, and a laboratory detergent is mixed with the sample. The chemical reaction forms bubbles

of gas and palynomorphs which are then easily separated. Clay dispersants are widely used in many protocols in palynology, such as sodium pyrophosphate ($\text{Na}_4\text{P}_2\text{O}_7$), as described by Brown (1960), who showed the effectiveness but stressed that when heated, $\text{Na}_4\text{P}_2\text{O}_7$ could destroy pollen grains by oxidation. However, other studies, for instance, Bates (1979) did not observe any oxidation in the clay dispersion process. The technique described by Eagar & Sarjeant (1963) for the extraction of dinoflagellate cysts consists of samples soaked in water, sieved, and boiled with sodium carbonate (Na_2CO_3) to deflocculate the clay. This process was repeated until the material was cleaned and the palynomorphs were extracted. After obtaining unsatisfactory results with traditional protocols in samples of Tertiary shales from Costa do Golf, USA, Felix (1963) described a technique without acids. His method consists of washing the samples with distilled water and a rotary washer for up to 48 hours. The disaggregated sample is centrifuged, and the organic fraction is dried in an oven at 70°C and macerated with mortar. The powder is placed in a solution of bromoform and toluene (C_7H_8) with a specific density of 2.3 g.cm^{-3} in a micro blender in which the residue is gently agitated, thus allowing vegetal and animal remains to float free of the heavier minerals. The study of the extraction of pollen from Quaternary sediments carried out by Dodson (1983) to test the effectiveness between the method that uses acid digestion and another by ultrasonic dispersion of clay in bromoform showed that the highest concentration of pollen and lowest amount of organic matter were found in the ultrasonic dispersion method compared to acid digestion. However, the ultrasonic process damaged thin-exine-pollen grains. Krukowski (1988) described a technique for extracting Conodont's microfossils from sediments from Mississippi and Pennsylvania, New Mexico, using sodium metatungstate ($\text{H}_2\text{O}_{40}\text{W}_{12}\cdot 6\text{Na}$), a non-toxic heavy liquid reaching an

effectiveness close to 100%. Clarke (1994) described an experiment involving a traditional acid digestion (HF, HCL, acetolysis) and two non-acidic techniques: the sieved and heavy liquid separation. According to the author, all three methods have advantages and disadvantages, but the traditional method with the use of acids generally proved to be the most effective technique. Acid-free preparation carried out by Riding (2004) on samples from the United Kingdom and Antarctica dating back to Jurassic to Quaternary. Sodium hexametaphosphate ($(\text{NaPO}_3)_6$) is used as a deflocculant and hydrogen peroxide (H_2O_2) to disaggregate the material. This technique requires a preliminary cleaning of the sample with water and detergent, a fragmentation and a maceration. The sample is first treated with sodium hexametaphosphate and the deflocculated clay is sieved. Then, the sample undergoes a brief hydrogen peroxide treatment for total disintegration. When comparing this method with the traditional acid digestion method, the obtained microfossils concentrations were similar for both methods. However, some differences were observed between the two procedures according to the type of samples. The sodium hexametaphosphate method proved to be more efficient than the acid digestion for upper Cretaceous (Maastrichtian) samples from Antarctica and Pleistocene glacial clay from North East England. In contrast, samples of the lower Campanian-Maastrichtian prepared with hydrochloric acid (HCl) were richer in palynomorphs than those treated without acid. Later this technique underwent additional tests by Riding & Kyffin-Hughes (2006) in samples of Ordovician, Carboniferous, Jurassic and Paleogene ages and proved to be as effective as treatment by acid digestion. In general, the results showed that the extraction of palynomorphs from consolidated rocks is more complicated than from sediments, requiring the use of acid digestion. Hydrogen peroxide process showed lower

palynomorph concentrations than the other treatments. Riding & Kyffin-Hughes (2011) studies on Upper Jurassic clay samples from northwest Scotland compared quantitative and qualitative results obtained with the acid digestion and two non-acid treatments (sodium hexametaphosphate and hydrogen peroxide) developed by Riding (2004) and Riding & Kyffin-Hughes (2006). Acid digestion and sodium hexametaphosphate treatments were equally efficient when the hydrogen peroxide method was significantly less effective. After using the sodium hexametaphosphate method the production of organic matter-free residues which does not require additional oxidation was signified. According to Riding & Kyffin-Hughes (2011), these results suggest that the sodium hexametaphosphate method is significantly superior to the hydrogen peroxide technique.

However, as most of the methods described above were applied to consolidated sediments or rocks dating back to early geological times, we decided to work out an acid free digestion approach which would be better adapted to our field area, meaning quaternary deposited samples and impossibility to buy the reagents generally needed for a classic acid digestion treatment.

3. Alkali treatment

With the acid digestion treatment, once the silica is destroyed, the pollen analyst usually proceeds to the alkali treatment which consists of eliminating the humic acids produced during oxidation by solutions of potassium hydroxide, sodium hydroxide, ammonium hydroxide, and potassium carbonate (Batten 1999; Bruch & Pross 1999; Riding 2021). As these solutions are destructive for the sporopollenin

(Southworth (1974), the need for a new protocol without acid would also contribute to remove one destructive step.

4. Material and Method

The method was tested on Holocene sediments collected in tropical lakes, peatlands, and swamps located in Brazil. The samples were divided into three groups, of five samples. In each group, the five samples are : two lacustrine sediment samples one composed of fine clay with organic matter and the other one composed of lightly oxidized fine clay with low content of organic matter; two palm-swamp sediment samples, one organic-rich clay sample and the other one composed of sand with low organic matter content; a peat sediment sample composed of fine organic clay, vegetal fibers, and carbonized particles.

Each group was submitted to different pollen extraction protocols. For the first group we used the classic pollen extraction protocol (Faegri & Iversen 1989), with hydrochloric acid (HCl, 10%), potassium hydroxide (KOH, 10%), hydrofluoric acid HF (40%), and acetolysis (9 parts of acetic anhydride $C_4H_6O_3$ and 1 part of sulfuric acid H_2SO_4 , 95%) to digest carbonates, humic acids, silica, and cellulose, respectively. The second group was treated without acids using sodium metaphosphate ($NaPO_3$). For the third group we used the same treatment without acids than for group 2 but with the substitution of sodium metaphosphate by sodium pyrophosphate ($Na_4P_2O_7$) in order to assess which clay deflocculant allows the best quality for the analyzes.

Description of the acid-free method

In a centrifuge tube, a tablet of an exotic marker *Lycopodium clavatum* (Stockmarr 1971) is added to 10 ml of distilled water and stirred until the tablet is

completely dissolved (Fig. 1). Then, 0.5 cm³ of sediment sample is inserted into the tube and homogenized using vortex equipment. The material is centrifuged for four minutes at 3000 rpm, and the supernatant is discarded.

To remove humic acids and part of the organic matter, 10 ml of KOH 10% is added to the residue, mixed, and left in a hot water bath at 80°C for six minutes, stirring the tube content every three minutes. The material is then centrifuged at 3000 rpm for four minutes, and the supernatant is discarded. This organic matter elimination step is repeated until the supernatant becomes clear after the centrifugation. To stop the KOH action and proceed to the next step, 10 ml of distilled water is added to the tube, then homogenized and centrifuged for four minutes at 3000 rpm, the supernatant is discarded. This washing process is repeated twice.

Density separation is more efficient when the sample is dry and after the last centrifugation, the tube containing the sample is turned over on a flat surface lined with absorbent paper for sixty minutes until the water excess drains.

Once the sample humidity has decreased, 10 ml of Zinc Chloride (ZnCl₂) with 1.9 g.cm⁻³ density is added to the tube, then homogenized and centrifuged for four minutes at 1000 rpm. This process causes the pollen residue to float as the supernatant phase, our material of interest, and the heavier material to be decanted. After centrifugation, the supernatant that contains the pollen residue is carefully transferred to a 100 ml beaker, and the decanted material is discarded. 10 ml of distilled water is added to the beaker to decrease the density of ZnCl₂, stirring the solution slowly. Then, 5 ml of this mixture is transferred back into the tube, and 10 ml of distilled water is added and centrifuged for four minutes at 3000 rpm. The

supernatant is discarded. An additional 5 ml of the beaker mixture is transferred to the tube with 10 ml of distilled water, centrifuged for four minutes at 3000 rpm, and the supernatant is discarded. This step was repeated until all the mixture of the beaker is centrifuged. To ensure that all ZnCl_2 is eliminated from the sample, we add 10 ml of distilled water to the sample tube, centrifuging at 3000 rpm for four minutes, and discarding the supernatant. This is repeated twice.

The flotation step is normally able to eliminate all the heaviest material. Anyhow, some organic matter aggregated to clay particles may still hinder the pollen content. Thus, to ensure the end of the previous step, 10 ml of distilled water is added to the tube, then homogenized and centrifuged for four minutes at 3000 rpm, the supernatant is discarded. The aggregated organic matter is eliminated using a solution of KOH 10% + sodium metaphosphate (NaPO_3). The solution is prepared by adding 10 ml of KOH 10% and 0.5 g of sodium metaphosphate in a beaker, homogenized until the sodium metaphosphate crystals get entirely dissolved. In the tube containing the residue, we add 10 ml of the KOH 10%+ sodium metaphosphate solution, which is homogenized and accommodated in a hot water bath at 80 °C for 30 minutes, stirring every 10 minutes. During this process, the sodium metaphosphate disassociates the clay particles, and the KOH 10% eliminates the organic matter that may still be aggregated to clay particles. The sample is centrifuged for four minutes at 3000 rpm, and the supernatant is discarded. The residue is rinsed with 10 ml distilled water, centrifuged for four minutes at 3000 rpm, and the supernatant is discarded. This is repeated two times.

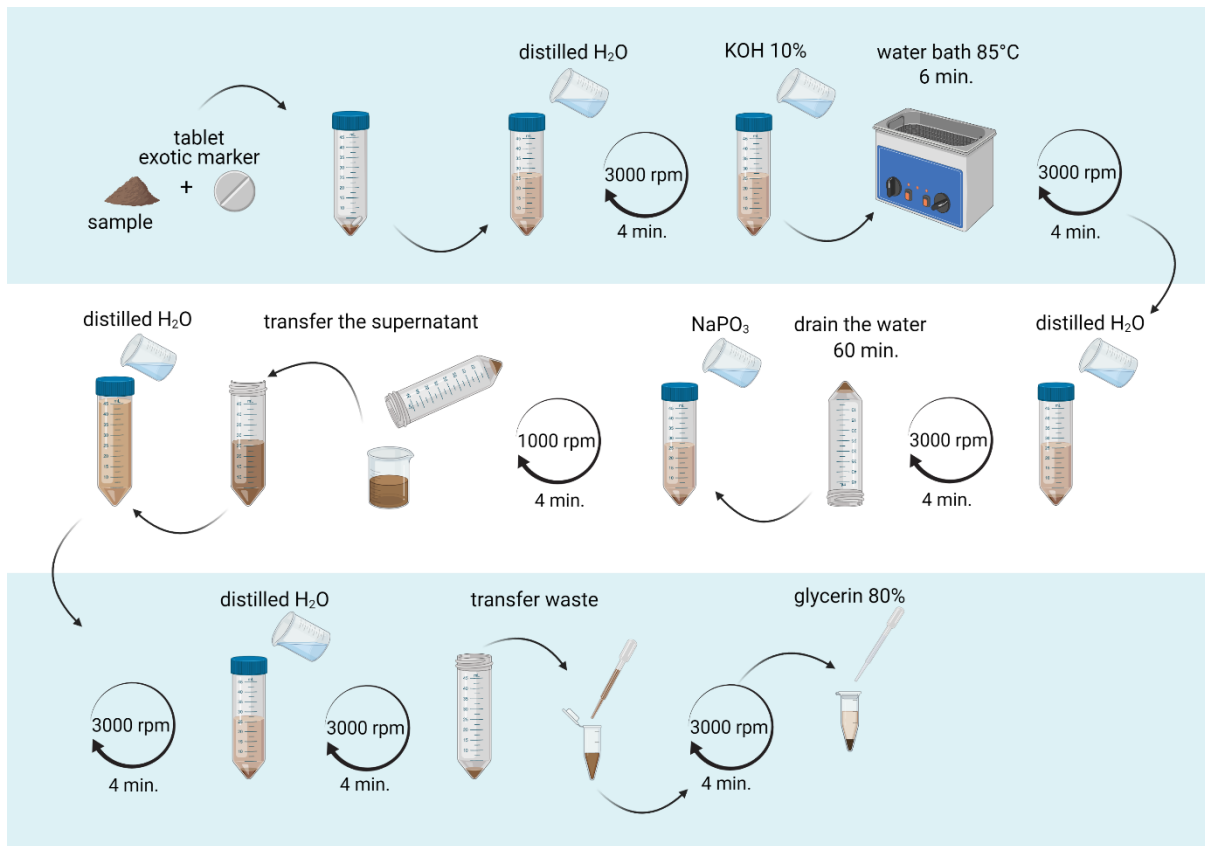


Figure 01: Summary of the different stages of the acid-free palynological processing

The residue is transferred to an Eppendorf tube using a pipette, centrifuged, and the supernatant discarded. Finally, Glycerin 80% is added to the Eppendorf tube at the exact or approximate volume of the sample and homogenized, making the sample ready for glass slide preparation.

The duration of the whole process depends on the capacity of the laboratory, but generally, it is possible to do the full treatment of 24 samples within six hours.

The protocol was developed with sediments from tropical regions of Brazil, where the diversity of pollen types is high, and the morphology of the grains varies broadly, including size. For this reason, the use of sieves was discarded in this experiment, because even using fine meshes to filter the sample and remove the

disintegrated clay, small grains such as *Cecropia* (Urticaceae) can be found, with the equatorial measurement between 5-7 µm and polar 6-11µm.

Preparing palynological slides

For the slide, 30 µl of the sample residue is deposited on the glass. A coverslip (24x50 mm) is positioned over the residue and glued with a thin layer of histolaque resin on both edges. The pollen residue will spread through the coverslip and finally we seal the resin-free edges.

Quantitative Analysis

The samples from the three different types of environment (palm swamp, lake, and peat) are then analysed until 300 grains of arboreal and non arboreal pollen grains are counted per sample. Concentration is based on the spike that was added in the sample at the beginning of the treatment and the calculation is:

$$concentration = \frac{conc. Lycopodium tablet}{num. Lycopodium counted} \times counted pollen grains$$

4. Results and quality control of the acid-free protocol

After each centrifugation, and at every step of the procedure, the discarded supernatant was carefully observed under the microscope to assess the eventual loss of pollen grain and spore. No pollen grain and spore were found in any of the supernatants. A greater attention was paid during the flotation step with ZnCl₂, as we suspected that a small concentration of pollen grain and spore could be accidentally discarded, but again the discarded material was clean of pollen and spores.

When observed under the microscope (Fig. 2) the residues of the tested samples mounted on glass slides showed good quality. The walls of the fossil pollen grains and spores do not show any accumulation of organic matter thus facilitating their morphological identification. This acid free palynological process also allow a good preservation of the algae cysts and carbonized particles.

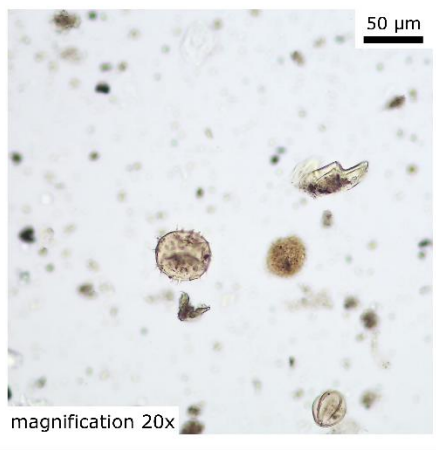
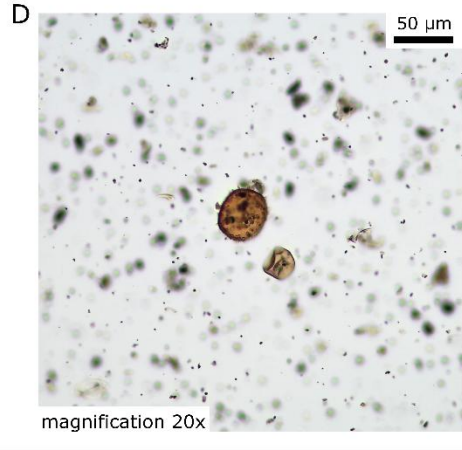
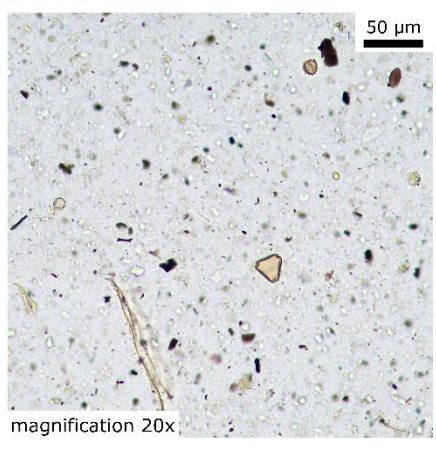
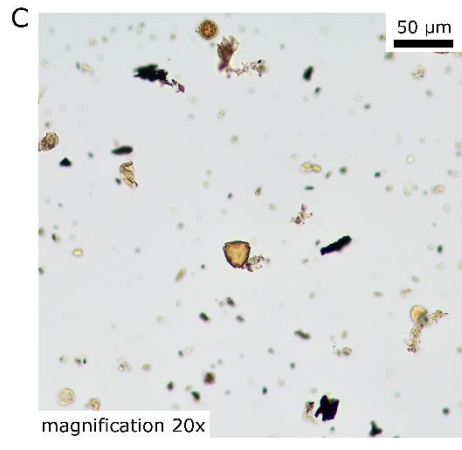
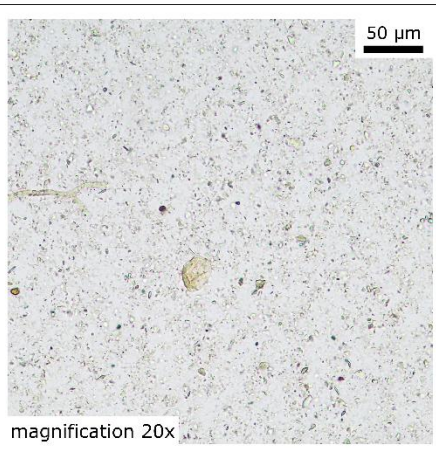
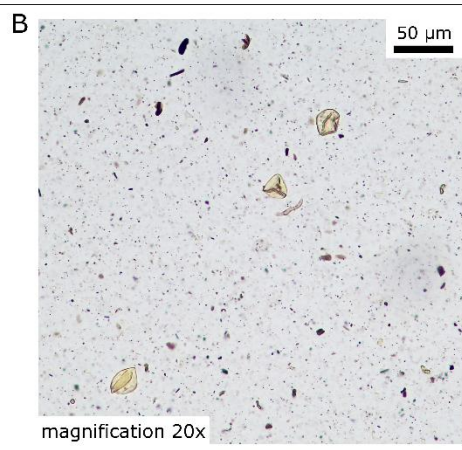
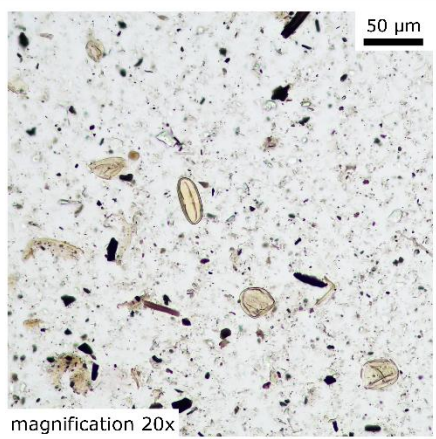
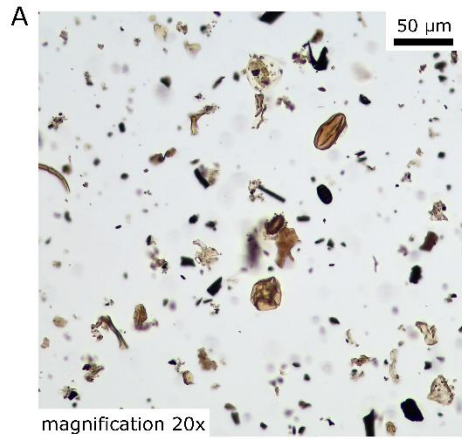


Figure 2: Visual comparison of the residues after treatment following the classic method (left) and the acid-free method (right) for: **(a)** lacustrine sample from tropical forest environment composed of fine clay, vegetal fibers, and carbonized particles; **(b)** lacustrine sample from dry forest environment composed of lightly oxidized plastic clay with low organic matter content; **(c)** peat sample from semi-deciduous forest environment composed of clayey sediment rich in organic matter, vegetal fibers, carbonized particles; and **(d)** palm swamp sample composed of sandy, organic clayey sediment with vegetal fibers and carbonized particles.

In addition, all the carbonate contained in the *Lycopodium clavatum* tablet was totally or partially dissolved and eliminated during the procedure. Regarding sand particles of the sand-rich samples, most of them were decanted at the bottom of the tube during the flotation step with $ZnCl_2$ and discarded after centrifugation.

The sodium metaphosphate was highly effective at deflocculating clay aggregates and the remaining organic matter, contributing to a perfect cleaning of the sample. The KOH 10% which is performed also contributes to eliminate almost completely the remaining organic matter. The test performed with sodium pyrophosphate was also effective with the deflocculating clay. However, the quality of the material compared to the method using sodium metaphosphate is lower as pyrophosphate seems to aggregate a large part of the remaining silica in the residue, making difficult the observation under the microscope (fig. 3).

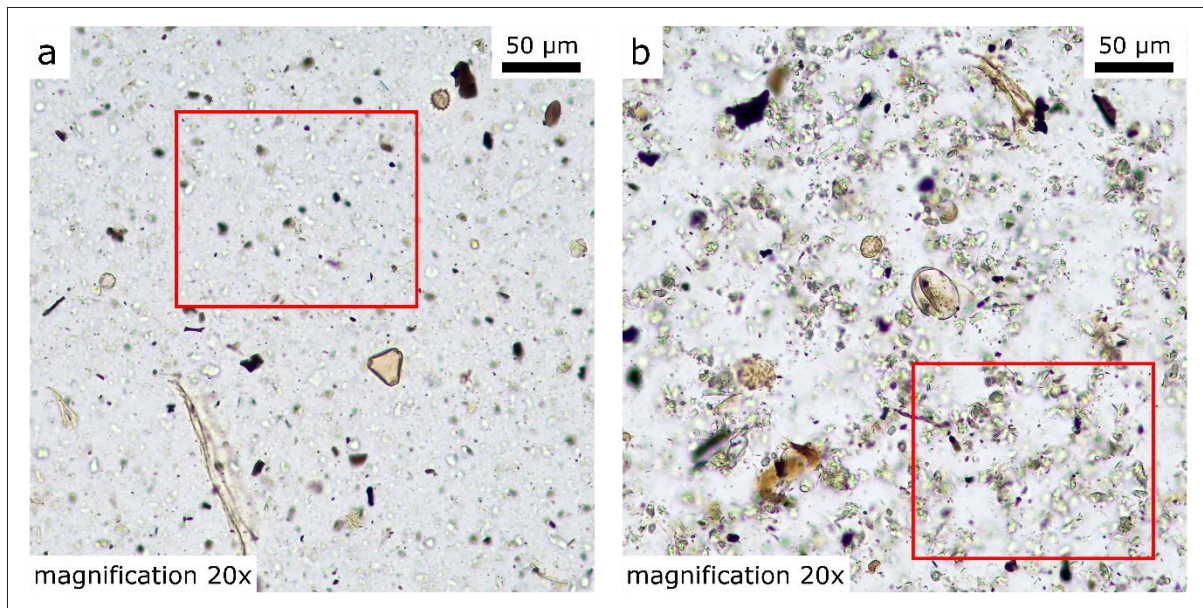


Figure 3: Two peat samples composed of organic clay with carbonized particles; **(a)** sample treated with sodium metaphosphate, red square shows the small clay particles disintegrated after treatment; **(b)** sample treated with sodium pyrophosphate, red square highlights the agglutination of materials together with the disaggregated clay particles.

The acid-free procedure showed low efficiency only when applied to a lacustrine sample characterized by a high concentration of silt with low organic matter content from the Brazilian semi-arid region, a region of high mean temperature with poor preservation of the pollen grains (de Medeiros et al. 2018). A 5 µm mesh sieve was used to reduce the clay volume and concentrate the pollen grains in this sample. However, the result was not satisfactory as small pollen grains passed through the sieve mesh and were found in the discarded material. Palynological analysis of these plastic clay samples is still possible without sieves but when the amount of clay is important it strongly compromises the quality of the observations.

In specific cases, the use of sieves may be recommended as for example, if the expected pollen grains of the study area encompass pollen grains with a

diameter at least 2 μm larger than the mesh used for the sieving. In this case, all the disintegrated clay is removed from the residue and a better quality of observation is possible.

We calculated the pollen concentration for the residues obtained with both methods and we can see on table 1 that the results are very similar ensuring the quality of the treatment for the recovery of the pollen and spore residues.

Table 2: Pollen grain concentrations per $\frac{1}{2} \text{ cm}^3$ measured for the three groups of samples treated with the classic palynological processing method and the alternative acid-free treatment.

Sample	Classic	Acid-free
Palm swamp	90.526	83.668
Lake	234.983	276.105
Peat	334.673	409.044

Moreover, although we discussed above that alkali treatment could generate the loss of pollen grains, our results show that it is not the case within our samples.

5. Cost comparison

In 2021, at the Institute of Evolutionary Sciences of Montpellier (ISEM), the cost of reagents per sample using the classic method is 1.10 € for the HF, HCL, KOH, $\text{C}_4\text{H}_6\text{O}_3$, H_2SO_4 , $\text{C}_2\text{H}_5\text{OH}$, and $\text{C}_3\text{H}_8\text{O}_3$ and 0.40 € per sample for the non-acid protocol using KOH, NaPO_3 , and $\text{C}_3\text{H}_8\text{O}_3$.

6. Conclusion

Our results show that the absence of acids as part of the palynological processing procedure does not compromise the extraction of pollen grains and spores from our tropical Quaternary sediments. The use of potassium hydroxide and sodium metaphosphate as a detergent agent was successful since most of the organic matter aggregated to the clay particles was eliminated after the clay deflocculation. Our method is very efficient and has excellent potential in tested samples from various sedimentary contexts, such as tropical peat, palm swamps, and lakes.

The acid-free protocol proposed here has important implication for making palynological research more accessible, achievable, cheaper and convenient in scenarios where the availability of heavy chemicals like hydrofluoric acid is strongly restricted by law. Besides, the lower cost will make palynological science more affordable in the context of the on-going environmental changes and the strong need for new data in poorly studied areas such as the inter tropical latitudinal band. Still, this protocol diminishes the risk of accidents during handling chemicals since most laboratory-related accidents are caused by exposure to highly hazardous acids. Finally, the duration for sample processing is much lower than classic protocols making palynological processing also less time-consuming.

Bibliography

Arms BC. 1960. A Silica Depressant Method for Concentrating Fossil Pollen and Spores. *Micropaleontology*. 6(3):327.

Assarsson G, Granlund E. 1924. En metod för pollenanalys av minerogena jordarter. *Gff*. 46(1–2):76–82.

Bates CD. 1978. A New Method for the Preparation of Clay-Rich Sediment Samples for Palynological Investigation. *New Phytol*. 81(2):459–463.

Batten D. 1999. Small palynomorphs. In: Jones TP, Rowe NP, editors. *Foss Plants Spores Mod Tech*. London: Geological Society; p. 15–19.

Bennett KD, Willis KJ. 2001. Pollen. In: *Track Environ Chang Using Lake Sediments* [Internet]. Dordrecht: Kluwer Academic Publishers; p. 5–32. http://link.springer.com/10.1007/0-306-47668-1_2

Brown CA. 1960. *Palynological Techniques*. Brown CA, editor. Louisiana: Baton Rouge.

Bruch A, Pross J. 1999. Palynomorph extraction from peat, lignite and coal. In: Jones TP, Rowe NP, editors. *Foss Plants Spores Mod Tech*. London: Geological Society; p. 26–30.

Clarke CM. 1994. Differential recovery of fungal and algal palynomorphs v. embryophyte pollen and spores by three processing techniques. In: Davis OK, editor. *Asp Archaeol Palynol Methodol Appl*. College Station: American Association of Stratigraphic Palynologists; p. 53–62.

Colinvaux PA, De Oliveira PE, Patiño JE. 1999. *Amazon Pollen Manual and Atlas*. I. Balboa, Republic of Panama: Smithsonian Tropical Research Institute.

Dodson JR. 1983. Pollen recovered from organic lake clays: a comparison of two techniques. *Pollen et Spores*. 25:131–138.

Eagar SH, Sarjeant WAS. 1963. Fossil hystrichospheres concentrated by Sieving techniques. *Nature*. 198(4875):80.

Erdtman G. 1986. *Pollen morphology and plant taxonomy: angiosperms*. 1st ed. Leiden: Brill Archive.

Faegri K, Iversen J. 1989. *Textbook of Pollen Analysis*. 4th ed. Chichester: John

Wiley and Sons.

Felix CJ. 1963. Mechanical Sample Disaggregation in Palynology. *Micropaleontology*. 9(3):337.

Heusser LE, Stock CE. 1984. Preparation techniques for concentrating pollen from marine sediments and other sediments with low pollen density. *Palynology*. 8(1):225–227.

Knox AS. 1942. The use of bromoform in the separation of non-calcareous microfossils. *Science* (80-). 95:307–308.

Krukowski ST. 1988. Sodium metatungstate: a new heavy-mineral separation medium for the extraction of conodonts from insoluble residues. *J Paleontol*. 62(2):314–316.

Lennie CR. 1968. Palynological Techniques used in New Zealand. *New Zeal J Geol Geophys*. 11(5):1211–1221.

de Medeiros VB, de Oliveira PE, Santos RA, Barreto AMF, de Oliveira MAT, Pinaya JLD. 2018. New holocene pollen records from the Brazilian Caatinga. *An Acad Bras Cienc*. 90(2):2011–2023.

Moore PD, Webb J, Collinson M. 1991. *Pollen Analysis*. 2nd ed. Oxford, UK: Blackwell Scientific Publications.

Moore PD, Weeb JA, Collinson ME. 1991. *Pollen analysis*. 2nd ed. Oxford: Blackwell.

Riding J. 2004. A review of the laboratory preparation of palynomorphs with a description of an effective non-acid technique. *Rev Bras Paleontol*. 7(1):13–44.

Riding JB. 2021. A guide to preparation protocols in palynology. *Palynology* [Internet]. 0(0):1–157. <http://dx.doi.org/10.1080/01916122.2021.1878305>

Riding JB, Kyffin-Hughes JE. 2006. Further testing of a non-acid palynological preparation procedure. *Palynology*. 30(1):69–87.

Riding JB, Kyffin-Hughes JE. 2011. A direct comparison of three palynological preparation techniques. *Rev Palaeobot Palynol*. 167(3–4):212–221.

Southworth D. 1974. Solubility of Pollen Exines. *Am J Bot*. 61(1):36–44.

Stockmarr J. 1971. Tablets with spores used in absolute pollen analysis. *Pollen et Spores*. XIII(September):615–621.

Traverse A. 1988. *Paleopalynology*. Boston: Allen and Unwin.

Traverse A. 2007. *Paleopalynology*. 2nd ed. Dordrecht, The Netherlands: Springer Science & Business Media.