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Sonication improves the efficiency, efficacy and safety of phytolith extraction

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ABSTRACT

Phytolith analysis is increasingly used in archaeological and paleoecological research, yet the methods used to extract phytoliths from some types of sediments are still not completely satisfactory. This paper reports on the effect of adding sonication to protocols frequently used for phytolith extraction. We compare two common methods of phytolith extraction, both with and without part of the process being carried out in an ultrasound bath. Results show that sonication permits the destruction of soil micro aggregates and, in doing so, improves the removal of both soil organic matter and clay. Adding sonication to commonly used protocols for phytolith extraction is inexpensive and reduces the processing time and the need to use dangerous products, even with the samples that are most difficult to treat. Sonication increases the purity of the extracted phytoliths as well as augmenting the quantity of recovered phytoliths.

1. Introduction

In recent years, the dramatic increase of archaeological and paleoecological reconstructions that rely on the analysis of phytoliths (Hart, 2016) attests the great potential of phytoliths as a proxy for studying such things as past use and domestication of plants (e.g. Iriarte et al., 2010; Madella et al., 2014; Piperno, 2009; Piperno and Stothert, 2003) and for the reconstruction of changes in vegetation cover (e.g. Barboni et al., 1999; Bremond et al., 2005; Strömberg, 2004; Strömberg and McInerney, 2011). Phytoliths are discrete bodies of biogenic silica produced within plant tissues, which have taxonomic significance and are produced in large quantities by many plant types (Ball et al., 2016; Piperno, 2006). After plant decay, phytoliths are accumulated in the soil, where the amount of biogenic silica stored as phytoliths can be as much as 1000 times the silica found in the living biomass (Conley, 2002). Phytoliths are the most durable of plant fossils known to science (Blinnikov et al., 2002; McInerney et al., 2011).

Phytolith analysis consists in identifying and counting phytoliths mounted on glass slides using transmitted light microscopy. It is a time consuming activity which can be very challenging and biased if the extraction of phytoliths is unsatisfactory. As noted in Madella et al. (1998), an extraction is successful if it permits: i) the recovery of a representative assemblage of the biogenic silica contained in the original sediment; ii) the concentration of the silica fraction; and iii) can be carried out economically, safely and quickly. The process of extracting phytoliths from sediments consists of four steps: the dissolution of carbonates; the oxidation of organic matter (OM); the removal of clay; and the gravimetric separation of biogenic silica from the mineral fraction. The most challenging of these four steps are the oxidation of organic matter and the removal of clay. (Boyd et al., 1998; Lentfer and Boyd, 1998; Parr, 2002; Zhao and Pearsall, 1998). The most common procedure for the oxidation of OM is treating the sediments with 30% H_2O_2 in a hot water bath (Lentfer and Boyd, 1998; Madella et al., 1998; Pearsall, 2015; Piperno, 2006). This procedure has the advantage of being relatively easy, safe and inexpensive. It has been used to extract phytoliths in studies of the oxygen isotope composition of phytoliths (Alexandre et al., 2012) and for radiocarbon dating (Madella et al., 2014). Radiocarbon dating and other analyses based on C isotopes are possible because between 0.2% and 2% of phytolith weight is OM (Piperno, 2006). Most of the phytolith OM is mixed with silica (Gallagher et al., 2015) and therefore is preserved within the phytoliths and can be used for the analysis of stable C isotope only if the extracted phytoliths are pure, i.e. soil organic matter (SOM) has been completely removed (Piperno, 2016; Santos et al., 2012). In some cases, H_2O_2 alone is not sufficient to oxidize all the OM; e.g. it has been shown that between 10% and 15% of SOM in cultivated soil is resistant to H_2O_2 oxidation (Leifeld and Kögel-Knabner, 2001; Plante et al., 2004). The reasons for this are not completely understood, but it seems that the formation of aggregates (<250 μm) of clay and OM is a key factor in the stabilization of SOM (Six et al., 2002; Theng et al., 1992), and, hence, in the accumulation of H_2O_2 resistant SOM (Leifeld and Kögel-Knabner, 2001; Plante et al., 2004). If not destroyed, these aggregates are extracted together with the phytoliths by gravimetric separation with heavy liquid

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(step 4). The quantity of clay aggregates may well be greater than the weight of the extracted phytoliths (Pansu and Gautheyrou, 2006). Therefore, if they are not completely destroyed, this reduces significantly the accuracy with which the concentration of phytoliths in the original sediments can be estimated. Current methods that improve the oxidation of OM include: pressurized microwave digestion (Parr, 2002); the use of strong acids (Corbineau et al., 2013; Pearsall, 2015; Piperno, 2016); burning the samples until all the organic matter is reduced to ash (Powers and Gilbertson, 1987); the application of acetolysis (Costa et al., 2016); or the use of a combination of strong acids and burning (Corbineau et al., 2013; McInerney et al., 2011). However, all these methods have drawbacks: the addition of further reactions and the use of strong acids make the process more complex, expensive, time consuming and dangerous; burning is not reliable if samples contain clay (Lentfer and Boyd, 1998); pressurized microwave digestion only allows the processing of very small samples (Parr, 2002), 0.25 g instead of the usual 5–10 g. Moreover, some of these methods (Corbineau et al., 2013; Costa et al., 2016) have only been tested for the extraction of phytoliths from modern plants and their effectiveness in destroying soil clay aggregates is still unknown.

In this article, we propose using ultrasonic vibration (or sonication) to improve the efficiency, efficacy and safety of phytolith extraction from sediments. As sonication could destroy articulated phytoliths (Katz et al., 2010), we do not recommend its use in those cases where they need to be preserved. Sonication is the process by which, through the application of ultrasound to a liquid, micro-bubbles form, grow and collapse causing extremely high temperatures and pressure during microseconds (Suslick, 1990). Water sonication has been used widely to destroy soil aggregates and improve the dispersion of the sediments since the mid 60's (Edwards and Bremner, 1967). With the exception of Katz et al. (2010), we are not aware of any protocol used for the extraction of phytoliths from sediments that includes sonication. Sonication can act in two different ways: on the one hand, it increases the dispersion of sediments by destroying clay aggregates (Pansu and Gautheyrou, 2006); on the other hand, it produces H^+ and OH^-, which can combine to form either H_2 or H_2O_2 (Henglein, 1987). Sonication has been shown to enhance the activity of H_2O_2, improving the oxidation of humic substances (Chemat et al., 2001). An ultrasonic bath is a relatively inexpensive piece of equipment and is part of any phytolith lab, as it is used regularly to clean samples of modern plants before processing them for reference collections or to extract phytoliths and starch residues from artifacts (Pearsall, 2015). Here, commonly used methods of phytolith extraction carried out without an ultrasound bath are compared with the same methods plus sonication. We discuss how sonication can improve the extraction of phytoliths for optical microscopy and for isotopic studies and suggest how to incorporate sonication in currently used protocols.

2. Material and methods

The samples used for the experiment come from early and mid-Holocene neotropical shell middens (Lombardo et al., 2013). We chose samples from these sites because, being old and rich in clay, organic matter and calcium, they contain a great amount of soil micro aggregates (Six et al., 2004). With these samples, standard methods of phytolith extraction are likely to give poor results and we can test whether the use of sonication provides better results. Eight samples were used, approximately 80 g each, taken from three different shell middens at different depths (See Table 1). Each of these samples was divided into two samples of approximately 40 g each and processed following the "phase 1" steps (Table 2). Each sample was divided into four sub-samples and each sub-sample was processed following a different protocol. Two of these four protocols did not include the use of sonication: Standard Oven (SO) and Standard Light (SL). The other two protocols, which include the use of sonication, are Ultrasound Oven (UO) and Ultrasound Light (UL). The SO protocol follows the burning method delineated in Lentfer and Boyd (1998), where organic matter is removed by burning the samples in a furnace after they have been shaken in 5% sodium hexametaphosphate solution and cleared from carbonates with 10% HCl. In the SL protocol, based on Madella et al. (1998), samples are processed with sodium hexametaphosphate and HCl as in the SO protocol, but organic matter is oxidized using 30% H_2O_2. The UO and the UL protocols include the same steps as SO and SL respectively, but several steps are performed in an ultrasound bath: the initial dispersion with sodium hexametaphosphate, the oxidation of organic matter with 30% H_2O_2 and the second dispersion with sodium hexametaphosphate. Samples were sonicated in 50 ml plastic tubes, which were placed inside the ultrasound bath (a 3 l ultrasound bath can contain up to 16 tubes). The intensity of the ultrasounds inside the ultrasonic bath is not homogeneous, therefore tubes were placed randomly, and their position inside the ultrasound bath was changed for each new step in the sonication process. Each of these four protocols is described in more detail in Table 2.

Table 1
Site and location of the samples.

Site	Coordinates of the site	Depth of the sample
SM1	14° 57.781′S 64° 38.501′O	60–70 cm
SM1		110–115 cm
SM3	14° 51.390′S 64° 42.186′O	Surface
SM3		80–84 cm
SM3		160–164 cm
SM4	14° 25.197′S 64° 45.418′O	Surface
SM4		50–60 cm
SM4		80–90 cm

The workflow was divided into 3 phases (see Table 2) and after each phase samples were dried and weighed to measure the effects of each protocol on removing carbonates, clay and organic matter. Moreover, a portion of the extract was examined under the microscope to better quantify these effects. In order to mount a known quantity of phytoliths on the microscope glass slides, the extract was diluted with a known amount of distilled water (Dw) and 0.5 μl of the suspension was placed on the glass slide, dried and mounted with Entellan®. Slides were analyzed with an Olympus BX51 transmitted light microscope at 500×, and all the identifiable biogenic silica bodies within a field of view were counted together with clay aggregates (micro and macro, here defined as 5–50 μm and >50 μm, respectively), charcoal fragments (>5 μm), unidentified opal (>5 μm), and mineral quartz (>5 μm). The number of broken phytoliths was recorded in order to assess to what extent the exposure to prolonged sonication damages the phytoliths. For each slide, a total of 250–300 particles was counted. The total number of extracted phytoliths (T_e) was calculated by multiplying the average number of phytoliths for each field of view by the number of field of views in one slide by Dw/0.5 μl. In order to compare the different methods, two indexes were defined. A purity index P_i was defined as $(T_c - T_p)/T_c$, where T_c is the total amount of counted elements (phytoliths plus clay aggregates), and T_p is the total amount of phytoliths counted in one slide. A fragmentation index F_i was defined as $(T_p - F)/T_p$, where F is the number of broken phytoliths.

3. Results and interpretation

After phase 1, part of the clay and SOM content had been eliminated by dispersion and the totality of carbonates dissolved with 10% HCl. The differences in weight between samples that had been shaken and samples dispersed with sonication (Fig. 1a) indicate that, in most cases, 20 min of sonication were more effective in removing clay and SOM than 24 h of shaking. Carbonates were removed with 10% HCl in the same manner for all the samples; therefore they have had little to no influence on the weight difference shown in Fig. 1a. The SOM and clay that