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.

Keywords

Ultrasound, microfossil, biogenic silica, organic matter, clay

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% H₂O₂ 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, H₂O₂ 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 H₂O₂ 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 H₂O₂ 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 (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 grams instead of the usual 5-10 grams. 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.

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 grams 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 grams 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% H₂O₂. 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% H₂O₂ and the second dispersion with sodium hexametaphosphate. Samples were sonicated in 50 ml plastic tubes, which were placed inside the ultrasound bath (a 3 liter 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.

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 500X, 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 $Dw/0.5 \mu l$. 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.

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 (Figure 1a) indicate that, in most cases, 20 minutes of sonication were more effective in removing clay and SOM than 24 hours 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 Figure 1a. The SOM and clay that were not washed out in phase 1, were further removed in the second phase. After phase 2, samples treated with ultrasound weighed far less than the rest (Figure 1b), even though most of these samples had already lost more material during phase 1.

UL outperformed SL in all the samples, with the exception of SM4 0, in which the results of UL and SL are almost equivalent. The same is true for the oven samples, with UO obtaining better results than SO in all the samples but one. The difference in the amount of material removed between ultrasound and the standard methods is, in some cases, quite substantial. In the case of the samples from SM1, the UL method removed up to three times more material than the SL method. Interestingly, the difference between SL and UL is smaller in the case of the modern samples (SM3 0 and SM4 0) and much larger in the case of the sub-surface (older) samples (Figure 1b). However, the combined results from phases 1 and 2 (Figure 1c) show that sonication improved the removal of clay and organic matter for all the samples. Moreover, several samples show a clear inverse relation between

the amount of material removed in phases 1 and 2 (Figure 1c) and the amount of material extracted with flotation (Figure 1d), indicating that a fraction of the clay aggregates that was not destroyed through SO and SL procedures had floated together with the phytoliths and ended up in the extract. This is further confirmed by looking at the Purity Index (P_i) (Figure 2a). The P_i shows that the material extracted using the UL method consists almost exclusively of phytoliths, whereas up to 30% of the material extracted using the SL method may consist of soil aggregates. P_i is consistent with the amount of phytoliths per gram of extract (Figure 2b), although the comparison between SO and SL is not easy to interpret. It seems that when performing the SO procedure most of the clay hardened in the oven forming large aggregates, containing part of the mineral fraction. These were discharged after the heavy liquid flotation. In contrast, in the case of SL, the clay was still in the form of micro aggregates and part of it was extracted together with the phytoliths. While the extract was drying, the clay hardened and formed new aggregates that consisted almost exclusively of clay and phytoliths. These phytoliths could not be counted because they were not visible under the microscope, but we did count the large clay aggregates. On average, 0.3 macro aggregates ($>50\text{ }\mu\text{m}$) per field view were counted on SL slides, while only 0.1 were counted on SO slides. The Fragmentation Index (F_i) shows that phytoliths can be exposed to sonication for long and continued periods without any damage (Figure 2c).

Discussion

The effects of sonication in the process of phytolith extraction can be assessed at each phase (Table 2). Phase 1 clearly shows that the first advantage of using sonication is saving time. During the first washing of the samples with sodium hexametaphosphate, 20 minutes of sonication offered similar or better results than 24 hours of shaking (Figure 1a). With respect to phase 2, the removal of SOM and clay, we did not measure the total amount of organic matter left in the extract, therefore we cannot be certain that the reaction with H_2O_2 in the ultrasonic bath was more efficient than outside the

ultrasonic bath. However, if we take into account that most of the SOM recalcitrant to H_2O_2 is enclosed with clay in micro aggregates (Leifeld and Kögel-Knabner, 2001; Plante et al., 2004), then, a more efficient removal of SOM should be accompanied by an increase in the amount of clay that is suspended in the supernatant. This is exactly what we observed after phase 2, where sonicated samples lost far more clay than non-sonicated ones (Figure 1b). This is also observed in the analysis of the material under the microscope, which shows a higher Purity Index (P_i) for sonicated samples. P_i is very close to 1 in the case of UL samples, which suggests that the extract is almost completely made of pure silica; hence, the SOM has been almost completely removed. Interestingly, Figure 1b shows that the difference between SL and UL depends on the samples, the difference being greater for the sub-surface (older) samples. This is consistent with the assumption that the H_2O_2 recalcitrant SOM is mostly found in clay micro aggregates, as these are more likely to be found in old and carbonate rich soils than in the topsoil (Six et al., 2004).

Following the definition of a successful extraction of phytoliths put forward by Madella et al. (1998), our data shows that sonication clearly improves several aspects of the extraction of phytoliths from sediments. We did not test whether sonication improved the representativeness of the biogenic silica contained in the original sediment, as we did not identify the extracted phytoliths. However, it can be argued that, at least for SL, which is one of the protocols most commonly used to extract phytoliths from sediments, the smallest phytoliths were more likely to be embedded in the clay aggregates that formed during the drying of the extract, as the smaller a phytolith is, the more likely it is to be deposited in the upper part of the precipitate, together with the clay. It is also interesting to note that UO has a higher P_i than SO, as well as more phytoliths per gram of extract. This difference is probably due to the fact that while the SO samples were vortexed with sodium hexametaphosphate, which is unlikely to break the clay aggregates that formed in the oven, the UO samples were repeatedly exposed to 10 min of ultrasound, which destroyed those clay aggregates, releasing the phytoliths that were trapped in them. This would explain why, even after 10 sodium hexametaphosphate/ultrasound cycles, the UO supernatant was still not clear. At each new

sonication, a new fraction of the clay aggregates was destroyed, the supernatant had more suspended clay and more phytoliths were released. In theory, repeated sodium hexametaphosphate/ultrasound cycles should result in a completely clear supernatant, producing high quality extracts, but we do not propose this procedure as it would be an extremely time consuming process. Instead, we recommend the UL procedure because, as shown in Figure 2a-b, this method increases the concentration of the silica fraction in the extract, producing almost pure extracts.

We think that adding sonication steps to the protocols currently used to extract phytoliths for isotopic studies and radiocarbon age would greatly reduce the chances that the extract is contaminated with SOM. However, we are aware that sonication might destroy silica skeletons; thus, we suggest it should not be used in those cases where articulated phytoliths need to be preserved. We propose that, in addition to using sonication before the flotation with SPT (Katz et al., 2010), sonication steps can also be added during sodium hexametaphosphate treatments and during H₂O₂ oxidation of OM, as we did in this experiment (UL method). Also, our results show that the quality of phytoliths extracted can be greatly improved without having to use dangerous acids or expensive equipment (e.g. the model of ultrasound bath we used in this study costs less than 100 US\$). We are currently working on further laboratory experiments to test whether sonication can help overcome some of the problems associated with obtaining reliable radiocarbon ages from phytoliths (Piperno, 2016; Santos et al., 2012).

Conclusions

This study shows that the standard protocols for extracting fossil phytoliths from sediments can be improved by carrying out some steps of these protocols in an ultrasonic bath. Sonication breaks down soil micro and macro aggregates, increasing the elimination of clay and soil organic matter from the mineral fraction. This results in increased quantity and quality: more phytoliths are

extracted and they are more pure. Sonication is an easy to use, inexpensive and safe tool even for those sediments that are most difficult to treat.

Acknowledgements.

The present study has been funded by the Swiss National Science Foundation (SNSF) grant no P300P2158459/1. CaSEs is a recognised and funded Research Group of the Generalitat de Catalunya (2014 SGR 1417). We would like to thank Elisa Canal-Beeby and the two anonymous reviewers who helped improve earlier versions of the manuscript.

References

- Alexandre, A., Crespin, J., Sylvestre, F., Sonzogni, C., Hilbert, D.W., 2012. The oxygen isotopic composition of phytolith assemblages from tropical rainforest soil tops (Queensland, Australia): validation of a new paleoenvironmental tool. *Clim. Past* 8, 307-324.
- Ball, T., Chandler-Ezell, K., Dickau, R., Duncan, N., Hart, T.C., Iriarte, J., Lentfer, C., Logan, A., Lu, H., Madella, M., Pearsall, D.M., Piperno, D.R., Rosen, A.M., Vrydaghs, L., Weisskopf, A., Zhang, J., 2016. Phytoliths as a tool for investigations of agricultural origins and dispersals around the world. *Journal of Archaeological Science* 68, 32-45.
- Barboni, D., Bonnefille, R., Alexandre, A., Meunier, J.D., 1999. Phytoliths as paleoenvironmental indicators, West Side Middle Awash Valley, Ethiopia. *Palaeogeography, Palaeoclimatology, Palaeoecology* 152, 87-100.
- Blinnikov, M., Busacca, A., Whitlock, C., 2002. Reconstruction of the late Pleistocene grassland of the Columbia basin, Washington, USA, based on phytolith records in loess. *Palaeogeography, Palaeoclimatology, Palaeoecology* 177, 77-101.
- Boyd, W.E., Lentfer, C.J., Torrence, R., 1998. Phytolith analysis for a wet tropics environment: Methodological issues and implications for the archaeology of Garua island, West New Britain, Papua New Guinea. *Palynology* 22, 213-228.

Bremond, L., Alexandre, A., Hély, C., Guiot, J., 2005. A phytolith index as a proxy of tree cover density in tropical areas: calibration with Leaf Area Index along a forest–savanna transect in southeastern Cameroon. *Global and Planetary Change* 45, 277-293.

Chemat, F., Teunissen, P.G.M., Chemat, S., Bartels, P.V., 2001. Sono-oxidation treatment of humic substances in drinking water. *Ultrason. Sonochem.* 8, 247-250.

Conley, D.J., 2002. Terrestrial ecosystems and the global biogeochemical silica cycle. *Global Biogeochemical Cycles* 16, 68-61-68-68.

Corbineau, R., Reyerson, P.E., Alexandre, A., Santos, G.M., 2013. Towards producing pure phytolith concentrates from plants that are suitable for carbon isotopic analysis. *Review of Palaeobotany and Palynology* 197, 179-185.

Costa, F.G.C.M.d., Souza, P.C.T., Klein, D.E., Bove, C.P., 2016. Application of acetolysis in phytoliths extraction. *Review of Palaeobotany and Palynology* 228, 93-97.

Edwards, A.P., Bremner, J.M., 1967. Dispersion of soil particles by sonic vibration. *Journal of Soil Science* 18, 47-63.

Gallagher, K.L., Alfonso-Garcia, A., Sanchez, J., Potma, E.O., Santos, G.M., 2015. Plant growth conditions alter phytolith carbon. *Frontiers in Plant Science* 6.

Hart, T.C., 2016. Issues and directions in phytolith analysis. *Journal of Archaeological Science* 68, 24-31.

Henglein, A., 1987. Sonochemistry: Historical developments and modern aspects. *Ultrasonics* 25, 6-16.

Iriarte, J., Glaser, B., Watling, J., Wainwright, A., Birk, J.J., Renard, D., Rostain, S., McKey, D., 2010. Late Holocene Neotropical agricultural landscapes: phytolith and stable carbon isotope analysis of raised fields from French Guianan coastal savannahs. *Journal of Archaeological Science* 37, 2984-2994.

Katz, O., Cabanes, D., Weiner, S., Maeir, A.M., Boaretto, E., Shahack-Gross, R., 2010. Rapid phytolith extraction for analysis of phytolith concentrations and assemblages during an excavation: an application at Tell es-Safi/Gath, Israel. *Journal of Archaeological Science* 37, 1557-1563.

Leifeld, J., Kögel-Knabner, I., 2001. Organic carbon and nitrogen in fine soil fractions after treatment with hydrogen peroxide. *Soil Biology and Biochemistry* 33, 2155-2158.

Lentfer, C.J., Boyd, W.E., 1998. A Comparison of Three Methods for the Extraction of Phytoliths from Sediments. *Journal of Archaeological Science* 25, 1159-1183.

Lombardo, U., Szabo, K., Capriles, J.M., May, J.-H., Amelung, W., Hutterer, R., Lehndorff, E., Plotzki, A., Veit, H., 2013. Early and Middle Holocene Hunter-Gatherer Occupations in Western Amazonia: The Hidden Shell Middens. *PLoS ONE* 8, e72746.

Madella, M., García-Granero, J.J., Out, W.A., Ryan, P., Usai, D., 2014. Microbotanical Evidence of Domestic Cereals in Africa 7000 Years Ago. *PLoS ONE* 9, e110177.

Madella, M., Powers-Jones, A.H., 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, 801-803.

McInerney, F.A., Strömberg, C.A.E., White, J.W.C., 2011. The Neogene transition from C3 to C4 grasslands in North America: stable carbon isotope ratios of fossil phytoliths. *Paleobiology* 37, 23-49.

Pansu, M., Gautheyrou, J., 2006. *Handbook of soil analysis*. Heidelberg: Springer-Verlag.

Parr, J.F., 2002. A comparison of heavy liquid floatation and microwave digestion techniques for the extraction of fossil phytoliths from sediments. *Review of Palaeobotany and Palynology* 120, 315-336.

Pearsall, D.M., 2015. *Paleoethnobotany: a handbook of procedures*, Third edition. Walnut Creek, California, US: Left Coast Press.

Piperno, D.R., 2006. *Phytoliths*. Oxford, UK: AltaMira Press.

Piperno, D.R., 2009. Identifying crop plants with phytoliths (and starch grains) in Central and South America: A review and an update of the evidence. *Quaternary International* 193, 146-159.

Piperno, D.R., 2016. Phytolith radiocarbon dating in archaeological and paleoecological research: a case study of phytoliths from modern Neotropical plants and a review of the previous dating evidence. *Journal of Archaeological Science* 68, 54-61.

Piperno, D.R., Stothert, K.E., 2003. Phytolith Evidence for Early Holocene Cucurbita Domestication in Southwest Ecuador. *Science* 299, 1054-1057.

- Plante, A.F., Chenu, C., Balabane, M., Mariotti, A., Righi, D., 2004. Peroxide oxidation of clay-associated organic matter in a cultivation chronosequence. *European Journal of Soil Science* 55, 471-478.
- Powers, A.H., Gilbertson, D.D., 1987. A simple preparation technique for the study of opal phytoliths from archaeological and quaternary sediments. *Journal of Archaeological Science* 14, 529-535.
- Santos, G.M., Alexandre, A., Southon, J.R., Treseder, K.K., Corbinea, R., Reyerson, P.E., 2012. Possible source of ancient carbon in phytolith concentrates from harvested grasses. *Biogeosciences* 9, 1873-1884.
- Six, J., Bossuyt, H., Degryze, S., Denef, K., 2004. A history of research on the link between (micro)aggregates, soil biota, and soil organic matter dynamics. *Soil and Tillage Research* 79, 7-31.
- Six, J., Conant, R.T., Paul, E.A., Paustian, K., 2002. Stabilization mechanisms of soil organic matter: Implications for C-saturation of soils. *Plant and Soil* 241, 155-176.
- Strömberg, C.A.E., 2004. Using phytolith assemblages to reconstruct the origin and spread of grass-dominated habitats in the great plains of North America during the late Eocene to early Miocene. *Palaeogeography, Palaeoclimatology, Palaeoecology* 207, 239-275.
- Strömberg, C.A.E., McInerney, F.A., 2011. The Neogene transition from C3 to C4 grasslands in North America: assemblage analysis of fossil phytoliths. *Paleobiology* 37, 50-71.
- Suslick, K.S., 1990. Sonochemistry. *Science* 247, 1439-1445.
- Theng, B.K.G., Tate, K.R., Becker-Heidmann, P., 1992. Towards Establishing the Age, Location, and Identity of the Inert Soil Organic Matter of a Spodosol. *Zeitschrift für Pflanzenernährung und Bodenkunde* 155, 181-184.
- Zhao, Z., Pearsall, D.M., 1998. Experiments for Improving Phytolith Extraction from Soils. *Journal of Archaeological Science* 25, 587-598.

Table Captions

Table 1 Site and location of the samples

Table 2 Phytolith extraction methods compared in this study. *In the case of the UO samples, the supernatant was never completely clear; we stopped removing the clay after 10 sodium hexametaphosphate cycles.

Figure Captions

Figure 1 Changes in the weight of the samples processed under the four protocols at different stages of the extraction process (see Table 2).

Figure 2 a) Purity Index, P_i ; b) Millions of Phytoliths per gram of extract; c) Fragmentation Index, F_i

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

Phase 1		SO	SL	UO	UL
Initial dispersion of sediments	Samples placed in 50 ml plastic tubes, tubes filled with 45ml of 5% sodium hexametaphosphate solution. Tubes shaken every half hour during 24 hours plus 24 hours resting. Centrifuged 1 time at 1500 rpm for 3 min, then supernatant removed with pipette	X	X		
	Samples placed in 50 ml plastic tubes, tubes filled with 45ml of 5% sodium hexametaphosphate solution. Tubes placed in ultrasound bath at 60°C for 10 min, centrifuged at 1500 rpm for 3 min, then supernatant removed with pipette (repeated twice)			X	X
Rinsing	Sample tubes filled with distilled water and centrifuged at 2500 rpm for 2 min, supernatant removed with pipette (repeated 3 times)	X	X	X	X
Remove carbonates	Samples placed in 50 ml plastic tubes, 10% HCl added to the sample. Additional 10% HCl added until no further reaction is observed after shaking. Samples rinsed 4 times at 2500 rpm for 2 min	X	X	X	X
Remove large particles	Samples sieved with a 250-micron mesh	X	X	X	X
Drying/weighing	Samples and large particles dried in oven at 90°C, then weighed	X	X	X	X
Phase 2					
	Samples divided into 4 sub-samples				
Remove organic matter	Sub-samples placed in crucibles and heated in oven at 550°C for 2 hours	X		X	
	Sub-samples in plastic tubes, added 20 ml of 30% H ₂ O ₂ , tubes placed in water bath at 60°C. Once reaction slowed down, tubes centrifuged at 2500 rpm for 2 min, added new 30% H ₂ O ₂ and waited till no reaction is observed. Sub-samples rinsed 4 times at 2500 rpm for 2 min		X		
	Sub-samples in plastic tubes, added 20 ml 30% H ₂ O ₂ , tubes placed in ultrasound bath at 60°C. Cycles of 30 min ultrasound "On" and 30 min ultrasound "Off" are repeated until no reaction is observed with ultrasound "Off". Sub-samples rinsed 4 times at 2500 rpm for 2 min				X
Remove clay	Sub-samples placed in 50 ml plastic tubes, tubes filled with 45ml of 5% sodium hexametaphosphate solution. Tubes vortexed and centrifuged at 1500 rpm for 3 min, then supernatant removed with pipette. Process repeated until supernatant is clear*. Sub-samples rinsed 4 times at 2500 rpm for 2 min	X	X		
	Sub-samples placed in 50ml plastic tubes, tubes filled with 45ml of 5% sodium hexametaphosphate solution. Tubes placed in ultrasound bath at 60°C for 10 min, centrifuged at 1500 rpm for 3 min, then supernatant removed with pipette. Process repeated until supernatant is clear. Sub-samples rinsed 4 times at 2500 rpm for 2 min			X	X
Drying/weighing	Sub-samples dried in oven at 90°C, then weighed	X	X	X	X
Phase 3					
Flotation	Sub-samples placed in 50 ml plastic tubes, added 15 ml of SPT at 2.3 specific gravity. Tubes centrifuged at 3000 rpm for 5 min. 10 ml of SPT with floating fraction moved to a second tube. Repeated twice. Second tube filled with water, centrifuged at 3000 rpm for 10 min, diluted SPT recovered with pipette. Sub-samples rinsed 4 times at 2500 rpm for 2 min	X	X		
	Same as above, but tubes placed in ultrasound bath for 10 min before floating			X	X
Drying/weighing	Extracted phytoliths moved to glass vials, dried in oven at 90°C, then weighed	X	X	X	X