Review of Palaeobotany and Palynology 197 (2013) 179–185

Contents lists available at ScienceDirect

Review of Palaeobotany and Palynology

journal homepage: www.elsevier.com/locate/revpalbo

Research paper

Towards producing pure phytolith concentrates from plants that are suitable for carbon isotopic analysis

Rémi Corbineau [a,b], Paul E. Reyerson [c], Anne Alexandre [a,*], Guaciara M. Santos [d]

[a] Centre Européen de Recherche et d'Enseignement des Géosciences de l'Environnement (UMR 7330), CNRS, Aix-Marseille Université, Europôle méditerranéen de l'Arbois BP 80, 13545 Aix en Provence cedex 04, France
[b] Laboratoire d'Archéologie Médiévale et Moderne en Méditerranée (UMR 7298), CNRS, Aix-Marseille Université, MMSH, 5 rue du Château de l'Horloge, BP 647, 13094 Aix-en-Provence, France
[c] Department of Geography, University of Wisconsin-Madison, 550 North Park Street, Madison, WI 53706, USA
[d] Earth System Science, University of California, Irvine, B321 Croul Hall, Irvine, CA 92697-3100, USA

ARTICLE INFO

Article history:
Received 28 June 2012
Received in revised form 6 June 2013
Accepted 9 June 2013
Available online 19 June 2013

Keywords:
Phytolith
AMS radiocarbon dating
Extraction protocol
Carbon isotopes

ABSTRACT

Phytoliths are micrometric particles of amorphous silica that form inside or between the cells of higher plant tissues throughout the life of a plant. Phytolith morphological assemblages extracted from sediments and buried soils are increasingly used as proxies of grassland diversity and tree cover density. When found in significant amounts in archeological sites they can be used for identifying food habits, cultural and agricultural practices. Phytoliths can contain small amounts of C occluded in their structure (phytC). It is generally assumed that the source of this phytC is atmospheric CO_2 that was fixed by the plant via photosynthesis. Isotopic analyses of phytoliths ($\delta^{13}C$, ^{14}C) were thus expected to inform respectively on the photosynthetic pathway or on the age of the mineralized host plants. However recent ^{14}C analyses of phytC from phytolith concentrates extracted from soils and harvested grasses yielded unexpected ^{14}C ages of several hundreds to kyr old. These ^{14}C phytC results raised the question of a possible source of refractory/old soil organic matter component taken up by roots, which can be attached or occluded in phytoliths. Simultaneously these results highlighted the need for setting standardized protocols leading to concentrates entirely devoid of organic residues, as well as for a robust method for checking phytolith purity. The goal of this work was thus to develop protocols for extracting phytoliths from plants, leading to 100% phytolith purity, as required for phytC analyses. Protocol 1 utilizes a multi-step process of dry ashing and acid digestion, while protocol 2 also uses acid digestion as well as a separate alkali immersion step which removes surface layers. Phytolith concentrate purity was gauged in a semi-quantitative fashion through the use of SEM–EDS analysis. This quality check for phytolith purity can reveal small C particulate contamination of phytolith concentrates that may considerably bias isotopic and quantitative analyses of phytC. Results indicate that the two protocols were able to entirely remove small C particulate contamination. Protocol 1 produced phytolith concentrates with well defined morphologies suitable for both morphological and isotopic analyses. However measurement of C yields showed that protocol 1 probably induced C leakage, leading to lower recovery. Protocol 2 is faster, leads to higher C yield but may lead to a beginning of dissolution. With these protocols on hand, sources of phytC can be properly investigated.

1. Introduction

Phytoliths are micrometric particles of amorphous silica (ASi) that form inside or between the cells of higher plant tissues throughout the life of a plant. Silicon (Si) is taken up by the roots in its dissolved form, translocated in the sap, and deposited in the cells where it can take the shape of the host cell. The concentration of phytoliths ranges from less than 0.01% of dry weight in many gymnosperms and dicotyledon angiosperms to more than 8% of dry weight in Poaceae, Arecaceae, and Equisetaceae (e.g. Geis, 1973; Bozarth, 1992; Webb and Longstaffe, 2002). With plant decay, phytoliths that are preserved in oxidizing environments are either incorporated into soils or exported to sediments via regional watersheds. Phytolith morphological assemblages extracted from sediments and buried soils are increasingly used as proxies of grassland diversity and tree cover density (e.g. Blinnikov et al., 2002; Strömberg, 2002; Boyd et al., 2005; Bremond et al., 2005a, b; 2008a,b; Piperno, 2006; Lentfer and Torrence, 2007; Neumann et al., 2009; Messager et al., 2010; Prasad et al., 2011). When found in significant amounts in archeological sites they can be used for identifying food habits, cultural and agricultural practices (e.g. Delhon et al., 2008; Li et al., 2010; Yost and Blinnikov, 2011). In parallel, phytoliths in plants, soils, and rivers were quantified for investigating the

---

* Corresponding author. Fax: +33 4 42 97 15 42.
E-mail addresses: corbineau@mmsh.univ-aix.fr (R. Corbineau), reyerson@wisc.edu (P.E. Reyerson), alexandre@cerege.fr (A. Alexandre), gdossant@uci.edu (G.M. Santos).

0034-6667/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.revpalbo.2013.06.001

biogeochemical cycle of Si, which itself is coupled to the global C cycle (e.g. Blecker et al., 2006; Struyf et al., 2009; Alexandre et al., 2011; Cornelis et al., 2011).

Phytoliths can contain small amounts of C occluded in their structure (phytC), which is thought to range from 0.1 to 2% of phytolith dry weight (Wilding, 1967; Prychid et al., 2003). Raman spectroscopy and GC–MS analyses of phytC evidenced the presence of aliphatic compounds and lignins (Perry et al., 1987; Smith and Anderson, 2001), as well as aromatic hydrocarbon and graphite or coal when phytoliths were burnt (Pironon et al., 2001). PCR associated with protein staining detected the presence of glycoproteins but could not find evidence of any DNA (Elbaum et al., 2009).

It is generally assumed that the source of this phytC is atmospheric CO_2 that was fixed by the plant via photosynthesis (Wilding, 1967; Kelly et al., 1991; Raven et al., 1999; Piperno, 2006; Carter, 2009). From this basis, the assumption that phytC may be a terrestrial sink of C in the global C cycle was recently suggested (Parr and Sullivan, 2005; Jansson et al., 2010). In parallel, carbon isotopic studies have investigated the potential of phytC $\delta^{13}C$ signatures for providing information about photosynthetic pathways (Kelly et al., 1991; Smith and White, 2004; Webb and Longstaffe, 2010; Strömberg and McInerney, 2011) or deriving a paleo-atmospheric CO_2 record (Carter, 2009). However, isotopic calibration studies of phytoliths from grasses showed that the difference between $\delta^{13}C_{tissue}$ and $\delta^{13}C_{phytC}$ values is not constant from a plant to another (Smith and White, 2004; Webb and Longstaffe, 2010) and that changes in $\delta^{13}C_{phytC}$ values are not related to expected variation in the $\delta^{13}C$ values of atmospheric CO_2 (Webb and Longstaffe, 2010).

Few studies have used ^{14}C ages of phytolith concentrates from soils and archeological sediments as chronological indicators (Piperno and Becker, 1996; Piperno and Stothert, 2003; McMichael et al., 2012). One of them found a modern or post-bomb age for phytolith concentrates extracted from superficial soil (Piperno and Becker, 1996). Thousand-year ages were also reported for topsoil phytoliths (McMichael et al., 2012). This was justified by to the long mean residence time of phytoliths in soils. Other studies failed in matching ^{14}C phytC values with expected or independent chronologies (Wilding, 1967; Kelly et al., 1991; McClaran and Umlauf, 2000; Prior et al., 2005; Rieser et al., 2007; Boaretto, 2009). Encountered difficulties were thought to be associated with stratigraphic inversions, preferential oxidation of younger phytoliths or mostly with ineffectiveness of phytolith extraction procedures (Wilding, 1967; Kelly et al., 1991; Prior et al., 2005; Rieser et al., 2007; Boaretto, 2009). Neither the phytolith chemical procedural blank assessment nor the reproducibility and accuracy checks on ^{14}C of large pools of phytC were ever attempted to corroborate or explain the ^{14}C results obtained. A recent study (Santos et al., 2010a) evaluated the background of phytolith chemical extractions and the reproducibility and accuracy of ^{14}C phytC on phytolith concentrates extracted from soils and harvested grasses. Surprisingly, the phytC from harvested grasses yielded unexpected ^{14}C ages of several kyr old (though bulk material from the same plants gave contemporary ^{14}C values), when using an established protocol (Kelly, 1990; Kelly et al., 1991). This case is supported by phytC ^{14}C-AMS data obtained from harvested bamboo leaves and underlying litter layers that were expected to reproduce contemporaneous atmospheric ^{14}C values (Sullivan and Parr, 2008, 2013). As recently discussed in Santos et al. (2012a,b) the dataset shows varying depletions of at least 5 pMC (percent modern carbon) relative to the values expected when taking into account the southern hemisphere bomb radiocarbon peak and its recent decreasing trend in the atmosphere (Santos et al., 2012b). The minimum 5 pMC offset is equivalent to 400 years, reflecting incorporation of a substantial amount of "old" carbon in the phytolith concentrates (Santos et al., 2012b). The offset is maximum for the harvested leaves (which yielded an age of 3.5 ka BP), and undisturbed green litter with minimal contact

Table 1
Main steps of published protocols originally set up for phytolith extraction from plants. These protocols are commonly used for morphological identification purposes; some of them have been used for phytC analyses.

Protocols	Original references	Used for phytC analyses
Wet oxidation		
Main oxidizing agent: H_2SO_4/H_2O_2		
Rinsing of plant material with HCl. Boiling samples in 70% ethanol, washing and drying. Oxidation with concentrated H_2SO_4 at 70°C. Addition of H_2O_2 until solution is clear at room temperature. Rinsing with distilled water.	Geis,1973, 1978 Kelly, 1990	Pironon et al., 2001 Smith and Anderson, 2001 Krull et al., 2003 Smith and White, 2004 Carter, 2009 Santos et al., 2010a Webb and Longstaffe, 2010
Main oxidizing agent: $HNO_3/KClO_3$		
Rinsing of plant material. Oxidation with concentrated HNO_3 and $KClO_3$ at 100°C. Removal of carbonates using HCl at room temperature. Rinsing with distilled water.	Rovner, 1972 Pearsall, 1989 Piperno, 2006	
Main oxidizing agent: $HNO_3/HClO_4$		
Rinsing of plant material with HCl. Two oxidation steps with a 1:1 HNO_3-$HClO_4$ mixture at 80°C. Addition of H_2O_2 at 80°C. Rinsing with distilled water.	Rovner, 1971	Elbaum et al., 2009 Santos et al., 2010a
Microwave digestion		
Oxidation with HNO_3, H_2O_2 and HCl in closed digestion tubes. Microwave irradiation for 30 min. Sieving at 250 µm Rinsing in ethanol	Parr et al., 2001b Parr, 2002	Krull et al., 2003 Parr et al., 2010 Parr and Sullivan, 2005, 2011
Ashing		
Rinsing of plant material with distilled water. Heating crucibles in muffle furnace at 500°C for 6h. Remove from crucibles to test tubes. Oxidation with HCl at 70°C for 20 min/rinsing. Oxidation with H_2O_2 or HNO_3 at 70°C for 20 min. Rinsing with distilled water.	Parr et al., 2001a	

with soil contaminants (1.8 ka BP). These ^{14}C results in addition to Santos et al.'s (2010a) findings raised the question of a possible source of refractory/old soil organic matter (SOM) component in soils taken up by roots, through nitrogen assimilation (amino acids or proteins) or in dissolved form (Santos et al., 2012a and references within), which can be attached to or occluded in phytoliths. Scanning Electron Microscope images coupled with Energy Dispersive Spectrometer analyses (SEM–EDS) from splits of the phytoliths of living grasses dated by Santos et al. (2010a) revealed the presence of particulate organic matter (OM) residues (Santos et al., 2012a) that may have contributed to bias in phytC ages. This fact emphasizes the need for setting standardized protocols for phytC analysis that can dissolve or oxidize all external organic remains.

Methods commonly used for extracting phytoliths from modern plant materials can be grouped into three categories: wet oxidation, dry ashing and microwave digestion (Table 1). Although they were originally set up for phytolith morphological identification which does not require 100% purity, a survey of the published literature shows that some of them were used for plant phytC isotopic analyses (Table 1). Very few reports are available to evaluate phytolith concentrate purity (Parr et al., 2001a,b). This type of assessment is mostly performed by optical microscope evaluations, sometimes including cross-polarized light (Parr et al., 2010; Parr and Sullivan, 2011) which does not allow for the identification of non-polarizing organic remains (other than cellulose). FT-IR spectroscopy can be used on phytolith concentrates to check mineral purity (Cabanes et al., 2011), however, it has a low detection limit of organic compounds (1%).

Here we used SEM–EDS analyses to check for OM remains in phytolith concentrates. Moreover, we developed two protocols for extracting phytoliths from plants, leading to 100% phytolith purity, as required for

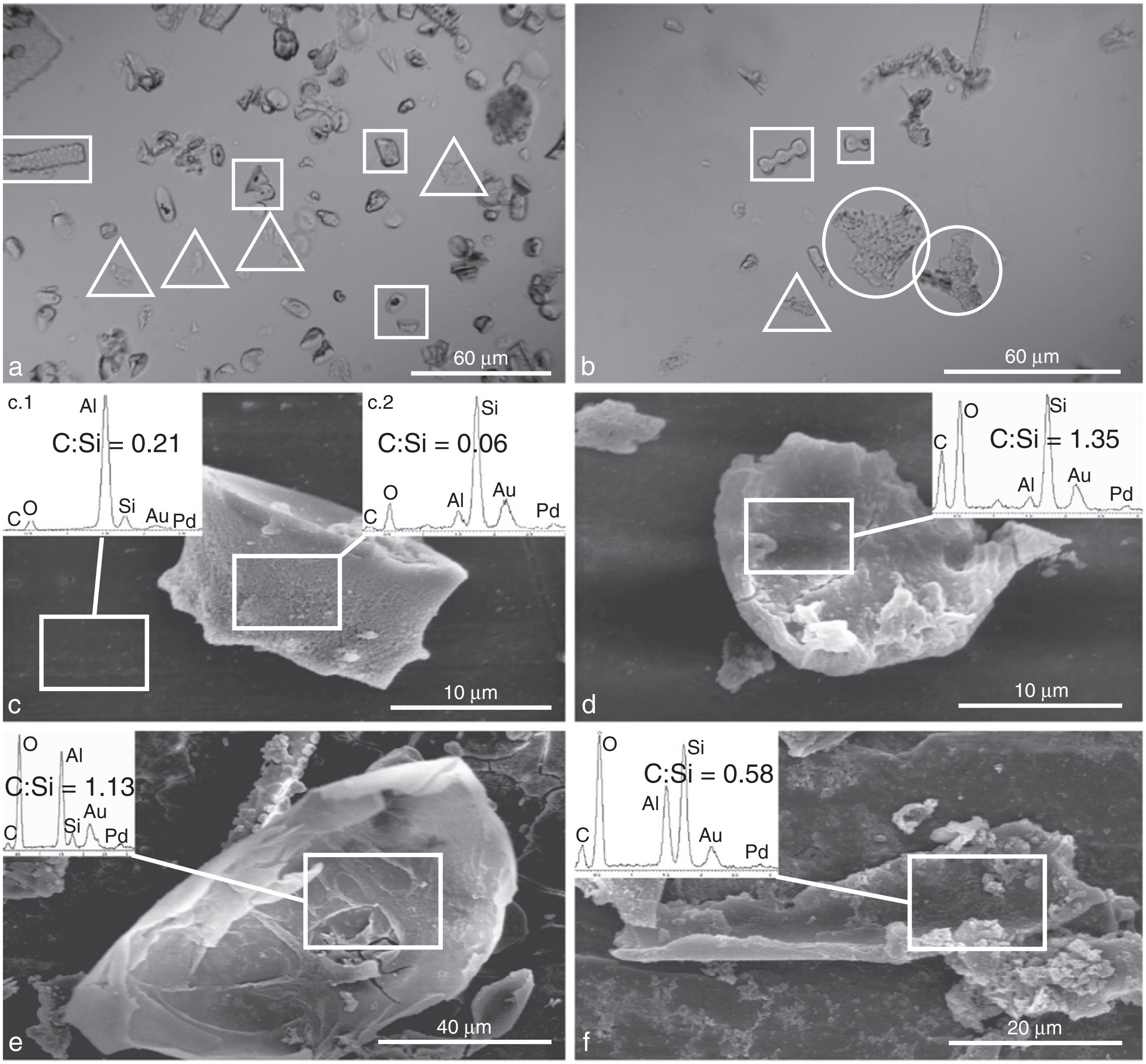

Fig. 1. Images of non-pure phytolith concentrates from Grass 1 and MN samples extracted following Kelly (1990). Optical microscopic images from (a) Grass 1, and (b) MN, showing highly refractive phytolith particles (white rectangles), poorly refractive particles without characteristic shape (white triangles) and tissue-like particles (white circles:). SEM images and EDS spectra of (c.1) Al sample holder, (c.2) rondel type phytolith, (d), (e) and (f) organic particles. 1-c, 1-d and 1-e are reproduced from Santos et al. (2012a) for the purpose of the comparison between pure and non-pure phytolith concentrates.

Table 2
Two protocols of phytolith extraction from plants assessed at CEREGE and UWM as leading to pure phytolith concentrates.

Protocol 1—wet digestion/dry ashing	Protocol 2—wet digestion
(1) Dry sample at 110 °C for 24 h then cut into cm-sized pieces.	(1) Dry sample at 110 °C for 24 h then cut into cm-sized pieces.
(2) Grind samples.	(2) Place sample with DW* in ultrasonic bath to remove particulate matter from the surface.
(3) Place sample with DW* in ultrasonic bath for 15 min to remove particulate matter from the surface.	(3) Immerse in 10% HCl to remove any carbonates and agitate for 30 min. Rinse with DW 3 times.
(4) Place sample in a porcelain crucible and oven dry at 110 °C for 24 h.	(4) Place samples in glass beaker and add concentrated H_2SO_4. Allow to react for 2 h at 70 °C then allow to sit unheated overnight.
(5) Place the crucible in a muffle furnace and increase temperature incrementally from 300 °C to 500 °C (300 °C–325 °C–350 °C–375 °C–500 °C) over 4 h. Hold for 4 h at 500 °C.	(5) Reheat to 70 °C then slowly add 30% H_2O_2 while stirring until the supernatant turns clear. Keep under heat for 2 h, stirring occasionally. Allow to sit, heated, for another hour. Pour off the supernatant then rinse the sample 3 times with DW.
(6) Place sample in a glass beaker and rinse/soak with 10% HCl for 30 min to remove any carbonates. Rinse with DW* 3 times.	(6) Add concentrated HNO_3 and heat at 70 °C for 2 h. Add a pinch of $KClO_3$ to facilitate digestion. Allow to sit unheated overnight. Decant then repeat this step to maximize organic matter oxidation. Rinse 3 times with DW.
(7) Add a 65% HNO_3/70% $HClO_4$ mixture (2:1) and place on sand bath at 80 °C for 16 h.	(7) Immerse the phytoliths in 0.001 M KOH solution ($pH = 11$) and heat at 70 °C for 15 min to remove any alkali-soluble forms of OM*.
(8) Rinse with DW three times. Place sample in a porcelain crucible and oven dry at 110 °C for 24 h.	(8) Extract material using PP filters. Rinse with DW, transfer to glass vials then dry at 110 °C overnight.
(9) Repeat steps (5) and (7).	(9) Check purity by SEM/EDS.
(10) Add H_2O_2 and place on sand bath at 80 °C for 16 h.	
(11) Rinse with DW 3 times.	
(12) Check purity by SEM/EDS. If some OM* still remains, repeat steps 2 to 6.	

* DW: distilled water; OM: organic matter.

phytC analyses. Regarding phytolith extraction from soils and sediments, we already have in hand a high purity protocol commonly used for phytolith oxygen isotopic analyses (Crespin et al., 2010; Alexandre et al., 2012).

2. Material and methods

Several combinations of the protocols listed in Table 1 were tested on random grasses of no relevance until consistently satisfactory results were achieved. The two final protocols were applied to 50 g of leaves of harvested sorghum (*Sorghum bicolor*), in preparation for ulterior ^{14}C-AMS analyses of phytC. The protocols were devoid of any organic chemicals that can sorb to the surface of clean silica (Schlechtriem et al., 2003; Santos et al., 2010a).

Final phytolith concentrates were observed under optical microscopy and analyzed using a SEM (Philips XL30) associated with an EDS system (Energy-dispersive X-ray spectroscopy, Oxford) according to the following steps: 1) coating with gold and palladium of the samples placed on

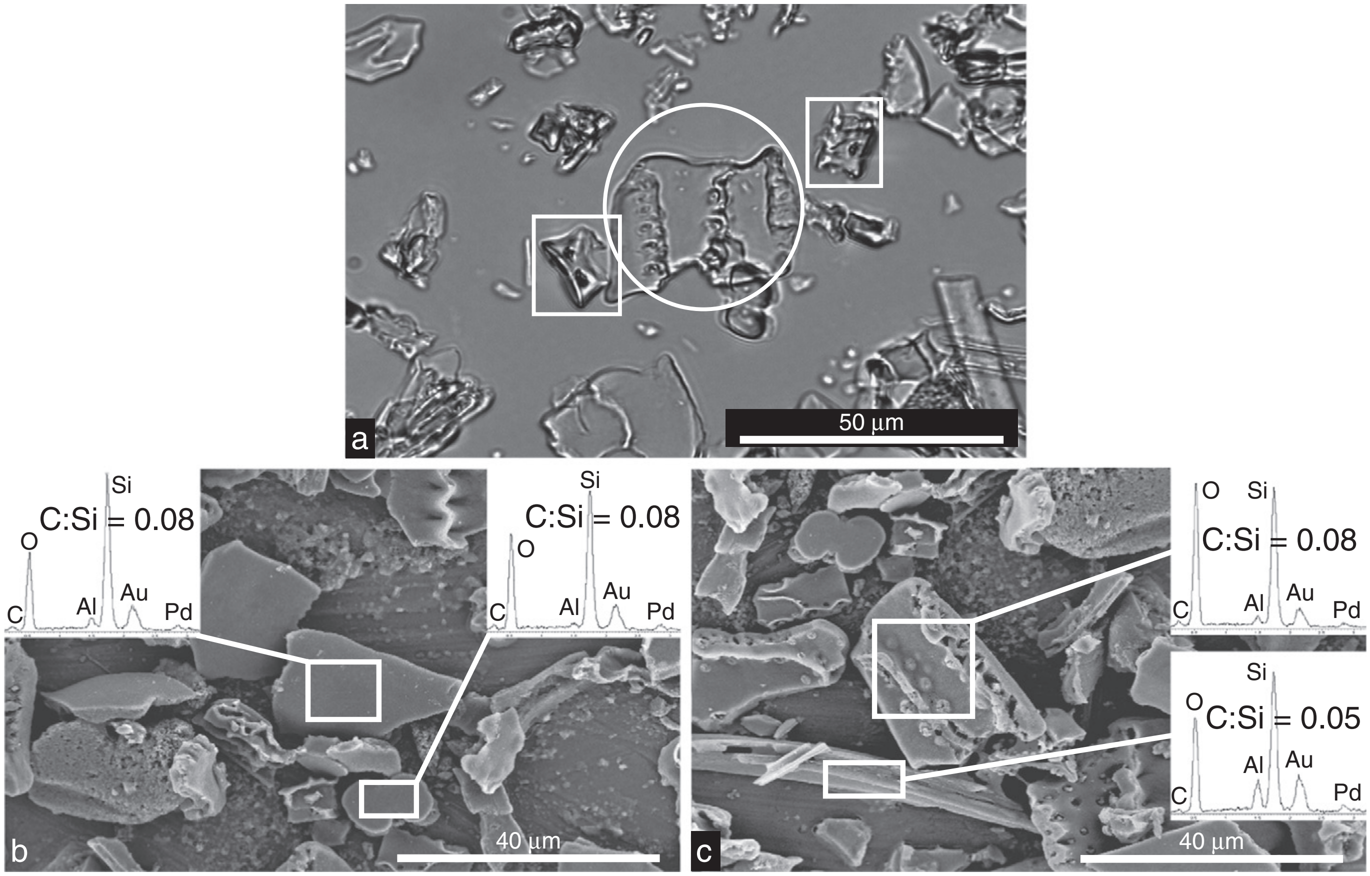

Fig. 2. (a) Optical microscopy images of phytoliths from *Sorghum bicolor* leaves, extracted following *Protocol 1*, showing highly refractive phytolith-like particles (white rectangles) and tissue-like particles (white circle). SEM images and EDS spectra of (b) phytoliths and (c) tissue-like particles.

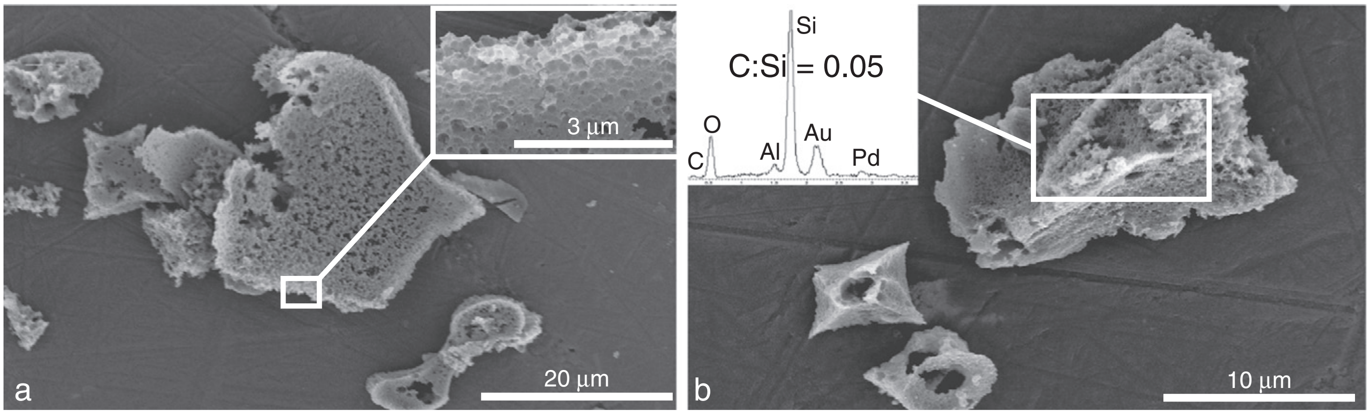

Fig. 3. (a) and (b) SEM images and EDS spectra of phytoliths particles from *Sorghum bicolor* leaves extracted following *Protocol 2*, including the use of KOH @ pH 13. Pits scattered on the phytolith surfaces evidence dissolution.

an Al holder; 2) SEM spotting of organic-like particles showing tissue-like or non-phytolith morphologies; 3) EDS semi-quantitative analyses of C, Si and Ca (indicative of the presence of C-bearing Ca-carbonates). Although SEM–EDS data are semi-quantitative and little accurate for C determination, C:Si % mass ratios, obtained from 10 to 30 µm spots were distinct enough to allow detection of silica particles and organic remains (Fig. 1).

For the purpose of comparison, two samples obtained by Santos and co-workers (Grass 1 and MN; Santos et al., 2010a) using the H_2SO_4/H_2O_2 protocol from which organic remains were previously evidenced (Santos et al., 2012a) were re-investigated using optical microscopy and SEM–EDS (Fig. 1).

In order to determine the carbon percentage of the phytC obtained by the optimized protocols, 100% pure phytolith concentrate samples were combusted at 900 °C in evacuated sealed 6 mm OD pre-baked quartz tubes in the presence of cupric oxide (Santos et al., 2010a), and the cryogenically cleaned CO_2 yield was measured manometrically at room temperature on a vacuum line (Santos et al., 2004).

3. Results and discussions

When observed in optical microscopy, non-pure phytolith concentrates obtained from Grass 1 and MN samples evidenced three categories of particles (Fig. 1): 1) highly refractive phytoliths; 2) poorly refractive particles without characteristic shape; and 3) tissue-like particles. SEM–EDS analyses (Fig. 1) allowed us to clearly categorize the observed particles into: 1) silica particles (or phytoliths) with values of C:Si % mass ratios equal or lower than values obtained for the Al sample holder (<0.5); 2) organic remains with values of C:Si % mass ratios higher than 0.5. Most of the tissue-like particles were silica (C:Si % mass <0.5). Organic remains (C:Si % mass >0.5) were mainly unshaped particles. No Ca was found by EDS analysis.

After several trial experiments to refine phytolith extraction protocols, two protocols were finally kept. They are described in Table 2. *Protocol 1* (Table 2) combines methods described in Rovner (1971), Parr et al. (2001a) and Piperno (2006). It includes multi-step wet oxidation using HNO_3 and $HClO_4$ along with dry ashing. *Protocol 2* combines methods described in Geis (1973), Pearsall (1989), Prior et al. (2005) and Piperno (2006). It is a wet digestion using H_2O_2, HNO_3, $KClO_3$, and KOH, sometimes used for soil phytolith extraction (Piperno, 2006). Several tests were made with three concentrations of KOH (0.001 M @ pH 11; 0.01 M @ pH 12 and 0.1 M @ pH 13). Methodic checking for carbon particulate contamination, using SEM–EDS analysis, revealed the absence of Ca and only the presence of particles with C:Si peak area ratios lower than 0.1 (Figs. 2 and 3). Thus, both protocols led to phytolith concentrates pure enough for phytC isotopic analyses. *Protocol 1* produced phytolith concentrates including tissue-like Si particles with well preserved ornamentation (Madella et al., 2005) (Fig. 2). Ornamentation features were well preserved when *Protocol 2* included the use of KOH @ pH 11 and 12 but started dissolve when KOH was used @ pH 13 (Fig. 4).

The carbon yields of phytolith concentrates from protocols 1 and 2 were 0.053 and 0.21%, respectively, and were reproducible among duplicates. From SEM–EDS analyses and phtyC CO_2 yields, we can conclude that both of these protocols show advantages and disadvantages: *Protocol 1* produces phytoliths with preserved ornamentation excellent for morphological counting prior to phytC analyses. However repeated ashing up to 500 °C may lead to C leakage as evidenced by the low CO_2 yield measured herein. This necessitates that a large amount of phytoliths (ca. 800 mg) need to be extracted from plant material (ca. 800 g assuming a phytolith content of 0.1% dry weight of bulk plant tissue) for obtaining large graphite targets of ~0.4 mg of C. However several AMS

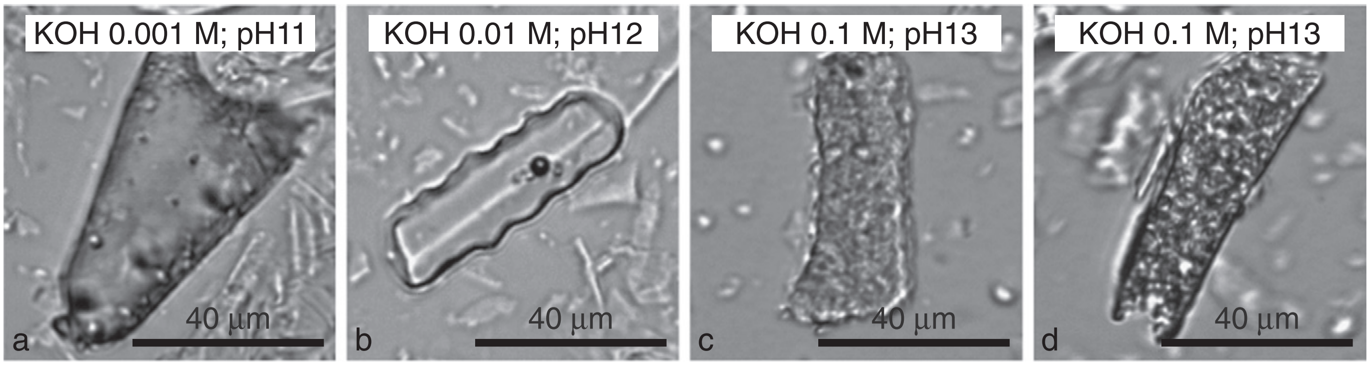

Fig. 4. Optical microscopy images of phytoliths obtained after *Protocol 2* using three concentrations of KOH: (a) 0.001 M @ pH11; (b) 0.01 M @ pH 12; (c) and (d) 0.1 M @ pH 13. Pits scattered on the phytolith surfaces evidence dissolution @ pH 13.

facilities are today able to measure samples as small as 0.010 mg C with 1 sigma errors of just 1% on close-to-modern targets (Santos et al., 2007; de Rooij et al., 2010; Fahrni et al., 2010; Ruff et al., 2010) which brings the required amount of phytolith to ca. 20 mg (using protocol 1) or 5 mg (using protocol 2). However, one should note that ^{14}C-AMS samples produced from some chemical extractions bear higher blanks (Santos et al., 2010a, b), and therefore slightly larger amounts of biosilica concentrates in order to produce larger graphite samples are recommended. Another drawback of protocol 1 is the use of $HClO_4$, which is dangerous to handle and requires the use of a specialized fume hood. *Protocol 2*, which does not utilize dry ashing, is faster and avoids the problem of C leakage. We were able to extract approximately four times more phytC with this approach, as compared to protocol 1. However the use of KOH concentrations above pH 12 should be avoided as it induces fast silica dissolution which needs to be carefully monitored. Finally, these protocols are both suitable for phytolith extraction prior to phytC isotopic analyses. The choice of one or another will depend on the amount of plant material, on available time and equipment, and on the amount of pure phytoliths required for accurate and reproducible analyses.

4. Conclusion

This study highlights the need of a robust method for checking phytolith purity prior to phytC quantification and isotopic analyses. SEM–EDS analysis of Ca, C and Si, although yielding semi-quantitative estimations, is suitable to be used as a routine method for phytolith concentrate purity evaluation. Organic remains, that may not be accounted for under light microscopy, can be clearly individualized from their high C:Si % mass ratios. This quality check for phytolith purity may reveal small C particulate contamination of phytolith concentrates that may considerably bias isotopic and quantitative analyses of phytC.

With the aim of phytC isotopic analyses, we have assessed two plant phytolith extraction protocols which yielded phytolith concentrates devoid of organic remains. Methodological advantages and drawbacks of both protocols were presented. With these protocols on hand, sources and concentration of phytC and possibly organic carbon involvement in biosilica synthesis of higher plants can be now properly investigated.

Acknowledgments

We thank Dr. Bruce A. Kimball and Dr. Michael J. Ottman from the U.S. Arid-Land Agricultural Research Center, Maricopa, AZ for providing samples of *Sorghum bicolor* plant tissue used in this work. Aspects of this work were partially supported by the NSF grant DEB-1144888, FIR 2010 (Aix-Marseille Université), ECCOREV 2011 and AIR Archéométrie 2011 (CNRS). We also thank two anonymous reviewers for their suggestions.

References

Alexandre, A., Bouvet, M., Abbadie, L., 2011. The role of savannas in the terrestrial Si cycle: a case-study from Lamto, Ivory Coast. Global and Planetary Change 78, 162–169.

Alexandre, A., Crespin, J., Sonzogni, C., Sylvestre, F., Hilbert, D.W., 2012. The oxygen isotopic composition of phytoliths from tropical rainforest soils (Queensland, Australia): application of a new paleoenvironmental tool. Climate of the Past 8, 307–324.

Blecker, S.W., McCulley, R.L., Chadwick, O.A., Kelly, E.F., 2006. Biologic cycling of silica across a grassland bioclimosequence. Global Biogeochemical Cycles 20 (3), GB3023.1–GB3023.11.

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 (1–2), 77–101.

Boaretto, E., 2009. Dating materials in good archaeological contexts: the next challenge for radiocarbon analysis. Radiocarbon 51, 275–281.

Boyd, W.E., Lentfer, C.J., Parr, J.F., 2005. Interactions between human activity, volcanic eruptions and vegetation during the Holocene at Garua and Numundo, West New Britain, PNG. Quaternary Research 64 (3), 384–398.

Bozarth, S.R., 1992. Classification of opal phytoliths formed in selected dicotyledons native to the great plains. In: Rapp, G., Mulholland, S.C. (Eds.), Phytolith Systematics. Plenum, New York, pp. 193–214.

Bremond, L., Alexandre, A., Hély, C., Guiot, J., 2005a. 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 (4), 277–293.

Bremond, L., Alexandre, A., Peyron, O., Guiot, J., 2005b. Grass water stress estimated from phytoliths in West Africa. Journal of Biogeography 32 (2), 311–327.

Bremond, L., Alexandre, A., Peyron, O., Guiot, J., 2008a. Definition of grassland biomes from phytoliths in West Africa. Journal of Biogeography 35 (11), 2039–2048.

Bremond, L., Alexandre, A., Wooller, M.J., Hely, C., Williamson, D., Schafer, P.A., Majule, A., Guiot, J., 2008b. Phytolith indices as proxies of grass subfamilies on East African tropical mountains. Global and Planetary Change 61, 209–224.

Cabanes, D., Weiner, S., Shahack-Gross, R., 2011. Stability of phytoliths in the archaeological record: a dissolution study of modern and fossil phytoliths. Journal of Archaeological Science 38 (9), 2480–2490.

Carter, J.A., 2009. Atmospheric carbon signatures in phytolith-occluded carbon. Quaternary International 193 (1–2), 20–29.

Cornelis, J.-T., Delvaux, B., Georg, R.B., Lucas, Y., Ranger, J., Opfergelt, S., 2011. Tracing the origin of dissolved silicon transferred from various soil–plant systems towards rivers: a review. Biogeosciences 8, 89–112.

Crespin, J., Sylvestre, F., Alexandre, A., Sonzogni, C., Paillès, C., 2010. Re-examination of the thermo-dependent relationship between δ^{18}O diatoms and δ^{18}O lake water. Implications for palaeoclimatic applications. Journal of Paleolimnology 44, 547–557.

de Rooij, M., van der Plicht, J., Meijer, H.A.J., 2010. Porous iron pellets for AMS ^{14}C analysis of small samples down to ultra-microscale size (10–25 μgC). Nuclear Instruments and Methods in Physics Research Section B 268, 947–951.

Delhon, C., Martin, L., Argant, J., Thiébault, S., 2008. Shepherds and plants in the Alps: multi-proxy archaeobotanical analysis of neolithic dung from "La Grande Rivoire" (Isère, France). Journal of Archaeological Science 35 (11), 2937–2952.

Elbaum, R., Melamed-Bessudo, C., Tuross, N., Levy, A.A., Weiner, S., 2009. New methods to isolate organic materials from silicified phytoliths reveal fragmented glycoproteins but no DNA. Quaternary International 193, 11–19.

Fahrni, S.M., Gaggeler, H.W., Hajdas, I., Ruff, M., Szidat, S., Wacker, L., 2010. Direct measurements of small C-14 samples after oxidation in quartz tubes. Nuclear Instruments and Methods in Physics Research Section B 268, 787–789.

Geis, J.W., 1973. Biogenic silica in selected species of deciduous angiosperms. Soil Science 116 (2), 113–130.

Jansson, C., Wullschleger, S.D., Kalluri, U.C., Tuskan, G.A., 2010. Phytosequestration: carbon biosequestration by plants and the prospects of genetic engineering. Bioscience 60 (9), 685–696.

Kelly, E.F., 1990. Method for extracting opal phytoliths from soil and plant material. Internal Report. Department of Agronomy, Colorado State University, Fort Collins.

Kelly, E.F., Amundson, R., Marino, B.D., Deniro, M., 1991. Stable isotope ratios of carbon in phytoliths as a quantitative method of monitoring vegetation and climate change. Quaternary Research 35 (2), 222–233.

Lentfer, C., Torrence, R., 2007. Holocene volcanic activity, vegetation succession, and ancient human land use: unraveling the interactions on Garua Island, Papua New Guinea. Review of Palaeobotany and Palynology 143 (3–4), 83–105.

Li, R., Carter, J.A., Xie, S., 2010. Phytoliths and microcharcoal at Jinluojia archeological site in middle reaches of Yangtze River indicative of paleoclimate and human activity during the last 3000 years. Journal of Archaeological Science 37 (1), 124–132.

Madella, M., Alexandre, A., Ball, T., 2005. International code for phytolith nomenclature 1.0. Annals of Botany 96, 253–260.

McClaran, M.P., Umlauf, M., 2000. Desert grassland dynamics estimated from carbon isotopes in grass phytoliths and soil organic matter. Journal of Vegetation Science 11 (1), 71–76.

McMichael, C.H., Bush, M.B., Piperno, D.R., Silman, M.R., Zimmerman, A.R., Anderson, C., 2012. Spatial and temporal scales of pre-Columbian disturbance associated with Western Amazonian lakes. The Holocene 22, 131–141.

Messager, E., Lordkipanidze, D., Delhon, C., Ferring, C.R., 2010. Palaeoecological implications of the Lower Pleistocene phytolith record from the Dmanisi Site (Georgia). Palaeogeography, Palaeoclimatology, Palaeoecology 288 (1–4), 1–13.

Neumann, K., Fahmy, A., Lespez, L., Ballouche, A., Huysecom, E., 2009. The Early Holocene palaeoenvironment of Ounjougou (Mali): phytoliths in a multiproxy context. Palaeogeography, Palaeoclimatology, Palaeoecology 276 (1–4), 87–106.

Parr, J.F., Sullivan, L.A., 2005. Soil carbon sequestration in phytoliths. Soil Biology and Biochemistry 37, 117–124.

Parr, J., Sullivan, L., 2011. Phytolith occluded carbon and silica variability in wheat cultivars. Plant and Soil 342 (1), 165–171.

Parr, J., Lentfer, C.J., Boyd, W.E., 2001a. A comparative analysis of wet and dry ashing techniques for the extraction of phytoliths from plant material. Journal of Archaeological Science 28, 875–886.

Parr, J.F., Dolic, V., Lancaster, G., Boyd, W.E., 2001b. A microwave digestion method for the extraction of phytoliths from herbarium specimens. Review of Palaeobotany and Palynology 116, 203–212.

Parr, J., Sullivan, L., Chen, B., Zheng, W., 2010. Carbon bio-sequestration within the phytoliths of economic bamboo species. Global Change Biology 16 (10), 2661–2667.

Pearsall, D.M., 1989. Paleoethnobotany: A Handbook of Procedures. Academic Press, San Diego (470 pp.).

Perry, C.C., Robert, J.P.W., Fry, S., 1987. Cell wall biosynthesis during silicification of grass hairs. Journal of Plant Physiology 126, 437–448.

Piperno, D.R., 2006. Phytoliths: A Comprehensive Guide for Archaeologists and Paleoecologists. AltaMira Press, New York (238 pp.).

Piperno, D.R., Becker, P., 1996. Vegetational history of a site in the central Amazon Basin derived from phytolith and charcoal records from natural soils. Quaternary Research 45 (2), 202–209.

Piperno, D.R., Stothert, K.E., 2003. Phytolith evidence for early Holocene *Cucurbita* domestication in southwest Ecuador. Science 229 (5609), 1054–1057.

Pironon, J., Meunier, J.D., Alexandre, A., Mathieu, R., Mansuy, L., Grosjean, A., Jardé, E., 2001. Individual characterization of phytoliths: experimental approach and consequences on paleoenvironment understanding. In: Meunier, J.D., Colin, F. (Eds.), Phytoliths: Applications in Earth Sciences and Human History. A.A. Balkema Publishers, Lisse, pp. 329–341.

Prasad, V., Strömberg, C.A.E., Leaché, A.D., Samant, B., Patnaik, R., Tang, L., Mohabey, D.M., Ge, S., Sahni, A., 2011. Late Cretaceous origin of the rice tribe provides evidence for early diversification in Poaceae. Nature Communications 2, 480. http://dx.doi.org/10.1038/ncomms1482.

Prior, C.A., Carter, J.A., Rieser, U., 2005. Are phytolith radiocarbon dates reliable? Poster Presented at the 10th International Conference on Accelerator Mass Spectrometry, Berkeley, USA, September 2005.

Prychid, C.J., Rudall, P.J., Gregory, M., 2003. Systematics and biology of silica bodies in monocotyledons. The Botanical Review 69, 377–440.

Raven, P., Evert, R., Eichhorn, S., 1999. Biology of Plants. W.H. Freeman and Company/ Worth Publishers, New York (944 pp.).

Rieser, U., Carter, J.A., Prior, C.A., 2007. Phytoliths: a chronometer for the late Quaternary. Poster Presented at the INQUA 2007 Conference, Cairns, Australia, July/August 2007.

Rovner, I., 1971. Potential of opal phytoliths for use in paleoecological reconstruction. Quaternary Research 1, 343–359.

Ruff, M., Fahrni, S., Gaggeler, H.W., Hajdas, I., Suter, M., Synal, H.A., Szidat, S., Wacker, L., 2010. On-line radiocarbon measurements of small samples using elemental analyzer and MICADAS gas ion source. Radiocarbon 52, 1645–1656.

Santos, G.M., Southon, J.R., Druffel-Rodriguez, K.C., Griffin, S., Mazon, M., 2004. Magnesium perchlorate as an alternative water trap in AMS graphite sample preparation: a report on University of California, Irvine. Radiocarbon 46 (1), 165–173.

Santos, G.M., Moore, R.B., Southon, J.R., Griffin, S., Hinger, E., Zhang, D., 2007. AMS ^{14}C sample preparation at the KCCAMS/UCI facility: status report and performance of small samples. Radiocarbon 49 (2), 255–269.

Santos, G.M., Alexandre, A., Coe, H.H.G., Reyerson, P.E., Southon, J.R., De Carvalho, C.N., 2010a. The phytolith ^{14}C puzzle: a tale of background determinations and accuracy tests. Radiocarbon 52 (1), 113–128.

Santos, G.M., Southon, J.R., Drenzek, N.J., Ziolkowski, L.A., Druffel, E., Xu, X., Zhang, D., Trumbore, S., Eglinton, T.I., Hughen, K.A., 2010b. Blank assessment for ultra-small samples: chemical extraction and separation vs. AMS. Radiocarbon 52 (3), 1322–1335.

Santos, G.M., Alexandre, A., Southon, J.R., Treseder, K.K., Corbineau, R., Reyerson, P.E., 2012a. Possible source of ancient carbon in phytolith concentrates from harvested grasses. Biogeosciences 9, 1873–1884.

Santos, G.M., Southon, J.R., Alexandre, A., Corbineau, R., Reyerson, P.E., 2012b. Interactive comment to reply the "Comment on: "Possible source of ancient carbon in phytolith concentrates from harvested grasses" by G. M. Santos et al. (2012) by L. A. Sullivan and J. F. Parr". Biogeosciences Discussions 9, C6114–C6124.

Schlechtriem, C., Focken, U., Becker, K., 2003. Effect of different lipid extraction methods on $\delta^{13}C$ of lipid and lipid-free fractions of fish and different fish feeds. Isotopes in Environmental and Health Studies 39 (2), 135–140.

Smith, F.A., Anderson, K.B., 2001. Characterization of organic compounds in phytoliths: improving the resolving power of phytolith $\delta^{13}C$ as a tool for paleoecological reconstruction of C3 and C4 grasses. In: Meunier, J.D., Colin, F. (Eds.), Phytoliths: Applications in Earth Sciences and Human History. A.A. Balkema Publishers, Lisse, pp. 317–327.

Smith, F.A., White, J.W.C., 2004. Modern calibration of phytolith carbon isotope signatures for C_3/C_4 paleograssland reconstruction. Palaeogeography, Palaeoclimatology, Palaeoecology 207 (3–4), 277–304.

Strömberg, C.A.E., 2002. The origin and spread of grass-dominated ecosystems in the late Tertiary of North America: preliminary results concerning the evolution of hypsodonty. Palaeogeography, Palaeoclimatology, Palaeoecology 177 (1–2), 59–75.

Strömberg, C.A.E., McInerney, F.A., 2011. The Neogene transition from C_3 to C_4 grasslands in North America: assemblage analysis of fossil phytoliths. Paleobiology 37 (1), 50–71.

Struyf, E., Smis, A., VanDamme, S., Meire, P., Conley, D., 2009. The global biogeochemical silicon cycle. SILICON 1, 207–213.

Sullivan, L.A., Parr, J.F., 2008. Bomb pulse dating of phytolith-occluded carbon for quantification of carbon sequestration in perennial vegetation. Progress Report no. AINGRA08061, AINSE - Australian Institute of Nuclear Science and Engineering.

Sullivan, L.A., Parr, J.F., 2013. Comment on "Possible source of ancient carbon in phytolith concentrates from harvested grasses" by G.M. Santos et al. (2012). Biogeosciences 10, 977–980.

Webb, E.A., Longstaffe, F.J., 2002. Climatic influences on the oxygen isotopic composition of biogenic silica in prairie grass. Geochimica et Cosmochimica Acta 66 (11), 1891–1904.

Webb, E.A., Longstaffe, F.J., 2010. Limitations on the climatic and ecological signals provided by the $\delta^{13}C$ values of phytoliths from a C_4 North American prairie grass. Geochimica et Cosmochimica Acta 74, 3041–3050.

Wilding, L.P., 1967. Radiocarbon dating of biogenetic opal. Science 156 (3771), 66–67.

Yost, C.L., Blinnikov, M.S., 2011. Locally diagnostic phytoliths of wild rice (*Zizania palustris* L.) from Minnesota, USA: comparison to other wetland grasses and usefulness for archaeobotany and paleoecological reconstructions. Journal of Archaeological Science 38 (8), 1977–1991.

Further reading

Geis, J.W., 1978. Biogenic silica in three species of *Gramineae*. Annals of Botany 42, 1119–1129.

Krull, E.S., Skjemstad, J.O., Graetz, D., Grice, K., Dunning, W., Cook, G., Parr, J.F., 2003. ^{13}C-depleted charcoal from C4 grasses and the role of occluded carbon in phytoliths. Organic Geochemistry 34, 1337–1352.

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.

Rovner, I., 1972. Note on a safer procedure for opal phytolith extraction. Quaternary Research 2, 591.