



Version 2 ▼

Sep 30, 2022

Phytolith extraction and counting procedure for modern plant material rich in silica skeletons V.2

Francesca D'Agostini^{1,2}, Javier Ruiz-Pérez¹, Marco Madella^{1,3,4}, Vincent Vadez^{2,5}, Carla Lancelotti^{1,3}

¹CaSEs Research Group, Department of Humanities, Universitat Pompeu Fabra. c/Trias Fargas 25-27, Barcelona 08005, Spain;

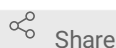
²DIADÉ Unit, IRD, University of Montpellier. Av. Agropolis 911, Montpellier 34394, France;

³ICREA-Catalan Institution for Research and Advanced Studies. Pg. Lluís Companys 23, Barcelona 08010, Spain;

⁴Department of Geography, Archaeology and Environmental Studies, University of Witwatersrand. 1 Jan Smuts Avenue, Braamfontein, Johannesburg 2000, South Africa;

⁵Crop Physiology Laboratory, ICRISAT. Patancheru 502324, Telangana, India

1 Works for me



Share

dx.doi.org/10.17504/protocols.io.q26g74mb8gwz/v2



fra.dagostini F D'Agostini
Universitat Pompeu Fabra, Université de Montpellier

ABSTRACT

Modern plant tissues are often processed for phytolith analysis. They represent a fundamental source of comparison for archeological and palaeoenvironmental phytolith assemblages; they efficiently serve for morphological studies of phytolith shapes and dimensions and, in the last two decades, they have been increasingly involved in physiological studies, which aim to understand the functioning of Si absorption in plants. Here we present a relatively fast, safe, and inexpensive phytolith extraction, combining a dry ashing technique followed by wet oxidation, and a counting methodology. This protocol offers an optimized strategy that achieves very pure samples, preservation of a high number of silica skeletons (phytoliths in anatomical connection), and a counting method which assures the richness and the evenness of the phytolith assemblage distribution. The methodology described in this paper is optimal for recognition and identification of morphotypes, isotope studies and concentration evaluations. This protocol has been developed to allow researchers to extract phytoliths from modern tissues of leaves, stem and chaff, and it is combined with a new strategy to count phytoliths in slides where several big silica skeletons (>50/100 cells each) are present.

References

- Andriopoulou, Nafsika C., and Georgios E. Christidis. 2020. 'Multi-Analytical Characterisation of Wheat Biominerals: Impact of Methods of Extraction on the Mineralogy and Chemistry of Phytoliths'. *Archaeological and Anthropological Sciences* 12 (186). <https://doi.org/10.1007/s12520-020-01091-5>
- Ball, Terry B. 2016. 'Morphometric Analysis of Phytoliths: Recommendations towards

- Standardization from the International Committee for Phytolith Morphometrics'. *Journal of Archaeological Science* 63: 106–11. <http://dx.doi.org/10.1016/j.jas.2015.03.023>
- Ball, Terry, John S. Gardner, and Jack D. Brotherson. 1996. 'Identifying Phytoliths Produced by the Inflorescence Bracts of Three Species of Wheat (*Triticum Monococcum* L., *T. Dicocon* Schrank., and *T. Aestivum* L.) Using Computer-Assisted Image and Statistical Analyses'. *Journal of Archaeological Science* 23 (July): 619–32. <https://doi.org/10.1006/jasc.1996.0058>
- Barboni, Doris, and Laurent Bremond. 2009. 'Phytoliths of East African Grasses: An Assessment of Their Environmental and Taxonomic Significance Based on Floristic Data'. *Review of Palaeobotany and Palynology* 158: 29–41. <https://doi.org/10.1016/j.revpalbo.2009.07.002>
- Braune, Caroline, Reinhard Lieberei, Douglas Steinmacher, and Thomas M. Kaiser. 2012. 'A Simple Microwave Extraction Method for the Isolation and Identification of Plant Opal Phytoliths'. *Biologia* 67 (5): 927–30. <https://doi.org/10.2478/s11756-012-0074-1>
- Bremond, Laurent, Anne Alexandre, Odile Peyron, and Joël Guiot. 2005. 'Grass Water Stress Estimated from Phytoliths in West Africa: Grass Water Stress Estimated from Phytoliths'. *Journal of Biogeography* 32: 311–27. <https://doi.org/10.1111/j.1365-2699.2004.01162.x>
- Brochier, Jacques Élie. 2002. 'Les sédiments anthropiques'. *Géologie de la Préhistoire: méthodes, techniques, applications*, 453–73.
- Cabanes, Dan, Yuval Gadot, Maite Cabanes, Israel Finkelstein, Steve Weiner, and Ruth Shahack-Gross. 2012. 'Human Impact around Settlement Sites: A Phytolith and Mineralogical Study for Assessing Site Boundaries, Phytolith Preservation, and Implications for Spatial Reconstructions Using Plant Remains'. *Journal of Archaeological Science* 39 (August): 2697–2705. <https://doi.org/10.1016/j.jas.2012.04.008>
- Cabanes, Dan, Steve Weiner, and Ruth Shahack-Gross. 2011. 'Stability of Phytoliths in the Archaeological Record: A Dissolution Study of Modern and Fossil Phytoliths'. *Journal of Archaeological Science* 38 (9): 2480–90. <https://doi.org/10.1016/j.jas.2011.05.020>
- Camargo, Mônica Sartori, Mariana Fernández Honaine, Margarita Osterrieth, Natália Ganzaroli Bozza, Vicente da Mota Silva, Maria Laura Benvenuto, and Marcelo de Almeida Silva. 2021. 'Silicon Fertilization Increases Gas-Exchange and Biomass by Silicophytolith Deposition in the Leaves of Contrasting Drought-Tolerant Sugarcane Cultivars under Well-Watered Conditions'. *Plant and Soil*. <https://doi.org/10.1007/s11104-021-05063-z>
- Carter, John A. 2009. 'Atmospheric Carbon Isotope Signatures in Phytolith-Occcluded Carbon'. *Quaternary International* 193 (January): 20–29. <https://doi.org/10.1016/j.quaint.2007.11.013>
- Chen, Iju, Kuang-ti Li, and Cheng-hwa Tsang. 2020. 'Silicified Bulliform Cells of Poaceae: Morphological Characteristics That Distinguish Subfamilies'. *Botanical Studies* 61 (5). <https://doi.org/10.1186/s40529-020-0282-x>
- Cooke, Julia, and Michelle R. Leishman. 2016. 'Consistent Alleviation of Abiotic Stress with Silicon Addition: A Meta-analysis'. Edited by Sue Hartley. *Functional Ecology* 30 (8): 1340–57. <https://doi.org/10.1111/1365-2435.12713>

- Corbineau, Rémi, Paul E. Reyerson, Anne Alexandre, and Guaciara M. Santos. 2013. 'Towards Producing Pure Phytolith Concentrates from Plants That Are Suitable for Carbon Isotopic Analysis'. *Review of Palaeobotany and Palynology* 197 (October): 179–85. <https://doi.org/10.1016/j.revpalbo.2013.06.001>
- Cornelis, Jean-Thomas, and Bruno Delvaux. 2016. 'Soil Processes Drive the Biological Silicon Feedback Loop'. Edited by Julia Cooke. *Functional Ecology* 30 (8): 1298–1310. <https://doi.org/10.1111/1365-2435.12704>
- Coskun, Devrim, Rupesh Deshmukh, Humira Sonah, James G. Menzies, Olivia Reynolds, Jian Feng Ma, Herbert J. Kronzucker, and Richard R. Bélanger. 2019. 'The Controversies of Silicon's Role in Plant Biology'. *New Phytologist*. <https://doi.org/10.1111/nph.15343>
- Elbaum, Rivka, Cathy Melamed-Bessudo, Noreen Tuross, Avraham A. Levy, and Steve Weiner. 2009. 'New Methods to Isolate Organic Materials from Silicified Phytoliths Reveal Fragmented Glycoproteins but No DNA'. *Quaternary International* 193 (January): 11–19. <https://doi.org/10.1016/j.quaint.2007.07.006>
- Elbaum, Rivka, Steve Weiner, Rosa M. Albert, and Michael Elbaum. 2003. 'Detection of Burning of Plant Materials in the Archaeological Record by Changes in the Refractive Indices of Siliceous Phytoliths'. *Journal of Archaeological Science* 30 (February): 217–26. <https://doi.org/10.1006/jasc.2002.0828>
- Ermish, Brendan J., and Shannon A. Boomgarden. 2022. 'Identifying Water Availability with Maize Phytoliths in Range Creek Canyon, Utah'. *Journal of Archaeological Science: Reports* 41. <https://doi.org/10.1016/j.jasrep.2021.103267>
- Fernández Honaine, Mariana, M. Laura Benvenuto, Lía Montti, Marcela Natal, Natalia L. Borrelli, M. Fernanda Alvarez, Stella Maris Altamirano, Mara De Rito, and Margarita L. Osterrieth. 2021. 'How Are Systematics and Biological and Ecological Features Related to Silica Content in Plants? A Study of Species from Southern South America'. *International Journal of Plant Sciences*. <https://doi.org/10.1086/712357>
- Frayse, Fabrice, Oleg S. Pokrovsky, Jacques Schott, and Jean-Dominique Meunier. 2009. 'Surface Chemistry and Reactivity of Plant Phytoliths in Aqueous Solutions'. *Chemical Geology* 258: 197–206. <https://doi.org/10.1016/j.chemgeo.2008.10.003>
- Frick, Daniel A., Rainer Remus, Michael Sommer, Jürgen Augustin, and Friedhelm von Blanckenburg. 2020. 'Silicon Isotope Fractionation and Uptake Dynamics of Three Crop Plants: Laboratory Studies with Transient Silicon Concentrations'. *Biogeosciences*. <https://doi.org/10.5194/bg-2020-66>
- Frick, Daniel A., Jan A. Schuessler, Michael Sommer, and Friedhelm Blanckenburg. 2019. 'Laser Ablation *In Situ* Silicon Stable Isotope Analysis of Phytoliths'. *Geostandards and Geoanalytical Research*. <https://doi.org/10.1111/ggr.12243>
- Gallaher, Timothy J., Sultan Z. Akbar, Phillip C. Klahs, Claire R. Marvet, Ashly M. Senske, Lynn G. Clark, and Caroline A.E. Strömberg. 2020. '3D Shape Analysis of Grass Silica Short Cell Phytoliths (GSSCP): A New Method for Fossil Classification and Analysis of Shape Evolution'. *New Phytologist*. <https://doi.org/10.1111/nph.16677>
- Ge, Yong, Houyuan Lu, Jianping Zhang, Can Wang, and Xing Gao. 2020. 'Phytoliths in Inflorescence Bracts: Preliminary Results of an Investigation on Common Panicoideae Plants in China'. *Frontiers in Plant Science* 10. <https://doi.org/10.3389/fpls.2019.01736>
- Gu, Yansheng, Hongye Liu, Hanlin Wang, Rencheng Li, and Jianxin Yu. 2016. 'Phytoliths as a Method of Identification for Three Genera of Woody Bamboos

- (Bambusoideae) in Tropical Southwest China'. *Journal of Archaeological Science* 68: 46–53. <https://doi.org/10.1016/j.jas.2015.08.003>
- Guerriero, Gea, Ian Stokes, Nathalie Valle, Jean-Francois Hausman, and Christopher Exley. 2020. 'Visualising Silicon in Plants: Histochemistry, Silica Sculptures and Elemental Imaging'. *Cells* 9. <https://doi.org/10.3390/cells9041066>
- Hartley, Sue E., Rob N. Fitt, Emma L. McLarnon, and Ruth N. Wade. 2015. 'Defending the Leaf Surface: Intra- and Inter-Specific Differences in Silicon Deposition in Grasses in Response to Damage and Silicon Supply'. *Frontiers in Plant Science* 6. <https://doi.org/10.3389/fpls.2015.00035>
- Harvey, Emma L., and Dorian Q Fuller. 2005. 'Investigating Crop Processing Using Phytolith Analysis: The Example of Rice and Millets'. *Journal of Archaeological Science* 32: 739–52. <https://doi.org/10.1016/j.jas.2004.12.010>
- Hodson, Martin J. 2019. 'The Relative Importance of Cell Wall and Lumen Phytoliths in Carbon Sequestration in Soil: A Hypothesis'. *Frontiers in Earth Science* 7 (July): 167. <https://doi.org/10.3389/feart.2019.00167>
- Hodson, Martin J., Adrian G. Parker, Melanie J. Leng, and Hilary J. Sloane. 2008a. 'Silicon, Oxygen and Carbon Isotope Composition of Wheat (*Triticum Aestivum* L.) Phytoliths: Implications for Palaeoecology and Archaeology'. *Journal of Quaternary Science* 23 (4): 331–39. <https://doi.org/10.1002/jqs.1176>
- Hodson, Martin J.. 2008b. 'Silicon, Oxygen and Carbon Isotope Composition of Wheat (*Triticum Aestivum* L.) Phytoliths: Implications for Palaeoecology and Archaeology'. *Journal of Quaternary Science* 23 (4): 331–39. <https://doi.org/10.1002/jqs.1176>
- Hurtado, Alexander Calero, Denise Aparecida Chiconato, Renato de Mello Prado, Gilmar da Silveira Sousa Junior, Dilier Olivera Viciado, Yanery Pérez Díaz, Kolima Peña Calzada, and Priscila Lupino Grato. 2020. 'Silicon Alleviates Sodium Toxicity in Sorghum and Sunflower Plants by Enhancing Ionic Homeostasis in Roots and Shoots and Increasing Dry Matter Accumulation'. *Silicon*. <https://doi.org/10.1007/s12633-020-00449-7>
- Jadhao, Kundansing Rajpalsing, and Gyana Ranjan Rout. 2020. 'Silicon (Si) Enhances the Resistance in Finger Millet Genotypes against Blast Disease'. *Journal of Plant Pathology*. <https://doi.org/10.1007/s42161-020-00622-2>
- Jenkins, Emma. 2009. 'Phytolith Taphonomy: A Comparison of Dry Ashing and Acid Extraction on the Breakdown of Conjoined Phytoliths Formed in *Triticum Durum*'. *Journal of Archaeological Science* 36 (October): 2402–7. <https://doi.org/10.1016/j.jas.2009.06.028>
- Jenkins, Emma, Khalil Jamjoum, Sameeh Nuimat, Richard Stafford, Stephen Nortcliff, and Steven Mithen. 2016. 'Identifying Ancient Water Availability through Phytolith Analysis: An Experimental Approach'. *Journal of Archaeological Science* 73: 82–93. <https://doi.org/10.1016/j.jas.2016.07.006>
- Jenkins, Emma, Lea Predanich, Sameeh Nuimat, Khalil Jamjoum, and Richard Stafford. 2020. 'Assessing Past Water Availability Using Phytoliths From the C4 Plant *Sorghum Bicolor*: An Experimental Approach'. *Journal of Archaeological Science* 33. <https://doi.org/10.1016/j.jasrep.2020.102460>
- Jones, L.H.P., Milne, A.A. Studies of silica in the oat plant. *Plant Soil* 18, 207–220 (1963). <https://doi.org/10.1007/BF01347875>
- Karoune, E., 2020. Pre-print of Assessing Open Science Practices in Phytolith

Research (preprint). Open Science Framework. <https://doi.org/10.31219/osf.io/fa7q3>

-Kameník, J., J. Mizera, and Z. Řanda. 2013. 'Chemical Composition of Plant Silica Phytoliths'. *Environmental Chemistry Letters* 11 (June): 189–95. <https://doi.org/10.1007/s10311-012-0396-9>

-Katz, Ofir, Dan Cabanes, Stephen Weiner, Aren M. Maeir, Elisabetta Boaretto, and Ruth Shahack-Gross. 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 (July): 1557–63. <https://doi.org/10.1016/j.jas.2010.01.016>

-Ksiaz, Mariem, Nèjia Farhat, Mokded Rabhi, Amine Elkhouni, Abderrazak Smaoui, Ahmed Debez, Cécile Cabassa-Hourton, Arnould Savouré, Chedly Abdelly, and Walid Zorrig. 2021. 'Silicon (Si) Alleviates Iron Deficiency Effects in Sea Barley (*Hordeum Marinum*) by Enhancing Iron Accumulation and Photosystem Activities'. *Silicon*, October. <https://doi.org/10.1007/s12633-021-01376-x>

-Kumar, Santosh, Yonat Milstein, Yaniv Bami, Michael Elbaum, and Rivka Elbaum. 2017. 'Mechanism of Silica Deposition in Sorghum Silica Cells'. *New Phytologist* 213: 791–98. <https://doi.org/10.1111/nph.14173>

-Kumar, Santosh, Milan Soukup, and Rivka Elbaum. 2017. 'Silicification in Grasses: Variation between Different Cell Types'. *Frontiers in Plant Science* 8 (March). <https://doi.org/10.3389/fpls.2017.00438>

-Leng, Melanie J., George E. A. Swann, Martin J. Hodson, Jonathan J. Tyler, Siddharth V. Patwardhan, and Hilary J. Sloane. 2009. 'The Potential Use of Silicon Isotope Composition of Biogenic Silica as a Proxy for Environmental Change'. *Silicon*, 65–77. <https://doi.org/10.1007/s12633-009-9014-2>

-Lombardo, U., Ruiz-Pérez, J., Rodrigues, L., Mestrot, A., Mayle, F., Madella, M., Szidat, S., Veit, H., 2019. Holocene land cover change in south-western Amazonia inferred from paleoflood archives. *Global and Planetary Change* 174, 105–114. <https://doi.org/10.1016/j.gloplacha.2019.01.008>

-Lux, Alexander, Miroslava Luxova, Taiichiro Hattori, Shinobu Inanaga, and Yukihiro Sugimoto. 2002. 'Silicification in Sorghum (*Sorghum Bicolor*) Cultivars with Different Drought Tolerance'. *Physiologia Plantarum* 115 (May): 87–92. <https://doi.org/10.1034/j.1399-3054.2002.1150110.x>

-Lv, Wanjie, Guomo Zhou, Guangsheng Chen, Yufeng Zhou, Zhipeng Ge, Zhengwen Niu, Lin Xu, and Yongjun Shi. 2020. 'Effects of Different Management Practices on the Increase in Phytolith-Occcluded Carbon in Moso Bamboo Forests'. *Frontiers in Plant Science* 11. <https://doi.org/10.3389/fpls.2020.591852>

-Ma, Jian Feng, Kazunori Tamai, Naoki Yamaji, Namiki Mitani, Saeko Konishi, Maki Katsuhara, Masaji Ishiguro, Yoshiko Murata, and Masahiro Yano. 2006. 'A Silicon Transporter in Rice'. *Nature* 440 (March): 688–91. <https://doi.org/10.1038/nature04590>

-Ma, Jian Feng, and Naoki Yamaji. 2006. 'Silicon Uptake and Accumulation in Higher Plants'. *Trends in Plant Science* 11 (8): 392–97. <https://doi.org/10.1016/j.tplants.2006.06.007>

-Ma. 2015. 'A Cooperative System of Silicon Transport in Plants'. *Trends in Plant Science* 20 (7): 435–42. <https://doi.org/10.1016/j.tplants.2015.04.007>

-Madella, M., M.K. Jones, P. Echlin, A. Powers-Jones, and M. Moore. 2009. 'Plant Water Availability and Analytical Microscopy of Phytoliths: Implications for Ancient

- Irrigation in Arid Zones'. *Quaternary International* 193 (1–2): 32–40. <https://doi.org/10.1016/j.quaint.2007.06.012>
- Madella, M., A.H. Powers-Jones, and M.K. Jones. 1998. 'A Simple Method of Extraction of Opal Phytoliths from Sediments Using a Non-Toxic Heavy Liquid'. *Journal of Archaeological Science* 25 (8): 801–3. <https://doi.org/10.1006/jasc.1997.0226>
- Madella, Marco, and Carla Lancelotti. 2012. 'Taphonomy and Phytoliths: A User Manual'. *Quaternary International* 275 (October): 76–83. <https://doi.org/10.1016/j.quaint.2011.09.008>
- Madella, Marco, Carla Lancelotti, and Juan José García-Granero. 2016. 'Millet Microremains—an Alternative Approach to Understand Cultivation and Use of Critical Crops in Prehistory'. *Archaeological and Anthropological Sciences* 8: 17–28. <https://doi.org/10.1007/s12520-013-0130-y>
- Markovich, Oshry, Santosh Kumar, Dikla Cohen, Sefi Addadi, Eyal Fridman, and Rivka Elbaum. 2019. 'Silicification in Leaves of Sorghum Mutant with Low Silicon Accumulation'. *Silicon* 11: 2385–91. <https://doi.org/10.1007/s12633-015-9348-x>
- Markovich, Oshry, Nerya Zexer, Boaz Negin, Yotam Zait, Shula Blum, Alon Ben-Gal, and Rivka Elbaum. 2022. 'Low Si Combined with Drought Causes Reduced Transpiration in Sorghum Lsi1 Mutant'. *Plant and Soil*, February. <https://doi.org/10.1007/s11104-022-05298-4>
- Mateos-Naranjo, Enrique, Luis Andrades-Moreno, and Anthony J. Davy. 2013. 'Silicon Alleviates Deleterious Effects of High Salinity on the Halophytic Grass *Spartina densiflora*'. *Plant Physiology and Biochemistry* 63 (February): 115–21. <https://doi.org/10.1016/j.plaphy.2012.11.015>
- Miller Rosen, Arlene. 1994. 'Identifying Ancient Irrigation: A New Method Using Opaline Phytoliths from Emmer Wheat'. *Journal of Archaeological Science* 21: 125–32.
- Mitani, N. 2005. 'Uptake System of Silicon in Different Plant Species'. *Journal of Experimental Botany* 56 (414): 1255–61. <https://doi.org/10.1093/jxb/eri121>
- Ngoc Nguyen, Minh, Stefan Dultz, and Georg Guggenberger. 2014. 'Effects of Pretreatment and Solution Chemistry on Solubility of Rice-straw Phytoliths'. *Journal of Plant Nutrition and Soil Science* 177 (June): 349–59. <https://doi.org/10.1002/jpln.201300056>
- Parr, Jeff F. 2006. 'Effect of Fire on Phytolith Coloration'. *Geoarchaeology* 21 (2): 171–85. <https://doi.org/10.1002/gea.20102>
- Parr, Jeffrey F., and Leigh A. Sullivan. 2014. 'Comparison of Two Methods for the Isolation of Phytolith Occluded Carbon from Plant Material'. *Plant and Soil* 374: 45–53. <https://doi.org/10.1007/s11104-013-1847-1>
- Parr, J.F., V. Dolic, G. Lancaster, and W.E. Boyd. 2001. 'A Microwave Digestion Method for the Extraction of Phytoliths from Herbarium Specimens'. *Review of Palaeobotany and Palynology* 116: 203–12. [https://doi.org/10.1016/S0034-6667\(01\)00089-6](https://doi.org/10.1016/S0034-6667(01)00089-6)
- Pearsall, Deborah M. 2016. *Paleoethnobotany- A Handbook of Procedures*. 3rd ed. Oxford: Routledge Taylor & Francis Group.
- Piperno, Dolores R. 2006. *Phytoliths*. Oxford: Altamira press.
- Prentice, Andrea J., and Elizabeth A. Webb. 2016. 'The Effect of Progressive Dissolution on the Oxygen and Silicon Isotope Composition of Opal-A Phytoliths: Implications for Palaeoenvironmental Reconstruction'. *Palaeogeography, Palaeoclimatology, Palaeoecology* 453: 42–

51. <https://doi.org/10.1016/j.palaeo.2016.03.031>

-Roy, Biswajit, Sutapa Patra, and Prasanta Sanyal. 2020. 'The Carbon Isotopic Composition of Occluded Carbon in Phytoliths: A Comparative Study of Phytolith Extraction Methods'. *Review of Palaeobotany and Palynology*. <https://doi.org/10.1016/j.revpalbo.2020.104280>

-Rudall, Paula J., Christina J. Prychid, and Thomas Gregory. 2014. 'Epidermal Patterning and Silica Phytoliths in Grasses: An Evolutionary History'. *The Botanical Review* 80 (March): 59–71. <https://doi.org/10.1007/s12229-014-9133-3>

-Santos, Guaciara M, Anne Alexandre, Heloisa H G Coe, Paul E Reyerson, John R Southon, and Cacilda N De Carvalho. 2010. 'The Phytolith ¹⁴C Puzzle: A Tale of Background Determinations and Accuracy Tests'. *Radiocarbon* 52 (1): 113–28. <https://doi.org/10.1017/S0033822200045070>

-Schulz-Kornas, Ellen, Caroline Braune, Daniela E. Winkler, and Thomas M. Kaiser. 2017. 'Does Silica Concentration and Phytolith Ultrastructure Relate to Phytolith Hardness?' *Biosurface and Biotribology* 3: 135–43. <https://doi.org/10.1016/j.bsb.2017.12.004>

-Shahack-Gross, Ruth, Aldo Shemesh, Dan Yakir, and Steve Weiner. 1996. 'Oxygen Isotopic Composition of Opaline Phytoliths: Potential for Terrestrial Climatic Reconstruction'. *Geochimica et Cosmochimica Acta* 60 (20): 3949–53. [https://doi.org/10.1016/0016-7037\(96\)00237-2](https://doi.org/10.1016/0016-7037(96)00237-2)

-Stamm, Franziska M., Merlin Méheut, Thomas Zambardi, Jérôme Chmeleff, Jacques Schott, and Eric H. Oelkers. 2020. 'Extreme Silicon Isotope Fractionation Due to Si Organic Complexation: Implications for Silica Biomineralization'. *Earth and Planetary Science Letters* 541. <https://doi.org/10.1016/j.epsl.2020.116287>

-Strömberg, Caroline A. E., Verónica S. Di Stilio, and Zhaoliang Song. 2016. 'Functions of Phytoliths in Vascular Plants: An Evolutionary Perspective'. Edited by Jane De Gabriel. *Functional Ecology* 30: 1286–97. <https://doi.org/10.1111/1365-2435.12692>

-Strömberg, Caroline A.E. 2009. 'Methodological Concerns for Analysis of Phytolith Assemblages: Does Count Size Matter?' *Quaternary International* 193: 124–40. <https://doi.org/10.1016/j.quaint.2007.11.008>

Twiss, P. C., Erwin Suess, and R. M. Smith. 1969. 'Morphological Classification of Grass Phytoliths'. *Soil Science Society of America Journal* 33: 109–15. <https://doi.org/10.2136/sssaj1969.03615995003300010030x>

-Tyler, Jonathan J., Melanie J. Leng, and Hilary J. Sloane. 2007. 'The Effects of Organic Removal Treatment on the Integrity of $\Delta^{18}\text{O}$ Measurements from Biogenic Silica'. *Journal of Paleolimnology* 37 (May): 491–97. <https://doi.org/10.1007/s10933-006-9030-9>

-Vatansever, Recep, Ibrahim Ilker Ozyigit, Ertugrul Filiz, and Nermin Gozukara. 2017. 'Genome-Wide Exploration of Silicon (Si) Transporter Genes, Lsi1 and Lsi2 in Plants; Insights into Si-Accumulation Status/Capacity of Plants'. *BioMetals* 30: 185–200. <https://doi.org/10.1007/s10534-017-9992-2>

-Watling, Kym M., Jeff F. Parr, Llew Rintoul, Christopher L. Brown, and Leigh A. Sullivan. 2011. 'Raman, Infrared and XPS Study of Bamboo Phytoliths after Chemical Digestion'. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* 80 (October): 106–11. <https://doi.org/10.1016/j.saa.2011.03.002>

-Webb, Elizabeth A., and Fred J. Longstaffe. 2002. 'Climatic Influences on the Oxygen Isotopic Composition of Biogenic Silica in Prairie Grass'. *Geochimica et Cosmochimica*

*Acta*66 (11): 1891–1904.[https://doi.org/10.1016/S0016-7037\(02\)00822-0](https://doi.org/10.1016/S0016-7037(02)00822-0)

Webb, Elizabeth A. 2010. 'Limitations on the Climatic and Ecological Signals Provided by the $\Delta^{13}\text{C}$ Values of Phytoliths from a C4 North American Prairie Grass'. *Geochimica et Cosmochimica Acta*74 (June): 3041–50.<https://doi.org/10.1016/j.gca.2010.03.006>

-Yang, Shilei, Qian Hao, Hailong Wang, Lukas Van Zwieten, Changxun Yu, Taoze Liu, Xiaomin Yang, Xiaodong Zhang, and Zhaoliang Song. 2020. 'A Review of Carbon Isotopes of Phytoliths: Implications for Phytolith-Occcluded Carbon Sources'. *Journal of Soils and Sediments*.<https://doi.org/10.1007/s11368-019-02548-4>

-Zancajo, Victor M. R., Sabrina Diehn, Nurit Filiba, Gil Goobes, Janina Kneipp, and Rivka Elbaum. 2019. 'Spectroscopic Discrimination of Sorghum Silica Phytoliths'. *Frontiers in Plant Science*10.<https://doi.org/10.3389/fpls.2019.01571>

-Zurro, Debora. 2018. 'One, Two, Three Phytoliths: Assessing the Minimum Phytolith Sum for Archaeological Studies'. *Archaeological and Anthropological Sciences*10 (October): 1673–91.<https://doi.org/10.1007/s12520-017-0479-4>

-Zurro, Débora, Juan José García-Granero, Carla Lancelotti, and Marco Madella. 2016. 'Directions in Current and Future Phytolith Research'. *Journal of Archaeological Science*68 (April): 112–17.<https://doi.org/10.1016/j.jas.2015.11.014>

DOI

dx.doi.org/10.17504/protocols.io.q26g74mb8gwz/v2

PROTOCOL CITATION

Francesca D'Agostini, Javier Ruiz-Pérez, Marco Madella, Vincent Vadez, Carla Lancelotti 2022. Phytolith extraction and counting procedure for modern plant material rich in silica skeletons. **protocols.io**
<https://dx.doi.org/10.17504/protocols.io.q26g74mb8gwz/v2>
 Version created by fra.dagostini F D'Agostini



KEYWORDS

Phytoliths, Phytoliths extraction, Modern plant tissues, Silica skeletons, Extraction methodology

LICENSE

————— This is an open access protocol distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

CREATED

Sep 30, 2022

LAST MODIFIED

Sep 30, 2022

MATERIALS TEXT

Consumables:

- Glass beakers (100 ml).
- Crucibles (20 ml) that can stand at least 500°C in the furnace and equipped with lids.
- Eppendorf tubes (5 ml).
- Sterile centrifuge tubes (5 ml) and racks to store them.
- Microscope slides and covers.
- Micro spatula or micro spoon.

Chemical products:

- Hydrochloric acid 10% v/v.
- Hydrogen peroxide 10% v/v.
- Entellan New® (mounting medium).
- Ethanol 10% v/v for cleaning tools.
- Distilled water.

Equipment:

- Precision scale (at least 0.0001 g).
- Sonicator (ultrasonic bath).
- Muffle furnace that can reach at least 500°C.
- Pipette (1-100 µl).
- Fume hood.
- High speed centrifuge (6000 rpm) for tubes of 5 ml.
- Laboratory drying cabinet (30-60°C).
- Optical microscope. The optimum setting includes a digital camera for photographing phytoliths. A good quality scanning can be done with a 400-600x magnification.

SAFETY WARNINGS

Label elements, including precautionary statements

Hydrochloric acid

(CAS-No.) 7647-01-0

(EC-No.) 231-595-7; 231-596-7

(EC Index-No.) 017-002-01-X

(REACH-no) 01-2119484862-27

H314 - Causes severe skin burns and eye damage.

H335 - May cause respiratory irritation.

P301+P330+P331 - IF SWALLOWED: rinse mouth. Do NOT induce vomiting.

P303+P361+P353 - IF ON SKIN (or hair): Take off immediately all contaminated

clothing. Rinse skin with water/shower.

P304+P340 - IF INHALED: Remove person to fresh air and keep comfortable for breathing.

P305+P351+P338 - IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.

P310 - Immediately call a POISON CENTER/doctor.

Hydrogen peroxide

(CAS-No.)7722-84-1

(EC-No.)231-765-0

(EC Index-No.)008-003-00-9

(REACH-no)01-2119485845-22

H271 - May cause fire or explosion; strong oxidiser.

H332 - Harmful if inhaled.

H302 - Harmful if swallowed.

H314 - Causes severe skin burns and eye damage.

P210 - Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking.

P280 - Wear protective gloves/protective clothing/eye protection/face protection.

P301+P330+P331 - IF SWALLOWED: rinse mouth. Do NOT induce vomiting.

P304+P340 - IF INHALED: Remove person to fresh air and keep comfortable for breathing.

P310 - Immediately call a POISON CENTER/doctor.

Entellan® New

(CAS-No.)1330-20-7

(EC-No.)1272/2008

H226 Flammable liquid and vapour

H312 + H332 Harmful in contact with skin or if inhaled

H315 Causes skin irritation

P210 Keep away from heat. Response

P302 + P352 IF ON SKIN: Wash with plenty of soap and water

P304 + P340 IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing

BEFORE STARTING

We tested the protocol on monocots tissues, which produce high concentrations of phytoliths in a reduced volume. However, the dimension of the glassware, crucible and tubes can be adapted to the plant material of interest.

The protocol takes up to 10 days to complete, from when plant tissues are collected from the plant to when samples can be observed at the microscope and depending on the plant material and humidity conditions. Skipping the first drying steps (tips 1-2)

allows for faster extractions. The number of samples that can be processed at one time depends on the laboratory space (mostly the furnace cabinet space and the centrifuge) and the experience of the practitioner.

Drying plant material

1

The first steps (points 1 and 2) aim to obtain very clean and dry samples to evaluate biomass production before the extraction and to store plant tissues for future use. If biomass evaluation or storage are not needed, start directly from point 3.

Collect the tissues of interest from the whole plant. Store each sample in a separate paper bag and put the paper bags in a dryer. Paper bags prevent the formation of fungi and bacterial infection, allowing the evaporation of tissues' humidity. Collect the tissues of interest from the whole plant. Store each sample in a separate paper bag and put the paper bags in a dryer. Paper bags prevent the formation of fungi and bacterial infection, allowing the evaporation of tissues' humidity.

- 2 Leave the plant tissues to dry at 60-70°C in a dryer (we use a IWC125 Leec drying cabinet). Check the bags once a day to be sure they do not develop any fungi infection because of the heat. Weigh the samples until no weight loss is observed to be sure to have obtained completely dry tissues. Our dried samples weigh on average 45% less of the fresh biomass. Considering that each species/treatment and tissue has its own level of humidity, we suggest testing the tissues for their consistency to make sure they are dry: they will be completely dry when they become brittle (try to crush the leaves with your hands to check their consistency).
- 3 Wash samples in an ultrasound bath (we used a Ulsonix Proclean 3.0) at room temperature for ^{5m} 00:05:00 to remove extraneous debris (such as soil particles). To wash many samples simultaneously, use small glass beakers: put each sample in a labeled beaker and cover it with distilled water. Put all the beakers in the ultrasound bath and fill the container with water while paying attention not to overflow in the beakers. Cut the samples into pieces to fit into the beakers. 100 ml beakers are large enough to contain samples of grasses. Calibrate beaker and sample sizes based on the species under analysis.



Multiple samples in the ultrasound bath using glass beakers.

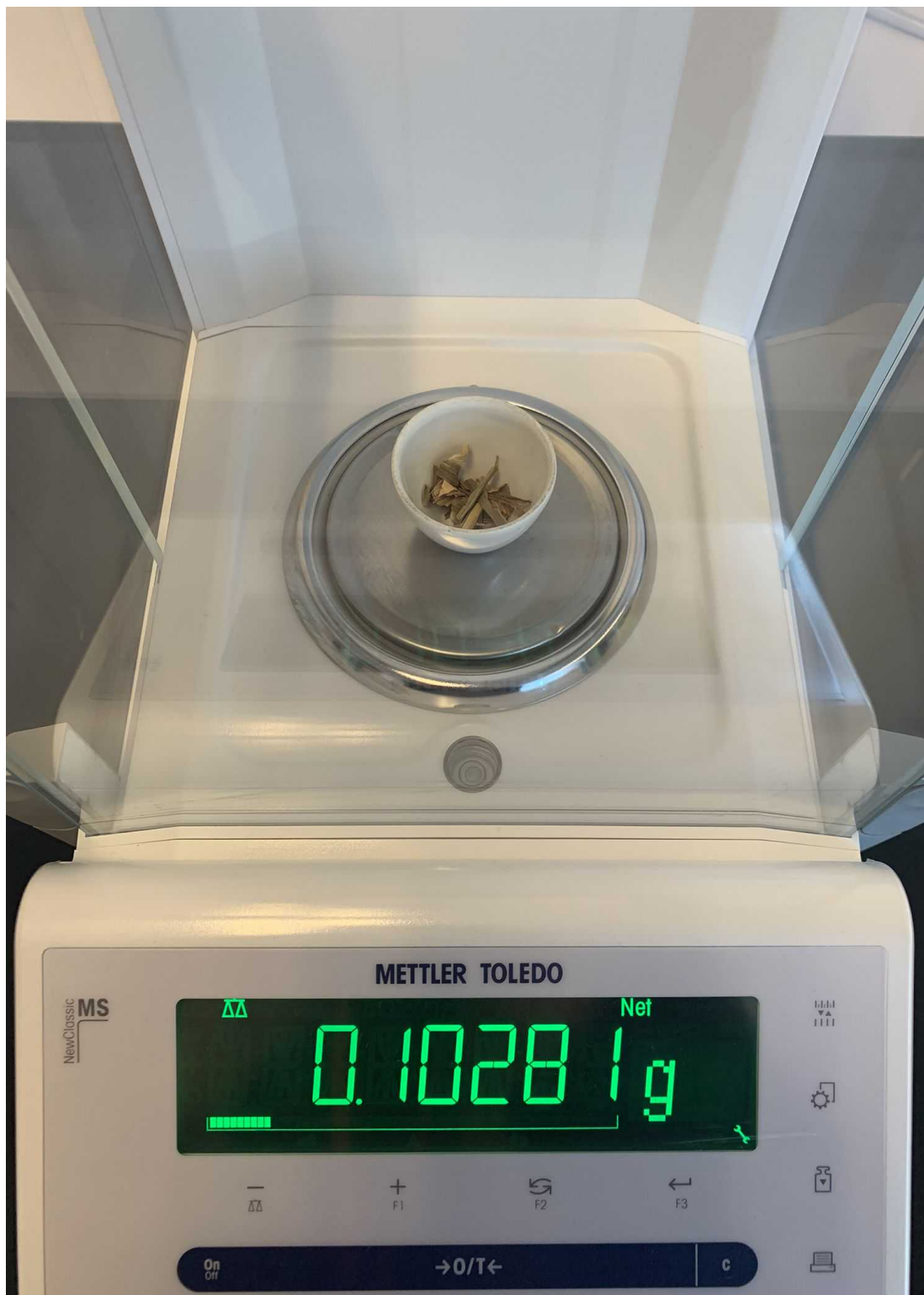
- 4 After 5 minutes, remove the samples from the beaker with a tweezer and place them on aluminum foil to dry.

Ashing procedure

- 5 0.1 g

The following steps ensure the complete decomposition and mineralization of the organic material.

Weigh the plant material (in our case we use a Mettler Toledo MS105DU to weight ca. **0.1 g**) in a labeled ceramic crucible



Plant material weighed with a precision scale inside the ceramic crucible.

- 6 Remove the crucible and cover it with a lid (ceramic lid or foil lid). Do not worry if the samples^{12h} are not completely sealed by the lid, if oxygen passes inside it will avoid carbonization. Place the crucibles in the furnace before turning it on and ash them at 500°C for 🕒 **12:00:00** (we use a Nabertherm C450 of 5 l capacity). The 12 hours include the time the oven takes to warm up til 500°C (in our case 1 hours on average).
- 7 Let the crucibles cool completely before removing the samples from the furnace.

Wet procedure

8

These steps are fundamental to digest carbonates and oxidize organic material left from the ashing procedure and any form of organic material that was not removed.

Remove the ashes from the crucible with a spatula and place them in an Eppendorf tube of 5 ml. Use a clean spatula for each sample so not to contaminate between samples.



Ceramic crucibles containing the samples, covered with a lid. a) shows the plant tissue before ashing and b) shows the white ashes obtained after 12 hours in the oven.

- 9 Add 🧊 **900 µL** of 10% v/v HCl and vortex the tube to stir the solution (we use an ES714R Maxi^{5h} Mixer). Leave the HCl to react for 🕒 **05:00:00** (or till the reaction stops) with the cap of the

tube open. Do not let the sample dry by adding more HCl solution if necessary.



Work in a fume cupboard. Wear a lab coat and gloves when dealing with Hydrochloric acid.

- 10 Dilute the solution with **450 µL** of distilled water added with a pipette and centrifuge the tubes at 6000 rpm for **00:05:00** (we use a Mini Spin Eppendorf®5453) after screwing on the lids of the centrifuge tubes. Discard the supernatant. Repeat this process for three more times to completely remove the HCl. ^{5m}

- 11 Add **900 µL** of H₂O₂ (10% v/v). Place the tube in a dryer or alternatively in a hot bath at 40°C ^{9h} until the reaction stops (in our experiments about **09:00:00** for chaff and leaves, at least 12 hours for stems) with the cap of the tube open. Every 2 hours rinse the samples with distilled water by repeating step 10 one time and add 900 µl H₂O₂. Do not let the sample dry by adding more H₂O₂ solution if necessary.



Work in a fume cupboard. Wear a lab coat and gloves when dealing with Hydrochloric acid

- 12 Dilute the solution with **450 µL** of distilled water added with a pipette and centrifuge the tubes at 6000 rpm for **00:05:00** after screwing on the lids of the centrifuge tubes. Discard the supernatant. Repeat this process for three more times to completely remove the H₂O₂. ^{5m}

- 13 Dry the extracts in a drying cabinet at 60°C. When the powder is completely dry, cool the extracts and weigh them. Repeat the weighing until no weight loss is observed to be sure to measure the completely dry silica weight produced by the plant. Furthermore, when samples are dry the colour of the extract is usually clearer than when it is humid and the powder is loose (shake the tubes to check if the powder is loose or if lumps are still present, in this case there is still residual humidity).

Dry mounting

- 14 Place **0.0001 g** of silica powder on a microscope slide with a clean spatula. Add 4 drops of Entellan New® on the top of the powder and gently stir using a micro clean spatula/micro

spoon to distribute the phytoliths homogeneously. Cover the slide: we used a cover slip of 24 x 32 mm but also a 24 x 24 can be used. Use the same cover slip size for all slides.



Wear gloves when dealing with Entellan New®

- 15 Leave the slide to dry for at least 30 minutes so that phytoliths set at the same depth. 12h
However, we suggest waiting about 🕒 **12:00:00** so that Entellan New® reaches the best density (neither too liquid nor too dry) to rotate phytoliths to guarantee correct descriptions/identifications.

Microscope observation and counting

- 16 Start counting and classify phytoliths. Always note the number of fields of view observed to calculate phytolith concentration later -it depends on how many fields of view are in one cover slip, indeed the measurement is related to the cover slip size. Rotate phytoliths to inspect their tridimensional shape and to assure a good classification of the morphotypes. Use the International Code for Phytolith Nomenclature (ICPN) 2.0 (Neumann et al. 2019) for the description of the morphotypes.

17

Synonyms

silica skeletons: multi-cells structure

disarticulated cells: single celled, silica monoliths, single phytolith

Count until 50 skeletons have been numbered and their phytolith cells classified. For each skeleton, annotate the total number of cells for each morphotype recognized. While counting skeletons, count and classify all the disarticulated phytoliths along the pathway separately. At the end of the procedure, the information about phytoliths in skeletons and disarticulated cells can be summarized together in a unique file to obtain the total phytoliths size and the absolute number of each morphotype, as well as the silica skeleton sizes.