Extraction of Nonstructural Carbon and Cellulose from Wood for Radiocarbon Analysis

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[Abstract] This method aims at isolating nonstructural organic carbon (NSC) pools, *i.e.* soluble sugars and starch, from wood for radiocarbon (^{14}C) analysis at natural abundance levels (≤1 ppt). Pools are operationally defined to 1) physically isolate pools - prohibiting the use of destructive methods, such as compound-specific enzyme digestion, and 2) minimize possible contamination with extraneous carbon form organic solvents.

Materials and Reagents

A. Nonstructural carbon and cellulose
   1. MQ H_2O
   2. Laboratory grade pure methanol for cleaning

B. Nonstructural carbon
   1. ^{14}C standards [international secondary standards: ANU sucrose (IAEA-C6), in-house laboratory standards, *e.g.* wheat flour, rye flour]
   2. Laboratory-grade ethanol
   3. HPLC-grade ethanol
   4. Analytical-grade 20% HCl [≈ 6 M HCl, 50:50 (v: v) conc. HCl]

C. Cellulose
   1. ^{14}C standards (international secondary wood standards: *e.g.* IAEA-C4, -C5)
   2. Laboratory-grade toluene
   3. Laboratory-grade glacial acetic acid (CH_3COOH)
   4. Technical grade sodium chlorite ($NaClO_2$)

Equipment

A. Nonstructural carbon and cellulose
   1. Aluminum (Al) foil (cover surfaces and prevent dust contaminating equipment and samples with extraneous carbon)
   2. Marker pen
   3. Spatula
   4. Wipes for cleaning (*e.g.* KCWW)
   5. Duster for cleaning (I L) (*e.g.* Innovera™)
   6. Pyrex beakers to hold reagents
   7. Graduated volumetric cylinder to prepare reagents

      Note: All glassware is baked and stored covered with Al foil to remove extraneous sources of carbon. Quartz is pre-combusted at 900 °C for 2 h, Pyrex at 550 °C for 3 h.

B. Nonstructural carbon
   1. Mill with mesh 10 or scalpel
   2. Graduated, adjustable volume, 5 ml pipette (*e.g.* VWR International, Eppendorf) or cylinder
   3. Disposable borosilicate glass culture tubes (13 x 100 mm) with plastic vented caps and snap caps (VWR International)
   4. Disposable, plastic (extended fine tip) transfer pipette (*e.g.* Thermo Fisher Scientific, Samco Scientific)
   5. Quartz combustion tubes (120 mm length, 9 mm O.D.)
   6. 50 ml reusable glass centrifugation tube with screw cap or plastic (*e.g.* PP) centrifuge tubes with screw cap
   7. Speedvac-dryer (Thermo Fisher Scientific, Savant, model: SC200 speedvac with RT400 cold trap) (operated at room temperature)
   8. Block heater with thermometer for 10 or 20 (13 x 100 mm) disposable borosilicate glass culture tubes

C. Cellulose
   1. General-purpose extra-long, stainless steel forceps
   2. Quartz combustion tubes (150 mm length, 6 mm O.D.)
   3. Heat sealer
   4. 1,000 ml pyrex beaker
   5. 1,000 ml pyrex round bottom flask (still pot)
   6. Soxhlet extraction apparatus (*e.g.* VWR International)

7. Heated magnetic stirrer & stir bar
8. Drying oven
9. Cork ring
10. Boiling chips
11. Fiber filter bags for sample digestion (ANKOM Technology, catalog number: F57)

Procedure

Part I: Extraction of nonstructural carbon

The sequential extraction protocol was developed for Reference 2, based on References 3 and 4. Following extractions, samples are dried in 9 mm O. D. quartz combustion tubes and combusted to CO_2 at 900 °C for 2 h with CuO (80 mg CuO for 1 mg C); the CO_2 is isolated cryogenically and converted to graphite (Xu *et al*. 2007). Ideally, sugar and starch concentrations should be known prior to this extraction to guide how much material should be extracted and combusted. Concentrations can be quantified as described in References 3 and 4. The target amount for a radiocarbon sample analyzed with accelerator mass spectrometry is typically 0.3-1.2 mg C.

A. Sample preparation

Samples are processed in batches of a maximum of 17 samples plus 3 standards.

1. Manually chop each dried wood core into small slices or pieces [or grind samples using a mill to mesh 10 (0.8 mm)]. Avoid producing powder to achieve clear separation into supernatant and precipitate. Clean all tools with laboratory grade methanol, then blow dry with an air duster. Cover all surfaces with Al foil.
2. Assign each sample and standard 2 different ID numbers (1 for soluble sugars, 1 for starch; except ANU which requires only 1 for soluble sugars).
3. Weigh each sample (entire core section) and standard [ANU (1.5-2 mg), flours (30 mg)] into a culture tube labeled with the ID numbers of both the sugar and the starch fraction. Each sample should not take up more than about 1/3 of the tube as larger samples will not easily absorb solutions or might float out of the tube.
4. Label combustion tubes with the ID numbers of either the sugar or the starch fraction and record the weight of the empty tubes.
5. Label centrifuge vials with the starch-fraction ID numbers.

B. Extraction of soluble sugars

1. At least 1 h before usage, switch on the speedvac drier to cool the cold-trap to its operating temperature (see operating manual for details).
2. Pre-heat block heater to 100 °C.

3. Add 5 ml MQ H_2O to each tube, close with a ventilated cap and place in block heater. Bring to boil and gently boil for 15 min. If sample floats, gently tap the tube.
4. Let solution cool, and then transfer the supernatant with a disposable pipette into a 50 ml glass centrifuge tube. If any sample is floating on the supernatant, centrifuge samples before transfer.

   Note: The disposable culture tubes break easily. They require specific adapters when used in a centrifuge.
5. Repeat boiling the sample in 5 ml MQ H_2O for 15 min TWICE and combine the supernatants in the same 50 ml centrifuge tube. This will yield ≤15 ml of liquid.
6. In batches of 1-2 ml, transfer 2 ml of supernatant to a combustion tube (labeled with sugar-fraction ID number) and speedvac-dry.

   Notes:

   a. *Ideally, sugar and starch contents should be known prior to ^{14}C analysis. Depending on the sugar yield and on the amount of other soluble compounds recovered with the sugars, samples may explode during combustion or not yield sufficient C for ^{14}C analysis. The target amount is 0.3-1.2 mg C. Samples may have to be combusted multiple times (trial and error) using 0.5-5 ml of supernatant.*
   b. *This should be done on the same day as the extraction, since samples are prone to growing mold.*

   While the sugar samples are drying over several hours, begin starch extraction. Alternatively, cover the solid precipitate remaining in the extraction tube with a snap cap and refrigerate.
7. Refrigerate remaining liquid sugar fraction.
8. After speedvac-drying, record the weight of the combustion tubes.

C. Starch extraction

Removal of nonpolar compounds, including lipids and pigments

1. Pre-heat block heater to 70 °C.
2. Add 5 ml ethanol to the sample in the culture tube, close with vented cap and gently boil for 30 min at about 70 °C.
3. Let solution cool and then discard the supernatant.
4. Repeat boiling the sample in fresh ethanol until the supernatant is clear and colorless.

   Note: It is critical that all of the ethanol is removed before proceeding to the starch extraction. If the sample size is very small and sample cannot be sucked very dry, sample might have to be washed with MQ H_2O to remove the ethanol.

Extraction of starch

5. Lower block heater temperature to 40 °C.
6. Add 1.5 ml 20% HCl to the sample in the culture tube, close with vented cap and keep at 40 °C for 30 min.

   Note: Make sure the sample is well suspended; samples of finer texture might require more liquid; keep ratios of acid and ethanol (see Part I, step C11) constant.
7. Switch off the block heater, remove samples and let them stand at room temperature for 8-12 h (over night). Pre-heat block heater to 40 °C.
8. Transfer supernatant from the culture tubes to a 50 ml centrifuge tube (labeled with the starch-fraction ID number). If any sample is floating in the supernatant, centrifuge samples before transfer.
9. Again, add 1.5 ml 20% HCl to wood sample in the culture tube (not to the supernatant) and keep at 40 °C for 30 min.
10. Transfer supernatant to the same 50 ml centrifuge tube - there should be about 3 ml of liquid in the tube. If any sample is floating, centrifuge samples and transfer the supernatant to a fresh tube.
11. Add 4.5 ml ethanol to the supernatant, vortex, close with a screw cap and let stand over night in the fume hood at room temperature. Starch will precipitate.
12. The wood sample remaining in the culture tube may be used for subsequent cellulose analysis. If this is required, wash the sample with MQ water until the pH is neutral and dry samples in the block heater or oven at <60 °C.

Removal of extraneous carbon (ethanol)

Note: Failure to remove (traces of) extraneous C will result in a lower ^{14}C content (unrealistically old ^{14}C ages). This removal process is validated by analyzing lab internal ^{14}C standards (flours) of known ^{14}C content alongside each batch of samples.

13. Switch on the speedvac-drier to cool the cold-trap to its operating temperature.
14. Centrifuge samples and discard the supernatant with a pipette.
15. Dissolve starch in 1.5 ml MQ H_2O (using vortex mixer) and then speedvac-dry sample within the centrifuge tube; this process will take several hours. Avoid completely drying the sample, as the starch will stick to the tube's walls and cannot be easily redissolved.
16. Repeat dissolution in 1.5 ml MQ H_2O and speedvac-drying.
17. Again, dissolve starch in 1.5 ml MQ H_2O, but transfer all of this liquid to a combustion tube (labeled with the starch fraction ID number) and dry. To ensure complete transfer, work with 0.5 ml MQ H_2O at a time and, or use vortex mixer. The target amount is 0.3-1.2 mg C.
18. After speedvac-drying, record the weight of the combustion tubes.

Part II: Extraction of Holocellulose

The holocellulose extraction protocol is based on Reference 1. Following extraction, samples are combusted to CO_2 at 900 °C for 2 h with CuO (80 mg CuO for 1 mg C); the CO_2 is isolated cryogenically and converted to graphite (Xu *et al*., 2007).

A. Sample preparation

Samples are processed in batches of up to 38 samples plus 2 standards (one fossil wood and one modern wood, with known ^{14}C age).

1. Assign each sample and standard a laboratory ID number.
2. After cleaning scalpel with methanol and blowing it dry with an air duster, manually slice or chop samples of dried wood into pieces. Cover all surfaces with Al foil.
3. Weigh ~100 mg of homogenized sample into a bag. Avoid producing very thin slices or powder, as this material cannot be recovered from the digestion bags or may pass through the bags. Record the initial sample weight. Each bag can hold up to four samples or standards. Up to 10 bags can be extracted in parallel.
4. Using a heat sealer, seal bags in unique patterns to allow sample identification following the extraction. Record sealing patterns and sample weight and ID in a notebook.

B. Lipid extraction

Note: Since the procedure takes about one week, it is best to start this extraction early Monday morning.

1. In a fume hood, assemble the soxhlet apparatus.

   Note: Make sure the soxhlet has a small enough thimble; otherwise more solvent is needed in a bigger still pot. In other words, make sure enough solvent is left in the still pot when the thimble volume is filled.
2. Using forceps, place (up to 10) digestion bags into the apparatus' thimble.
3. Fill the still pot (1,000 ml round bottom flask) with 600 ml of 2:1 (v: v) toluene:ethanol mixture; add boiling chips.

   Note: This solution may be used for up to three soxhlet extractions; discard earlier if mixture is discolored.
4. Turn the hot plate on and bring the solvent to a gentle boil. Wrap the still pot and its neck with Al foil loosely to keep it from cooling. Run the extraction for 24 h.
5. Turn the hot plate off; let apparatus cool. Remove the bags from the thimble and let dry on Al foil in the fume hood for 2 h.
6. Repeat the extraction with 600 ml of ethanol for 24 h.

   Note: This ethanol may be used for up to three soxhlet extractions; discard earlier if mixture is discolored.

7. Dry the bags again for 2 h.

C. Bleaching

1. Measure out 4 g sodium chlorite.
2. Place digestion bags into a 1,000 ml beaker.
3. Add 600 ml MQ H_2O, partially cover it with a watch glass and boil the samples on a hot plate for 2 h.
4. Switch off the hot plate; let the solution cool and discard it.
5. Add 600 ml MQ H_2O, add a stir bar and stir the solution while heating it to 70 °C. Cover it with a watch glass.
6. Add 4 g sodium chlorite and 2 ml glacial acetic acid to the water at about the same time, and continue stirring this solution at 70 °C.
7. Change this solution every 3-4 h by adding additional 2 ml glacial acetic acid and 4 g sodium chlorite to the MQ H_2O until the samples are white. This process can take 1-2 days.
8. When the samples are white (like paper), rinse samples at least 4 times with 600 ml MQ H_2O over a 3-4 h period. Continue stirring this solution at 70 °C. Make sure no acetic acid smell remains.
9. Dry samples in a drying oven at 60 °C to constant weight, typically over night.
10. Record final sample weight.
11. Transfer sample to combustion tube, labeled with sample ID.

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