ISOFYS cellulose extraction

This is the cellulose extraction protocol of the ISOFYS lab at Ghent University. The procedure is based upon the protocol as developed by Dr. Gerhard Helle at GFZ Potsdam, but adaptations and optimizations have been made to deal with small changes in the available equipment and sample sizes.

For any inquires on the protocol please e-mail Dr. Koen Hufkens at koen.hufkens@gmail.com

1. Sample preparation:

- Cut the wood samples of a year ring as small as possible, this will increase the contact surface for the solvents.
- Use pliers or a scalpel to cut the wood samples on a clean surface (glass plate). The pliers are necessary for hard wood.
- Weigh the samples for a later check of the effectiveness of the extraction. **(min. 3 mg – max. 25 mg)**
- **The above weights can be adjusted upward if the protocol is adjusted as well, the alternative protocol is marked with a ***

2. Solutions (reagents):

Making a 5% NaOH solution:

Location of reagents in storage: 4.1

Add 50g NaOH to 1000g distilled water

Making a 7,5% NaClO2 Solution:

Location of reagents in strorage: 15.5

ONLY MAKE THIS SOLUTION RIGHT BEFORE YOU USE IT!!!

w_1 = *80% NaClO2*
m_1 = *NaClO2 for a 7.5% solution*
w_2 = *distilled water, so 0%*
$m_{ges.}$ = *1000g*
$w_{ges.}$ = *7.5%*

$m_1 \times w_1 + m_2 \times w_2 = m_{ges} \times w_{ges}$
$m_1 \times 80\% + \cancel{m_2 \times 0\%} = 1000g \times 7{,}5g$
$m_1 = 1000g \times 7{,}5g / 80\%$
$m_1 = 93.75g$ *with 1000g distilled water = 7.5% NaClO2 solution*

Tune the pH value of the NaClO2 solution with 100% acetic acid (location of reagents: B 1) => After the complete dissolving of the NaClO2 in water, add acetic acid. The pH should be between 4 and 5.

100ml of reagents will be required to fill a complete plate (20 vials), adjust reagents quantities according to this estimate (only valid in the ISOFYS Ugent configuration)

3. Water bath setup and timer:

Before you start any wet procedures, setup your water bath under a ventilated hood. Fill the water bath with hot distilled water. Set the timer to stop after x hours, and start again before the next dcycle if preheating is necessary (see manual timer).

This procedure will be void when using the new Mennert auto-refilling hot water bath.

4. vial plates and template sheets:

Setup the necessary vial plates using the glass vials and the rubber o-rings. Put an outlet tube on the outlet position (last place on the a row). Finally, distribute all samples over the plates. For consistency reasons we fill plates row wise. To prevent mixing of samples fill out a vial plate template using the appropriate plate and sample numbers for the vial locations you use on every plate. There is both a visual template as well as an exell sheet prepared (templates). Use these templates but save the files using a different name for each and every project / extraction.

5. Extraction:

Day 1: Add **5% NaOH** solution to each sample for **2 x 2 hours** (4 hours total) in the **water bath at 60°C** (removes resins, fatty acids and tannins). (*** 4 x 2 hours for samples > 25mg)**

Wash the samples at least **3 times with boiling water** until the samples are pH-neutral.

Add **7,5% NaClO2** solution with a **pH of 4-5** to each sample in the **water bath at 60°C** .

A **total of 9-18 hour**s is needed to remove lignin as well as different dyes. Because the NaClO2 solution is only reactive for max. 3-6 hours it is necessary to change the solution several times. To prevent total evaporation of the water in the water bath a time switch is used (to switch off the heaters), which will also be used to warm up the water bath in the morning. When the Menner bath is used we can leave the warm water bath running as evaporation will be limited due to the hood and the auto refill function.

Day 2: **Change the 7,5% NaClO2 solution:**
Make the 7,5% NaClO2 solution and add acetic acid as described above. If necessary fill the water bath, so that the samples can react for another **3-6 hours at 60°C**.
(Day 1: 3-6 hours + day 2: 3-6 hours = 6-12 hours with NaClO2 solution)

Day 3:**Change the 7,5% NaClO2 solution two times:**
Change the solution and leave the samples for **3-6 hours at 60C**, afterwards change the NaClO2 solution again and leave the samples for **another 3-6 hours**. Fill the water bath if necessary.
(Day 1: 3-6 hours + day 2: 3-6 hours + day 3: 3-6 hours = 9-18 hours with NaClO2 solution)

Day 4: Wash the samples at least **8 times with boiling water** until the samples are **pH-neutral.**

6. Removal of the samples from the vials:

When the cellulose extraction is finished you are left with white pieces of cellulose at the bottom of the vials. These will have to be removed from the glass vials and put into eppendorf vials for subsequent homogenization and drying.

Equipment needed:

- tweezers (smooth)
- squeeze bottle of distilled water
- eppendorf vials
- a beaker for rinse water

Procedure:

1. rinse the tweezers above the beaker with demineralized water
2. put a small amount of demineralized water in a **2 ml eppendorf** eppendorf vial (~0.5 ml).
3. remove the cellulose sample from the glass vial with the clean tweezers
4. put the sample into the eppendorf vial (the small amount of water will help loosen the sample from the tweezers as the cellulose tends to stick to the tweezers
5. squeeze some water over the tweezers and into the eppendorf to remove any left behind cellulose into the eppendorf (do not fill the eppendorf to more than 2 ml as this will make homogenization difficult)

6. repeat from step 1. for all samples

While you remove the vials to get the samples into the eppendorfs put them in a beaker with distilled water.

7. Clean up of the cellulose extraction setup

Separate the rubber seals from the glass vials you put into distilled water in step 6. In addition remove any rubber seals for empty vial locations from the plates.

Rinse both in a bath of distilled water.

Put the glass vials in a beaker (several if necessary) and make a Potassium persulfate ($K_2S_2O_8$) solution using 10g $K_2S_2O_8$ / 100ml distilled water. Roughly ~30 vials will fit into a 500ml beaker.

Put the beaker with the solution on a hot plate at 90 degrees for one hour. Cover the top of the beaker with tin foil. Never measure the temperature in the solution, always use a separate beaker with distilled water. Place the setup under a vented hood as the fumes are noxious. The reagents is extremely oxidizing so be careful! Use proper protection.

After the treatment transfer all vials one by one to a new beaker with distilled water to rinse them. Make sure to do this while the solution is still hot. Once all vials are transferred you can turn of the heat and dispose of the solution.

The rinsed vials can be left to dry in free air (on a flat surface covered with some tissue) or in the vacuum oven (see below).

8. Homogenization:

When all samples are in eppendorf vials proceed to homogenize the samples. We will use the BRANSON sonic mixing device to accomplish this.

Equipment needed:

- (BRANSON) sonic homogenizer / mixer
- (BRANSON) small homogenization mixer tip (found in the BRANSON cabin/box)
- ear protection (annoying high pitch noises can be heard even with the encasement closed)

Procedure:

1. install the small mixer tip if not already installed

2. rinse the mixer tip with distilled water (capturing the water in a small beaker)
3. put the fine extender mixer tip into the eppendorf vial (not to deep as it shouldn't overflow during mixing), set the timer to 1 minute (no marker is present – eyeball between 0 and 2), output control to 2 (higher values will cause sample material to spill out of the eppendorf), duty cycle to 50% for small and large samples respectively. Let pulse for 1 minute, the operation will stop automatically. The resulting fluid should show no big sample pieces with all material suspended, sometimes even showing gel like properties.
4. remove the eppendorf and close properly
5. repeat from step 2. for all samples

9. Drying of the cellulose samples:

As the sample material is in suspension we need to remove all water before a stable isotope analysis is possible.

To do this we place the eppendorf vials with open lids, but COVERED WITH TIN FOIL (make small cuts in the tin foil with a knife to let the moist air out) into a vacuum drying oven. This will take ~24h at 75 ºC to be on the safe side. When the samples aren't properly dried manipulation of the samples will become difficult. After drying the resulting sample material should resemble cotton balls or paper.

10. Stable isotope preparation:

The final step is the standard preparation of any stable isotope analysis, being the packing of the sample material in tin or silver cups depending on the analysis (^{13}C or ^{18}O respectively).

11. Storage:

To make sure that all samples are properly stored, mark all vials in a precise manner and always put all extracted or unextracted material in the square eppendorf boxes that are available in the lab. Always use NEW boxes to avoid confusion of samples due to multiple markings on the box. Store your samples in a dry place.