Chlorophyll *a*+*b* extraction from chlorophyte macroalgae

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This guide refers to the measurement of chlorophyll *a*+*b* in chlorophytes using ethanol. This methodology can be adapted to other orders of algae/solvents using the formulas given in Ritchie 2006.

Ethanol is a very suitable solvent for most extractions due to its low toxicity and non-reactivity with plastics, making it much more versatile and easy to work with than other solvents such as acetone, see Ritchie 2006 for a discussion.

Macroalgae have very tough thalli, so many suggest the use of strong solvents such as acetone mixed with DMSO. We have found that these still leave the vast majority of pigment within the thalli, therefore we recommend grinding the thalli. This adds time to the process but vastly increases the yield of pigment extracted.

Preparation

- Make sure you have been trained to use the Eppendorf 5414D centrifuge, HITACHI 6200 spectrophotometer and have completed all relevant risk assessments + COSHH.
- Label one microcentrifuge tube (MCT) for each sample you plan to take.
- Use a razor blade to cut a 4-5mm section off the bottom of another MCT.
- Hold the piece cut off using tweezers and use it to scoop a set quantity of acid washed 212-300 µm glass beads (Sigma G9143-250G) into each labelled MCT (these beads are necessary for grinding the algae – make sure to add sufficient amount so that the pestle will reach the glass beads for grinding).
- Fill the black dewar with liquid nitrogen.

Sampling and storage

- Pigment samples should be taken as soon as the algae are taken from culture since pigment contents adapt quickly to new environments.
- Dab the thalli dry between pieces of blue roll to remove excess water, then use a scalpel to cut a 2.0-5.0mg piece (less than 5mmx5mm) from a non-apical piece of thalli. Avoid reproductive structures and midribs.
- Use tweezers to transfer it onto the balance and record to the nearest 0.1 mg (=0.0001 g).
- Immediately place in a labelled centrifuge tube and snap freeze by dropping into the dewar.
- Once all samples are collected go to room 4.32 and use a small fishing net to remove algae from the dewar into a labelled container and immediately place it in the -80^{O}C.

Note: snap freezing seems to solidify the membrane and prevent any pigment extraction without grinding. It is best to deal with samples fresh when possible. Thin algae such as *Ulva lactuca* may not require grinding if processed fresh as pigments readily diffuse out of the thalli upon submersion in solvent.

Sample extraction

- Remove all samples from the -80^{o}C and quickly organise in a polystyrene rack.
- Dispense 1ml of 90% ethanol into each MCT a.s.a.p.
- Grind each piece of algae using a micropestle within the MCT, until there are no large fragments left.
- Use tweezers to resuspend thalli pieces that sink into the layer of beads.
- Rinse off the micropestle with dH_2O and pat dry with tissue between samples to prevent carry over.
- Once all have been ground, leave to extract in the dark.

Sample measurement

- With grinding the samples could be measured between 3-6 hours following the start of extraction. Otherwise, incubate in fridge overnight.
- Turn on the HITACHI 6200 spectrophotometer in 5.33 and leave to warm up for 30 mins.
- Vortex all the MCTs prior to centrifugation.
- Centrifuge samples for 15mins at 4000 rpm using the Eppendorf 5415D centrifuge in 3.07 (this may not always be necessary, do a test first, if it makes no difference to the measurement this step can be excluded).
- Keep all samples shielded from light and transport up to 5.33.
- Set the spec to scan between 640 and 760nm at 300nm sec^{-1} with a 2nm slit. This catches all the wavelengths needed so means you do not need to keep resetting the wavelength to measure at.
- Run the baseline using 90% ethanol.
- For each sample add 0.5ml from the MCT followed by 2.5ml of 90% ethanol to the cuvette. Mix the contents using a fresh piece of Parafilm to cover the cuvette. After taking the reading empty the cuvette into a waste conical flask, rinse it with 0.5ml of ethanol, tap out as much solvent as you can onto a white lens tissue and wipe clean the outside window edges before adding the next sample.

Note: Excess solvent in the MCT should be emptied into the waste conical and the MCT should then be closed before being thrown away (if they are not closed the fumes may intoxicate you and those in the vicinity).

Data processing

- The data files should then be processed to allow then to be opened in Excel.
- Enter these formulas (substituting each ʎ for a cell descriptor eg B6)

Chl *a* (µg^{-1} ml) = -5.201(649nm-750nm)+13.528(665nm-750nm)
Chl *b* (µg^{-1} ml) = 22.433(649nm-750nm)-7.074(665nm-750nm)

- Then factor these x 3 = µg chlorophyll in whole cuvette
  x 2 = µg chlorophyll in sample
  ` /mg of algal sample = µg of chlorophyll^{-1} mg of algal thalli

References

Ritchie RJ (2006). Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents. Photosynthesis Research 89: 27-41. (available online via library).