Analysis of chlorophyll-a using UV-vis spectrophotometry

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1. PURPOSE
This procedure is used to determine chlorophyll-a, b and c using a trichromatic spectrophotometric method.

2. PRINCIPLE
A small volume of algal culture (approx. 10-20mls depending on the density of the culture) is filtered onto a glass fibre filter pad (GF/F) and extracted into organic solvent. The extract is measured for absorbance at wavelengths that absorb chlorophyll and a calculation is performed to calculate the concentration of the different chlorophylls in the extract. This is used to calculate chlorophyll concentrations in the culture media, or in dry weight or per cell. There are two basic spectrophotometric methods, monochromatic and trichromatic. The monochromatic methods have been developed to correct chlorophyll a for pheopigment-a useful for senescent cultures or where cultures have been significantly affected by grazing. In the monochromatic method acidification degrades all chlorophyll-like pigments into pheopigments by eliminating the magnesium ion from the tetrapyrrole complex. The drop in absorbance allows both chlorophyll a and pheopigment to be calculated. The trichromatic method is most suitable for algal cultures where there the cultures are not senescent or degrading and provides estimates on chlorophyll-a, b and c. Here we describe the trichromatic method.

3. REQUIREMENTS
3.1. EQUIPMENT AND MATERIALS
- Vacuum system (3-4 psi)
- Glass Fibre (Whatman GF/F type) (25 mm or 47 mm in diameter)
- Filter forceps
- 15ml graduated glass centrifuge tubes
- 250 mL filter flask with sidearm
- Nalgene Tubing
- 200 mL volumetric flask
- (5) 100 mL volumetric flask
- Aluminum Foil
- Parafilm
- Spectrophotometer with a 2nm spectral bandwidth.
- Disposable glass pipets
- High purity grade acetone (1 L)
- De-ionised water

3.2. REAGENTS
90% (v/v) acetone: 900 mL of acetone in 1 L volumetric flask

4. PROCEDURE
4.1. Filtration
- Carry out filtration within one hour of collection of the sample. Place a glass-fibre filter (Whatman GF/F type, 25 mm or 47 mm in diameter) on the filter holder, using forceps.
- Gently mix the algal culture sample.
- Vacuum filter at < 50mm Hg
- Gently suck the last part of the algal culture through the filter.
- Take off the filter from holder. The filter is then folded once with the algae inside, blotted gently with absorbent paper to remove excess media, and placed in a properly labelled clean container.

Precautionary notes
- If the samples are not filtered immediately after collection they can be kept for a few hours in cold and dark storage, in a refrigerator or an ice bath. The time between sampling and filtering must be as short as possible, and no longer than 24 hours.
- Filtration should be carried out under subdued light.
- Magnesium carbonate should not be used as a filter aid.
- All handling of the filters should be done using forceps.
- The filtration time should be kept as short as possible. Clogging of the filters should be avoided.
- Check that the filtration funnels are well seated on the base, and be sure that the filters (Whatman GF/F) are in place. Improperly placed filters or loose funnels will result in loss of sample. The chlorophyll samples are volumetric and should sample loss occur, replace the filter with a new one and redraw the sample.

4.2. Pigment Extraction
- Carry out extraction by grinding the filters in a few mls of 90 % acetone in a glass homogenizer with a motor-driven Teflon pestle, for 1 minute, in an ice bath and under subdued light.
- After grinding, carefully transfer the extract to a stoppered and graduated centrifuge tube rinse the glass homogenizer and the pestle with 90 % acetone and add rinsing volumes to the centrifuge tube.
- Make up the extract volume in the centrifuge tube to exactly 10 ml 90 % acetone (i.e. 10 ml + dead volume of filter) and stopper the tube.

Precautionary notes
- Soaking of the filters overnight is not recommended unless the extraction efficiency of this procedure is thoroughly checked against grinding for the actual working conditions.
- If the extracts are not measured immediately after grinding, for instance, if the measurements are done in batches, they can be kept tightly stoppered in cold and dark storage for up to one hour.

Working with solvents such as acetone has a health risk. Therefore all work should be carried out in well-ventilated conditions, preferably under a hood.
4.3 Measurement.

Use a spectrophotometer of 2 nm maximum bandwidth and stoppered matched glass or quartz cuvettes with path-length of 1cm. A path-length of up to 5cm can be used for dilute cultures.

Switch on spectrophotometer and leave to warm up for 45 min before using.

Transfer the sample extracts from the centrifuge tubes to the sample cuvette by careful pipetting. Place in the sample cuvette holder. Pipette 90 % acetone (blank) into the reference cuvette and place in the reference cuvette holder. Measure the absorbance of the sample extract at 750, 664, 647, and 630 nm referenced against the blank. Rinse carefully and thoroughly between samples.

**Precautionary notes**

Use matched cuvettes and ensure the cuvettes are clean. Ensure correct cuvette holders fitted.

5. CALCULATION OF RESULTS

**Trichromatic method**

Calculate the concentration of chlorophyll a, b and c, according to the equations of Jeffrey and Humphrey (1975):

Chlorophyll a = (11.85* (E664 – E750) – 1.54* (E647 – E750) – 0.08 (E630–E750))*Ve/L*Vf

Chlorophyll b = (−5.43* (E664 – E750) + 21.03* (E647 – E750) – 2.66 (E630–E750))*Ve/L*Vf

Chlorophyll c = (−1.67* (E664 – E750) − 7.60* (E647 – E750) + 24.52 (E630–E750))*Ve/L*Vf

Where:

L = Cuvette light-path in centimetre.
Ve = Extraction volume in millilitre.
Vf = Filtered volume in litre.

Concentrations are in unit mg m⁻³. (= µg L⁻¹).

If dry weight and cell numbers are known this can then be converted to µg chlorophyll g⁻¹ dry weight or pg chlorophyll cell⁻¹.

6. QUALITY CONTROL

- If the samples are not filtered immediately after collection they can be kept for a few hours in cold and dark storage, in a refrigerator or an ice bath. The time between sampling and filtering must be as short as possible and no longer than 24 hours.

7. ERRORS, CALIBRATION AND INTERFERENCES

- As the pigments are both photo- and heat-sensitive, care to protect them from direct sunlight and from warming must be taken at each step of the procedure.

- Interferences – chlorophyll degradation products can seriously interfere with measurements of chlorophyll-a. If for example the algae are in a senescence stage then the reading will not be accurate. Chlorophyllides, not spectroscopically distinct from chlorophyll-a, lead to an overestimation of chlorophyll a.
• Storage of frozen filters at -18°C to -20°C is only acceptable for short periods (not exceeding several weeks). Filters can be stored in liq N₂ (-196°C) or at -80°C for up to and over one year.

• **Vacuum pressure**: To avoid damaging cells during filtration, vacuum pressure should be limited. While Jeffrey et al. (1997) mention keeping residual pressure under the filter not lower than 0.5 bar, most users recommend that it be kept higher than 0.7 bar.

• **Solvent choice**. If acetone is used, it is strongly recommended to grind the filters instead of sonicating or soaking overnight. In a glass homogeniser with a motor-driven teflon pestle, complete disruption of the filter is obtained in about 1 minute. Extraction time may be prolonged to 30–60 minutes after transfer into the centrifuge tubes kept tightly closed and protected from heat and light (Lorenzen, 1967).

8. WASTE STREAM AND PROPER DISPOSAL
Dispose of acetone on appropriate container – not down the sink.

9. HAZARDS AND PRECAUTIONARY STATEMENTS
Working with solvents such as acetone has a health risk. Therefore all work should be carried out in well-ventilated conditions, preferably under a hood.

10. REFERENCES and FURTHER READING


11. CONTRIBUTIONS
- SOP prepared by: Carole Llewellyn (PML - EnAlgae project)