PHOTOSYNTHETIC PIGMENT DETERMINATION USING ETHANOL EXTRACTION

Principle

A water sample of known volume containing photosynthetic organisms is filtered through glass fibre filter paper and chlorophyll is extracted in a refrigerator overnight or at 60^{o}C for 1 hour in ethanol (preferred over methanol for safety reasons). The absorbance of the chlorophyll extract is read spectrophotometrically and readings used to calculate the concentration of chlorophyll *a*, phaeophytin and carotenoid accessory pigments.

Procedures

1. Filter 1 litre of water (volume depends on the amount of suspended material) through a pre-weighed Whatman 47 mm GF/C filter paper, using slight vacuum (no more than 0.5 Bar=30cm. Hg).

2. Weigh filter and place in a stoppered centrifuge tube,eg Hach tube. Freeze filter, or for preference put immediately into extractant. (Water content of the filter determined by weighing the filter dry and after filtration: normally is ~0.4-0.5ml)

3. Extract each filter in 19 volumes of 100% ethanol (industrial methylated spirit); eg. if water content was 0.5g, add 9.5 ml. (This procedure ensures that the water content of the final mix is not greater than 5%). Mark the solvent level on the tube, invert several times to mix.

   Solutions : 100% and 95% ethanol (industrial methylated spirit) saturated with $MgCO_3$

4. Wrap tubes in aluminium foil to exclude light: light degrades chlorophyll.

5. Place tubes in a waterbath at 60^{o}C for 1 hour. This speeds extraction and destroys chlorophyllase.

6. Remove tubes, invert several times, and allow to cool on ice. They can be stored overnight in a fridge or freezer. Any evaporative loss should be made up with 100% ethanol, topping up to the mark again. (extraction for 24 hours is recommended)

7. Centrifuge without removing the filter at full speed (about 3500 rpm) in a bench centrifuge for 15 minutes. Decant or pipette out the extract: check volume. Alternatively the filter may be removed (squeeze out the solvent on the side of the tube) before spinning.
   The volume of solvent must be greater than 8 ml to read in a 4 cm path length spectrophotometer cell. For convenience the volume may be adjusted to exactly 10 ml; otherwise record the volume to the nearest 0.1 ml for use in subsequent calculation.

   Cells used should be read against the reference, containing the same blank solution*, before use at all wavelengths, as not all cells are perfectly matched: subtract any differences from the extract readings. **If acidifying extracts, read acidified blank cells also.**

8. Read the absorbance against a suitable reference [*$MgCO_3$-saturated 95% ethanol which has been centrifuged or filtered (Whatman No. 1)] at the following wavelengths, in a 4 cm glass cell : 750, 665, 510 and 480 nm. Return wavelength to 665.
   The OD at 665 should be between 0.2 and 0.8: it may be necessary to dilute more concentrated samples (or read them in a shorter path length cell) -see Marker et al, 1980.

9. <u>To determine phaeophytin **</u>
   Acidification of chlorophyll degrades it to phaeophytin with a change in Specific Absorbance Coefficient (SAC), which can be used to estimate the amount of phaeophytin present before acidification. In methanol (ethanol) the extract must be neutralised before re-reading absorbance, in order to prevent complications due to differences in pH. (pH after neutralisation can be checked with pH paper)

   The method assumes an extract volume of 10 ml.

   (a) Add directly to the cuvette 0.1 ml of 1 M HCl (10%), mix.
   (b) Leave for 2 minutes
   (c) Add 0.13 ml* of 1 M 2-phenylethylamine in ethanol (12.1g in 100 ml), mix.
   (d) Read absorbance at 665 and 750 nm.

   * with new solutions, check the amount of base needed to neutralise 0.1ml acid: add 0.1ml base and check by spotting on pH paper, then add 10µl aliquots until pH7 is reached

10. Calculations:

Subtract cuvette blanks at each wavelength from all readings.
Subtract A_{750} (turbidity blank) from all other readings.

Equations refer to corrected values: (Parsons, Maita and Lalli (1984) equations for acetone**)

chlorophyll *a* ($\mu g\ l^{-1}$) = $\frac{29.11^{**}\ [A665_o - A665_a]\ s\ d}{V\ \rho}$

** modification to factor if used in ethanol: change to 29.11 from 26.7. Not rigorously tested yet

phaeophytin *a* ($\mu\ gl^{-1}$) = $\frac{29.11^{**}[1.7\ (A665_a) - A665o]\ s\ d}{V\ \rho}$

d = dilution factor (if necessary to reduce A665 to <0.8) eg. for 1in 5 dilution, d = 5
ρ = path length of cell, cm
s = solvent extract volume, ml
V = sample volume, litres
$A665_o$= absorbance at 665 nm before acidification
$A665_a$ = absorbance at 665 nm after acidification and neutralisation.

Notes

Method for phaeophytin is according to the recommendations of Marker, Crowther and Gunn (1980) and Marker *et al.* (1980) for methanolic extracts. Webb *et al.* (1992) found that spectrophotometric methods greatly overestimate phaeophytin compared to chromatographic methods, and therefore do not recommend its use. As an alternative, calculate chlorophyll a according to this equation:

Chlorophyll a ($\mu g\ l^{-1}$) = $\frac{11.99\ (A665-A750)s}{V_p}$

Marker *et al.* (1980)
Recommended equation for ethanol
(cf. constant in methanol=12.99)

Carotenoids: calculate as 'microscopic pigment units' =μspu.

Most formulae given refer to acetone extracts, e.g. Strickland and Parsons (1968):

(a) μ spu l^{-1} = $\frac{4.0\ (A480-A750)\ s}{V\rho}$ if predominantly Chlorophyta or Cyanophyta

(b) = $\frac{10.0\ (A480-A750)\ s}{V\rho}$ if predominantly Chrysophyta or Pyrrophyta

Parsons, Maita and Lalli (1984) give this formula:

(c) μ spu l^{-1} = $\frac{7.6\ [(A480-A750) - 1.49\ (A510-A750)]\ s}{V\rho}$

[Foy (1987) used formula (a) with methanolic extracts of Cyanophyta.]

NB. Extraction from stones:

Drain excess moisture from the stones and place each stone in a resealable plastic bag (double-wrap for security), weigh.

Add an appropriate volume of extractant for the size of the stone, seal and re-weigh. Avoid exposure to bright light. Appropriate volumes:- eg. 50 ml., depending on size of stone and amount of algal cover. With dense mats estimate the water content and use 9.5 volumes of 100% alcohol, diluting further with 95% if needed.

Place in a water bath at 60°C for 1 hour, cool (on ice). Re-weigh to check no evaporative loss (or gain though leakage).

Remove 10ml to a Hach tube, centrifuge. Extracts may require dilution: A_{663} should not exceed 0.8.

Measure absorbance as with filtered samples.

Calculate chlorophyll yield per unit area (cm^2) of stone surface. Stone surface covered by algae can be determined by wrapping the area with foil and comparing the foil weight with a known area. Area takes the place of sample volume (V) in the formula.

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