Analysis of chlorophyll and carotenoid compounds in eucalyptus leaves

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Method Article

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Abstract

This protocol aims to extract and measure the amount of chlorophyll and carotenoids in leaf material using spectrophotometry. An ethanol extraction is used to analyse both chlorophyll a and b and carotenoids in leaf material with compounds quantified using absorbance across a range of wavelengths. This protocol uses ethanol as the extracting solution due to its affordability and safety compared to other reagents. We report a methodology that extracts pigments from whole leaf discs. The combination of extraction from leaf discs and ethanol extraction ensures this protocol is safe and rapid to complete. The protocol includes three phases: a preparation phase where samples are punched and added to ethanol (approximately 3 hours for 100 samples), an extraction phase where samples are left overnight, and a measurement phase where absorbance at a range of wavelengths is recorded using spectrophotometry (approximately 1 hour for 100 samples).

Introduction

Photosynthetic pigments such as chlorophyll and carotenoids are important plant components that are useful for understanding photosynthetic activity in a plant (Ritchie 2006, Nayek et al. 2014). Further, they can provide insights into local environment conditions including climate and shade effects (Nayek et al. 2014). This protocol describes pigment extraction from eucalyptus leaves using ethanol as the reagent. As outlined in Ritchie (2006), ethanol is an effective extraction solution for chlorophyll pigments in laboratory assays. Whilst carotenoids are known to be polar and are easier to extract in polar solvents, sufficient extracting power was found in ethanol over a 24 hour period (Nayek et al. 2014). Due to the safety and efficacy of ethanol we selected it as an extracting solvent over alternatives; in addition, ethanol allows for high sample throughput, which is desired for some applications. We opted to identify absorbance across the full available spectrum (350-900nm) to ensure that we were identifying the peak maxima appropriate for our species. If it is possible to measure the whole available spectra this is recommended as the peak absorbance values can vary ± 5 nm. Obtaining the whole available spectra enables researchers to ensure they are recording absorbance at the peak maximum. Overall, the protocol provides guidance on pigment extraction from eucalypt leaves and highlights the importance of inspecting spectra when quantifying pigments in separate species. For further reference, see the protocol “Leaf collection and handling in the field” for instructions on how to collect and store leaves prior to analysis.

Reagents

Ethanol (100%), spectrophotometric grade CAS-No. 64-17-5

Equipment

- Fresh leaf samples
- Hole punch
- Digital Balance (to four decimal places)
- 5-ml graduated centrifuge tubes (e.g. Eppendorf or similar)
- explosion-proof tabletop centrifuge (e.g. Beckman Coulter Allegra X-12 R)
- UV-VIS spectrophotometer/microplate reader (e.g. SPECTROstarNano BMG Labtech)
- Vortex (e.g. Lab Co Vortex)
- 96 well microplate
- Auto-pipette and pipette tips (100-1000 µL)

**Procedure**

This method follows Ritchie (2006) and Nayek et al. (2014).

**Pigment Extraction:**

1. Punch 5 holes from a eucalypt leaf sample avoiding mid veins and signs of damage (figure 1)
2. Weight the discs using a precision balance and add them to a microcentrifuge tube.
3. Add 2 mL of ethanol to the sample.
4. Vortex for 30 seconds and leave in the fridge (4°C) for 24 hours.
5. Vortex again briefly to homogenise the solution the next day.
6. Centrifuge for 5 min at 3500 rpm in an explosion-proof tabletop centrifuge at 4°C.

**Spectroscopic analysis:**

1. Transfer 200 µl of extract of each sample to the microplate. Include an ethanol blank at the beginning and every 15-20 samples.
2. Using a spectrophotometer, take absorbance readings of the leaf extract against an ethanol blank across the full spectra (350 nm to 1000 nm)
3. Extract absorbance values (A) for the following wavelengths:
   4. 470 nm (total carotenoids).
   5. 649 nm (chlorophyll a maximum using 100% ethanol).
   6. 664 nm (to allow for calculation of carotenoids).
   7. 665 nm (chlorophyll b maximum using 100% ethanol).

**Calculation of Pigment Concentrations:**

1. Apply measured absorbance values to equations 1, 2, and 3 to determine pigment content of chlorophyll a (Ca), chlorophyll b (Cb), and total carotenoids (Cx+c) (Sumanta et al., 2014). Alternatively see (Ritchie, 2006).
(1) \( C_a (\mu g/ml) = 13.36 A_{664} - 5.19 A_{649} \)

(2) \( C_b (\mu g/ml) = 27/43 A_{649} - 8.12 A_{664} \)

(3) \( C_{x+c} (\mu g/ml) = (1000 A_{470} - 2.13 C_a - 97/63 C_b)/209 \)

**Troubleshooting**

Figure 2 contains some common problems that may occur whilst conducting this protocol and provides potential solutions.

**Time Taken**

Preparing leaf extraction – 100 samples in 3 hours

- Hole punch leaves, weigh the punches and record the weight
- Add ethanol and vortex the sample for 30 seconds

Leave overnight to extract – 24 hours

Spectrophotometry – 1 hour

- Vortex all samples briefly and aliquot out samples into 96-well plate
- Record absorbance across the full range of available wavelengths

**Anticipated Results**

Using this protocol we obtained a range of results for all three pigments of interest from eucalyptus leaves. Chlorophyll a ranged from 0.24-27.5 µg/mL, chlorophyll b ranged from 0.62-19.96 µg/mL, and carotenoids ranged from 0.03-7.97 µg/mL. As peak absorbance for the pigments varied ± 5 nm compared to published studies, measuring the whole absorbance spectra for samples will ensure that pigment absorbance peaks are identified and quantified accurately.

**References**


Figures

Figure 1

Example of a eucalyptus leaf with punches removed to create discs for pigment analysis. Note that the midvein and areas of any damage have been avoided.
<table>
<thead>
<tr>
<th>Step</th>
<th>Common Issues</th>
<th>Potential Reasons</th>
<th>Solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Difficult to obtain an accurate weight on the 4.d.p balance</td>
<td>The vessel you are weighing the leaf punches in is too heavy</td>
<td>We recommend using weighing paper or the lightest vessel you can find. As the 5 leaf punches do not weigh a lot, to obtain accurate data it is important that the vessel on which the plant material is weighed is light.</td>
</tr>
<tr>
<td>8</td>
<td>Spectrophotometer output reads 'overflow'</td>
<td>The concentration of the extract is too high for certain wavelengths of light to pass through</td>
<td>This has not been an issue at the wavelengths of interest for this protocol. It is recommended that you check that this is the case for your samples. Otherwise samples can be diluted and analysis can be run with adjustments made to the results for the diluted samples.</td>
</tr>
</tbody>
</table>

**Figure 2**

Common issues and problems when conducting analysis of leaf pigments using a spectrophotometer. Potential solution are suggested. Steps numbers indicate which step in the procedure the issue may occur.