Journal of Plant Nutrition, 31: 1321–1332, 2008

ISSN: 0190-4167 print / 1532-4087 online
DOI: 10.1080/01904160802135092

Taylor & Francis
Taylor & Francis Group

Rapid Measurement of Chlorophylls with a Microplate Reader

C. R. Warren

School of Biological Sciences, The University of Sydney, Sydney, Australia

ABSTRACT

This paper describes a robust and rapid procedure for measuring chlorophyll using a microplate reader. The procedure enables direct translation of any published equation developed on a 1-cm pathlength spectrophotometer to any format microplate with any sample volume. Chlorophylls were extracted with methanol because it is compatible with cheap polystyrene plates; however, the principles apply to any solvent system. Keys to obtaining accurate and precise results include determining pathlength in the same solvent used to extract chlorophylls, and avoiding evaporation. After taking these precautions, there was a strong linear 1:1 relationship ($R^2 = 0.98$–0.99) between microplate and spectrophotometer estimates of chlorophyll concentrations.

Keywords: Chlorophyll, high throughput, microplate, spectrophotometer, methanol

INTRODUCTION

The chlorophyll content of plants is a fundamental measurement that underpins many branches of the plant sciences. Chlorophyll is central to plant function and thus it is not surprising that chlorophyll content has been measured as an indicator of developmental stage, plant health and potential growth (Mencarelli and Saltveit, 1988; Blackbourn et al., 1990). Chlorophyll content is also used as an indirect indicator of nitrogen (N) content (Chapman and Barreto, 1997; Wang et al., 2004) owing to the significant proportion of leaf N in pigment-protein complexes. Chlorophylls are involved in photosynthesis and thus rates of photosynthesis are often expressed per unit of chlorophyll (Porra, 2002;

Received 26 March 2007; accepted 15 May 2007.
Address correspondence to C.R. Warren, School of Biological Sciences, Heydon-Laurence Building A08, The University of Sydney, Sydney NSW 2006, Australia. E-mail: charles.warren@bio.usyd.edu.au

Fischer et al., 2006; Ritchie, 2006) and chlorophyll content is used to drive models of radiation interception and net primary production (Sari et al., 2005).

The past decade has seen increasing requirements for high-throughput measurements in plants. In part this has come about due to the rapid rise of metabolomics, but also as part of a more general recognition of the need for greater replication. High-throughput technologies for small molecules are generally well established (e.g., GC- and LC-MS methods; Oikawa et al., 2004), but most existing methods for chlorophylls are unsuited to high-throughput measurements. The most tried and true methods for measuring chlorophylls involves extraction with an organic solvent and subsequent measurement on a spectrophotometer (Arnon, 1949; Porra, 2002; Ritchie, 2006). These methods are widely used owing to their accuracy; however, they are slow because samples are measured one at a time.

Portable chlorophyll meters (e.g., SPAD) permit rapid relative measures of chlorophyll content and thus meet one criterion for high-throughput methods (Chang and Robison, 2003; Madeira et al., 2003; Bonneville and Fyles, 2006). However, relative measures of chlorophyll content can only be translated into actual chlorophyll content once a robust calibration is established (e.g., by comparison with spectrophotometric measurements). This requirement for calibration is problematic because calibrations often vary among genotypes or with growth conditions (Chang and Robison, 2003; Wang et al., 2004), and thus multiple calibrations must be established for studies comparing genotypes or growing the same genotype under different conditions.

Microplate readers are common in plant science laboratories and are widely used to speed up spectrophotometric methods, and thus it is surprising that microplate methods are not routinely used to measure chlorophylls. More than ten years ago Martinez and Damidaux (1993) proposed a microplate method for measuring chlorophylls but this method has not been adopted widely. One reason the method of Martinez and Damidaux (1993) has not been widely adopted is that they derived a set of new equations valid only for a microplate with a 1 cm optical pathlength. Pathlength is the crux of translating spectrophotometer methods to microplates. With a microplate reader light passes vertically through the solution, and thus changes in sample volume or plate design will affect the pathlength. What this means is that the equations of Martinez and Damidaux (1993) cannot be applied to the same microplate with a different sample volume or microplates with inherently different pathlength (e.g., half versus full volume, or 96 versus 384 well).

The use of absorbance to estimate concentration is described by the Beer-Lambert equation: Absorbance = extinction coefficient X concentration X pathlength. Equations for estimating chlorophylls are more complicated due to the presence of two absorbing molecules (chlorophyll *a* and chlorophyll *b*), but such detail is unimportant for our purposes here (for a full discussion and derivation of equations for chlorophylls see Martinez and Damidaux, 1993; Ritchie, 2006).

Pathlength in spectrophotometers has been standardised to 1 cm and thus spectrophotometric equations for estimating chlorophyll implicitly assume a 1 cm pathlength. If absorbance of a chlorophyll solution is measured in a device with a pathlength different to 1 cm (e.g., in a microplate reader), then the standard spectrophotometric equations can only be used if they are corrected to 1 cm.

This paper describes a robust procedure for rapidly measuring chlorophyll using a microplate reader and cheap polystyrene plates. The procedure is based on correcting for differences in pathlength so as to directly translate any published equation developed on a spectrophotometer to any format microplate with any sample volume. Being able to use published equations (e.g., Arnon, 1949; Porra, 2002; Ritchie, 2006) eliminates the need to purify chlorophylls and develop a standard curve.

MATERIALS AND METHODS

Extraction of Chlorophylls

Eight plant species, each from a different family, were used so as to test the ubiquity of the method and obtain a large range in chlorophyll concentrations. Species used were: *Acacia terminalis* (sunshine wattle), *Allium sativum* (garlic), *Araucaria bidwillii* (bunya pine), *Avicennia marina* (mangrove), *Eucalyptus regnans* (mountain ash/swamp gum), *Gossypium hirsutum* (cotton), *Marsilea hirsuta* (water clover), and *Schleffera actinophylla* (umbrella plant).

Chlorophylls can be extracted from fresh, frozen (−80°C) or freeze dried samples. In this experiment freeze dried samples were used because they are easier to grind in a matrix mill. Fresh or frozen samples can only be ground in matrix mill if they are maintained at LN_2 temperatures.

Two freeze dried leaf discs (0.56 cm^2 each) were placed in a 2-mL microfuge tube (Safelock, Eppendorf, Hamburg, Germany) along with a 5 mm stainless steel ball bearing. Samples were ground to a fine powder by shaking for one minute at 30 hz in a vibratory ball mill (TissueLyser, Qiagen Inc, Doncaster, Australia). Chlorophylls were extracted from the ground samples by adding 1.00 mL of methanol and shaking for two minutes at 30 hz. Samples were centrifuged for 2 minutes at 16873 g (Microcentrifuge 5418, Eppendorf, Hamburg, Germany) and the supernatant was transferred to a second microcentrifuge tube. The pellet was re-extracted with a second 1.00-mL aliquot of methanol by adding 1.00 mL of methanol to the pellet, shaking for another two minutes, centrifuging and removing the supernatant. The pellet was discarded while the two supernatants were pooled and used for measurement of chlorophylls.

Microplate and Spectrophotometer Measurements

Microplate measurements were made by pipetting 200 μL of sample (or blank) into a 96-well flat bottom polystyrene plate (Greiner Bio-One, Frickenhausen, Germany), which was then read with a monochromator-based microplate reader with 2.4 nm bandwith (Synergy 2, BioTek, Winooski, USA). Microplate measurements were generally made with the lid on the microplate so as to reduce evaporation. Spectrophotometer measurements were made by transferring 200 μL of sample (or blank) into a 1-cm pathlength quartz cell and reading absorbance in a spectrophotometer with 1 nm bandwidth (UV-2550, Shimadzu, Kyoto, Japan).

Calculation of Chlorophyll Concentration

The absorbance of 200 μL of sample in a microplate ($A_{652,\text{microplate}}$ $A_{665,\text{microplate}}$) was converted into a 1-cm pathlength corrected absorbance using the measured pathlength:

$$A_{652,1\text{ cm}} = (A_{652,\text{microplate}} - \text{blank})/\text{pathlength}$$
$$A_{665,1\text{ cm}} = (A_{665,\text{microplate}} - \text{blank})/\text{pathlength}$$

Chlorophyll concentration was calculated from 1-cm corrected pathlength using recently published formulae of Ritchie (2006):

$$\text{Chl } a\ (\mu\text{g/mL}) = -8.0962\ A_{652,1\text{ cm}} + 16.5169\ A_{665,1\text{ cm}}$$
$$\text{Chl } b\ (\mu\text{g/mL}) = 27.4405\ A_{652,1\text{ cm}} - 12.1688\ A_{665,1\text{ cm}}.$$

RESULTS AND DISCUSSION

There are several methods of correcting pathlength to 1 cm. All are based on comparing absorption in a microplate reader with (measured or known) absorption in a 1-cm spectrophotometer. The methods are:

1. Using a sample that is aqueous and correcting each well based on water's measured absorption at 977 nm in a microplate reader and known 1-cm absorption.
2. Using a sample dissolved in organic solvent (e.g. methanol) and correcting each well based on the solvent's measured absorption in a microplate reader and spectrophotometer.

3. Not correcting individual samples but applying a constant pathlength correction to all samples based on pathlength determined with an equal volume of water.
4. Not correcting individual samples but applying a constant pathlength correction to all samples based on pathlength determined by measuring a sample in a 1-cm spectrophotometer and a microplate reader.

The following discussion deals with each method in turn.

Some of the newer microplate readers incorporate a pathlength correction function that corrects each well for differences in pathlength (e.g., due to differing sample volumes). This is based on a microplate reader measuring the absorption of water at 977 nm (Figure 1) and then comparing this to the known absorption of water at a fixed 1 cm pathlength. However, chlorophyll is extracted with organic solvents (e.g., methanol, ethanol, acetone, DMSO, and DMF), and thus a correction procedure based on water's properties will not work. The only scenario in which a correction based on water's properties might work is when chlorophylls are extracted with 80% acetone (i.e., 20% H_2O). This approach was not used for two reasons: 1) the presence of acetone reduces the size of the 977 nm peak from 0.18 to 0.03 and this would have resulted in an unacceptably small signal-to-noise ratio; 2) Acetone is incompatible with cheap polystyrene microplates, and acetone-compatible plates are many times more expensive.

It was then tested whether it was possible to use the spectrum of a solvent for pathlength correction of each individual well. A large number of solvents

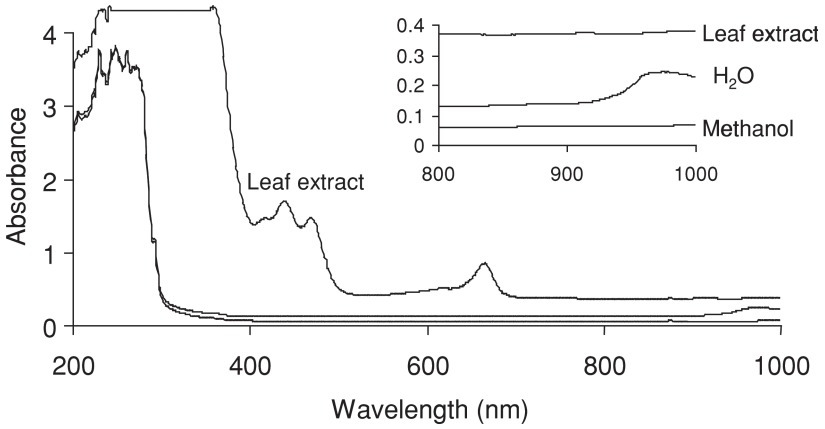

Figure 1. UV-Vis spectra of methanol, water and a leaf extract of *Schefflera actinophylla* in 96-well polystyrene microplates Absorbance was measured at 1 nm with a microplate reader. Please note that the y-axes have been offset so as to allow better comparison of spectra in the near IR region (see inset). The strong absorbance of methanol and H_2O at wavelengths shorter than 300 nm is due to the polystyrene microplate.

have been used to extract chlorophylls (e.g., methanol, ethanol, acetone, DMSO, and DMF), but only those that were compatible with polystyrene microplates were of interest. This reduced the list to the two alcohols, methanol and ethanol. Methanol was chosen for further experiments because it is used more commonly than ethanol and is a common solvent for HPLC analyses of chlorophylls. Before searching for a solvent absorption peak that could be used for blank correction it was first necessary to determine the absorption spectrum of leaf extracts (Figure 1). A spectrum scan from 200 to 1000 nm at 1 nm intervals showed that leaf extracts absorb strongly throughout the visible and ultraviolet (UV). Polystyrene plates also absorb strongly in the UV, and thus the only suitable region for pathlength correction was the near infrared (IR). Methanol has small absorption peaks in the near IR region (Figure 1). The peak at 880 nm was around 0.005, the peak at 915 nm was even smaller at 0.003, while the ramp from 950 to 1000 nm was in the order of 0.01. These peaks were too small to be used for pathlength correction because they were not much larger than microplate absorbance resolution of 0.001 and would have resulted in an unacceptably small signal-to-noise ratio.

It is theoretically possible to determine pathlength of any volume of solvent by measuring the absorbance of an equal volume of water at 977 nm (the water peak in Figure 1) and 900 nm (to blank the plate) and using the formula

$$\text{Pathlength} = (A_{977} - A_{900})/0.18,$$

where 0.18 is the known absorbance of 1 cm of water. This method requires that the volume used to determine pathlength (e.g., 200 μL) is the same as the volume of samples, and that the volume of samples does not vary (i.e., there are no pipetting errors). The measured pathlength of 200 μL of water was 0.58 (Table 1) and this was used to create a 1-cm corrected value by dividing the experimental absorbance (blanked) by pathlength:

$$A_{652,1\text{ cm}} = (A_{652,\text{microplate}} - \text{blank})/0.58,$$

$$A_{665,1\text{ cm}} = (A_{665,\text{microplate}} - \text{blank})/0.58$$

However, when pathlength derived from 200 μL of water was applied to 200 μL of methanolic leaf extract, the absorbance corrected to 1 cm was 13% smaller than the actual 1 cm absorbance (determined by spectrophotometer) (Figure 2). This led to an underestimation of chlorophyll concentrations of 13%. To determine whether this was a matrix effect (water vs. methanol) or a peculiarity of chlorophyll, the pathlength of an aniline blue solution (absorbance peak = 600 nm) dissolved in water or methanol (Table 1) was determined:

$$\text{Pathlength} = (A_{600,\text{microplate}} - \text{blank})/(A_{600,1\text{ cm}} - \text{blank})$$

Table 1

Absorbance of 200 μL solution in a 96-well polystyrene microplate or in a 1-cm pathlength spectrophotometer. Pathlength of the microplate was determined as (microplate absorbance-microplate blank) / (spectrophotometer absorbance–blank). Data are means (n = 6–12), with one standard error in parentheses

	Microplate		Spectrophotometer		
	Absorbance	Blank	Absorbance	Blank	Microplate Pathlength
Water A_{997}	0.190 (0.001)	0.085 (0.001)	0.18[1]		0.58 (0.01)
Aniline blue in water A_{600}	0.905 (0.001)	0.079 (0.001)	1.420 (0.008)	0.037 (0.002)	0.59 (0.01)
Aniline blue in methanol A_{600}	0.692 (0.002)	0.079 (0.002)	1.228 (0.009)	0.045 (0.001)	0.51 (0.01)
Leaf extract in methanol A_{652}	0.797	0.079 (0.003)	1.42 (0.01)	0.045 (0.002)	0.51 (0.01)
Leaf extract in methanol A_{665}	1.255	0.079 (0.003)	2.36 (0.02)	0.045 (0.002)	0.51 (0.01)

[1]Blanked value is assumed to be 0.18.

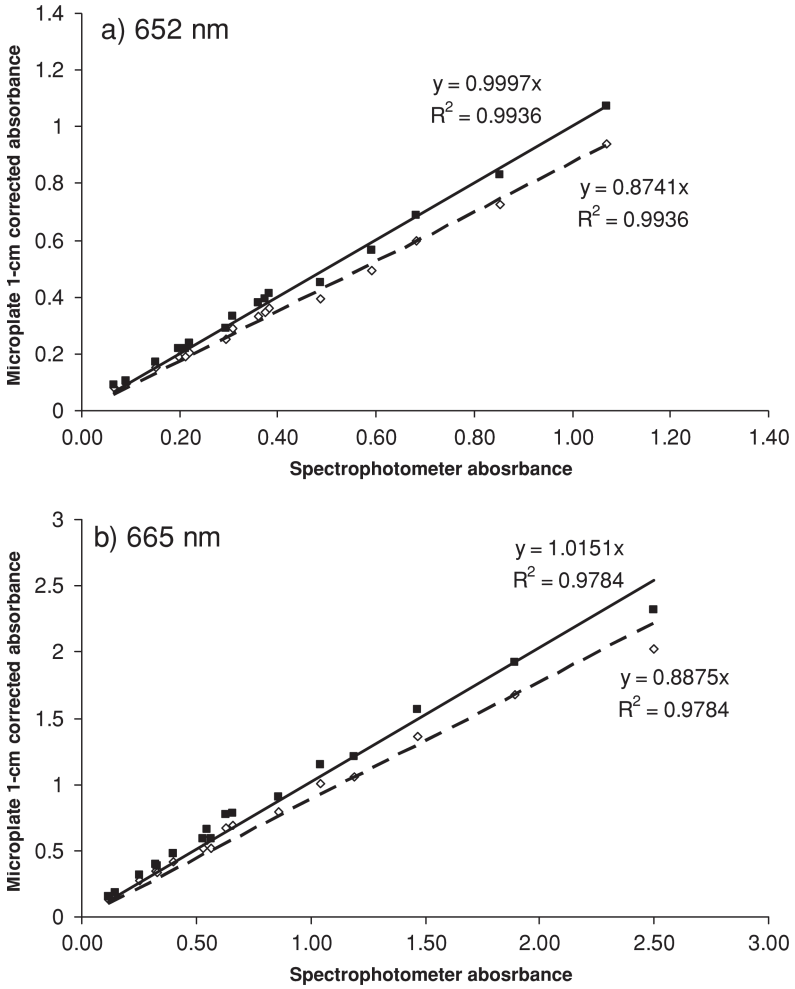

Figure 2. The relationship between absorbance measured on a spectrophotometer with absorbance measured with a microplate reader and corrected to 1 cm pathlength. Pathlength was corrected to 1 cm by applying a constant derived from water (dashed line and hollow diamonds) or by measuring pathlength of a leaf extract in methanol (solid line and filled squares)(see Table 1). Samples are methanolic leaf extracts. There are a total of 18 extracts, comprising 2–3 samples from eight species. Data are for a) 652 nm and b) 665 nm: the two wavelengths used to calculate the amounts of chlorophylls *a* and *b*.

The pathlength of 200 μL of aniline blue dissolved in water was 0.58, which was the same as that determined for pure water from water's absorption at 977 nm. The pathlength of 200 μL of aniline blue dissolved in methanol was approximately 13% smaller at 0.51. These findings suggest that the pathlength of a 200 μL solution of water is 13% longer than the pathlength of 200 μL of

methanol, perhaps because the meniscus of methanol bends downward in the centre to a greater degree than water which results in methanol having a shorter pathlength than water.

The most direct way of determining pathlength that avoids matrix effects (water vs. methanol) is to measure absorbance of 200 μL a methanolic leaf extract in a microplate and compare this with the same solution measured in a 1-cm pathlength spectrophotometer (Table 1). The pathlength of 200 μL of a methanolic leaf extract was 0.51 at 652, and 665 nm, i.e., the same as that determined for 200 μL of aniline blue in methanol. Microplate absorbance corrected to 1 cm by dividing absorbance (blanked) by 0.51 was almost identical to absorbance measured in a spectrophotometer (Figure 2). Hence, chlorophyll concentrations measured in a microplate reader were nearly identical to concentrations of the same samples measured in a spectrophotometer (Figure 3). Chlorophyll *a* and *b* tended to be overestimated slightly (slope = 1.004 and 1.002), but these deviations from a strict 1:1 relationship are well within the standard deviation for samples.

After establishing accuracy of microplate measurements of chlorophylls it was necessary to determine precision. There was good reason to expect precision of microplate analyses to be worse than for a spectrophotometer because microplate measurements are affected by the precision of pipetting since it affects pathlength, whereas spectrophotometer measurements are not affected by pipetting since pathlength is fixed. Precision was determined by repeated analysis of the same methanolic leaf extract from *Schleffera actinophylla*. Relative standard deviation (standard deviation/mean) of analysis of chlorophyll *a* (concentration =21.7 μg mL^{-1}) was 1.7 %, while relative standard deviation for analysis of chlorophyll *b* (concentration = 7.6 μg mL^{-1}) was 2.0%. These relative standard deviations are comparable to those determined with a spectrophotometer (typically 1–2%) and indicate that microplate estimates are not only as accurate, but also as precise as spectrophotometer measurements.

Correcting for differences in pathlength permits accurate and precise determination of chlorophylls, but rapid evaporation of methanolic extracts can be problematic unless precautions are taken. Initial experiments with uncovered microplates showed that chlorophyll concentrations decrease rapidly (Figure 4) because of the rapid evaporation of methanol that results in a smaller pathlength and a proportion of chlorophylls being left behind on the microplate wall. This rapid loss of chlorophyll and subsequent erroneous estimates could be overcome by keeping the microplate covered before and while making measurements (Figure 4). Microplates with and without lids gave identical estimates of chlorophyll at time zero (data not shown), and thus it is highly recommended that measurements are always made with the lid on.

In summary, this research described a simple means of transferring spectrophotometric equations for estimating chlorophylls to a microplate format. The method is very rapid and permits extraction and measurement of > 500 samples per day. Methanol or ethanol are recommended as solvents because

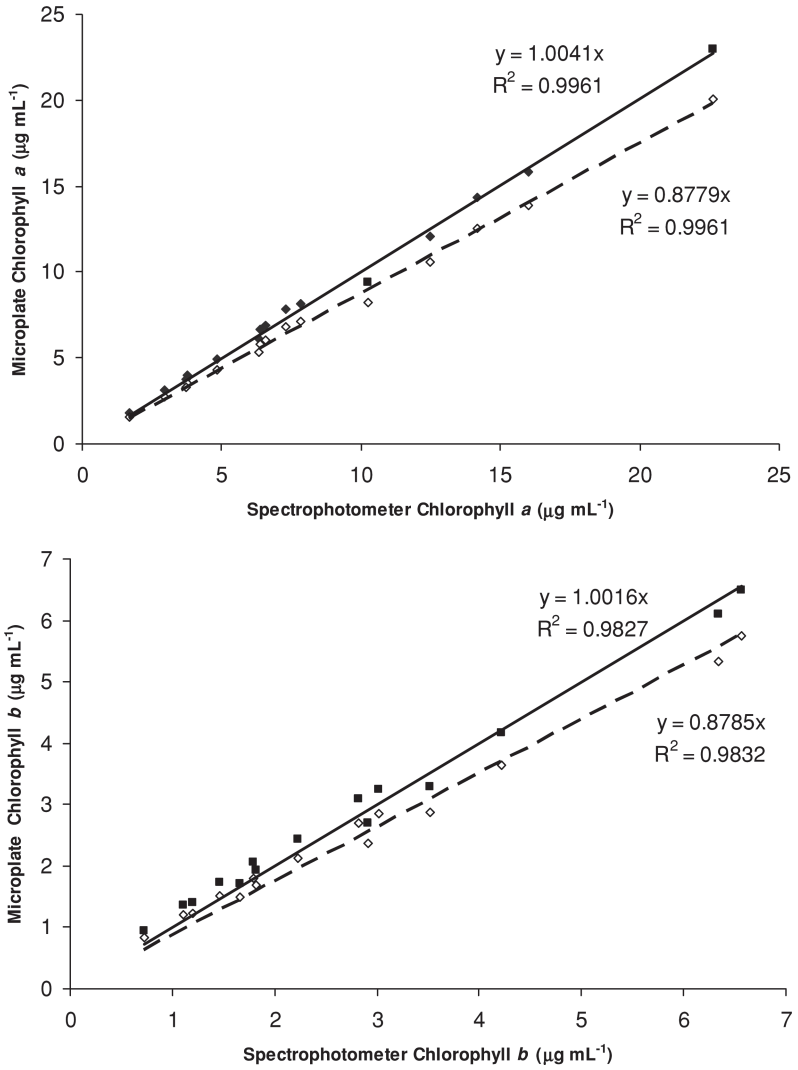

Figure 3. The relationship between chlorophyll concentration measured on a spectrophotometer with chlorophyll *a* and *b* concentrations measured with a microplate reader and corrected to 1 cm pathlength. Pathlength was corrected to 1 cm by applying a constant derived from water (dashed line and hollow diamonds) or by measuring pathlength of a leaf extract in methanol (solid line and filled squares)(see Table 1). Samples are methanolic leaf extracts. There are a total of 18 extracts, comprising 2–3 samples from eight species.

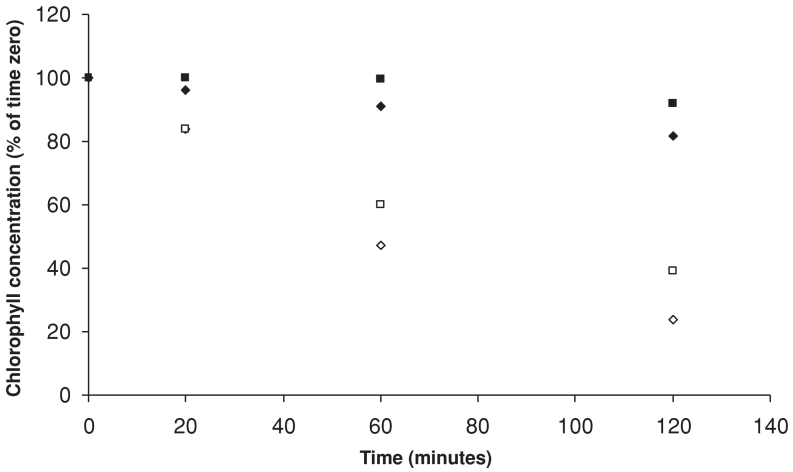

Figure 4. The apparent decrease in chlorophyll concentration with time in microplates with lids (filled symbols) and microplates without lids (empty symbols). Chlorophyll a (diamonds) and chlorophyll b (squares) are expressed as a percentage of the initial value at time zero. After making the first reading, microplates were left in a dimly lit laboratory at 22°C.

they are compatible with cheap polystyrene microplates. The crux of transferring spectrophotometer methods to a microplate is the accurate measurement of pathlength. It is highly recommended that pathlength of the microplate is determined using the same solvent as is used to extract chlorophylls (e.g., methanol) because use of water will give rise to an erroneous estimate of pathlength and erroneous estimates of chlorophyll.

ACKNOWLEDGMENTS

This work was supported by a Discovery Grant from the Australian Research Council. The Australian Research Council is also thanked for a QEII Fellowship. Charles Warren warmly acknowledges the generous financial support of The University of Sydney.

REFERENCES

Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts—polyphenoloxidase in beta-vulgaris. *Plant Physiology* 24: 1–15.

Blackbourn, H. D., M. J. Jeger, P. John, and A. K. Thompson. 1990. Inhibition of degreening in the peel of bananas ripened at tropical temperatures. 3. Changes in plastid ultrastructure and chlorophyll-protein complexes

accompanying ripening in bananas and plantains. *Annals of Applied Biology* 117: 147–161.

Bonneville, M. C., and J. Fyles. 2006. Assessing variations in SPAD-502 chlorophyll meter measurements and their relationships with nutrient content of trembling aspen foliage. *Communications in Soil Science and Plant Analysis* 37: 525–539.

Chang, S.X., and D. J. Robison. 2003. Nondestructive and rapid estimation of hardwood foliar nitrogen status using the SPAD-502 chlorophyll meter. *Forest Ecology and Management* 181: 331–338.

Chapman, S. C., and H. J. Barreto. 1997. Using a chlorophyll meter to estimate specific leaf nitrogen of tropical maize during vegetative growth. *Agronomy Journal* 89: 557–562.

Fischer, B. B., M. Wiesendanger, and R. I. L. Eggen. 2006. Growth condition-dependent sensitivity, photodamage and stress response of *Chlamydomonas reinhardtii* exposed to high light conditions. *Plant and Cell Physiology* 47: 1135–1145.

Madeira, A.C., A. Ferreira, A. de Varennes, and M.I. Vieira. 2003. SPAD meter versus tristimulus colorimeter to estimate chlorophyll content and leaf color in sweet pepper. *Communications in Soil Science and Plant Analysis* 34: 2461–2470.

Martinez, J., and R. Damidaux. 1993. Rapid methods to assess the chlorophyll and carotenoid content of leaves and fruits using a microplate reader. *Annals of Applied Biology* 123: 673–684.

Mencarelli, F., and M. E. Saltveit.1988. Ripening of mature-green tomato fruit slices. *Journal of the American Society for Horticultural Science* 113: 742–745.

Oikawa, A., A. Kimura, A. Kuroda, K. Yoshimura, N. Ohta, S. Takenaka, and D. Ohta. 2004. Metabolomics and proteomics by LC-MS, GC-MS, and FT-MS - Analyses of detergent-insoluble complexes in *Arabidopsis thaliana*. *Plant and Cell Physiology* 45: S148–S148.

Porra, R.J. 2002. The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls a and b. *Photosynthesis Research* 73: 149–156.

Ritchie, R. J. 2006 Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents. *Photosynthesis Research* 89: 27–41.

Sari, M., N. K. Sonmez, and A. Kurklu. 2005. Determination of seasonal variations in solar energy utilization by the leaves of Washington navel orange trees (*Citrus sinensis* L. Osbeck). *International Journal of Remote Sensing* 26: 3295–3307.

Wang, Q. B., M. J. Chen, and Y. C. Li. 2004. Nondestructive and rapid estimation of leaf chlorophyll and nitrogen status of peace lily using a chlorophyll meter. *Journal of Plant Nutrition* 27: 557–569.