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SPECTROPHOTOMETER 6505 Application note: A09-001A

Spectrophotometry of chlorophylls *a* and *b*

Introduction

The photosynthetic pigment chlorophyll is present in most plants, algae and cyanobacteria. It can be measured both by spectrophotometry and fluorimetry as an indicator of the abundance of photosynthetic organisms in fresh and salt water. In fresh water, levels of chlorophyll are also an important factor in determining water quality. Chlorophyll pigments may be present in several forms in varying ratios, the most common being chlorophyll *a*. Here we demonstrate the spectra of chlorophylls *a* and *b* and how the two can be distinguished in a mixture by acidification of the sample. We also show that chlorophyll *a* and *b* concentrations can be calculated to an approximate value using a simple equation based on their absorbance maxima.

Methods

Chlorophyll *a* and *b* from spinach were purchased from Sigma, part codes C5753 and C5878. The contents of the vials, containing 1mg of pure chlorophyll, were each dissolved in 50ml of 90% acetone (spectrophotometric grade, Sigma 154598) to give 20mg/l stock solutions. These were stored in aliquots in the dark at -20ºC until required.

Stock solutions of chlorophylls *a* and *b* were diluted 1 in 20 in 90% acetone to give solutions of 1mg/l. Prior to measurement, these were further diluted and mixed in a 1ml quartz cuvette, as shown in **Table 1**.

Chl *a* (ml)	Chl *b* (ml)	90% acetone (ml)	Conc. *a*:*b* (mg/l)
1.0	-	0	1.0:0.0
0.8	-	0.2	0.8:0.0
0.6	-	0.4	0.6:0.0
0.4	-	0.6	0.4:0.0
0.2	-	0.8	0.2:0.0
-	1.0	0	0.0:1.0
-	0.8	0.2	0.0:0.8
-	0.6	0.4	0.0:0.6
-	0.4	0.6	0.0:0.4
-	0.2	0.8	0.0:0.2
0.9	0.1	-	0.9:0.1
0.8	0.2	-	0.8:0.2
0.6	0.4	-	0.6:0.4
0.5	0.5	-	0.5:0.5

Table 1: Dilutions and mixtures of chlorophyll (Chl) *a* and *b* solutions. The final volume was 1ml in each case.

Photosynthetic pigments were extracted from a sample of slime algae isolated from a domestic marine fish tank. The algae were ground in a mortar containing ceramic beads with 90% acetone. The extract was filtered through Whatman No. 1 filter paper into a volumetric flask and made up to a volume of 50ml. The extract was stored in aliquots in the dark at -20ºC until required.

The model 6505 spectrophotometer was set up in Spectrum mode to scan between 600nm and 700nm in 0.2nm steps. It was connected to a PC and Jenway's DataWay data acquisition software was used to collect and store the spectral data. The data was copied into Microsoft Excel™ for plotting and analysis.

The spectra of each of the dilutions and mixtures listed in **Table 1** were recorded, as was that of the unknown algae sample. After the initial recording, 30µl of 0.1N HCl was added to the cuvette to give a final concentration of 0.003N, mixed, and the spectrum recorded again after 90s.

Results

The spectra for pure solutions of chlorophyll *a* and *b* are shown in **Figure 1**. The absorbance maximum for chlorophyll *a* was determined to be 662.6nm and for chlorophyll *b*, 645.6nm.

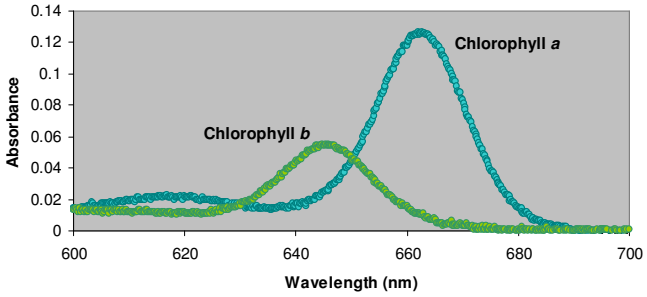

Figure 1: Absorbance spectra of chlorophylls *a* and *b*. Both samples were at a concentration of 1mg/l.

In certain methods of chlorophyll determination, acidification of the chlorophyll samples can help to remove the interferences of degradation products. When chlorophylls are acidified, the magnesium ion is lost from the porphyrin ring, resulting in the production of pheophytin. The ratio of pure chlorophyll *a* before acidification to that after is around 1.7 (1). Presence of degradation products and other chlorophylls have the effect of lowering the ratio.

Figure 2 shows the effect of adding HCl to a final concentration of 0.003N to a sample of chlorophyll *a*. The degradation was virtually complete within 90 seconds (2). The absorbance maximum fell by approximately 45% and the peak shifted approximately 2nm to the red. The ratio was calculated to be 1.8, slightly higher than the expected value of 1.7. This may indicate further degradation of pheophytin by the acid.

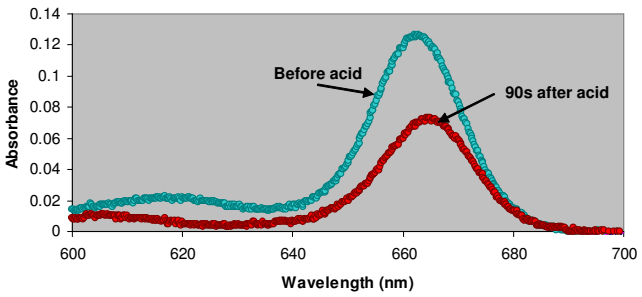

Figure 2: Degradation of chlorophyll *a* to pheophytin following acidification.

Chlorophyll *b* on the other hand does not degrade at such a rapid rate; after 90 seconds the absorbance maximum fell by only approximately 15%

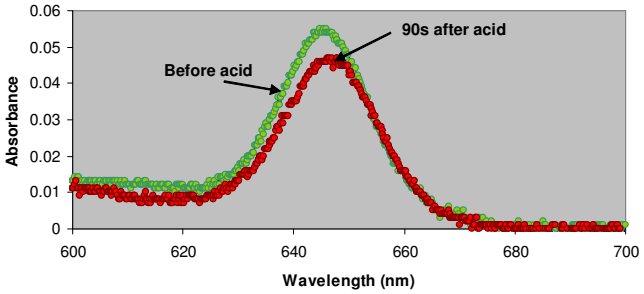

Figure 3: Degradation of chlorophyll *b* following acidification.

Due to this acid effect, the presence of chlorophyll *b* in a mixture with chlorophyll *a* can be determined as the relative amount increases by observing the presence of a shoulder at around 646nm on the chlorophyll *a* peak.

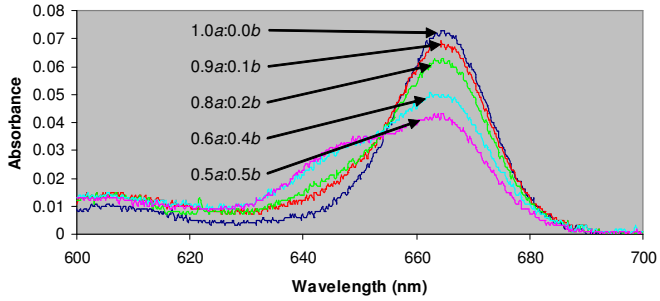

Figure 4: Spectra of various mixtures of chlorophyll *a* and *b* after 90 seconds of acidification.

The approximate amounts of chlorophyll *a* and *b* present in a sample can be calculated by a number of different formulae. The formulae of Lichtenthaler and Wellburn (3) are based on the absorbance maxima of each pigment and are dependent on the solvent used. The formulae for samples dissolved in acetone are as follows:

$C_a = 11.75\ A_{662.6} - 2.35\ A_{645.6}$

$C_b = 18.61\ A_{645.6} - 3.96\ A_{662.6}$

where C_a and C_b are the concentrations of chlorophyll *a* and *b* respectively. **Table 2** shows the results of these calculations for the chlorophyll *a* and *b* dilutions and mixtures.

Expected conc. Chl *a* (mg/l)	Expected conc. Chl *b* (mg/l)	Calculated conc. Chl *a* (mg/l)	Calculated conc. Chl *b* (mg/l)
0.0	0.0	-0.01	0.00
1.0	0.0	1.42	-0.02
0.8	0.0	1.13	-0.02
0.6	0.0	0.85	-0.04
0.4	0.0	0.58	-0.05
0.2	0.0	0.29	-0.04
0.0	1.0	-0.01	0.97
0.0	0.8	-0.03	0.78
0.0	0.6	-0.02	0.58
0.0	0.4	-0.03	0.40
0.0	0.2	0.00	0.22
0.9	0.1	1.33	0.12
0.8	0.2	1.18	0.24
0.6	0.4	0.93	0.40
0.5	0.5	0.75	0.50

Table 2: Nominal chlorophyll *a* and *b* concentrations based on dilutions and mixtures and the calculated concentrations based on the absorbance values at 662.6nm and 645.6nm.

The values calculated for chlorophyll *a* are somewhat higher that expected, which may indicate that the original dilution was inaccurate, however, there is very good linear correlation between the expected and calculated values as shown in **Figure 4**. Similar results were obtained for chlorophyll *b* with calculated concentrations very close to the expected values.

The sample of photosynthetic pigments extracted from the slime algae was also measured in this way. The sample was diluted 1 in 20 in 90% acetone and the spectrum measured over the range 600 to 700nm. The calculated chlorophyll *a* and *b* concentrations were 0.91mg/l and 0.19mg/l respectively, therefore the ratio of chlorophyll *a* to *b* in this sample is 1:0.21.

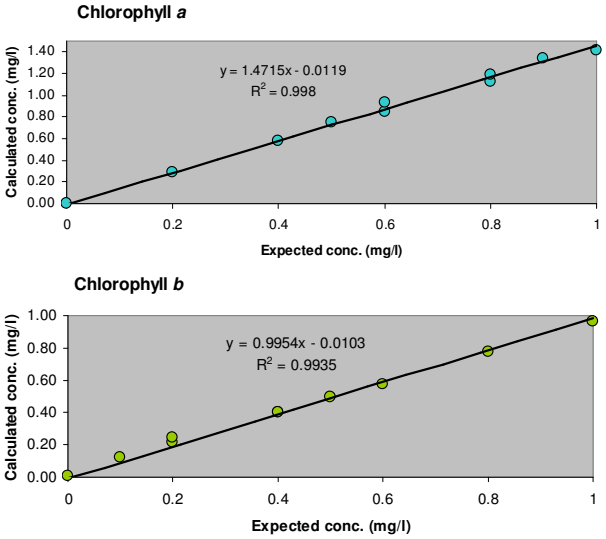

Figure 4: Correlation of expected concentration against calculated concentration for chlorophylls *a* and *b* using the formulae of Lichtenthaler and Wellburn.

Conclusions

We have demonstrated here that samples containing mixtures of chlorophyll *a* and *b* can be distinguished by their spectra in the presence of hydrochloric acid using a spectrophotometer with a narrow band width such as the model 6505 (1.8nm). In addition, the amounts of chlorophylls *a* and *b* can be approximated from simple equations based on their maximum absorbance peaks. Although numerous equations have been developed in order to calculate the concentration of chlorophyll a in the presence of other forms and derivatives, due to interference of spectra and the presence of degradation products and other pigments, it is important to remember that most of these give at best only an estimation of the total chlorophyll a content.

References

(1) Lorenzen. C. J., Determination of chlorophyll and pheopigments: Spectrophotometric equations. Limnol. Oceanogr. **12**: 343-346, (1967)

(2) Method based on EPA Method 445.0: In vitro determination of chlorophyll *a* and pheophytin *a* in marine and freshwater algae by fluorescence. Arar, E. J. and Collins, G. B. (1997).

(3) Lichtenthaler, H. K., and Wellburn, A.R., Determination of total carotenoids and chlorophylls *a* and *b* of leaf in different solvents. Biol. Soc. Trans. **11**: 591-592 (1983).