Extraction and determination by fluorescence of Phycocyanin

Kristel Sanchez[1]

[1]University of Michigan - Ann Arbor

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Duffy Lab, EEB, University of Michigan

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This protocol details the chemical extraction of phycocyanin from phytoplankton cells and the quantification of phycocyanin by fluorescence using the Turner Trilogy Laboratory Fluorometer.

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Extraction and determination by fluorescence of *Phycocyanin*

Author: Kristel Sánchez

The protocol below comes from Kasinak *et al.*, 2015.

Summary.

This protocol details the chemical extraction of phycocyanin from phytoplankton cells and the quantification of phycocyanin by fluorescence using the Turner Trilogy Laboratory Fluorometer.

Materials.

Apparatus & Equipment	Labware	Reagents & Standards
Turner Trilogy Fluorometer	Graduated cylinders (1L)	50 mM Phosphate buffer *
Centrifuge	Volumetric flaks (25, 50,100mL & 1L)	C-Phycocyanin standard
Teflon pestle	Glass rods	Nano pure water
GF/F Whatman filters (47 mm Pall A/E filters can be used too)	Pasteur pipets and bulb	
Aluminum dishes	Glass cuvette (for fluorometer)	
Aluminum foil	1L Filtration flask	
Kimwipes (lab tissue)	Fritted disk base	
Vacuum pump	Filter tower	
Tweezers or forceps	50 & 15 mL Centrifuge tubes (falcon tubes) with screw caps	
Turner snap-on module for Phycocyanin extracted or in-vivo	Glass bottle with screw cap	
Analytical balance	Magnetic stir bar	
Stirring plate	Weight boat	
25-mm inline filter (VWR #28145-485);	Spatula	
0.20 µm filter (VWR #28145-483)	pH paper	
	Glass rod	
	5 or 10mL syringe	

***** 50mMPhosphate buffer can be purchased or prepared as described below.

Preparation of reagents.

Phosphate buffer

Using an analytical balance, weight the following compounds in a weight boat.

Reagents	Amount in grams	Molarity
Sodium phosphate dibasic (mw: 268.07 g/mol)	7.744 g	0.0289 M
Sodium phosphate monobasic (mw: 137.99 g/mol)	2.913 g	0.0211 M

In a 1 L volumetric flask add 500 mL of nano-pure water and a magnetic stir bar. Add both weighted compounds into the volumetric flask and place the flask on top of a stirring plate. Begin stirring until all the crystals have dissolved. If crystals are not dissolving add an additional 200 mL of nano-pure water. Once all crystals have dissolved check the pH of the solution using pH paper (Insert clean glass rod into

the solution and add a few drop into the pH paper).The pH of the solution should be pH 7 ±0.1. If needed adjust pH by adding HCL if need to bring the pH down or NaOH if need to bring pH up. Once pH has been adjusted add nano-pure water to the flask until the 1L water mark (make sure to remove magnetic stir bar before adding water).

Phycocyanin standard solution PSS

In subdue light, transfer the phycocyanin standard to a 50 mL volumetric flask. Add ~ 30 mL of phosphate buffer to the flask and mixed to dissolve the dry phycocyanin. Once dissolved, add additionally phosphate buffer until the 50 mL mark. The concentration of the solution must be determined spectrophotometrically using a multiwavelength spectrophotometer. The following formula must be used to calculate the concentration in mg/mL:

C-Phycocyanin (mg/mL) =

Where A_{615} is the absorbance the phycocyanin standard at 615 nm and A_{652} is the absorbance at 652nm. The phycocyanin standard can be stored in 2 °C wrapped in aluminum foil for up to 6 months.

Calibration curve

Prepare a 2000 ug/L phycocyanin solution in a 100 mL flask using the PSS and phosphate buffer. The calibration curve will be determined by analyzing 6 calibration standards ranging from 0 ug/L to 2000 ug/L. Note that the Turner Trilogy fluorometer can only save up to 5 calibration readings within a curve so you may adjust the calibration points according to the needs of your experiment. Prepare the rest of the calibration standards, 0, 10, 50, 100, 500, and 1000 ug/L, following the dilution series in the table below:

Standard concentration	Amount of phycocyanin solution	Amount of phosphate buffer
1000 ug/L	50 mL of 2000 ug/L phycocyanin solution	50 mL
500 ug/L	50 mL of 1000 ug/L phycocyanin solution	50 mL
100 ug/L	20 mL of 500 ug/L phycocyanin solution	80 mL
50 ug/L	50 mL of 100 ug/L phycocyanin solution	50 mL
10 ug/L	20 mL of 50 ug/L phycocyanin solution	80 mL
Blank	0	100 mL

Calibration standards can also be prepared inside 100 mL volumetric flasks by adding the amounts provided in the table above of phycocyanin standards and filling the rest of the flask until the meniscus

mark with phosphate buffer.
Once samples are prepared keep them away from light (you may also wrap them with aluminum foil for extra protection) and at a consistent room temperature (±3 °C) or in the fridge if need to use at a later time.
Snap-in the Phycocyanin module into the Turner Trilogy fluorometer (the module is labeled as Trilogy module: PC, GIU selection: Orange). Turn-on the fluorometer and on the main screen choose “Orange” and press OK. On the next screen, press “Calibrate” found at the bottom of the screen. Select “Run New Calibration.” Next, select the unit of measurement “ug/L.” Rinse the borosilicate or glass cuvette with phosphate buffer. Once rinsed, add between 2-3mL of phosphate in the cuvette, this will be your blank point. Open the lid of the fluorometer and insert the cuvette with the sample and close the lid immediately. Press “OK” in the screen and wait for the fluorometer to take the reading. After the measurement is complete, open the lid and remove the cuvette. Obtain a new cuvette or empty the previously used cuvette and rinse the glass cuvette with the calibration sample of the lowest concentration, then add 2-3mL of the same low concentration sample into the cuvette. Open the lid of the fluorometer and insert the cuvette with the sample and close the lid immediately. On the fluorometer screen, enter the concentration of the first standard and click “OK” and press “OK” again to indicate that the sample is inserted. Repeat the steps in the order of increasing concentration to enter the measure the rest of the standards. Once you have finished all 5 standards name and save the calibration curve for future use.

Preparation of samples

Water samples should be collected in clean acid-free containers. As soon as samples are collected, ***samples should remain in subdue light at all times*** for the rest of the preparation and measurement procedures. Once water samples have been collected, assemble the filtration apparatus by connecting a 1L filtration flask to a vacuum pump. Then place the fritted disk base on the mouth of the 1L filtration flask and place a GF/F whatman disk on the disk base using tweezers or forceps. Place the filter tower on top of the fritted disk base. Add your first sample to the filter tower (make sure to not add so much water that it overflows) and turn on the vacuum pump. Note the amount of water volume you are filtrating per sample (either with a graduated cylinder or graduated pipet). Make sure the pump does not exceed 20kPa or adjust if it does (higher pressure may damage cells and phycocyanin). Once the samples have been completely filter, remove the whatman disk using tweezers or forceps and place it in a labeled falcon tube. Repeat the same procedure for the rest of the water samples rinsing in between the filter tower with nano pure or distilled water. Cover samples with aluminum foil to protect from the light and place samples in the freezer if extraction will be done on a different day.

Phycocyanin extraction

To each tube containing a whatman filter with phytoplankton, add 10 mL of phosphate buffer. Obtain one tube and macerate the filter paper with a Teflon pestle. Once the filter is sufficiently macerate rinse the pestle with 10 mL of phosphate buffer using a volumetric pipet making sure you collect the rinse inside the falcon tube (for a total of 20mL per tube). Cap the tube and shake the tube vigorously. Rinse again the Teflon pestle well with phosphate buffer using a squirt bottle and repeat procedure with the remainder of samples. Samples should then be allowed to steep for 2 hours in 4 °C and then an additional 2 hours at room temperature in the dark. Longer extraction times have been shown to reduce the amount of phycocyanin observed.
After steeping is complete, sample need to filter-sterilized to remove particulates prior to fluorescence measurement. With a syringe, obtain 5mL of aliquot of a sample and filter the aliquot through a 25-mm inline filter and then through a 0.20 µm syringe filter. Rinse filters with 10 mL of fresh phosphate buffer and repeat procedure with the next sample.

Sample measurement

Turn-on the fluorometer (make sure the PC/ORANGe module is snap-in place) and allow the machine to warm-up for 20 minutes. Obtain a clean cuvette and rinse it with phosphate buffer. Once rinsed add 2-3 mL of phosphate buffer solution to the cuvette and place the cuvette inside the fluorometer. Press measure absorbance and make sure the sample read as 0.000 Ab or note the reading to later subtract from samples. Next, obtain a new cuvette or discard the sample from the previous cuvette and rinse the cuvette with your first sample to measure. Once rinsed, add 2-3mL of your sample in the cuvette. Insert the cuvette inside the fluorometer. Press measure absorbance on the fluorometer screen and record the raw fluorescence unit and/or the concentration. Repeat the same steps for the rest of the samples. For every 10 samples read a new blank in case there is a change of room temperature or something that may influence the sensitivity of the instrument and use this number to calculate the actual fluorescence of the next 10 samples.

Concentration calculation

Only do this step if not using the calibration step for the fluorometer. Before running your samples, measure your 5 standards plus the blank and record the raw fluorescence unit (RFU). In excel, create a line graph using the known concentrations as your x-values and the RFU as your y-values. Generate the equation of the line for your given values. To calculate the concentration of your samples, insert the RFU for y in the formula and solve for x.