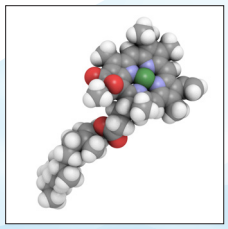
HORIBA Scientific

Fluorescence

Quantitative Determination of Chlorophyll A with the Duetta Dual Fluorescence/ Absorbance Spectrometer



Application Note
Life Sciences
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Chlorophyll A (Chl A) is one of the most important molecules on the planet as it is responsible for the energy fixation in plant cells during photosynthesis. Besides its importance in photosynthesis, the quantitative determination of Chl A content is the most common method of estimating algal biomass in water reservoirs.

There are several optical methods for Chl A detection and quantification. The most common are spectrophotometric, which require no prior calibration. Fluorescence-based methods offer higher sensitivity allowing to go to much lower concentrations than can be attained by the absorbance based methods. However, the fluorescence assays require a prior calibration with Chl A standards of known concentrations.

HORIBA's Duetta, a CCD-based dual fluorescence/ absorbance spectrometer is a very capable instrument for the determination of Chl A concentration by fluorescence due to its inherent sensitivity and ability to automatically correct for the inner filter effect (IFE), which ensures a very high dynamic range for such analysis. It also can be used to quantify the concentration of the standard stock solution by absorbance, thus eliminating a need for a dedicated UV-VIS spectrometer.

This note illustrates Chl A determination based on a modified EPA protocol [1]. The protocol includes extracting the chlorophyll and related compounds using 90% aqueous acetone from algae. The concentration of the pigments is determined by measuring fluorescence of the extract before and after acidification. The calibration procedure with the use of a commercial Chl A standard is described in detail.

Reagents

- Chl A standard: Chl A standard Anacystis nidulans algae C6144-1MG (Sigma-Aldrich)
- 90% aqueous acetone solution: mix 450 ml HPLC grade acetone (Sigma-Aldrich) with 50 ml type I water (v/v)
- Type I water
- 1N hydrochloric acid

Standard stock solution preparation

The Chl A standard (1 mg solid) was carefully transferred from the ampule into a vial. The 90% acetone solution was used to dissolve and bring the volume up to 10 ml to make a 100-ppm standard stock solution (SSS). The SSS vial was wrapped in aluminum foil and stored at 4°C in the dark.

Calibration standards preparation

The SSS was diluted with 90% acetone to make an intermediate standard solution (ISS) 1 ppm. Using serial dilution, five Chl A calibration standards (S1 – S5) with the approximate concentrations of 100, 50, 20, 10, 5 ppb were prepared in 9 ml disposable glass tubes with 90% acetone. The exact concentration of ISS was determined spectrophotometrically (see below). The exact concentrations of calibration standards were then determined from ISS based on the dilution factors (Table 1).

Standard	Approx. Concentration	Dilution Factor
SSS	100 ppm	-
ISS	1 ppm	100x from SSS
S1	100 ppb	10x from ISS
S2	50 ppb	2x from S1
S3	20 ppb	2.5x from S2
S4	10 ppb	2x from S3
S5	5 ppb	2x from S4

Table 1

Determining the concentration of standard stock solution (ISS)

Using the Duetta's absorbance mode, the optical density of an aliquot of ISS was measured at 750 nm and 665 nm in a 1 cm path length quartz cuvette. Optical density values were recorded as U750 and U665, respectively. For acidification of the ISS sample, 9 µl of 1 N of the HCl was added to the cuvette. After gently agitating the cuvette and waiting for 60 seconds, the absorbance after acidification was measured at 750 nm (A750) and 665 nm (A665), respectively. The acidification step allows the determination of the amount of pheophytin present in the original sample. The blank used for absorbance measurements was the 90% acetone solution, for both before and after acidification. The corrected Chl A concentration was determined using the following equation:

$$\text{Chl A (ppb)} = 1000 \times (27.3 \times ((U665 - U750) - (A665 - A750))/PL) \quad (1)$$

where

27.3 is the absorbance conversion factor and PL is the path length (1 cm).

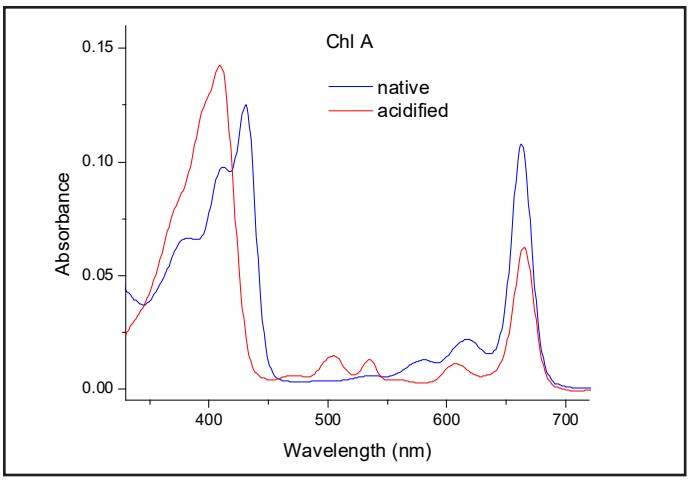

Figure 1: Absorbance spectrum of ISS before and after acidification measured with the Duetta

Fluorescence measurements of calibration standards

The fluorescence intensities of five Chl A calibration standards were measured with the Duetta using the Capture Value mode with the following parameters: ex = 631 nm, em = 670 nm, ex and em bandpass = 5 nm, int time = 0.1 s, 20 accumulations and 8-pixel binning (4 nm). The total acquisition time per sample was 2 s. Each sample was subsequently acidified and the measurement repeated.

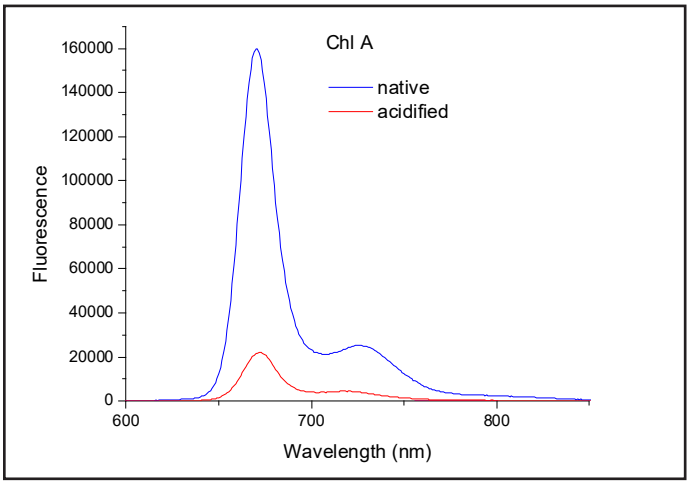

Figure 2. Fluorescence spectrum of ISS before and after acidification measured with the Duetta

Figure 3 shows the Chl A calibration curve based on the fluorescence measurements of the 5 standard samples. It should be noted that the fluorescence intensity vs. Chl A exhibits excellent linearity with R2 = 0.9997.

For the actual measurement, the ISS stock solution was diluted 200x and 6 independent fluorescence measurements of un-acidified and acidified samples were made at 670 nm, in addition to a blank measurement. The fluorescence intensity was calculated as U – A with background values subtracted (Table 2).

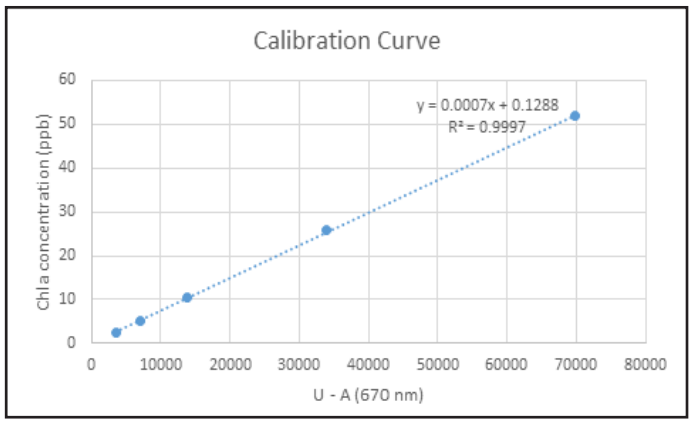

Figure 3: The calibration curve for Chl A determined from fluorescence measurement of un-acidified (U) and acidified (A) standard samples at 670 nm.

Sample	U (Fluor 670 nm)	A (Fluor 670 nm/HCl)	U-A	PChl (ppb)
sample1	3895	543	3252	2.549
sample2	3855	531	3224	2.528
sample3	3859	550	3209	2.517
sample4	3876	531	3245	2.543
sample5	3971	554	3317	2.597
sample6	3835	555	3180	2.495
blank	151	51		
			STDEV	0.034
			MDL	0.11

Table 2

The Method Detection Limit (MDL) is calculated as

$$MDL = t \times STDEV$$

where STDEV is the standard deviation of replicate measurements, t = 3.365 is the Student's t value for the 99% confidence level and the standard deviation estimate with n-1 = 5 degrees of freedom.

Conclusion

The MDL = 0.11 ppb compares very favorably with the expected detection limit (EDL) of 96 ppb for filter fluorometry and 80 ppb reported in the EPA study [1]. This is the result of excellent performance characteristics of the Duetta, such as the sensitivity, stability and reproducibility. The Duetta is a very capable and versatile instrument for a variety of analytical applications including Chl A determination and quantification.

References

1. EPA Method 445.0 In Vitro Determination of Chlorophyll A and Pheophytin A in Marine and Freshwater Algae by Fluorescence
2. ASTM D3731 Standard Practices for Measurement of Chlorophyll Content of Algae in Surface Waters

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