

Evaluation of Zinc Pthalocyanine as an Internal Standard for the Routine HPLC Analysis of Chlorophylls in Environmental Water Samples

Patrick Kohler, David Struve and Yelena Guthman

April 29, 2011

Abstract

The commercially available dye, zinc-pthalocyanine is tested as a potential internal standard for the routine analysis of water samples for chlorophylls by HPLC analysis. The internal standard is evaluated to determine its relative stability compared to chlorophyll *a* and chlorophyll *b* and to determine if suitable recoveries can be obtained during the routine extraction process which involves filtration on a glass-fiber filter followed by solvent extraction. Chlorophyll *a*, chlorophyll *b* and zinc-pthalocyanine were separated by HPLC using a C₈ reverse-phase column and photodiode array detection.

1.0 Introduction

The South Florida Water Management District is an agency with diverse roles including environmental monitoring. The District covers an area of approximately 1,800 miles and included within its borders are three areas of special interest, Lake Okeechobee, the Kississimee River and the Everglades. As part of its monitoring efforts, water quality is monitored at approximately 1800 locations. Water quality measurements include determination of nutrient concentrations, physical parameters, common anions and cations, metals, and carbon. Additionally, many samples are also routinely monitored for Chlorophyll concentrations as a measure of phytoplankton biomass and as an indicator of primary production. The District lab receives 100-200 samples for this test on a weekly basis. [1]

Given that the common methods for determination of chlorophyll concentrations are labor intensive, the District lab has sought to utilize methods that are highly accurate, rapid and that can be automated as much as possible. This has led the lab to acquire instrumentation and develop methods for the rapid determination of Chlorophylls by High Performance Liquid Chromatography. The method utilized for the determination is a modified version of EPA Method 447 [2]. Principal modifications of this method are the eluent composition and the column type. The modified method utilizes a binary gradient composed of tetrabutylammonium acetate (TBAA) and methanol to elute chlorophylls from a column containing a C8 stationary phase [3]. Additionally, a photodiode array (PDA) detector is used rather than a fixed wavelength detector so that the entire absorption spectrum of each peak can be recorded and compared to reference spectrum stored in the system software. The method is optimized for fast elution of chlorophyll a, b and pheophytin a and the chromatogram is recorded at 665 nm, as absorption at this wavelength is limited to the compounds of interest.

High Performance Liquid Chromatography is a fast and accurate method for analyzing chlorophyll a and b, as well as pheophytin a, however, the steps necessary to extract these compounds from the water sample prior to analysis on the chromatographic system can cause degradation of the target compounds and increased measurement uncertainty. Given this fact, the inclusion of an appropriate internal standard, prior to sample extraction, could lead to more accurate determinations and reduce measurement uncertainty. While a number of potential internal standards have been identified [4], Bohn and Walczyk [5] have suggested using a commercially available dye, zinc-pthalocyanine (ZnPc). An ideal internal standard for routine chlorophyll analysis would be one that is structurally similar, does not co-elute with a target compound, shows similar rates of degradation to the target compounds and is not otherwise discriminated against during sample processing. Here we show, that ZnPc (Fig. 1) meets these requirements as an internal standard, and that it can be used for conventional analysis of water samples but only by using the extraction solvent DMF, as ZnPc is not sufficiently soluble in the more commonly used 90% acetone extraction solvent.

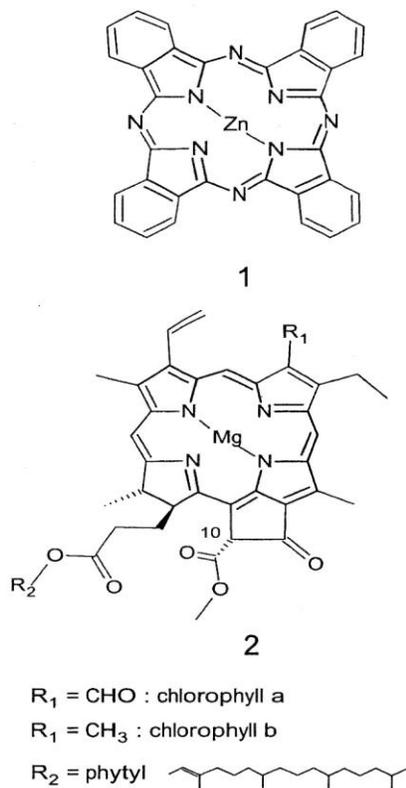


Fig. 1. Structure of zinc-phthalocyanine (1), chlorophyll *a* and chlorophyll *b* (2). Reprinted from [5].

2.0 Experimental Section

2.1 Standards and Chemicals

Chlorophyll *a* and chlorophyll *b* (Sigma, <97%), and zinc phthalocyanine (Sigma, >97%) were obtained in solid form. Other reagents and solvents were of analytical grade or better. Chlorophyll stock solutions were prepared by dissolving approximately 1 mg of chlorophyll *a* and chlorophyll *b* in 100 mL of a 90% aqueous acetone solution. The exact concentrations of the stock solutions were determined by measuring the absorbance of the solutions at an appropriate wavelength using a 1cm cell with the following extinction coefficients (Chl *a* Abs_{664.3}/87.67, Chl *b* Abs_{646.8}/51.36). A stock solution of zinc phthalocyanine was prepared by dissolving ~3 mg of ZnPc in 100 mL of DMF. Working standards of chl *a*, chl *b* and ZnPc were prepared by volumetric dilution of the stock solutions using class A glassware.

Previous experience has shown that the chlorophyll stock solutions are stable for 28 days if stored at -20 °C. Bohn and Walczyk indicated that ZnPc solutions were stable for at least six months. [5]

To initially investigate the chromatographic performance of ZnPc using our HPLC methodology, solutions of ZnPc in 90% aqueous acetone were prepared at concentrations expected to produce peaks of sufficient area to minimize variability. After initial experimentation solutions in the range of 100-1000 µg/L were found to be acceptable although some band broadening was noted. Ultimately solutions

containing similar concentrations in DMF were utilized to investigate the degradation properties of ZnPc as ZnPc was found to form aggregates in acetone solutions.

2.3 HPLC analysis

Samples were analyzed by reverse phase chromatography with photodiode array detection. The HPLC system was composed of a refrigerated autosampler (Shimadzu SIL-20A), 2 solvent delivery modules (Shimadzu LC-20AT), an eluent degassing system (Shimadzu DGU-20A3), high pressure solvent mixer (Shimadzu , dwell volume 2.8 mL), column oven (Shimadzu CTO-20A), photodiode array detector (Shimadzu SPD M20A), and chromatography data system (Shimadzu LCsolution version 1.54). A 4.6mm x 150mm C8 column (Kromasil, 3.5µm particle size) equipped with a guard column (Kromasil, KR100-5-C8-4.6XGC, 10mm, 5 µm) was used for all separations. Flow rate was 2.0 mL/min using a linear binary gradient where A was 28mM TBAA and B was MeOH. The gradient was run from 85% B to 100% B over 5.5 minutes, held at 100% B for 1 minute and then returned to initial conditions over 1 minute. Column temperature was 60 C°. Chromatographs were recorded at 665 nm (20 nm bandpass) with the diode array detector set to scan from 300nm – 700nm at a frequency of 1.85 Hz.

Response factors for chl a, chl b, and ZnPc were determined by linear regression of the peak areas for a series of working standards using Excel. Relative response factors for chl b/ZnPc and chla/ZnPc were determined by dividing the appropriate individual response factors.

2.4 Evaluation of the Internal Standard for Routine Analysis of Water Samples

For routine analysis of water samples, 300-500 mL of a water sample was first filtered under subdued lighting through a 47mm Whatman type GF glass fiber filter. After filtration the filter funnel and filter holder were rinsed with deionized water (about 20 mL) and then preserved by addition of 1 mL of a saturated magnesium carbonate solution. Filters were then placed in 13 mL Sarstedt tubes (#62.515.006) and 100 µL (2.6 µg) of internal standard in DMF was added onto the filter using a fixed volume eppendorf pipette. Tubes were sealed and placed in -20C° storage until extraction.

The sample filters were extracted in a fume hood by placing approximately 8 mL of DMF into the tube with the filter and then sonicated (Misonix Sonicator 3000 with sonication probe (#420,.5-15m, 1/8", 3.2mm Intensity very high Aplitude 250um) for 2 minutes at a power setting of 8 (18-24watts) using 2 seconds on 0.5 second off pulse. Tubes were kept in an ice bath while sonicating to keep the extract cool.

Multiple replicates of sample and blank filters were conducted to determine the IS recoveries after sample extraction.

2.5 Degradation Studies

Sample extracts along with the internal standard (ZnPc) in DMF were subjected to repeated sonication cycles to investigate the degradation characteristics of the internal standard relative to the target compounds. Approximately 10 mL of extract was sonicated for 45 seconds and subsampled, this represented one typical extraction cycle. The extracts were further sonicated for 4 additional cycles to

encourage further degradation of the compounds. Sub samples of each test solution were subjected to HPLC analysis and the recoveries of ZnPc, chl a and chl b were computed for each cycle.

3. Results

3.1 Chromatographic Separation of ZnPc

Initial studies with prepared solutions containing ZnPc in DMF showed that the compound elutes near 4.07 minutes and is well resolved from the target compounds (Fig. 2). However, the peak shape for ZnPc solution in DMF showed signs of extreme band broadening with our typical 50 uL injection volumes (Fig 2.) so injection volumes were reduced to 20 uL to maintain better peak shapes with minimal tailing. A typical chromatograph of a 20 uL injection of a DMF sample extract with ZnPc as an internal standard is shown in Fig. 3. The response factor for ZnPC was determined to be 3.727878×10^{-3} and the relative response factor for chl b/ZnPc was determined to be 8.84070 and for chl a/ZnPc to be 3.855124.

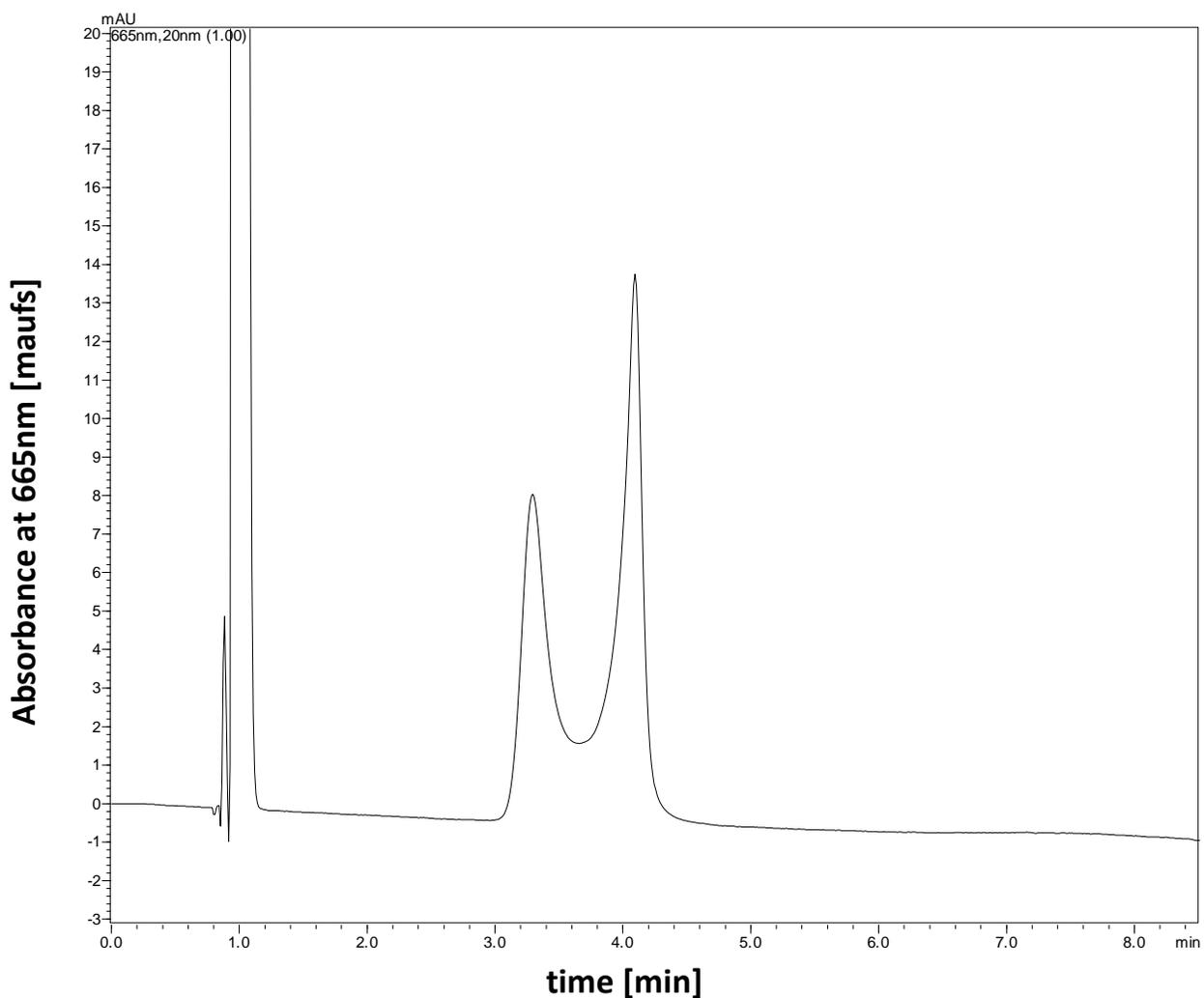


Fig. 2. Chromatogram of a 50 uL injection of ZnPc in DMF. (1) ZnPc

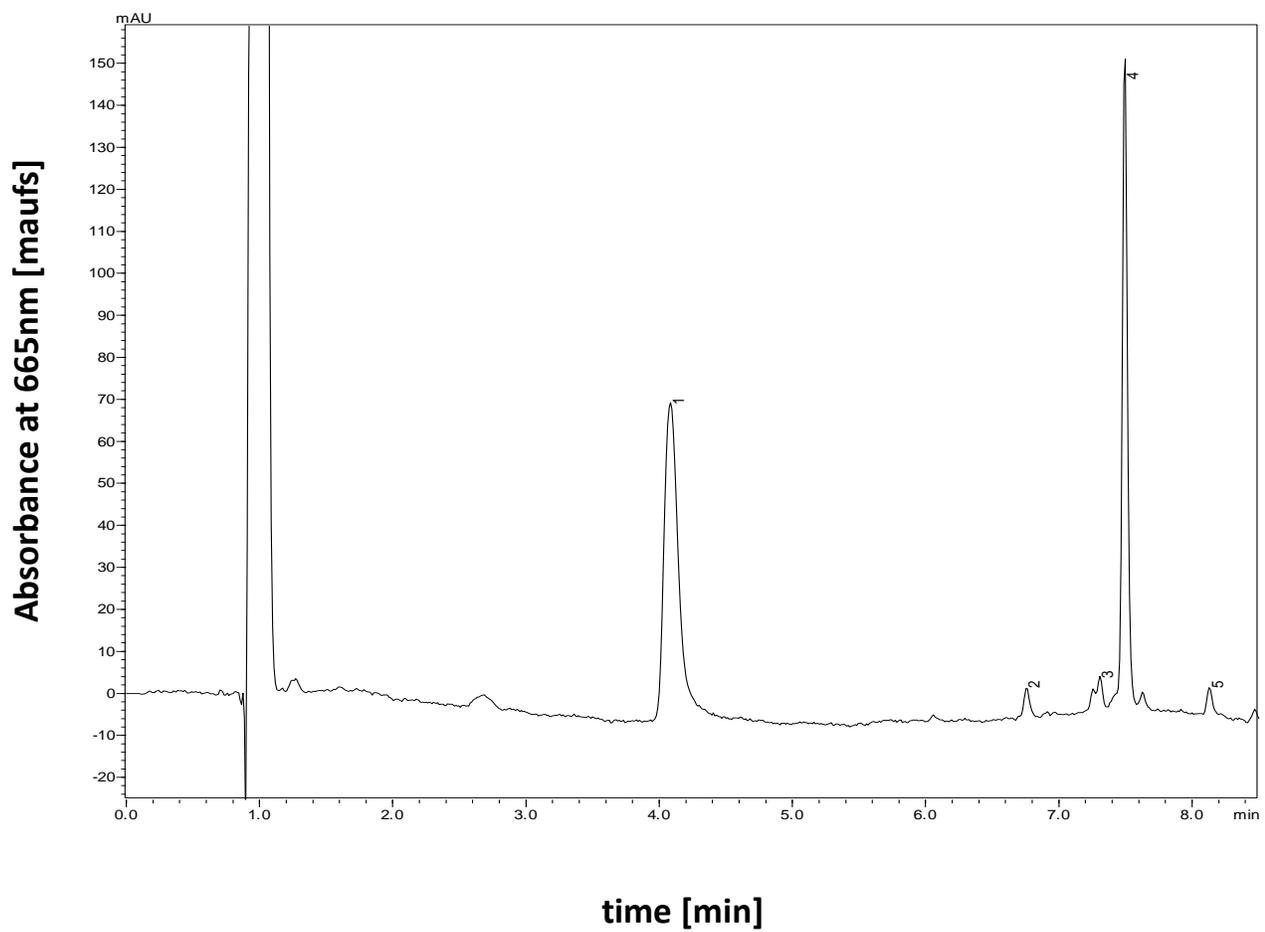


Fig. 3. Chromatogram of a 20 μ L injection of a sample extract containing ZnPc. (1) ZnPc, (2) chl b, (4) chl a

3.2 Degradation Studies

The average recoveries of ZnPc , chl a and chl b from four samples are shown in Table 1.

Cycles	ZnPc	Chl a	Chl b
AVG (1c)	99%	100%	100%
AVG (2c)	74%	93%	102%
AVG (3c)	60%	85%	97%
AVG (4c)	56%	80%	95%
AVG (5c)	58%	75%	89%

Table 1. Average recoveries of ZnPc, chl a and chl b after repeated sonication cycles.

4. Discussion

While ZnPc has structural similarities to chl a and chl b its solubility in 90% aqueous acetone is low and ZnPc tends to form aggregates in this solvent [6] that are easily removed by filtration. As a result, only when a solvent with a much higher dipole moment, DMF, was used were we able to fully dissolve the compound. DMF is considered the “benchmark” solvent for extraction of chlorophylls [5] although it induced severe band broadening during HPLC analysis that was minimized by using a smaller injection volume.

Degradation studies of ZnPc in DMF were very promising and showed that initial recoveries of ZnPc were excellent. While the compound did not degrade in equivalent amounts to chl a and chl b after repeated sonication cycles, recoveries of ZnPc were deemed acceptable under these extreme conditions.

5. Conclusion

More testing is needed to fully evaluate ZnPC as an internal standard but the results so far are acceptable. ZnPc is not compatible with the typical solvent system of 90% acetone used by most labs for chlorophyll extraction. ZnPc is compatible with DMF as an extraction solvent although some band broadening is induced in the HPLC analysis requiring that smaller injection volumes be used.

Note: This paper is currently a work-in-progress and will be submitted for publication once further experimentation is completed.

References

- [1] South Florida Water Management District. (2010). Executive Summary 2010 South Florida Environmental Report (v.12).
- [2] EPA Method 447.0, Determination of Chlorophylls a and b and Identification of Other Pigments of Interest in Marine and Freshwater Algae Using High Performance Liquid Chromatography with Visible Wavelength Detection, Methods for Determination of Chemical Substances in Marine and Estuarine Matrices - 2nd Edition (EPA/600/R-97/072)
- [3] South Florida Water Management District. (2010). Standard Operating Procedure for the Determination of Chlorophylls and the Related Pigments by HPLC. SFWMD-LAB-SOP-3140-003, CHLOROPHYLL AND CAROTENOID.
- [4] Van Heukelem, L., & Thomas, C. (2001). Computer-assisted high-performance liquid chromatography method development with applications to the isolation and analysis of phytoplankton pigments. *Journal of Chromatography*, 910, 31-49.
- [5] Jeffrey, S.W., Mantoura, R.F.C., & Wright S.W. (1997). *Phytoplankton pigments in oceanography: Monographs on oceanographic methodology*. Paris: United Nations Educational, Scientific and Cultural Organizations
- [6] Bohn, T., & Walczyk T. (2004). Determination of Chlorophyll in plant samples by liquid chromatography using zinc-phthalocyanine as an internal standard. *Journal of Chromatography*, 1024, 123-128.
- [7] Ogunsipe, A., Maree, D. and Nyokong, T. (XXXX) Solvent Effects on the Photochemical and Fluorescence Properties of Zinc Phthalocyanine Derivatives. XXXXXXXXXXXXXXX