

Detection and Quantification of Heme and Chlorophyll Precursors Using a High Performance Liquid Chromatography (HPLC) System Equipped with Two Fluorescence Detectors

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[Abstract] Intermediates of tetrapyrrole biosynthetic pathway are low-abundant compounds, and their quantification is usually difficult, time consuming and requires large amounts of input material. Here, we describe a method allowing fast and accurate quantification of almost all intermediates of the heme and chlorophyll biosynthesis, including mono-vinyl and di-vinyl forms of (proto) chlorophyllide, using just a few millilitres of the cyanobacterial culture. Extracted precursors are separated by High Performance Liquid Chromatography system (HPLC) and detected by two ultra-sensitive fluorescence detectors set to different wavelengths.

Materials and Reagents

1. Methanol (HPLC grade)
2. Pyridine reagent Plus >99% (Sigma-Aldrich, catalog number: P57506)
3. H₂O (HPLC grade) (Merck KGaA)
4. Acetonitrile (HPLC grade)
5. HPLC solvent A (see Recipes)
6. HPLC solvent B (see Recipes)

Equipment

1. 0.5 ml polypropylene tubes
2. HPLC 2 ml glass vials and 200 µl glass inserts
3. HPLC system with an autosampler (Agilent, model: 1200) (Figure 1)



Figure 1. The Agilent 1200 HPLC system equipped with an autosampler and two fluorescence detectors marked as FLD #1 and FLD #2

4. Two HPLC fluorescence detectors (Agilent, model: 1200) (see Note 1)
 The original Agilent 1200 detectors offer adequate sensitivity for emission wavelengths ranging 300-600 nm, however for longer wavelengths the sensitivity quickly falls almost to zero. This issue also applies to the newer 1260 version of the detector. As sensitivity to longer wavelengths is essential for detection of several heme/chlorophyll precursors, the original Agilent photomultiplier was replaced with the R928 photomultiplier tube (Hamamatsu; catalog number: R928) in both detectors. This modification significantly improved sensitivity within the entire wavelength range, and dramatically improved the sensitivity to wavelengths >600 nm. The replacement for the photomultiplier was provided by Agilent's technical service.
5. Tabletop centrifuge (Eppendorf, MiniSpin plus)
6. Reverse phase column (ReproSil 100 C8-AB, 5- μ m particle size, 4 x 250 mm) (Dr. Maisch, catalog number: r15.8b.s2504)

Procedure

1. The protocol requires 2 ml of a culture of cyanobacterial cells at the exponential phase of growth. The protocol works very well for the cyanobacterium *Synechocystis* PCC 6803 grown at 30 °C in the liquid medium BG11 (Rippka *et al.*, 1979) with optical density at 750 nm ~ 0.4 (see Note 2). The BG11 medium is used frequently for various cyanobacterial strains, it contains a balanced mix of macro- and microelements required for photoautotrophic grow and its preparation is described in detail in Rippka *et al.* (1979).
2. All further steps should be carried out under dim or green light to prevent degradation of photo labile tetrapyrroles. Alternatively, the tubes can be covered by aluminum foil.
3. Harvest cells by centrifuging at room temperature and maximal speed ($\geq 12\ 000 \times g$) for 5 min. Remove the supernatant and resuspend cells in 200 μ l of ultrapure H₂O. Transfer the cell solution into a 500 μ l tube, spin cells down again and carefully remove all water by pipette. At this point, cells can be frozen in liquid nitrogen and stored in -70 °C for up to a month.
4. Resuspend the pellet from the end of step 3 in 25 μ l of ultrapure water, then add 75 μ l of methanol, mix thoroughly and incubate in dark and at room temperature for 15 min to extract tetrapyrroles. Pellet cell debris by centrifuging for 5 min at maximal speed.
5. Transfer the supernatant (~ 90 μ l) to a new 500 μ l tube.
6. Resuspend the pellet from the end of step 3 in 20 μ l of ultrapure water, add 80 μ l of methanol, mix thoroughly and incubate again in dark and at room temperature for 15 min. Pellet cell debris by centrifuging for 5 min at maximal speed.
7. Pool both supernatants (~ 180 μ l) in the 500 μ l tube, mix thoroughly and centrifuge again for 5 min at maximal speed to remove any traces of cell debris. Transfer the supernatant to the 200 μ l insert and inject it immediately into the HPLC system.

8. Separation is performed on a reverse phase column with 35% methanol and 15% acetonitrile in 0.25 M pyridine (solvent A) and 50% methanol in acetonitrile as solvent B. Tetrapyrroles are eluted with a gradient of solvent B (35 to 50% in 5 min) followed by 50 to 64% of solvent B in 30 min at a flow rate of 0.8 ml/min at 40 °C (Table 1). The program continues by column washing (36-45 min) and equilibration (36-45 min).

Table 1. Setting of HPLC pumps for a single run

Time (min)	% of the solvent B
0	35
5	50
35	64
36	100
45	100
46	35
51	35

9. Each run is finished using 100% of solvent B for 10 min to wash out more hydrophobic and abundant pigments like chlorophylls or carotenoids. The column is finally equilibrated for 5 min by 35 % of the solvent B (Table 1 for the complete pump program).
10. Eluted pigments are detected by their specific fluorescence emission maxima. Settings of fluorescence detectors, the elution times and the excitation/emission maxima of individual precursors are summarized in Table 2 and also indicated graphically in Figure 2.

Table 2. Settings of fluorescent detectors and the list of detectable heme/chlorophyll precursors, including their excitation and emission maxima and approximate elution times

Detector	Detector Setting Ex/Em (nm), Time	Precursor, Ex/Em maxima (nm)	Elution time
FLD #1	400/620, 0-10 min	Coproporphyrin IX 400/420	6 min
	440/660, 11-22 min	DV ^a -chlorophyllide 440/670	17 min
		MV ^b -chlorophyllide 440/670	18 min
		DV -protochlorophyllide 440/640	20 min
		MV-protochlorophyllide 440/640	21 min
	400/630, 13-30 min	Protoporphyrin IX 400/630	26 min
FLD #2	416/595, 0-30 min	Mg-protoporphyrin IX 416/595	18 min
		Mg-protoporphyrin IX ME ^c 416/595	24 min

a-divinyl

b-monovinyl

c-monomethyl-ester

Representative data

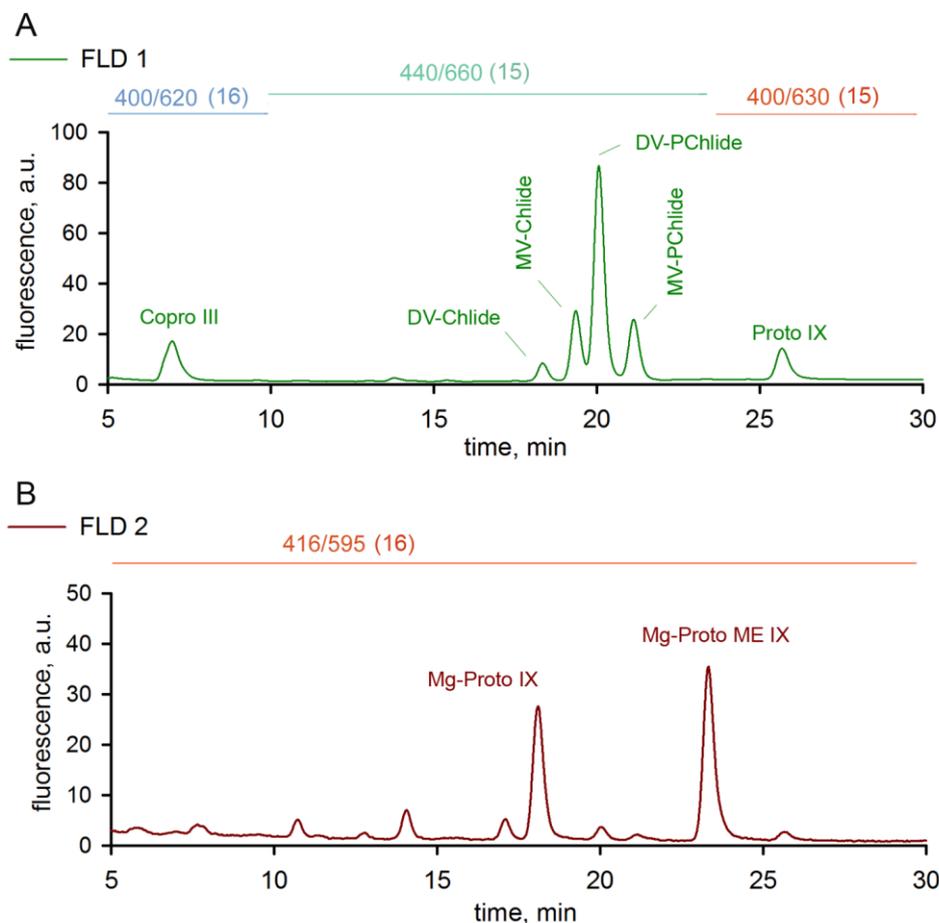


Figure 2. Representative chromatograms of separated precursors extracted from the cyanobacterium *Synechocystis* PCC 6803. A. Chromatogram recorded by the first fluorescent detector (FLD). B. Chromatogram recorded simultaneously by the second FLD. Settings of excitation and emission wavelengths for FLD #1 and #2 are shown above the each chromatogram. The number in round brackets indicates the FLD sensitivity (gain; the maximum = 16) set for the given wavelengths (see Note 3). Copro III = Coproporphyrin IX; DV-PChlide = divinyl-protochlorophyllide; MV-PChlide = monovinyl-protochlorophyllide; DV-Chlide = divinyl-chlorophyllide; MV-Chlide = monovinyl-chlorophyllide; Proto IX - Protoporphyrin IX; Mg-Proto = Mg-protoporphyrin IX; Mg-Proto ME = Mg-protoporphyrin IX monomethylester.

Notes

1. The protocol should work using any HPLC system with binary or quaternary pump system and with at least one fluorescence detector sensitive enough for the longer wavelengths (up to 680 nm). To detect the whole spectrum of precursors using a system with one fluorescence detector, each sample needs to be measured twice with different setting of excitation/emission wavelengths.
2. Apart from the cyanobacterium *Synechocystis* PCC 6803 the described protocol has already been successfully tested for the green alga *Chlamydomonas reinhartii*, the moss *Physcomitrella patens*, the plant *Arabidopsis thaliana*, and for *Escherichia coli*. Coproporphyrin IX and Protoporphyrin IX should be detectable virtually in any organism.
3. Sensitivity of the Agilent fluorescence detector can be set from 1-16 (max). This particular setting (numbers) is specific for the Agilent detectors but a similar increasing/decreasing of the sensitivity should be possible to achieve with virtually any HPLC fluorescence detector.

Recipes

1. HPLC solvent A
35% methanol and 15% acetonitrile in 0.25 M pyridine
2. HPLC solvent B
50% methanol in acetonitrile

Acknowledgments

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