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Comprehensive Determination of Chlorophyll Derivatives by Chromic Acid Oxidation

Yuhi Satoh,[1] Shinya Nomoto,[2] and Takeo Hama*[3]
[1]*Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572*
[2]*Faculty of Pure and Applied Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8571*
[3]*Faculty of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572*

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It is difficult to measure the total amount of chlorophyll derivatives due to their variety. We applied a new method for comprehensive determination of all derivatives by ethylmethylmaleimide (EMMi) derived from chlorin which is a ring structure of chloropigments. Relationships between the amount of applied chloropigments and the yield of EMMi through the chromic acid oxidation were examined for three species of chloropigments. The yield of EMMi from chloropigments was almost constant regardless of the amount or pigment species, indicating the applicability of this method for comprehensive deterimination of chlorophyll derivatives.

Accumulation of chlorophyll and its degradative derivatives is well known to reflect the past dynamics of various biological and crustal thermodynamic phenomena, especially in aquatic environments.[1] Chloropigment contents in marine water column and sediments have attracted considerable interest. High-performance liquid chromatograph (HPLC) equipped with photodiode array or fluorescence detector has been most commonly used for quantitative analysis of chlorophyll derivatives. However, the presence of over 70 species of chlorophyll derivatives in the environment[2] makes their accurate determination difficult using a photometric detector because of the variability in photometric properties of each pigment.

The tetrapyrrole ring, chlorin, gives a specific structure to a chlorophyll derivative. The structure of chlorin of chlorophyll *a* (Chl *a*), which is the foremost chloropigment of the plant kingdom, has already been elucidated (Chart 1). One of the pyrrole rings in chlorin, the B-ring, is of interest since it contains no reactive moieties but methyl and ethyl groups. This implies that the B-ring is likely conserved through diagenetic processes, suggesting that the amount of B-ring allows one to estimate the total chlorophyll derivatives existing in a particular environment. Although some pigments such as chlorophyll c_3 or phycobilin are likewise known to contain the B-ring, their production is significantly lower than Chl *a*.[3] Thus, the amount and the distribution of B-ring likely reflect the dynamics of chlorophylls.

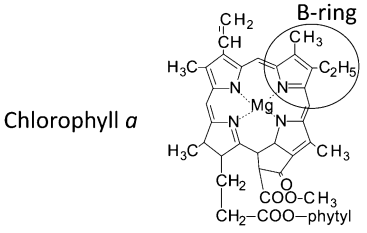
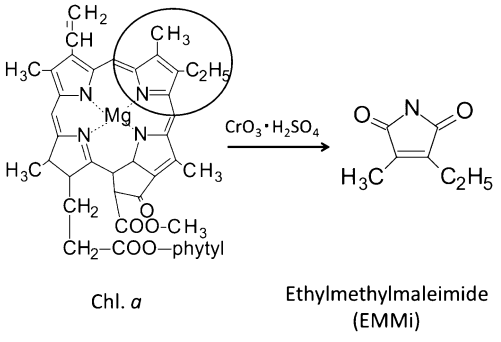


Chart 1.

In the present study, the method to determine the concentration of B-ring using chromic acid was examined. In the acidic condition, the B-ring is oxidized to ethylmethylmaleimide (EMMi) by chromic acid oxidation (Scheme 1). The concentration of EMMi was analyzed by gas chromatography/mass spectrometry (GC/MS), and the quantitative evaluation of EMMi produced by chromic oxidation was examined using authentic chlorophyll derivatives.

Three authentic derivatives were prepared (Chl *a*, pheophytin *a*: Pheo *a*, and pyropheoforbide *a*: Pyrobide *a*; Wako Pure Chemical Industries, Ltd., Chart 2), and EMMi was synthesized in our laboratory.[4] These derivatives were dissolved in acetone (0.01–10 nM), and the oxidizing reagent (5 mL) mixed with chromium trioxide (10% aq, w/v) and sulfuric acid (25% aq, w/v) at a ratio of 1:1 (v/v) was simultaneously added to the derivative solution. At this time, the volume of the oxidizing agent was at least 10-fold larger than acetone to maintain a sufficient oxidative effect. The solutions were left to stand for 2 h in ice and then for 2 h at room temperature in the dark for oxidation. Na_2SO_3 was added to the mixture to stop the reaction. The reaction mixture was then extracted with dichloromethane:acetone (9:1, v/v, 3 mL) three times and the combined extract was passed through silica gel (10 mL, particle size 125–



Scheme 1.

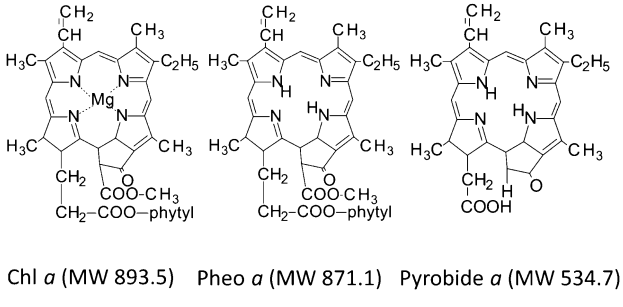


Chart 2.

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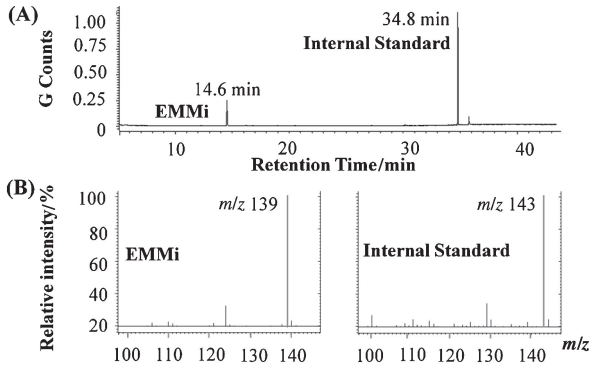

Figure 1. Total ion monitor mass chromatogram (A) and fragmentation pattern (B) of EMMi and IS analyzed by GC/MS. Scan range was from m/z 100 to 145.

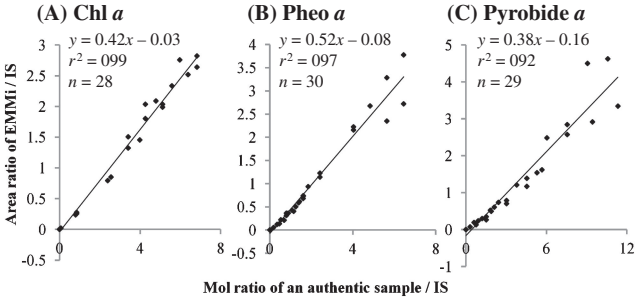

Figure 2. Relationships between the amount of molar ratio of authentic samples to IS and the area ratio of derived EMMi to IS.

450 μm^3, Wako Pure Chemical Industries, Ltd.; packed into a 30 mmϕ × 120 mm column) to remove remaining water and washed with the same solvent (30 mL). The eluates were added with henicosanoic acid methyl ester (purchased from Wako Pure Chemical Industries, Ltd.) as internal standard (IS, 2–4 nM) and subsequently concentrated to about 100 μL. Care was taken not to allow it to dry because the recovery ratio declined when the dried residue was dissolved. A GC/MS (Varian, 300 series) system equipped with a capillary column (VF-5 ms, VARIAN, 30 m × 0.25 mm i.d.) was used for analysis.[5] Identification of EMMi and IS was done by comparison of the retention time with authentic samples and the fragment pattern (Figure 1). Selected ion monitor (SIM) mode was applied for the detection of EMMi and IS at m/z 139 and 143. Blank test of EMMi was done by measurements of the samples composed of acetone containing no authentic derivatives. A little peak was measured for the blank and was included in Figure 2. The accuracy of repeated measurements through the entire operation was within 10%.

Table 1 and Figure 2 show the proportional relationship between the applied amount of molar ratio of authentic samples to IS and the area of EMMi derived by chromic acid oxidation to IS. All regression lines showed strong correlations, suggesting the constancy of this oxidative reaction regardless of chloropigment species. Although the same molar quantity of EMMi with the applied chloropigment should be produced on chemical reaction (Scheme 1), the amount of EMMi derived from chloropigments by chromic acid oxidation was lower than the theoretical value, probably due to oxidative degradation. Therefore, we estimated the yield constant of EMMi derived from

Table 1. Summary of oxidation examination of authentic derivatives

	Slope	Intercept	Correlation coefficient (r^2)	Yield constant[a] /%
Chl *a*	0.42	0.03	0.99	37.6
Pheo *a*	0.52	0.08	0.97	39.7
Pyrobide *a*	0.38	0.16	0.92	36.1

[a]Yield constants were estimated by each linear regression in Figure 2 and the value of authentic EMMi detected by GC/MS.

chloropigments by measurement of the authentic EMMi. The slopes of the regression lines of the molar ratio of EMMi/IS and the area ratio of EMMi/IS were obtained from the oxidation of chloropigments (Figure 2) by the slopes obtained from the analysis using the authentic EMMi. The resultant yield constants are shown in Table 1.

The yield constants were almost the same for the three species of chloropigments, suggesting that the difference of physicochemical characteristics such as molecular weight (MW, 534.7–893.5 in Chart 2), hydrophobicity (the presence or absence of phytol chain), and chelated state (the presence or absence of Mg) in chloropigments has little effect on the efficiency of the oxidation. Moreover, this relation was applicable to a comparatively wide range (0.01–10 nM). The yield constants reported by previous study varied over a rather wide range; the yield producing ketone by chromic acid oxidation using various low molecular compounds ranged from 25 to 75%,[6,7] while EMMi using etioporphyrin from 10 to 20%.[8] This variability in the yield probably reflects the difference in the organic chemicals applied and in oxidation reaction procedure. Since the conditions were strictly controlled in the present study, such as use of reducing agent, Na_2SO_3, to stop the oxidation, constant yields were likely obtained. A quantitative loss of EMMi during the refining process through silica gel column is likely possible but it is included in the yield constant estimated. Thus, the mean yield constant (37.8%) was applied to estimate the concentration of chloropigments from the measurement of EMMi in the samples obtained from aquatic environments.

We examined the reproducibility of the analysis of natural particulate organic matter (POM) collected from coastal seawater. POM on the glass fiber filter (Whatman GF/F, 0.7 μm pore size) was soaked in the oxidizing agent with sonication for 2 min in ice-cold water, then left to stand for 2 h in ice-cold water followed by 2 h at room temperature. Table 2 summarized the results of Chl *a*, particulate organic carbon (POC), and EMMi for POM obtained from Tokyo Bay and Oarai Coast.

The existence of free EMMi in natural water samples produced in natural environments should be considered since it possibly causes the overestimation of EMMi produced by chromic acid oxidation. The amount of free EMMi should be subtracted from the concentration determined by GC/MS to obtain that of chlorophyll derivatives.

$$\text{Chlorohyll derivatives} = (\text{CrEMMi})/Y - R(\text{fEMMi}) \quad (1)$$

where, Y is the yield constant of EMMi produced by chromic acid oxidation (37.8%), (CrEMMi) is a measured value of

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Table 2. Concentration of Chl *a*, POC, EMMi, and chlorophyll derivatives in particulate matter collected from Tokyo Bay and Oarai Coast

	Chl *a*[a] /nM	POC[b] /μM	EMMi[c] /nM	Variation coeffecient of EMMi
Tokyo bay	15.0	217	19.2	12.0% ($n = 7$)
Oarai coast	2.0	17.9	1.3	6.8% ($n = 5$)

[a]Chl *a* was measured by a fluorometer (Turner design) as an indicator of biomass. [b]POC (particulate organic carbon) was measured by an elemental analyzer (Fisons EA 1108). [c]These values were estimated from a calibration line using a relationship between the applied amount of mole ratio of authentic EMMi to IS and the area of EMMi to IS.

EMMi after chromic acid oxidation, R is the residual ratio of free EMMi through chromic acid oxidation (we obtained the value of 55.7% for R by chromic oxidation of authentic EMMi), and (fEMMi) is a measured value of free EMMi. (fEMMi), which can be determined using a nonoxidized sample. The concentrations of fEMMi were 0.2 and 0.1 nM for the samples from Tokyo Bay and Oarai Coast, respectively. These account for only 0.4 and 2.9% of chlorophyll derivatives in both samples, showing that a small amount of free EMMi exists in the water column. This low contribution of free EMMi to chlorophyll derivatives suggests that a very small fraction of chlorin derived from Chl *a* is decomposed to monomer of pyrrole within the water column. It is expected that the free EMMi concentrations are likely high in the samples mainly composed of degraded organic matter like sediment.[9]

The concentrations of chlorophyll derivatives which are corrected by the concentration of free EMMi in Tokyo Bay and along the Oarai Coast were 50.7 and 3.4 nM, respectively. The contribution of Chl *a* to chlorophyll derivatives in both samples (29.6 and 58.5%) suggests that substantial amounts of chlorophyll derivatives exist in the water column. The contribution of Chl *a* to total chlorophyll derivatives was higher in eutrophic Tokyo Bay, indicating that the biogeochemical properties of POM, such as the contribution of living phytoplankton and the physiological state, differ between the two stations.

We hereby describe a new method to determine the concentration of chlorophyll derivatives using the oxidation reaction. The concentration of EMMi was determined quantitatively by conchromic acid oxidation and it is applicable to chlorophyll derivatives determination. This method can afford significant biogeochemical information on the diagenetic processes of plant organic matter, which has not been elucidated by ordinary methods such as HPLC.

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