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A compound-specific isotope method for measuring the stable nitrogen isotopic composition of tetrapyrroles

Yoshito Chikaraishi [a,*], Yuichiro Kashiyama [a], Nanako O. Ogawa [a], Hiroshi Kitazato [a], Mitsuru Satoh [b], Shinya Nomoto [b], Naohiko Ohkouchi [a]

[a] *Institute for Research on Earth Evolution, Japan Agency for Marine-Earth Science and Technology, 2-15 Natsushima-cho, Yokosuka 237-0061, Japan*
[b] *Department of Chemistry, University of Tsukuba, Tsukuba 305-8571, Japan*

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Abstract

The stable nitrogen isotopic signature of natural tetrapyrroles such as chloro- and pheo-pigments and alkylporphyrins is a potentially valuable proxy for the reconstruction of the nitrogen cycle of modern aquatic environments and of the geological past. However, its application has been limited due to analytical difficulties. In this study, we developed a method to determine stable nitrogen isotopic composition of tetrapyrroles by a combination of chemical conversion into monopyrrole units (*i.e.* maleimides) and their isotope analysis by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS). Two authentic (pyropheophorbide *a* and mesoporphyrin IX methylester) and four natural (chlorophyll *a*, deoxophylloerythroetioporphyrin (DPEP), and two 17-nor-DPEP) tetrapyrroles were converted to maleimides by HCl treatment and chromic acid oxidation. The stable nitrogen isotopic composition of the maleimides can be determined by GC/C/IRMS, with a standard deviation (1σ) of better than $\pm 0.5‰$ and the minimum sample amount of 0.8 nmol N. The isotopic composition of the maleimides is consistent with that of the original tetrapyrroles, suggesting nitrogen isotopic fractionation during the chemical treatment was insignificant. Further, no substantial difference is observed in the isotopic composition among the maleimides derived from a single tetrapyrrole. The developed method is applicable for the nitrogen isotope analysis of natural tetrapyrroles in various biological and geological samples.

1. Introduction

Since chloropigments such as chlorophylls and bacteriochlorophylls are representatives of natural tetrapyrroles directly related to the photosynthetic processes of photoautotrophs (*e.g.* Blankenship, 2002; Grimm et al., 2006), they are definitive biomarkers of photoautotrophic activity in the aquatic surface environment (*e.g.* Sanger, 1988; Nakajima et al., 2003). Moreover, the chloropigments are long preserved as pheo-pigments such as pheophytins and bacteriopheophytins in oceanic and lacustrine sediments (*e.g.* Sanger, 1988; Keely et al., 1990; Prowse and Maxwell, 1991; Airs et al., 2001; Squier et al., 2002; Tolosa et al., 2004) and as metallo-alkylporphyrins such as VO deoxophylloerythroetioporphy-

* Corresponding author. Tel.: +81 46 867 9778; fax: +81 46 867 9775.
E-mail address: ychikaraishi@jamstec.go.jp (Y. Chikaraishi).

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rin (VO DPEP) even in sedimentary rock and petroleum (*e.g.* Marriott et al., 1984; Baker and Louda, 1986; Van Berkel et al., 1989; Keely et al., 1994; Gibbison et al., 1995; Callot and Ocampo, 2000; Mawson et al., 2004). Also, since tetrapyrroles contain hydrogen, carbon, and nitrogen, their isotopic compositions could record information on the hydrologic and nutrient cycles in the environment where the photoautotrophs grow (*e.g.* Bidigare et al., 1991; Sachs et al., 1999; Chikaraishi et al., 2005). Therefore, the molecular isotope signatures of natural tetrapyrroles and their natural degradation products (*e.g.* maleimides) have sometimes been used as tools for the reconstruction of the environment in the euphotic zone (for chloro- and pheo-pigments: Qian et al., 1996; Sachs and Repeta, 2000; Ohkouchi et al., 2005; York et al., 2007; for alkylporphyrins: Hayes et al., 1987; Boreham et al., 1989, 1990; Ocampo et al., 1989; Chicarelli et al., 1993; Ohkouchi et al., 2006; for maleimide: Grice et al., 1996, 1997; Pancost et al., 2002). Particularly, the nitrogen isotopic signature is highly useful as a proxy for the reconstruction of the nitrogen cycle in the aquatic surface environment (Chicarelli et al., 1993; Qian et al., 1996; Sachs and Repeta, 2000; Ohkouchi et al., 2005, 2006; York et al., 2007).

However, molecular isotope studies of tetrapyrroles have been limited due to analytical difficulties. One of the most powerful approaches in molecular isotopic studies is compound specific isotope analysis (CSIA) by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS), which allows a rapid and precise determination of isotopic compositions of specific molecules with a minimal amount of material (nanomolar amount of the element) even in a complex mixture (*e.g.* Hayes et al., 1990; Sessions, 2006). The small sample size is highly advantageous as it facilitates high resolution studies for the reconstruction of paleoenvironment. However, low volatility and conjugated cyclic structures of tetrapyrroles require high temperature (more than 360 °C) for GC elution, and prevent quantitative conversion into CO_2 and N_2 in the combustion furnace of GC/C/IRMS. Alternatively, such problems are not encountered in conventional approaches such as isotope analysis by elemental analyzer/isotope ratio mass spectrometry (EA/IRMS). However, it generally requires purified compounds and larger (micromolar) amounts of carbon or nitrogen for the precise determination of isotopic compositions of these elements. This requirement is particularly problematic for routine analyses of geological samples. So far, stable carbon and nitrogen isotope analyses of natural tetrapyrroles were achieved by the latter approaches (*e.g.* Ohkouchi et al., 2005, 2006; York et al., 2007), and the use of the GC/C/IRMS is limited to only the stable carbon isotope analysis of maleimides in free polar fraction (Grice et al., 1996, 1997). Grice (1995) also attempted chromic acid oxidation of porphyrins into maleimides to obtain stable carbon isotopic composition of them. However, she found a significant carbon isotopic fractionation of the observed maleimides during the chromic acid oxidation, and reverted back to the isotope analysis of free maleimides at that time (K. Grice, personal communication). Thus, the GC/C/IRMS method has never been applied for the stable isotope analysis of tetrapyrroles.

In this study, we report a method for determining stable nitrogen isotopic composition of tetrapyrroles, by the use of GC/C/IRMS. To overcome analytical problems related to low volatility and conjugated cyclic structures, we apply chemical treatment (HCl treatment and chromic acid oxidation) to degrade tetrapyrroles into monopyrrole units, namely maleimides, prior to the isotope analysis. Here, we demonstrate that the nitrogen isotopic composition of two authentic and four natural tetrapyrroles can be determined by GC/C/IRMS at the nanomolar level.

2. Materials and methods

2.1. Samples

As listed in Table 1, nine pure maleimide standards were synthesized in the laboratory (Nomoto et al., 2001a; Nomoto and Kigoshi, 2005). Briefly, 2-methylmaleimide (MM), 2,3-dimethylmaleimide (DMM), 4-methylphthalimide (4MP) and 3,4,5,6-tetrahydrophthalimide (THP) were synthesized by imidation of the corresponding maleic anhydride (Nomoto and Kigoshi, 2005). 2-Ethylmaleimide (EM) and 2-ethyl-3-methylmaleimide (EMM) were synthesized from the corresponding 1-ethoxycarbonyl-2-alkyl-propane-1-diethylphosphonate and alkanoic acid ethylester (Nomoto and Kigoshi, 2005), and the EMM was synthesized three times to obtain those with different isotopic composition. 3-Methylphthalimide (3MP) was synthesized from 1,2,3-trimethylbenzene *via* 3-methylphthalic anhydride (Nomoto et al., 2001a). The purity of these maleimides was checked by nuclear magnetic reso-

Table 1
Nitrogen isotopic composition of maleimides (synthesized standards) determined by EA/IRMS and GC/C/IRMS

Compounds	Abbreviation	EA/IRMS average ± 1σ (‰)	GC/C/IRMS average ± 1σ (‰)	Difference $\delta_{GC/C/IRMS} - \delta_{EA/IRMS}$ (‰)
2-Methylmaleimide	MM	−5.7 ± 0.2 ($n = 2$)	−5.7 ± 0.5 ($n = 13$)	±0.0
2,3 -Dimethylmaleimide	DMM	−7.0 ± 0.0 ($n = 2$)	−6.7 ± 0.4 ($n = 16$)	+0.3
2-Ethylmaleimide	EM	+1.7 ± 0.0 ($n = 2$)	+1.7 ± 0.6 ($n = 3$)	±0.0
2-Ethyl-3-methylmaleimide (#1)	EMM (#1)	−8.3 ± 0.1 ($n = 2$)	−8.1 ± 0.1 (n = 20)	−0.1
2-Ethyl-3-methylmaleimide (#2)	EMM (#2)	+9.4 + 0.1 ($n = 2$)	+9.8 + 0.4 ($n = 3$)	+0.4
2-Ethyl-3-methylmaleimide (#3)	EMM (#3)	+7.7 ± 0.1 ($n = 2$)	+7.6 ± 0.2 ($n = 3$)	−0.1
3-Methylphtalimide	3MP	+3.6 ± 0.1 ($n = 2$)	+3.3 ± 0.5 ($n = 14$)	−0.2
4-Methylphtalimide	4MP	+20.8 ± 0.5 ($n = 2$)	+20.8 ± 0.2 ($n = 3$)	±0.0
3, 4, 5, 6-Tetrahydrophthalimide	THP	+5.7 ± 0.2 ($n = 2$)	+5.7 ± 0.4 ($n = 14$)	±0.0

nance (NMR; JEOL JNM-EX270), gas chromatograph/mass spectrometer (GC/MS; Agilent Technologies 6890N GC/5973A MSD system) and elemental analyzer (EA; Yanagimoto CHN Corder MT-6). Two authentic standards of tetrapyrroles, pyropheophorbide *a* and mesoporphyrin IX dimethylester were purchased from Wako Pure Chemical Industries, Ltd. As representatives of natural tetrapyrroles, chlorophyll *a* was isolated and purified from corn leaf by the improved procedure of Chikaraishi et al. (2005, 2007a), and VO DPEP and VO 17-nor-DPEP were isolated and purified from Miocene sedimentary rocks (Onnagawa Formation, Japan) by the improved procedure of Kashiyama et al. (2007). Stable nitrogen isotopic composition of these maleimides and tetrapyrroles was determined by the conventional technique using a Flash EA coupled to an IRMS (ThermoFinnigan Delta plus XP) *via* ConFlo III interface. The isotopic composition was expressed in the conventional δ notation against atmospheric N_2. Analytical error (1σ) of the isotopic measurement was better than 0.5‰ (~0.1‰ on average) at the minimum sample amount of 30 μg N (~2 μmol N).

2.2. Chemical degradation treatment

As illustrated in Fig. 1, pyropheophorbide *a* was esterified using HCl/CH_3OH at 100 °C for 1 h to form pyropheophorbide *a* methylester before chromic acid oxidation. Mesoporphyrin IX dimethylester was used without further derivatization. The chlorophyll *a* was transformed to pyropheophorbide *a* by HCl treatment at 100 °C for 1 h (Baker et al., 1968), and the pyropheophorbide *a* was methylesterified using the above method before chromic acid oxidation. The VO DPEP and VO 17-nor-DPEP were also used without further derivatization. The prepared tetrapyrroles were degraded to maleimides by chromic acid oxidation according to the method described in Nomoto et al. (2001b), with 10% CrO_3 (1 ml) and 25% H_2SO_4 (1 ml) at 0 °C for 1 h and then at room temperature for 1 h. The obtained maleimides were extracted with benzene (2 ml × 5), and subsequently methylesterified using above method. Great care was taken to concentrate solvent under a N_2 stream in order to prevent scatteration of the maleimides.

The obtained maleimides were identified by GC/MS by comparison with their retention times and mass spectra of standards or the published spectra in previous studies (*e.g.* Ellsworth and Aronoff, 1968; Nomoto and Kigoshi, 2005). The GC was equipped with a DB-1HT capillary column (30 m × 0.32 mm i.d., 0.1 μm film thickness, Agilent Technologies) with a programmable temperature vaporizing (PTV) injector. The GC oven temperature was programmed as follows: 40–20 °C at a rate of 5 °C min^{-1} after 10 min at the initial temperature and then heated to 320 °C at a rate of 20 °C min^{-1} and held isothermally at 320 °C for 12 min. Carrier gas (He) flow through the GC column was 1.5 ml min^{-1}. The PTV temperature was programmed as follows: 50 °C to 350 °C at a rate of 600 °C min^{-1} after 0.2 min at the initial temperature and held isothermally at 350 °C for 10 min.

2.3. Stable nitrogen isotopic measurements of maleimides by CSIA

Stable nitrogen isotopic compositions of the maleimides were determined by GC/C/IRMS using ThermoFinnigan Delta plus XP coupled to a gas chromatograph (GC; Agilent Technologies 6890N) *via* combustion and reduction furnaces (*e.g.* Hayes et al., 1990; Brand et al., 1994; Merritt and Hayes,

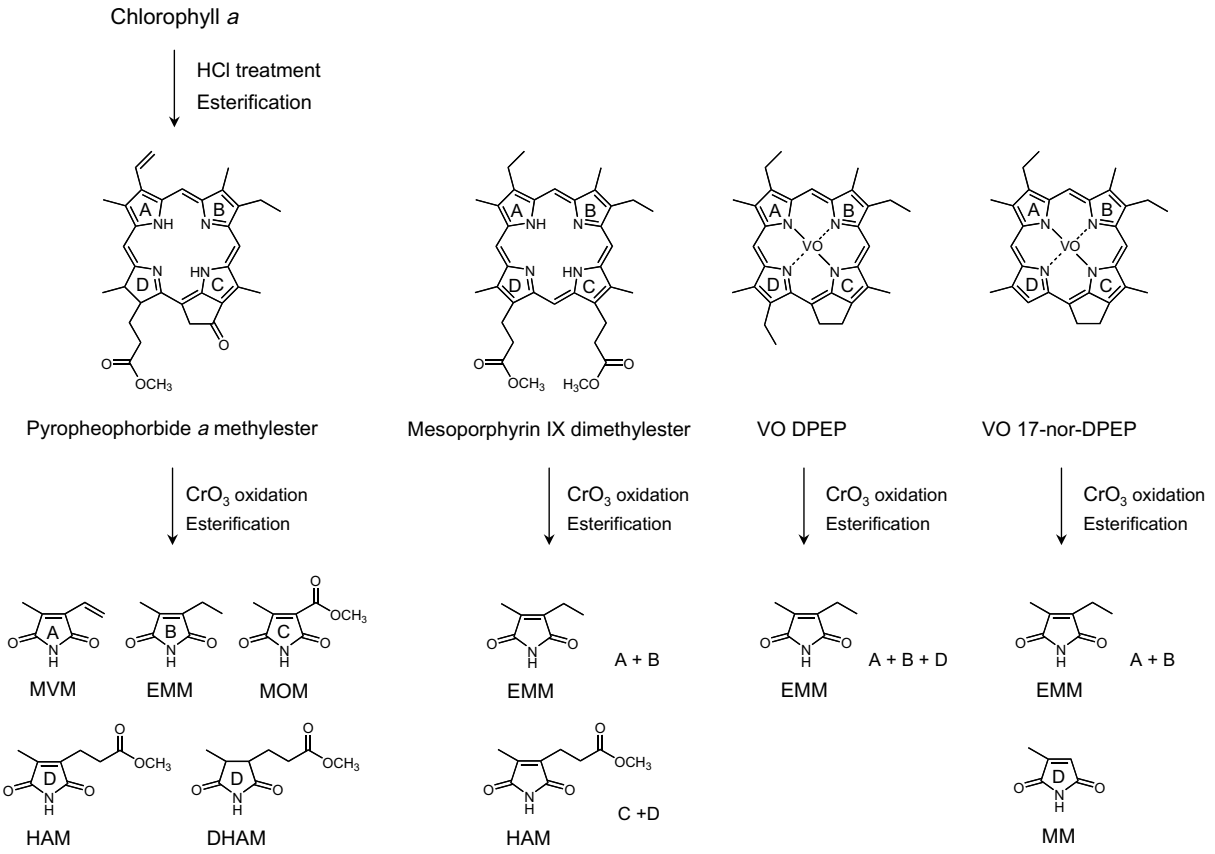

Fig. 1. Schematic illustration of chemical degradation treatment for chlorophyll *a*, mesoporphyrin IX dimethylester, VO DPEP and VO 17-nor-DPEP.

1994; Sessions, 2006). The conditions for GC/C/IRMS were optimized for nitrogen isotope analysis and allow quantitative conversion into N_2 in the combustion/reduction furnaces without CO generation (Merritt and Hayes, 1994; Chikaraishi et al., 2007b). The combustion was performed in a microvolume ceramic tube with CuO, NiO, and Pt wires at 1000 °C, and the reduction was performed in a microvolume ceramic tube with reduced Cu wires at 550 °C. The GC was equipped with an Ultra-2 capillary column (25 m × 0.32 mm i.d., 0.52 μm film thickness, Agilent Technologies) with a PTV injector. The GC oven temperature was programmed as follows: 40–220 °C at a rate of 8 °C min^{-1} after 6 min at the initial temperature and then heated to 260 °C at a rate of 20 °C min^{-1} and held isothermally at 260 °C for 10 min. Carrier gas (He) flow through the GC column was 1.3 ml min^{-1}. The PTV temperature program was the same as GC/MS condition. The H_2O and CO_2 generated in the combustion furnace were eliminated by a counter-current drier (Permeable membrane, Nafion™) and a liquid nitrogen trap, respectively.

3. Results and discussion

3.1. Stable nitrogen isotope analysis of maleimide standards

Stable nitrogen isotopic composition and its standard deviation (1σ) for replicate analysis ($n = 3$–20) of the maleimide standards determined by GC/C/IRMS are summarized in Table 1, with the value independently determined by conventional EA/IRMS. For all maleimides with the isotopic composition between −8.3‰ and +20.8‰, the $\delta^{15}N$ value determined by GC/C/IRMS is identical to that determined by EA/IRMS within ±0.5‰ (Fig. 2). The m/z 28 intensity required for the accurate and precise $\delta^{15}N$ measurement is lager than 40 mV, which corresponds to maleimide with more than 11 ng N (0.8 nmol N, Fig. 3). Thus, the nitro-

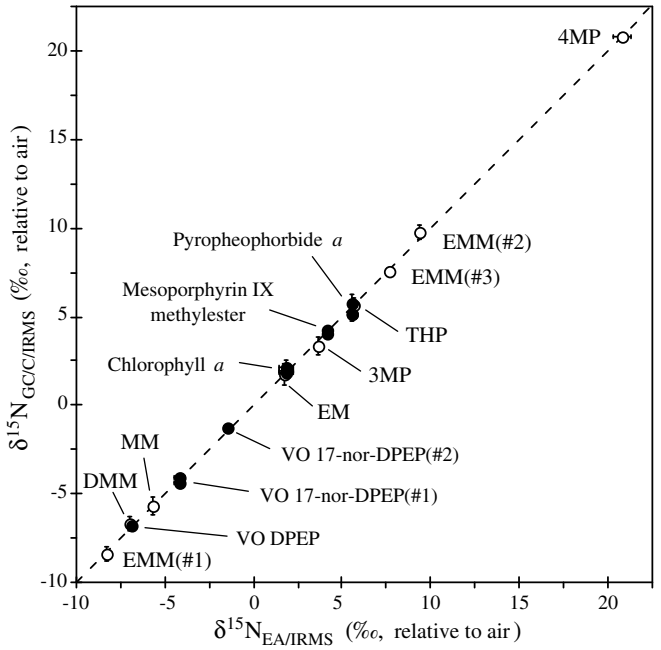

Fig. 2. Nitrogen isotopic compositions of maleimides (open symbol) and tetrapyrroles (filled symbol) determined by GC/C/IRMS and EA/IRMS. Bar represents standard deviation (1σ) for replicate analysis.

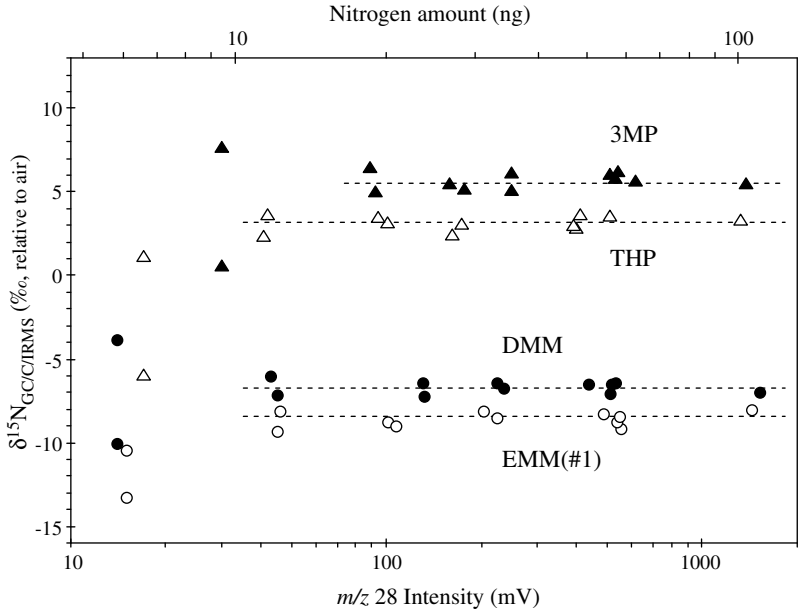

Fig. 3. Nitrogen isotopic composition of maleimide standards, DMM, EMM (#1), 3MP and THP with respect to varying sample amount. Dashed line represents an average of the δ^{15}N value with more than 11 ng of nitrogen.

gen isotopic composition of maleimides can be determined by GC/C/IRMS for the sample amount larger than 0.8 nmol N, with the accuracy and precision essentially equal to those obtained in EA/IRMS ($<\pm 0.5‰$).

3.2. Degradation products of tetrapyrroles

During chromic acid oxidation, degradation of tetrapyrroles proceeds *via* attack at the methine bridge carbons between pyrrole rings, and mono-

pyrrole units are obtained as the corresponding maleimides (*e.g.* Ellsworth, 1970; Bogacheva et al., 1980; Quirke et al., 1980). As illustrated in Fig. 1, EMM, 2-methyl-3-vinyl maleimide (MVM), 2-methyl-3-oxycarbonyl maleimide (MOM), hematic acid methylester (HAM) and dihydrohematic acid methylester (DHAM) are obtained as chemical degradation products of standard pyropheophorbide *a* and sample chlorophyll *a*. The MVM, EMM and MOM are derived from the A-, B- and C-rings of the original tetrapyrroles, respectively, and the HAM and DHAM are derived from the D-ring of the original tetrapyrroles (*e.g.* Ellsworth and Aronoff, 1968; Ellsworth, 1970; Suzuki et al., 1999). For other tetrapyrroles (mesoporphyrin IX dimethylester, VO DPEP and VO 17-nor-DPEP), EMM is commonly obtained as chemical degradation products, and HAM and MM are obtained from mesoporphyrin IX dimethylester and VO 17-nor-DPEP, respectively. For two DPEPs, the EMM is derived from the A-, B- and D-rings of the VO DPEP and the A- and B-rings of the VO 17-nor-DPEP, and the MM is derived from the D-ring of the VO 17-nor-DPEP. However, maleimide derived from the C-ring of the DPEPs is not observed in this study. It may be due to greater resistance of the C-ring structure of the DPEPs to chemical oxidation.

On the mass chromatogram, these maleimides are resolved well enough to identify them (Fig. 4a), although they cannot be recovered quantitatively (Table 2). This recovery limitation is probably due to incomplete or excessive degradation of tetrapyrroles during the chemical oxidation (*e.g.* Furlong and Carpenter, 1988). High hydrophilicity of maleimides could also cause loss during the liquid–liquid separation. For EMM, the degradation efficiency is 40–60% in our procedure and the extraction efficiency with benzene from aqueous phase is 40–45% for one time (65–80% for five times) extraction. The overall recovery is thus 30–45%.

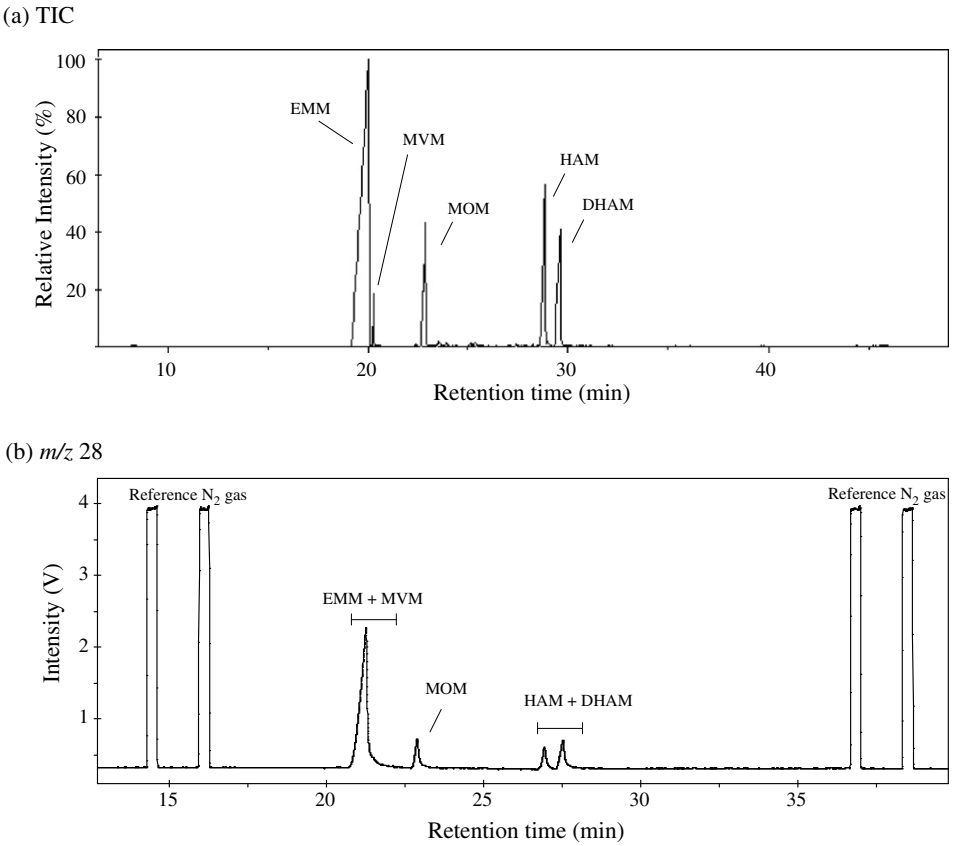

Fig. 4. (a) Total ion chromatogram (TIC) on the GC/MS analysis and (b) *m/z* 28 chromatogram on GC/C/IRMS analysis for maleimides from pyropheophorbide *a*.

Table 2
The yield of maleimides from investigated tetrapyrroles

Compounds	Abbreviation	Yield (%)	Required (nmol)[a]
Pyropheophorbide *a*			
2-Ethyl-3-methylmaleimide	EMM	43.6	1.8
2-Methyl-3-vinylmaleimide	MVM	<1	–
2-Methyl-3-oxycarbonylmaleimide	MOM	5.2	15.4
Hematic acid methylester	HAM	10.5	7.6
Dihydrohematic acid methylester	DHAM	4.8	16.7
Mesoporphyrin IX dimethylester			
2-Ethyl-3-methylmaleimide	EMM	42.5	0.9
Hematic acid methylester	HAM	14.6	2.7
VO deoxophylloerythroetioporphyrin	VO DPEP		
2-Etfayl-3-methylmaleimide	EMM	32.0	0.8
VO 17-nor-Deoxophylloeiythroetioporphyrin	VO 17-nor-DPEP		
2-Methylmaleimide	MM	19.1	4.2
2-Ethyl-3-methylmaleimide	EMM	34.0	1.2

[a] Minimum sample amount required for a single GC/C/IRMS measurement of $\delta^{15}N$ (nmol).

3.3. Stable nitrogen isotope analysis of tetrapyrroles

We also determined stable nitrogen isotopic composition of the maleimides from standard and sample tetrapyrroles by GC/C/IRMS (Table 3 and Fig. 4b). For standard pyropheophorbide *a* and sample chlorophyll *a*, the $\delta^{15}N$ values of EMM and MVM and of HAM and DHAM are reported as combined values, because these maleimides were not resolved completely with baseline resolution (Fig. 4b).

For all tetrapyrroles, the nitrogen isotopic composition of the maleimides can be determined by GC/C/IRMS with an analytical error (1σ) of less than $\pm 0.5‰$ (Table 3). The isotopic composition determined by GC/C/IRMS is consistent with that

Table 3
Nitrogen isotopic composition of tetrapyrroles determined by EA/IRMS and GC/C/IRMS

Compounds	EA/IRMS average $\pm 1\sigma$ (‰)	GC/C/IRMS average $\pm \sigma$ (‰)	Difference $\delta_{GC/C/IRMS} - \delta_{EA/IRMS}$ (‰)
Authentic standards			
Pyropheophorbide *a*	$+5.6 \pm 0.0$ ($n = 2$)		
EMM + MVM		$+5.2 \pm 0.5$ ($n = 5$)	−0.4
MOM		$+5.8 \pm 0.5$ ($n = 4$)	+0.2
HAM + DHAM		$+5.2 \pm 0.2$ ($n = 4$)	−0.4
Mesoporphyrin IX dimethylester	$+4.1 \pm 0.1$ ($n = 2$)		
EMM		$+4.0 \pm 0.1$ ($n = 2$)	−0.1
HAM		$+4.2 \pm 0.0$ ($n = 2$)	+0.1
Natural samples			
Chlorophyll *a*	$+1.8 \pm 0.4$ ($n = 2$)		
EMM + EMVM		$+1.9 \pm 0.3$ ($n = 5$)	+0.1
MOM		$+2.1 \pm 0.4$ ($n = 5$)	+0.3
HAM + DHAM		$+1.8 \pm 0.3$ ($n = 5$)	±0.0
VO DPEP	-6.9 ($n = 1$)		
EMM		-6.8 ± 0.1 ($n \pm 3$)	+0.1
VO 17-nor-DPEP (#1)	-4.2 ± 0.3 ($n = 3$)		
MM		-4.1 ± 0.0 ($n = 2$)	+0.1
EMM		-4.4 ± 0.2 ($n = 3$)	−0.2
VO17-nor-DPEP (#2)	-1.5 ± 0.2 ($n = 3$)		
MM		n.d	
EMM		-1.3 ± 0.1 ($n = 3$)	+0.2

of the original tetrapyrroles independently determined by EA/IRMS (Fig. 2). These results clearly suggest that the isotopic composition of tetrapyrroles is accurately and precisely determined by the developed method. Moreover, no substantial difference is observed in the $\delta^{15}N$ value among the maleimides produced from a single tetrapyrrole, which indicates that the four nitrogen atoms within a single tetrapyrrole have identical isotopic composition, and that the isotopic analysis of tetrapyrroles is represented by that of a single maleimide produced from them. This finding is consistent with a theoretical consideration on the tetrapyrrole biosynthesis that the four nitrogens in tetrapyrrole are commonly derived from an intermediate precursor (*i.e.* δ-aminolevulinic acid) in the biosynthetic pathway (*e.g.* Buchanan et al., 2000; Blankenship, 2002).

As mentioned earlier, recovery for the maleimides during the chemical degradation and subsequent processes is somewhat low. However, the consistency in the isotopic composition between the maleimides and original tetrapyrroles suggests no substantial fractionation of nitrogen isotopic composition during the chemical degradation treatment. By chromic acid oxidation, degradation of tetrapyrroles proceeds *via* attack at the methine bridge carbons between pyrrole rings, and carbon–nitrogen bonds are unaffected (*e.g.* Ellsworth, 1970). This degradation model is in agreement with the observed finding that the nitrogen isotopic composition is retained during the chemical degradation.

4. Implications

By applying the developed method, the nitrogen isotopic composition of tetrapyrroles can be determined at the nanomolar level. It means that the sample size required for the isotope analysis is dramatically decreased compared with the conventional methods. For example, in this study, the minimum sample amount required for the analysis of the isotopic measurement is 1.8 nmol (~1.6 μg) for chlorophyll *a* and 0.8 nmol (~0.4 μg) for VO DPEP, about three orders of magnitude smaller than that of the conventional EA/IRMS. Thus, the developed method has overcome a significant problem on the nitrogen isotope analysis of tetrapyrroles, and allows easy access to the isotopic composition of them in various biological and geological samples.

One of the potential fields for the application of the developed method is reconstruction of nitrogen cycle in the geological past as well as modern aquatic environment. So far, the stable nitrogen isotopic signature of bulk sediments has often been used for this purpose, because the isotopic composition between sediments and particulate organic matter (POM) in the overlying surface water is known to vary in parallel (Altabet and Francois, 1994). In contrast, we know that the nitrogen isotopic composition of bulk organic matter is substantially altered in the water column and sediments based on the field observations and laboratory experiments (*e.g.* Wada, 1980; Saino and Hattori, 1987; Freudenthal et al., 2001a,b; Lehmann et al., 2002). Alternatively, stable nitrogen isotopic composition of chloropigments and their derivatives (*e.g.* pheo-pigments in sediment and alkylporphyrins in sedimentary rock) has been used as a potential proxy, which directly records the cellular isotopic composition of source photoautotrophs as well as their utilization of different nutrients (*e.g.* NO_3^-, NH_4^+ and N_2) (*e.g.* Sachs and Repeta, 1999; Ohkouchi et al., 2005, 2006; York et al., 2007). However, its application has been limited, mainly due to the requirement of a large amount of sample for the isotope analysis of tetrapyrroles by conventional EA methods. By applying the GC/C/IRMS method, for example, nitrogen isotopic composition of chlorophyll *a* and VO DPEP can be determined from 18 l of seawater containing 0.1 nmol l^{-1} of chlorophyll *a* and 0.5 g of sedimentary rock containing 1 nmol g^{-1} of VO DPEP, respectively. These sample sizes are in the realistic range for routine analysis of the nitrogen isotopic composition of chloropigments and their derivatives in various samples (*e.g.* seawater, sediment, and sedimentary rock). Thus, the GC/C/IRMS method is suitable for the reconstruction of nitrogen cycle in aquatic environment in the modern to geological past.

This method is also useful for evaluating isotopic relationship between chloropigments and cells of photoautotrophs, which provides indispensable background information for the interpretation of the isotopic signature of chloropigments and their derivatives in geological samples. So far, several studies have reported the correlation for phytoplankton (Sachs et al., 1999), higher plants (Kennicutt et al., 1992; Chikaraishi et al., 2005) and photosynthetic bacteria (Beaumont et al., 2000). For example, Sachs et al. (1999) reported in marine phytoplankton chlorophyll *a* is $5.1 \pm 1.8‰$ (mean $\pm 1\sigma$) depleted in ^{15}N relative to cell, and Beaumont et al. (2000) reported in purple nonsulfur

bacterial bacteriochlorophyll *a* is $8.7 \pm 1.6‰$ depleted in ^{15}N relative to cell. However, these relationships derive from a limited number of species of photoautotrophs, and we are still not certain whether the isotopic shift is commonly observed for other photoautotrophs as well as the same photoautotrophs with different growth parameters (*e.g.* nutrients, light intensity, etc.). By applying the GC/C/IRMS method, the nitrogen isotopic shift in natural and cultivated photoautotrophs can be readily determined, which allows knowledge of the isotopic shift to be readily expanded. Moreover, the low detection limits of this method will enable the nitrogen isotope analysis to be extended to the minor chloropigments such as chlorophylls *b*, *c*, and *d*, enabling further clarification of the isotopic relationship among different chloropigment species within a single group of photoautotroph as well as the systematic isotopic fractionation mechanisms associated with the whole biosynthetic pathway of chloropigments.

Furthermore, the developed method is applicable for the isotope analysis of other natural tetrapyrroles such as phycobilins (open chain tetrapyrroles) and hemes (cyclic tetrapyrroles complexed to iron). Phycobilins are alternative antenna pigments found in cyanobacteria, red algae and some cryptophytes, and are definitive biomarkers of these photoautotrophs (*e.g.* Blankenship, 2002). Therefore, stable nitrogen isotopic composition of phycobilins could be another potential tool for the reconstruction of nitrogen cycle in the environment. On the other hand, hemes are biological functional molecules found in most organisms, and closely related to various vital systems such as enzymatic catalysis and transport of electron and gases (*e.g.* Blankenship, 2002; Ajioka et al., 2006; Tsiftsoglou et al., 2006). Therefore, stable nitrogen isotope analysis of hemes may be used for the evaluation of these biochemical processes.

Overall, the developed method 'GC/C/IRMS method' will be applicable for the nitrogen isotope analysis of natural tetrapyrroles in various samples, and contribute to the progress in various biological and geological studies.

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