

An improved method for isolation and purification of sedimentary porphyrins by high-performance liquid chromatography for compound-specific isotopic analysis

Yuichiro Kashiya^{a,b,*}, Hiroshi Kitazato^b, Naohiko Ohkouchi^b

^a Department of Earth and Planetary Science, University of Tokyo, Tokyo 113-0033, Japan

^b Institute for Research on Earth Evolution, Japan Agency for Marine-Earth Science and Technology, Yokosuka 237-0061, Japan

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Abstract

We describe an improved method for purification of sedimentary vanadyl and nickel porphyrins (i.e., naturally occurring metalloalkylporphyrins). For the purpose of compound-specific isotopic analyses, various sedimentary porphyrins were purified from the complex natural mixtures by the dual-step high-performance liquid chromatography (HPLC) method. The high-sample-capacity reversed-phase HPLCs by adding *N,N*-dimethylformamide to the mobile phase allow an efficient collection of fractions containing the target compounds even using analytical-scale columns. Furthermore, this method achieved improved chromatographic resolutions but significantly reduced the overall retention time down to 60% compared with the previous work. The target compounds were then isolated with the normal-phase HPLC with the baseline-resolution, which is necessary to avoid chromatographic isotopic fractionation. One of the advantages of this method is that it requires neither derivatization nor demetallation. The purity of these isolated compounds was demonstrated by various HPLC online detection methods utilizing a photodiode-array detector, a mass selective detector. The overall recoveries of Ni porphyrin, VO porphyrin, and porphyrin-free base, respectively, were estimated to be approximately 50–60%, 65%, and 85%.

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1. Introduction

Sedimentary porphyrins (i.e., geoporphyrins, petroporphyrins, etc.; Fig. 1) are the tetrapyrrole compounds (mostly alkylporphyrins and porphyrin acids) extracted from geological samples including sediments and petroleum. The majority of these compounds are thought to originate from various chloropigments produced by phototrophic organisms of the geological past [1–3]. Structural comparisons of chloropigments, sedimentary porphyrins, and their presumable intermediates have resolved their precursor-product relationships and the transformation pathways [4–9]. Source-specific porphyrins derived from chlorophyll *c* (specific for algae including Cryp-

tophyta, Dinophyta, Chromophyta, and Haptophyta) [10–14] and bacteriochlorophylls *d* and *e* (specific for anaerobic green sulfur bacteria) [15–17] have been identified and used for reconstructing the paleoenvironments based on their structural grounds [18–23].

Meanwhile, isotopic analyses of purified, individual sedimentary porphyrins have been conducted to extract further information on the paleoenvironment [14,20,24–29]. Stable nitrogen and carbon isotopic compositions of chloropigments reflect not only those of nitrogen and carbon sources in the environment but also isotopic fractionations associated with the biochemical processes involved in their synthesis [30,31]. Therefore, together with the structural inferences, the isotopic compositions of individual porphyrin varieties should not only enhance the understanding of the precursor-product relationships [14,20,24–27] but also provide the information regarding biogeochemical processes related to the photoautotrophs involved in the primary production in the past ([24,25,28,29]; see also [30–33]). Significantly, unlike any other biomarkers ever known, porphyrins have

* Corresponding author at: Institute for Research on Earth Evolution, Japan Agency for Marine-Earth Science and Technology, 2-15 Natsushima-cho, Yokosuka 237-0061, Japan. Tel.: +81 468679805; fax: +81 468679775.

E-mail address: chiro@jamstec.go.jp (Y. Kashiya).

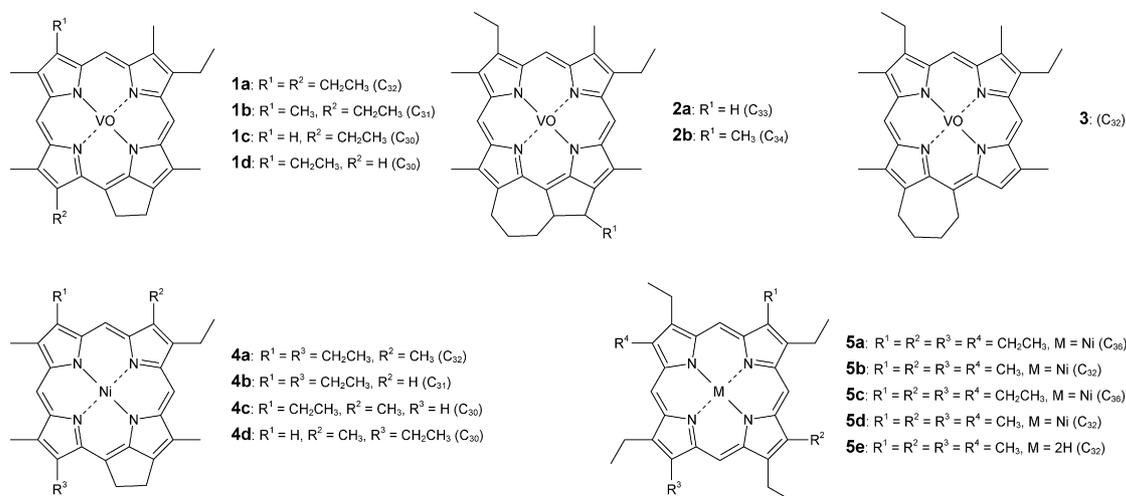


Fig. 1. Structures of sedimentary metalloalkylporphyrins (1–4) and standards (5) used in the present work.

nitrogen in their structures. Because the photoautotrophs play a key role in the ocean nitrogen cycle, therefore, the nitrogen isotopic compositions of sedimentary porphyrins should be able to resolve the nitrogen cycle in the past oceans. Such information is otherwise hardly inferred from other traditional sedimentological and geochemical proxies.

Despite such significance in the paleoenvironmental researches, analytical difficulties have prevented the progress of this subject. In particular, establishment of an efficient purification technique for individual porphyrins is essential; paleoenvironmental studies often require a quantity of datasets for a meaningful discussion, so the method must be affordable for routine analysis of numbers of geological samples. Sedimentary porphyrins occur as complex mixtures of numerous varieties so that considerable time and cost have been required for the purification of each target compound for the isotopic analysis. In particular, analysis of nitrogen isotopic composition of sedimentary porphyrins, which requires a large quantity of purified compounds (4 mg or more; [25]), has been limited to only one study [25].

High-performance liquid chromatography (HPLC) has been a method widely applied to the analysis of sedimentary porphyrins, and so has the preparative-scale HPLC for the purification. The sedimentary porphyrins are generally complexed with metals such as nickel(II) and vanadium(IV) (as VO) and analyzed by HPLC either as metalloporphyrins or demetallated free bases (Fig. 1). Thus, depending on the complexing metals, various HPLC conditions have been employed. In the analysis of free-base alkylporphyrins, the normal-phase HPLC method on the silica gel column [34] gives a fairly high chromatographic resolution. This method has been repeatedly adopted in many works ([20,21,38–41] among others including purifications for isotopic analyses [20,25]). Alternatively, the metal-complexed alkylporphyrins could directly be analyzed by a variety of reversed-phase HPLC methods without demetallation. For vanadyl alkylporphyrins, an efficient separation was achieved by the reversed-phase analysis on the octadecylsilica column with the mobile phase of the acetonitril–methanol–water mixture in various proportions [35]. For nickel alkylporphyrins,

methanol or methanol–acetonitrile mixture with or without addition of a small amount of pyridine have been used as the mobile phase [15,36,42–44]. These methods are also employed in many previous works [18,22,37,45–49] including purifications for isotopic analyses [14,26,27].

The HPLC method in the present work was designed for purification of intact sedimentary porphyrins for the purpose of compound-specific isotopic analyses. Therefore, direct analysis/purification of metalloporphyrins by HPLC is particularly essential for this purpose because of the following drawbacks related to the demetallation process. First, in the study of sedimentary porphyrins on paleoenvironmental reconstruction, the information on metal complexing may not be ignored, although we have not yet understood the significance of it well. Second, recovery in the demetallation procedure is considerably low (less than 75%) [35,37]. Some alkylporphyrins, such as C_{33} cycloheptanoDPEP (DPEP is an abbreviated form of deoxyphylloerythroetioporphyrin, commonly used in geochemical works [50] 2a), are particularly susceptible to acid treatment for the demetallation [22], so the information on the natural abundance of sedimentary porphyrins should be obscured by preferential losses through this process. Third, isotopic effects may be present during the demetallation processes, which must be avoided.

In the isotopic analysis of individual compounds, special attention must be paid to the isotopic effect during preparation and purification, in particular, the chromatographic isotopic fractionation [51]. It has been well known that the isotopic composition of a single, pure compound varies significantly across a peak on the HPLC chromatogram [51,52]. Consequently, to obtain unbiased isotopic information, the entire peak of the target compound must be recovered. Therefore, the compounds must be separated from others with base-line resolution in the chromatogram during the purification. However, since natural metalloalkylporphyrins are rather complex mixtures, and since each variety has close chemical properties, separation of a single porphyrin with the base-line resolution would be hardly possible even by the best-resolving HPLC method reported so far. Furthermore, co-eluting background impurities are potentially

associated in the analysis of such natural extracts. We found that a dual-step purification using two different HPLC conditions is a straightforward and, eventually, the most efficient solution to this problem.

Besides the chromatographic resolution, improvement in the solubility of analyte (i.e., metalloalkylporphyrins) to the mobile phase is key for the efficiency in the HPLC purification. The metalloalkylporphyrins have low solubility to the solvents commonly used for HPLC mobile phases, such as *n*-hexane, acetone, acetonitrile, and water [53], which causes precipitation of the analyte upon injection in the initial mobile phase resulting in poor chromatographic separations and poor reproducibility of the chromatogram [54]. It would also be a critical problem particularly when the same method is applied for purification where the solubility would limit the sample capacity for each HPLC run, requiring a tremendous amount of time for purification of sedimentary porphyrins for the purpose of isotopic analyses. This problem can be potentially resolved by increasing the scale of analysis (i.e., preparative-scale HPLC). However, considering the cost and the convenience, purification by the analytical-scale HPLC is of choice for applications in the paleoenvironmental study.

We report here an improved method for direct HPLC analyses of natural mixtures of metalloalkylporphyrins from rock extracts. We have developed high-efficiency reversed- and normal-phase HPLC methods for each of vanadyl and nickel alkylporphyrins (they are the two most important groups of sedimentary porphyrins; hereafter VO porphyrins and Ni porphyrins, respectively). Using these improved methods, we demonstrate an efficient purification of microgram quantities of natural metalloalkylporphyrins by a dual-step, analytical-scale HPLC for purpose of the compound-specific isotopic analyses.

2. Experimental

2.1. Samples and standards

The sample used in this study was an organic-rich, dark siliceous mudstone constituting a thick, pelagic sequence of the middle Miocene Onnagawa Formation, northern Japan [55,56]. The Onnagawa Formation consists of bedded siliceous rock that generally exhibits rhythmical alternations of dark-colored, organic-rich clayey layers and light-colored, biogenic silica-rich layers reflecting periodically variable flux of diatomaceous silica [56]. The sample was obtained from the stratigraphic position GJ01-3 [56] in the Gojome Area. The detailed geological settings of the sample were described in [56]. Authentic standards of porphyrins were purchased from the following companies, respectively: Ni octaethylporphyrin (97% purity), VO octaethylporphyrins (95% purity) and Ni etioporphyrins I (purity not reported by the supplier) from Aldrich Chemical Co. (Milwaukee, Wisconsin, USA); etioporphyrins I free base (purity not reported by the supplier) from Frontier Scientific (Logan, Utah, USA); and VO etioporphyrin I (purity not reported by the supplier) from Wako Chemicals (Osaka, Japan). We confirmed the purities of these standards by HPLC without suppliers' purity reports to be better than 95%.

2.2. Extraction and separation of metalloporphyrins

The surface of the samples was carefully removed and rinsed with dichloromethane (DCM)/methanol (1:1 v/v) before being pulverized. The pulverized samples were then Soxhlet extracted with chloroform/methanol (7:3, v/v) for 72 h. The total lipid was dried with a rotary evaporator.

The preparative separation procedure is summarized in the schematic diagram (Fig. 2). First, a low-polarity fraction containing alkylporphyrins was separated from the total extract by a silica gel column chromatography. The sample was dissolved in approximately 10 ml of DCM and placed onto the

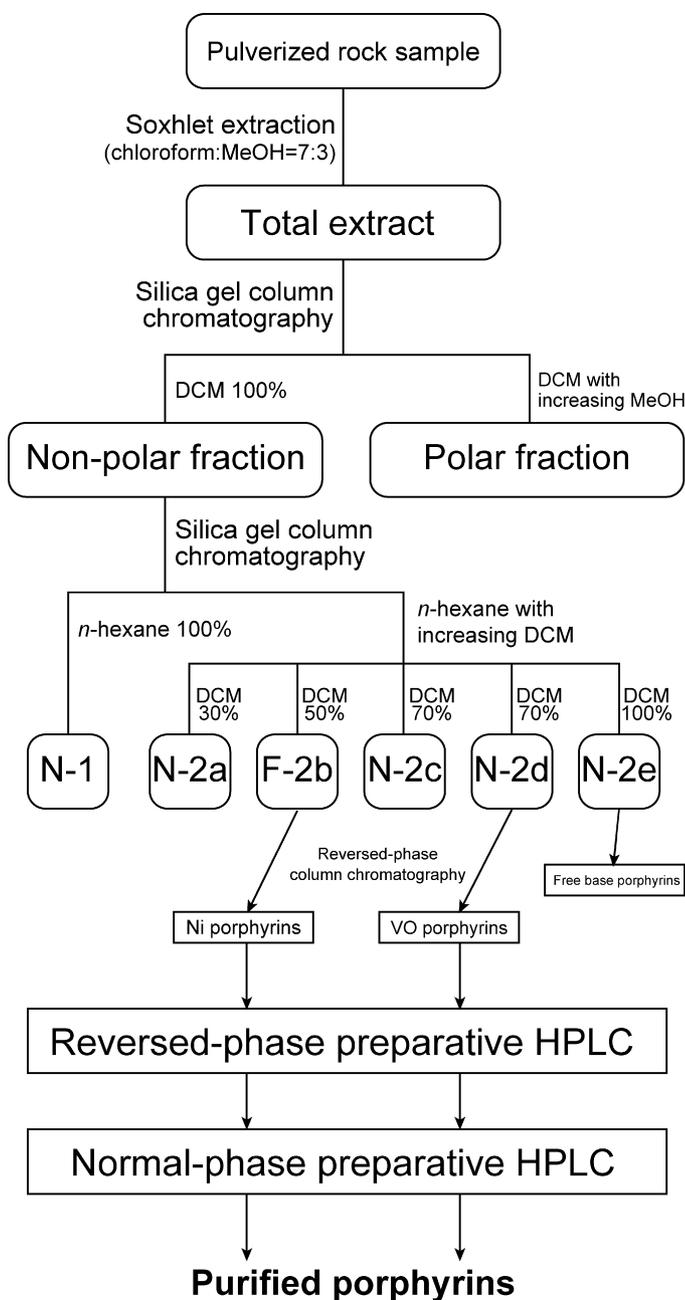


Fig. 2. Analytical scheme for purification of metalloalkylporphyrins from sedimentary rocks.

silica gel column (Sigma–Aldrich, St. Louis, Missouri, USA; 200–400 mesh; 80 mm in the column of 41.4 mm in internal diameter). The low-polarity fraction was then eluted with 100% DCM (400 ml) until the eluting solvent was colorless. The eluted fraction was dried with a rotary evaporator. Then, the low-polarity fraction was further separated into six fractions by another silica gel column chromatography. The low-polarity fraction was re-dissolved in 2 ml of DCM and mixed into 20 ml of *n*-hexane filled over the silica gel column (Sigma–Aldrich; 200–400 mesh; deactivated by adding 1 wt.% of H₂O; 80 mm in the column of 26 mm in internal diameter). After the N-1 fraction was eluted with the injected solution and an additional 30 ml of *n*-hexane (50 ml), five fractions (N-2a, N-2b, N-2c, N-2d, and N-2e) were eluted with *n*-hexane/DCM (7:3, v/v; 50 ml), *n*-hexane/DCM (1:1, v/v; 40 ml), *n*-hexane/DCM (1:1, v/v; 80 ml), *n*-hexane/DCM (3:7, v/v; 120 ml), and DCM (100 ml), respectively. Each fraction was dried by a N₂ blow-down evaporator (45 °C). Nickel porphyrins, VO porphyrins, and free-base alkylporphyrins were found in the fractions N-2b, N-2d, and N-2e, respectively.

Prior to HPLC analyses, each fraction containing specific metalloporphyrins was further processed with reversed-phase column chromatography. Each fraction was re-dissolved in 0.5 ml of DCM and mixed into 5 ml of the eluting solvent (described below) filled over the gel column (Wako Chemicals, Osaka, Japan; Wakogel 100C18, 63–212 μm; 40 mm in the column of 15.4 mm in internal diameter; flushed by nitromethane before use). The fraction N-2b was eluted with 30 ml of 100% *N,N*-dimethylformamide to recover the Ni porphyrins (N-2b-α). The fraction N-2d was eluted with 30 ml nitromethane/*N,N*-dimethylformamide (1:1, v/v) to recover the VO porphyrins (N-2d-α). Each eluted fraction was dried by a N₂ blow-down evaporator (75 °C). All solvents used above were “dioxin analysis grade” (Wako Chemicals). All glasswares were cleaned by heating at 450 °C for 5 h and carefully rinsed several times with both methanol and DCM immediately before use.

2.3. High-performance liquid chromatography

The HPLC system comprised a binary pump (Agilent, Santa Clara, California, USA; G1312), an on-line degasser (Agilent; G1322), an autosampler (Agilent; G1313A), a thermostated column compartment (Agilent; G1316A), an on-line photodiode-array detector (DAD; Agilent; G1315), optionally equipped with a fraction collector (Agilent; G1364a) during isolation/purification of metalloporphyrins, a mass selective detector (MSD; Agilent; G1946D) connected through an atmospheric pressure chemical ionization (APCI) interface for the HPLC/APCI-MS analysis, or an evaporative light scattering detector (ELSD; Polymer Laboratories, Church Stretton, Shropshire, UK; PL-ELS 2100) for the HPLC/ELSD analysis. This system was coupled to a personal computer with Agilent Chemstation software. The APCI condition was set as follows: drying gas flow: 6.0 l min⁻¹; nebulizer pressure: 345 kPa; drying gas temperature: 350 °C; vaporizer temperature: 500 °C; capillary voltage (positive): 4000 V; and corona current: 5.0 μA. The frag-

mentor voltage of MSD was set as 200 V with which protonated molecular ions (*M* + 1)^{•+} were best monitored for metalloalkylporphyrins. Analytical conditions for ELSD were set as follows: nebulizer: 90 °C; evaporator: 100 °C; and gas flow: air, 1.0 SLM (standard liter per minute; 1 min⁻¹ at 25 °C, 414 kPa in the mass flow controller). The flow rate of the mobile phase was 1 ml min⁻¹ during all the HPLC modes. All solvents used for the mobile phase were “HPLC grade” (pyridine: Aldrich; all others: Wako Chemicals). The mobile phase was ultrasonicated for 30 min before use.

Reversed-phase HPLC analyses of “the high-resolution HPLC analysis mode (Mode A)” and the high-sample-capacity HPLC purification mode (Mode B)”, respectively, were performed using three analytical-scale columns (ZORBAX SB-C18, Agilent; 4.6 mm × 250 mm; 5 μm silica particle size) connected in series with a guard column (ZORBAX SB-C18, Agilent; 4.6 mm × 12.5 mm; 5 μm silica particle size) set in front. The mobile phase used in Mode A, which was designed for HPLC/APCI-MS and HPLC/ELSD analyses, was acetonitrile/water mixture with acetic acid and pyridine, where the relative amount of water was gradually decreased through the analyses. In Mode B, which was designed exclusively for purification of metalloporphyrins, 20% of *N,N*-dimethylformamide was added to the mobile phase. Therefore, efficiency in the purification was significantly improved with Mode B by increasing the solubility of both VO and Ni porphyrins and by reducing the retention time of the whole chromatogram without significantly losing its resolution. However, *N,N*-dimethylformamide considerably reduces the sensitivity of the APCI-MS. The solvent gradient programs employed in Mode A and Mode B reversed-phase HPLCs for the Ni porphyrin (N-2b-α) and VO porphyrin (N-2d-α) fractions, respectively, are summarized in Table 1. During Mode A, 5 μl or less volume of highly concentrated samples dissolved in chloroform was injected for each analysis, however, when a large quantity of analyte was injected from highly concentrated sample solution, front tailing and splitting of the peaks were observed. During Mode B, depending on the requirement in each experiment, 2–5 μl of highly concentrated samples dissolved in chloroform was injected for each run. Excess injection of chloroform resulted in broadening of the peaks. Alternatively, VO porphyrin was dissolved in *N,N*-dimethylformamide that can be injected up to 30 μl during Mode B without significant broadening of the peak. The column temperature was set at 40 °C. Collected fractions during Mode B were dried by a N₂ blow-down evaporator (75 °C).

Normal-phase HPLC analyses (Mode C), which were used both for HPLC/APCI-MS analysis and purification of metalloporphyrins, were performed using three analytical-scale columns (ZORBAX SIL, Agilent; 4.6 × 250 mm; 5 μm silica particle size) connected in series with a guard column (ZORBAX SIL, Agilent; 4.6 mm × 12.5 mm; 5 μm silica particle size) set in front. The mobile phases for the isocratic analyses for Ni and VO porphyrins were *n*-hexane/acetone/*N,N*-dimethylformamide/acetic acid/pyridine (95:3:1:0.5:0.5, v/v/v/v/v) and *n*-hexane/DCM/*N,N*-dimethylformamide/acetic acid/pyridine (88:10:1:0.5:0.5, v/v/v/v/v), respectively. During

Table 1
Gradient programs for binary solvent system used in the reversed phase HPLC analyses

Time (min)	Solvent	
	A (%)	B (%)
(a) Mode A for VO porphyrins		
0	100	0
30	80	20
80	20	80
100	20	80
	C (%)	D (%)
(b) Mode B for VO porphyrins		
0	100	0
20	80	20
50	20	80
60	20	80
65*	100	0
75*	100	0
	A (%)	B (%)
(c) Mode A for Ni porphyrins		
0	40	60
30	20	80
80	0	100
100	0	100
	C (%)	D (%)
(d) Mode B for Ni porphyrins		
0	40	60
20	20	80
50	0	100
60	0	100
65*	40	60
75*	40	60

Solvents: A: acetonitrile/H₂O/pyridine/acetic acid (90:10:0.5:0.5); B: acetonitrile/pyridine/acetic acid (100:0.5:0.5); C: acetonitrile/*N,N*-dimethylformamide/H₂O/pyridine/acetic acid (70:20:10:0.5:0.5); D: acetonitrile/*N,N*-dimethylformamide/pyridine/acetic acid (80:20:0.5:0.5).

* re-equilibration.

Mode C, 2 μ l or less volume of highly concentrated samples dissolved in chloroform was injected. Excess injection of chloroform, particularly in the case of Ni porphyrin analysis, may result in splitting of the peak. When a large quantity of analyte was injected from highly concentrated sample solution during Mode C HPLC purification, front tailing of the peak was observed. However, peak separation may not be significantly impaired unless an extreme amount was injected. The column temperature was set at 40 °C. Collected fractions were dried by a N₂ blow-down evaporator (55 °C).

3. Results and discussion

Fig. 3a and 4a illustrate the reversed-phase HPLC/DAD chromatograms (Mode A) of Ni and VO porphyrins of the Onnagawa dark dolomitic siliceous shale sample (GJ01-3). The chromatogram of VO porphyrins (Fig. 3a) exhibited peak separations comparable to those previously reported

with slightly shortened retention time (e.g. [22,35,37,48,49]). On the other hand, the chromatogram of Ni porphyrins (Fig. 4a) was improved both in peak separation and retention time (30–40% reduction) compared with the previously published chromatograms (e.g. [18,46]). With our method, as low as a few nano grams of porphyrin can be detectable by DAD.

The HPLC/APCI–MS spectra of metalloalkylporphyrins show molecular ions ($M+1$)^{•+} as base peaks, which allows estimation of the carbon number and the basic structures such as ETIO-type (i.e., alkylporphyrins without an external ring represented by etioporphyrin III in natural samples), monocycloalkano-type, or bicycloalkano-type porphyrins (Figs. 3a and 4a). Most VO porphyrins in all samples of the Onnagawa siliceous shale/porcelanite are monocycloalkano-type, most likely DPEP-type (13,15-cyclopentanoporphyrin represented by DPEP), and bicycloalkano-type, but ETIO-type porphyrins are nearly absent. In contrast, Ni porphyrins are rich in ETIO-types, particularly among those having carbon numbers lower than 30, but bicycloalkano-type porphyrins are insignificant.

Some important improvements for efficient isolation of porphyrins were achieved in the Mode B HPLC (Figs. 3c and 4c). Using *N,N*-dimethylformamide in the mobile phase, our method successfully avoided precipitation of metalloalkylporphyrins in the mobile phase [54] even when a large amount of analyte was injected during Mode B purification and minimized unwilling phenomena related to overloading such as front tailing, splitting, or broadening of the peak. In addition, the overall retention time was further reduced (approximately 40% reduction compared to Mode A), but eluting pattern of porphyrin peaks was maintained identical early. These allowed efficient purification of metalloalkylporphyrins. For example, the shaded peak area in the Fig. 3c (Peak a) corresponds to 50 μ g of VO DPEP (**1a**). Thus, at best, several consecutive cycles were eventually adequate to collect over 100 μ g of each of major VO porphyrins.

The reversed-phase HPLC generally separates porphyrins of pseudohomologous series with different carbon numbers well, but structural isomers with the same carbon numbers are relatively resolved poorly. In contrast, the normal-phase HPLC for both Ni and VO porphyrins tends to separate positional isomers. For example, Fig. 3d is a normal-phase HPLC chromatogram (Mode C) of an unresolved VO alkylporphyrin fraction obtained by a reversed-phase HPLC chromatography (the highlighted fraction in Fig. 3c) and shows that many of VO porphyrins having the identical molecular weight, indicating structural isomers, were apparently resolved.

3.1. Isolation

We isolated six VO porphyrins (**1a**, **1c**, **1d**, **2a**, **2b**, **3**) and four varieties of Ni porphyrins (**4a–d**) for the purpose of isotopic analyses [57]. An example is shown in Fig. 5. First, using the reversed-phase HPLC (Mode B), several fractions that contain the entire peaks of porphyrins of interest were collected (Fig. 5a and e). Subsequent normal-phase HPLC/APCI–MS chromatog-

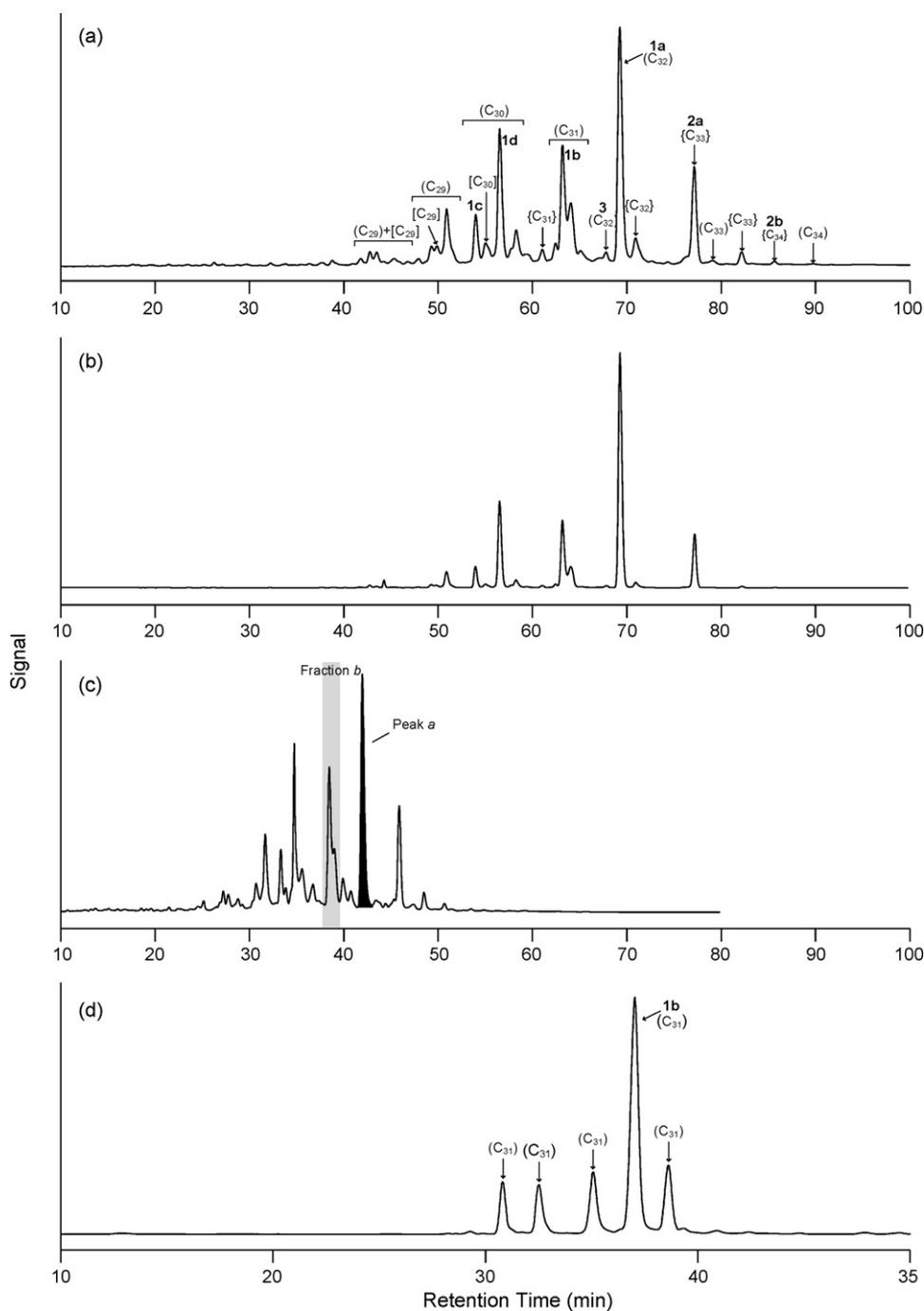


Fig. 3. HPLC chromatograms of VO porphyrins (fraction N-2d- α ; GJ01-3): (a) by Mode A reversed-phase HPLC/DAD (572 nm); carbon numbers are based on mass spectra obtained by simultaneous HPLC/APCI-MS analysis: $[C_{32}]$ ETIO-type; (C_{32}) monocycloalkano-type; $\{C_{32}\}$: bicycloalkano-type; the carbon numbers assigned were determined by LC/MS mass spectra; the structure of **2b** and **3** was temporary identified based on mass fragment patterns; all other structures were determined by X-ray crystallography; (b) by Mode A reversed-phase HPLC/ELSD simultaneously obtained with the DAD chromatogram of (a); (c) by Mode B reversed-phase HPLC/DAD (572 nm); see text for explanation for peak a and fraction b; (d): Mode C normal-phase HPLC chromatogram of a fraction of VO porphyrins (DAD absorption at 408 nm; fraction b of (c)).

raphy reveals that unresolved impurities of porphyrins in these fractions were mostly structural isomers of the target compounds with the same molecular weight, and, significantly, the target compounds were, in most of the cases, separated from impurities with base-line resolution. Thus, they were finally isolated using the normal-phase HPLC (Mode C; Fig. 5b and f).

3.2. Purity

High purity is a prerequisite for any compound-specific analysis of natural stable isotopic compositions. Impurity of non-porphyrin compound is, however, unlikely to be coeluted with the target metalloalkylporphyrins during the final isolation step after the dual-step HPLC purification. Such compounds may

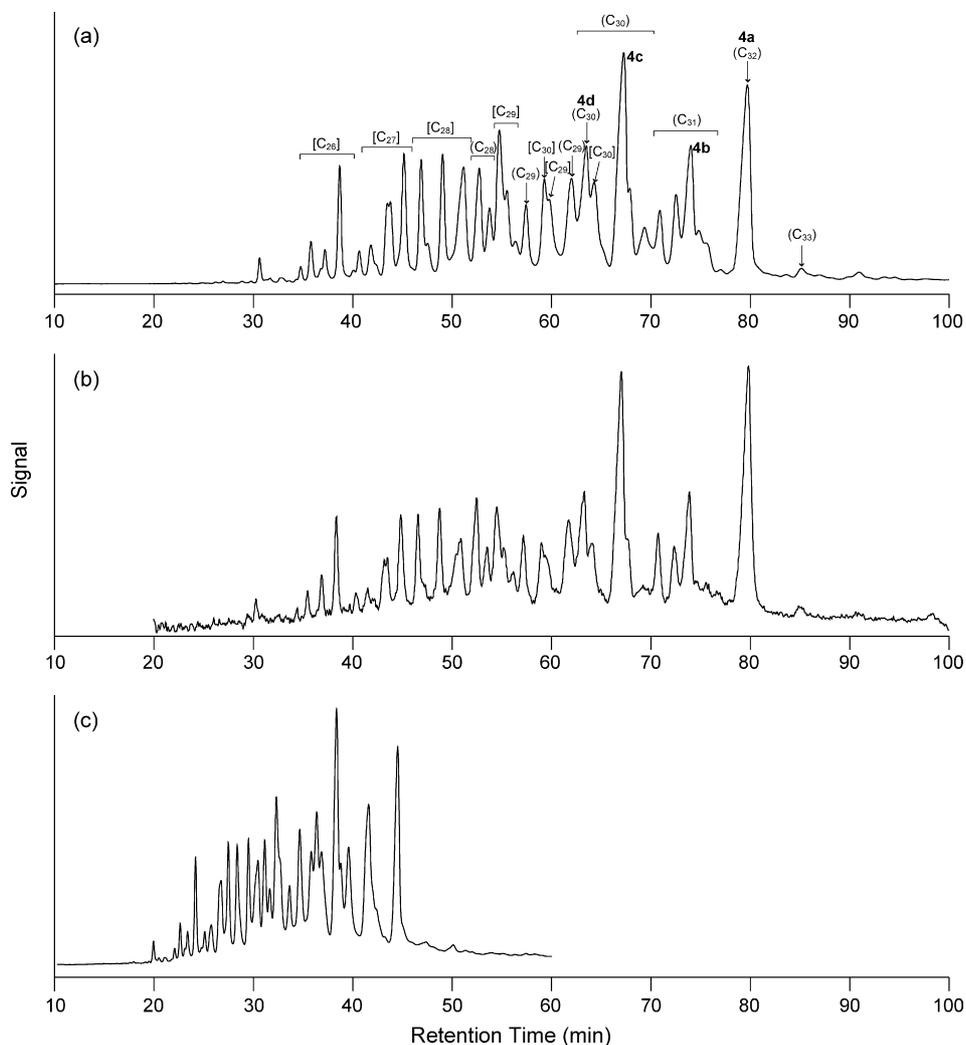


Fig. 4. HPLC chromatograms of Ni porphyrins (fraction N-2b- α ; GJ01-3): (a) by Mode A reversed-phase HPLC/DAD (550 nm); carbon numbers are based on mass spectra obtained by simultaneous HPLC/APCI-MS analysis: [C₃₂] ETIO-type; {C₃₂} monocycloalkano-type; {C₃₂} bicycloalkano-type; all structures indicated are temporary identified based on mass spectra and comparison of retention times to those of chromatograms in the previous works; (b) by Mode A reversed-phase HPLC/ELSD simultaneously obtained with the DAD chromatogram of (a); (c) by Mode B reversed-phase HPLC/DAD (550 nm).

not be detected by DAD and/or may not be ionized by APCI (little polar), hence not detected by MSD, but should be monitored by ELSD. The ELSD nebulizes the eluate, evaporates only the volatile mobile phase, and generates a continuous stream of solute particles that is optically detected by scattered light. Figures 3c and 4c illustrate chromatograms of a reversed-phase HPLC/ELSD analysis with Mode A. Based on the comparison with DAD results, non-porphyrin impurity was apparently absent in these fractions. Indeed, because metalloalkylporphyrins have rather unique chemical properties relative to geolipids that have similar polarity (so eluted into the same fraction by the silica gel column), non-porphyrin impurities were probably not eluted with the HPLC conditions tuned for metalloalkylporphyrins. Therefore, the non-porphyrin impurities are likely to be eliminated after two isolations by HPLC with distinct conditions.

Porphyrins surviving as impurities with isolated target compounds can be seen by HPLC/DAD that is highly sensitive for porphyrins unless the impurity has exactly the same reten-

tion times in both reversed- and normal-phase HPLCs. Such impurities may be porphyrins eluted in the normal-phase HPLC with identical retention time as the target compound but can be resolved to elute with slightly different retention time by the reversed-phase HPLC of the isolated compounds (see Fig. 5c and g for example). There were also impurities seen in the normal-phase HPLC chromatogram (Fig. 5d and h). This may be due to the limitation of the preparation scheme where, although isolated apparently by baseline-resolution, the ends of the tails of the adjacent peaks were accidentally isolated with the target compound by the reversed-phase HPLC (Mode B). If the porphyrin impurity has nearly the same retention time as the target compound, it may still be identified by MSD spectrum if the molecular weight is different from that of the target compound.

3.3. Recovery

We conducted an experiment to isolate alkylporphyrins from the standard mixture to estimate the recovery through the

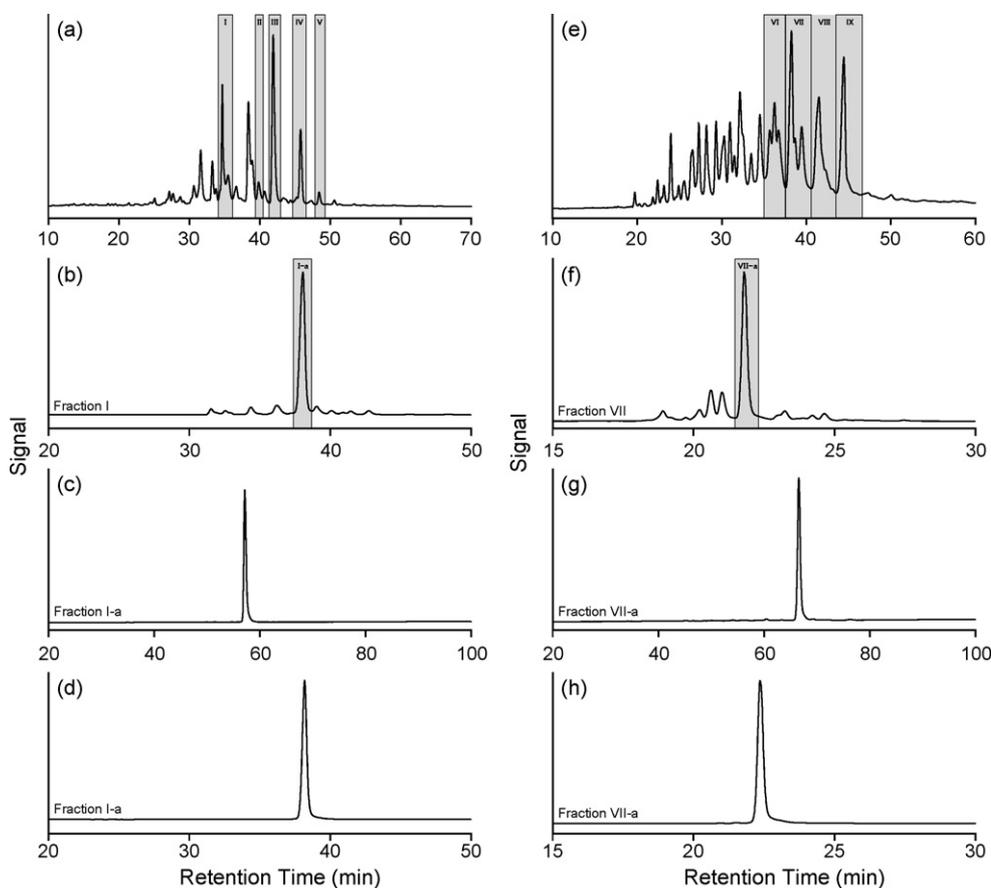


Fig. 5. HPLC/DAD chromatograms illustrating purification of VO porphyrins (a)–(d) and Ni porphyrins (e)–(h); (a) and (e): Mode B reversed-phase HPLC/DAD chromatogram (at 572 and 550 nm) of fractions N-2d-α and N-2b-α, respectively; shaded bands (fractions I–IX) were collected for further purification; (b) and (f): Mode C normal-phase HPLC/DAD chromatograms (at 572 and 550 nm) of the fractions I and VII, respectively; peaks I-a, and VII-a are isolated with baseline-resolution; (c) and (g) Mode A reversed-phase HPLC/DAD chromatograms (at 408 and 392 nm) of the isolated peaks I-a and VII-a; (d) and (h) Mode C normal-phase HPLC/DAD chromatograms (at 408 and 392 nm) of the isolated peaks I-a and VII-a.

presented procedure. The standard sample was prepared as a mixture of Ni octaethylporphyrin (Ni–OEP; **5a**), Ni etioporphyrin I (Ni–ETIO; **5b**), VO octaethylporphyrin (VO–OEP; **5c**), VO etioporphyrin I (VO–ETIO; **5d**), and etioporphyrin I free base (free-ETIO; **5e**). Aliquots of the sample presented in each step of the procedure were portioned to quantitate by the normal-phase HPLC/DAD with Mode C, with which recovery of each standard was calculated.

Fig. 6 summarizes recoveries of the standard compounds along with the purification procedure. The overall recovery of the entire procedure significantly varies among standard compounds. Relatively poor recoveries were observed among Ni porphyrins (58.5% for Ni–OEP; **5a**; and 47.2% for Ni–ETIO; **5b**) and the free base (63.9% for free-ETIO; **5e**). In contrast, the recovery was generally good among VO porphyrins (85.5% for VO–OEP; **5c**; and 83.7% for VO–ETIO; **5d**). Figure 6b illustrates recoveries of these compounds in each step of the procedure. Major losses occurred during the reversed-phase HPLC (Mode B) and the normal-phase HPLC (Mode C) where the recoveries were 75.0% and 88.3% on average, respectively, whereas early separation steps with open column chromatography show minimal losses. The variability in overall recovery

is mostly the consequence of large variability in recovery during the reversed-phase HPLC (Mode B) where poor recoveries were found among Ni porphyrins (64.6% for Ni–OEP; **5a**; and 58.5% for Ni–ETIO; **5b**) in contrast to those among VO porphyrins (90.2% for VO–OEP; **5c**; and 88.0% for VO–ETIO; **5d**).

The unexpectedly low recoveries in the HPLC steps, rather than in the conventional open column chromatographic steps, may have resulted from unwilling reactions between the organic solvents used in the mobile phase, particularly *N,N*-dimethylformamide, and porphyrins in the HPLC systems. Our supplementary experiment demonstrated relatively good recoveries of Ni porphyrins during the Mode A reversed-phase HPLC where *N,N*-dimethylformamide was not used in the mobile phase. In the step of reversed-phase open column chromatography where *N,N*-dimethylformamide was used as the eluting solvent, however, recoveries were not significantly reduced. Therefore, environments within the HPLC apparatus, such as the surface of stationary phases of the column (heated at 40 °C) or stainless capillary tubes, might catalyze such reactions. More likely, irradiation by UV light on the mobile phase at DAD may cause decomposition of porphyrin under the presence of *N,N*-dimethylformamide.

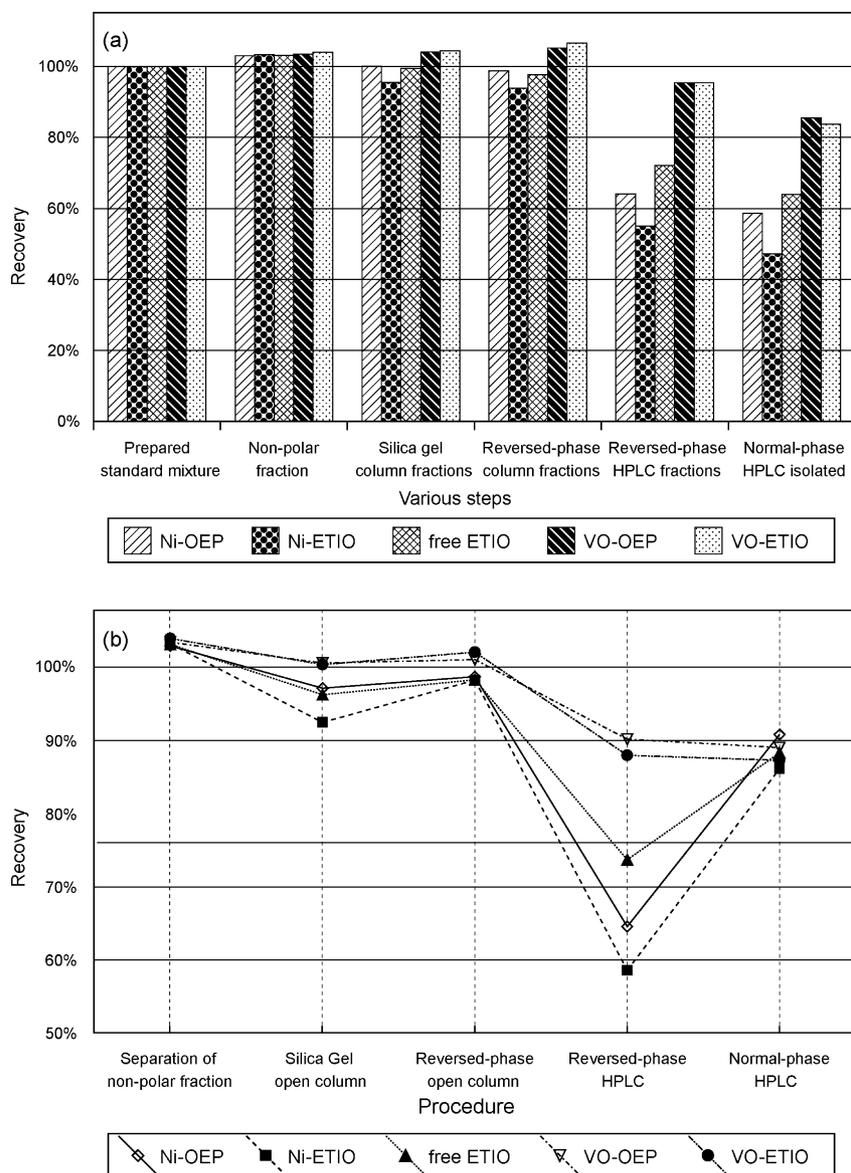


Fig. 6. (a) Recovery of the standard compounds in fractions obtained in various steps along the purification procedure. Recovery of each standard was expressed as the quantity present after each step was divided by the quantity present in the starting mixture; (b) recovery rates in each step of the procedure, expressed as the quantity presented after a step divided by the quantity presented before the step.

Overall, the variability in recoveries (or losses) is more significant between those with different complexing metals (i.e., poorer recoveries among Ni porphyrins) but minor among different structures (i.e., OEP and ETIO). Such selective losses may be caused by changes in reactivity (such as ease of oxidation) depending on the kind of complexing metals (or free of metal). Metal complexing alters the electronic properties and three dimensional arrangements of porphyrins and may affect their overall reactivity. For example, the small size of nickel(II) ion particularly distorts and twists the macrocyclic structure to fit it to the central cavity [58], which is likely to destabilize the molecule, whereas VO porphyrins are more or less planar and hence likely to be quite stable. However, changes in the degree of distortion of the macrocycle can also be caused by changes in peripheral structures such as formation of an additional five-

membered (i.e., ring E of DPEP; **1a**) or seven-membered ring (for example, C₃₃ cycloheptanoDPEP; **2a**). Thus, it predicts that the recovery through the purification procedure, particularly those during Mode B, may vary considerably among alkylporphyrins having various peripheral rings.

4. Conclusions

Here, we proposed an improved method to purify intact metalloalkylporphyrins using the high-resolution reversed- and normal-phase HPLCs for the purpose of compound-specific isotopic analyses. By the reversed-phase HPLC, the metalloalkylporphyrins were mostly separated according to the carbon numbers among the pseudohomologous series. By adding *N,N*-dimethylformamide to the mobile phase, the sample

capacities for the metalloalkylporphyrins were significantly improved, allowing efficient purification using analytical-scale columns. In contrast to the reversed-phase HPLC, the structural isomers were well separated in the normal-phase HPLC. Thus, the target compounds were isolated with baseline-resolution after the dual-step isolation using these HPLC methods so that the chromatographic isotopic effect could be excluded.

This protocol is suited for the preparation for the isotopic analysis of alkylporphyrins because it does not require demetallation or derivatization in preparation such as acetylation to isolate target compounds (for example [25]). The demetallations of VO porphyrins particularly result in poor recovery and loss of the original composition by preferential decomposition. Significantly, acetylation must, and demetallation may also, associate with the isotopic fractionation, which must be avoided in the isotopic analyses. Finally, our HPLC method allows rapid and low-cost purifications using the analytical-scale columns. Therefore, isotopic studies of sedimentary porphyrins can now be open to various paleoenvironmental problems where handling a large number of samples is often required.

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