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Quantitative Analysis of Coenzyme F430 in Environmental Samples: A New Diagnostic Tool for Methanogenesis and Anaerobic Methane Oxidation

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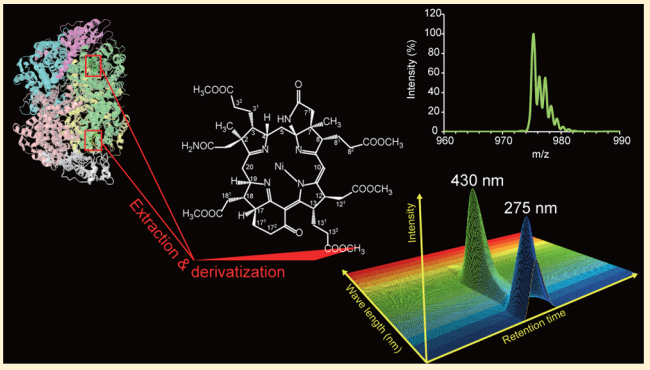
Supporting Information

ABSTRACT: Coenzyme F430 is a nickel hydrocorphinoid and is the prosthetic group of methyl−coenzyme M reductase that catalyzes the last step of the methanogenic reaction sequence and its reversed reaction for anaerobic methane oxidation by ANME. As such, function-specific compound analysis has the potential to reveal the microbial distribution and activity associated with methane production and consumption in natural environments and, in particular, in deep subsurface sediments where microbiological and geochemical techniques are restricted. Herein, we report the development of a technique for high-sensitivity analysis of F430 in environmental samples, including paddy soils, marine sediments, microbial mats, and an anaerobic groundwater. The lower detection limit of F430 analysis by liquid chromatography/mass spectrometry is 0.1 femto mol, which corresponds to 6×10^2 to 1×10^4 cells of methanogens. F430 concentrations in these natural environmental samples range from 63×10^{-6} to 44 nmol g^{-1} and are consistent with the methanogenic archaeal biomass estimated by microbiological analyses.



Coenzyme F430 is the hydrocorphinoid nickel complex[1−3] (Figure 1), which is a prosthetic group of methyl−coenzyme M reductase (MCR) that catalyzes the reduction of methyl−coenzyme M to methane.[4−6] Given that the last step of methanogenic reactions is common in all methanogenic pathways[7] (Figure 1), all methanogens should possess F430. In addition, recent studies have suggested that anaerobic methane oxidizing archaea (ANME) also use F430 for reverse methanogenesis.[8−10] Thus, F430 is a function-specific compound (FSC) and should be a robust biomarker for methanogens and ANME.

Given that F430 is directly involved in methanogenesis and the anaerobic oxidation of methane, quantitative analysis of F430 may be better able to indicate in situ methanogenic and anaerobic methane oxidation activity than gene and lipid biomarker-based diagnostic tools. In particular, such function-specific compound analysis (FSCA) might be a powerful tool to investigate microbial activity associated with methane production and consumption in deep subsurface sediments where microbiological and geochemical techniques are restricted.

The most common technique to detect F430 is high-performance liquid chromatography (HPLC) coupled with a photometric detector. However, F430 detection in environmental samples with this technique has been restricted to analysis of microbial mats in the Black Sea where ANME is present in high cell densities (1×10^{10} to 4×10^{10} cells cm^{-3} mat).[8,9,11] Improved sensitivity is required to detect F430 from most environmental samples that have lower microbial populations. A recent study using offline mass spectrometry was successful in detecting F430 in methanogenic marine sediment from offshore Shimokita Peninsula, which indicates that technical advances do allow for higher sensitivity.[12]

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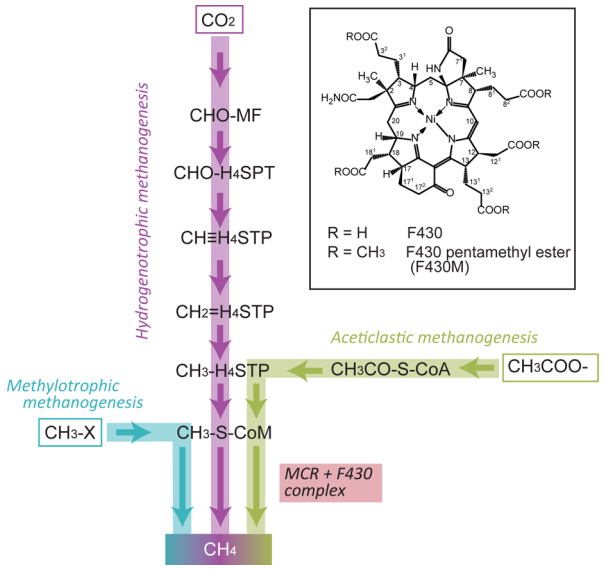

Figure 1. Chemical structure of F430 and its role in methanogenic pathways (modified after ref 7). CHO−MF, formyl−methanofuran; CHO−H_4SPT, formyl−tetrahydrosarcinapterin; CH≡H_4STP, methenyl−tetrahydrosarcinapterin; CH_2═H_4STP, methylene−tetrahydrosarcinapterin; CH_3−H_4STP, methyl−tetrahydrosarcinapterin; CH_3−S−CoM, methyl−coenzyme M.

In this study, we have developed a method for the quantitative analysis of F430 that involves extraction, derivatization, and analysis using a HPLC coupled to a triple quadrupole (QQQ) mass spectrometer. We have applied this method to the analysis of F430 in a range of environmental samples in which the presence of methanogens and ANME has previously been reported.

EXPERIMENTAL SECTION

Samples. Samples used in this study are listed in Table 1. A granule sample consisting of high-density methanogenic archaea and other prokaryotic cells (code MBK)[12] was supplied from Sumitomo Heavy Industry Ltd., Yokosuka, Japan. Anjo E2[13,14] was collected from the Anjo Research and Extension Station, Japan (34°48′N, 137°3′E) on September 26th, 2006. Two soil samples (Chikugo CF and RSC)[13−15] were collected from the National Agricultural Research Center for Kyushu Okinawa Region, Japan (33°12′N, 130°29′E) on September 21st, 2005. A further soil sample (Kawatabi) was collected from the Field Science Center at Tohoku University, Japan (38°44′N, 140°45′E) on August 4th, 2009.[16] Shimokita 11-4 was collected from 97 m below the seafloor (Site 9001; core section 11-4) off the Shimokita Peninsula, Japan (41°10.6380′N, 142°12.08′E) during the shakedown cruise CK06-06 of the D/V *Chikyu*.[17] Peru 9H1 was collected from 70 m below the seafloor (Site 1229; core section 9H1) from the Peru Margin (10°58.572′S, 77°57.459′W) during Ocean Drilling Program (ODP) Leg 201 by the R/V *JOIDES Resolution*.[18] ANME pink and black mats were collected from the northwestern Black Sea (44°46.46′N, 31°59.50′E) cruise M72 Leg 1 by the R/V *Meteor* in 2007.[19] An anaerobic groundwater sample (Ita-wari) was collected from a deep aquifer associated with an accretionary prism in the Shizuoka Prefecture, Japan (34°52.283′N, 138°09.150′E) on July 15th, 2013.[20] The water sample was centrifuged (× 10000*g* for 15 min), and the precipitant was used for F430 extraction.

Table 1. Methanogen and Environmental Samples Used in This Study and Measured F430 Concentrations

sample name	sample type	concentration		references
MBK	granules	3.30×10^7	fmol/g wet	12
Anjo E2	paddy soil	2.02×10^3	fmol/g wet	13, 14
Kawatabi	paddy soil	8.70×10^2	fmol/g wet	16
Chikugo CF	paddy soil	3.08×10^2	fmol/g wet	13−15
Chikugo RSC	paddy soil	1.06×10^3	fmol/g wet	13−15
Shimokita 11−4	marine sediments	6.33×10^1	fmol/g wet	12, 17, 31
Peru 9H1	marine sediments	1.92×10^3	fmol/g wet	18, 32
ANME pink mat	microbial mat	8.31×10^5	fmol/g wet	8, 9, 19, 35
ANME black mat	microbial mat	4.43×10^7	fmol/g wet	8, 9, 19, 35
Ita-wari	groundwater	8.11×10^2	fmol/L	20

Extraction and Derivatization. Samples (wet) were extracted with 1% formic acid (pH 2; Wako Pure Chemical Industries Ltd.) by ultrasonication for 30 min on ice, followed by centrifugation (× 10000*g*; 30 min at 4 °C) to recover the supernatant. This step was repeated three times. The combined supernatant was introduced to an anion exchange column (Q Sepharose column; GE Healthcare) that had been equilibrated with 50 mm of Tris/HCl (pH 7.5) and washed with deionized water prior to use. The recovered eluent was introduced to a C_{18} SPE column (Sep-Pack; Waters) that had been equilibrated with methanol and conditioned with 1% formic acid. An absorbed yellowish band (F430 fraction) on the column was eluted with 100% methanol. The recovered F430 fraction was dried and stored at −20 °C prior to further treatment. The dried F430 fraction was reacted with BF_3/methanol (Supelco) in a closed vial at 40 °C for 3.5 h to convert F430 to its pentamethyl ester (F430M). Water was added to the vial and the aqueous phase was extracted three times with dichloromethane (DCM). The organic phase was recovered after centrifugation and dried under N_2 stream. The recovery of F430M was >95% (Figure S-1 of the Supporting Information).

HPLC Analysis. HPLC conditions for F430 (pentacarboxylic acid) and F430M analysis were modified after ref 9. For F430 analysis, the F430 fraction was dissolved in H_2O/acetonitrile (85/15 v/v). Compounds were separated using an Agilent 1200 series HPLC equipped with HYPERCARB (4.6 × 100 mm; 5 μm p.s.; Thermo Scientific) and a guard column. Mobile phases were $HClO_4/H_2O$ (pH1.0; Wako Pure Chemical Industries Ltd.; A) and acetonitrile (HPLC-grade; Wako Pure Chemical Industries Ltd.; B). The flow rate was 0.5 mL min^{-1}. The gradient condition was started at 0% B followed by 30% B after 3 min and then 90% B after 90 min. For F430M analysis, the F430 fraction was dissolved in H_2O/acetonitrile (85/15 v/v). Compounds were separated using an Agilent 1200 series HPLC equipped with a ZORBAX Eclipse XDB-C_{18} (4.6 × 250 mm; 5 μm p.s.; Agilent Technologies) and a guard column. Mobile phases were 100 mM $NaClO_4$ (Kanto Chemical Company Incorporated; A) and acetonitrile (HPLC grade; Wako Pure Chemical Industries Ltd.; B). The flow rate was 0.5 mL min^{-1}. The gradient condition was started at 0% B followed by 30% B after 3 min and then 90% B after 90 min. A fraction collector was used for isolation of F430M from its

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epimers and other matrices. For both HPLC analyses, a signal of 430 and 560 nm was monitored using a photodiode array detector (DAD).

LC−MS/MS Analysis. LC−MS/MS was performed using an Agilent HPLC 1260 Infinity coupled to a 6460 Triple Quadrupole (QQQ) LC/MS system. F430M was analyzed in positive ion mode by electrospray ionization (ESI) and an Agilent jet stream. Source and sheath gas temperatures were set at 300 and 250 °C, respectively. Source and sheath gas flow rates were set to 5 and 11 L min^{-1}, respectively. Capillary and nozzle voltages were set at 3500 and 500 V, respectively. For MRM analysis, the fragmentor voltage was 180 V and the collision energy was 0 V. Both precursor and product ions of F430 were set to m/z 975.4 for F430M, 1021.4 for methylthio-F430. Compound separation by HPLC was conducted using a ZORBAX Eclipse XDB-C_{18} (4.6 × 250 mm; 5 μm p.s.). Mobile phases were 10 mM ammonium acetate (Wako Pure Chemical Industries Ltd.; A) and acetonitrile. The flow rate was 0.5 mL min^{-1}. The gradient condition was started at 0% B followed by 30% B after 3 min and then 90% B after 90 min.

Nuclear Magnetic Resonance Spectroscopy (NMR). For identification of isolated F430M, the ^{1}H NMR spectrum was recorded on a Bruker Avance-III spectrometer operated at 400 MHz with a 5 mm type BBO probe. Purified F430M was dissolved in CD_2Cl_2 (Cambridge Isotope Laboratories Inc.) and 2,2,2-trifluoroethanol-d_3 (TFE-d_3; Sigma-Aldrich) (4/1, v/v) in a 5 mm tube. Chemical shifts were referenced to the signals of TFE (3.92 ppm). The obtained spectra were compared with previously reported spectra and chemical shifts.[6,21] Repetitive NMR analysis was conducted to examine the stability of F430M. The samples were stored at −20 °C between analyses.

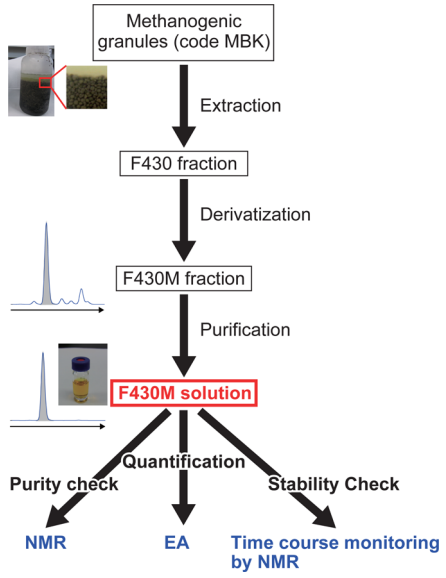
Elemental Analysis (EA). Carbon and nitrogen contents in the F430M solutions were determined with an online system of a Finnigan Delta Plus XP isotope ratio mass spectrometer.[22] They were determined by calibrated ion currents of m/z 44 and 28, respectively. The analytical error estimated by repeated analysis of our laboratory standard (nickel octaethylporphyrin) is 5%.

RESULTS AND DISCUSSION

Preparation of the F430M Standard Solution. Given that F430 and F430M are not commercially available, a standard solution of known concentration needed to be prepared for use as an external standard to quantify F430 in environmental samples. Purification and preservation of F430 are difficult due to the unstable nature of F430.[6,23] In our study, we prepared a F430M standard (see below) and F430 from samples that were also converted to F430M before analysis.

The F430M solution was prepared from the sample MBK by preparative HPLC (Scheme 1 and Table 1). The purity of the isolated F430M solution was confirmed by NMR spectroscopy. Assignment of chemical shifts by comparison with previously reported data (especially based on the chemical shift of H13) indicates that the isolated compound was F430M and not its epimers such as 13-epi-F430M and 12,13-diepi-F430M (Figure S-2 of the Supporting Information). The carbon-to-nitrogen atomic ratio determined by elemental analysis was 6.6 ± 0.2, which is consistent with the theoretical value of 6.7. The concentration of the F430M solution was determined to be 0.79 ± 0.03 μmol mL^{-1} by EA. Over 300 days, we monitored the F430M solution stored in the dark at −20 °C by ^{1}H NMR and found no viable degradation, which suggests that the prepared F430M solution is appropriate for use as a standard for quantification.

Scheme 1. Schematic of the Preparation of F430M Standard Solution from Methanogenic Granules (Code MBK)[12]



Quantification by LC−MS/MS. For the quantification of F430M, we conducted multiple reaction monitoring (MRM) with QQQ. In general MRM mode, the selected ion (precursor ion) in MS1 is fragmented to product ions at a collision cell followed by detection of an ion of interest in MS2. Even if a high collision energy was used for the fragmentation, the yield of product ions was low and a precursor ion (m/z 975.4) is abundant (Figure S-3 of the Supporting Information). Therefore, in this study, the ionized F430M (m/z 975) path through MS1 was redetected in MS2 without breakdown in the collision cell (zero-collision energy) in order to obtain higher sensitivity. The lower detection limit of the zero-collision energy MRM analysis of F430M is ∼0.1 fmol, which is 5 orders of magnitude lower than that of conventional photometric analysis (Figure 2).

An amount of 0.1 fmol of F430 corresponds to 6 × 10^2 to 1 × 10^4 cells of methanogens based on the F430 content of various methanogen cultures and prokaryotic cell weight.[23−25] Given the low methanogen population (<0.1% of prokaryotic biomass[26−28]), it is estimated that methanogens account for 3 × 10^2 to 1 × 10^7 cells in the global plot of prokaryotic biomass in marine sediments (0.001−1000 m sediment depth[29]). Thus, the sensitivity of our method of F430M analysis by zero collision energy MRM should enable the detection of F430 in marine sediments.

Analytical Artifacts during the Experiments on F430 and its Homologues. F430 is an unstable compound, particularly upon heating and in contact with oxygen. It has previously been reported that heating of F430 generates thermodynamically more stable forms, such as 13-epi-F430 and 12,13-diepi-F430[6,23] and contact with oxygen generates 12,13-didehydro-F430 (F560) as the main product.[6] In this study, extraction and subsequent chromatography (ion exchange and C_{18} solid extraction) were performed under ice cooling. Under these conditions (∼1 °C and pH 2), epimerization was not observed for 7 d (Figure S-4 and Table S-1 of the Supporting Information). During methyl esterification, F430 was heated at 40 °C for 3.5 h. However, this process does not affect the

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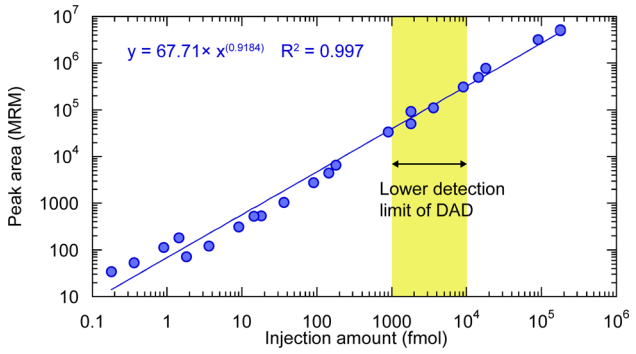

Figure 2. Regression curve for a plot of injection amount vs peak area obtained by LC–MS/MS analysis.

original concentration of F430 (Figure S-5 and Table S-2 of the Supporting Information), given that rapid methyl esterification of F430 takes place to produce more stable F430M.

During our entire experimental procedure, F430 is in contact with air and may generate 12,13-didehydro F430 (F560). Indeed, F560M-like peaks were detected by LC-DAD and LC–MS/MS analyses in the F430M fraction of the MBK sample (Figures S-6 and S-7 of the Supporting Information). However, given the signal intensity (<10% relative to F430M), the contribution of the F560-like compound is minor.

Therefore, we consider that epimerization and dehydrogenation during our experimental procedures has an insignificant effect on the quantification of primary F430 concentrations in our samples.

F430 Detection in Environmental Samples. Paddy fields account for ca. 10% of annual global methane emissions to the atmosphere.[30] In the Anjo and Chikugo fields, it has been reported that Methanomicrobiales, Methanosarcinaceae, Methanosaetaceae, and Methanocellales (rice cluster-I) were the predominant methanogens,and that the estimated total methanogenic population is 10^7 to 10^9 and 10^7 to 10^8 cells g^{-1} dry, based on real-time PCR analyses of the 16S rRNA gene and lipid biomarker analysis, respectively.[13−15] In the Kawatabi field, it has been reported that Methanomicrobiales, Methanosarcinaceae, Methanosaetaceae, Zoige cluster I, and Methanocellales (rice cluster-I) are the predominant methanogens and their population estimated from real-time PCR analysis of the *mcrA* gene, encoding α subunit of methyl-coenzyme M reductase, is 10^7 to 10^8 cells g^{-1} dry.[16] F430 was detected in all soils at concentrations ranging from 308 to 2019 fmol g^{-1} wet (Figure 3and Table 1), which equal from 496 to 2915 fmol g^{-1} dry based on water contents of 30.7% for Anjo E2, 33.5% for Kawatabi, 37.8% for Chikugo CF, and 45.7% for Chikugo RSC. This concentration range corresponds from 3×10^6 to 3×10^8 cells g^{-1} dry of methanogens. This is broadly consistent with the previously reported number of cells in these samples.

In anoxic marine sediments, biological methanogenesis is a crucial metabolic process, although its distribution and activity remain poorly understood.[27,28] In a sediment core collected from offshore Shimokita Peninsula, methanogens belonging to the genus Methanobacterium, Methanosarcina, Methanococcoides, and Methanobrevibacter have been isolated from sediment at a depth of 107 m using a newly developed continuous-flow bioreactor.[31] Furthermore, F430 has been detected using offline mass spectrometry at the same depth.[12] In the present study, we detected F430 from an adjacent

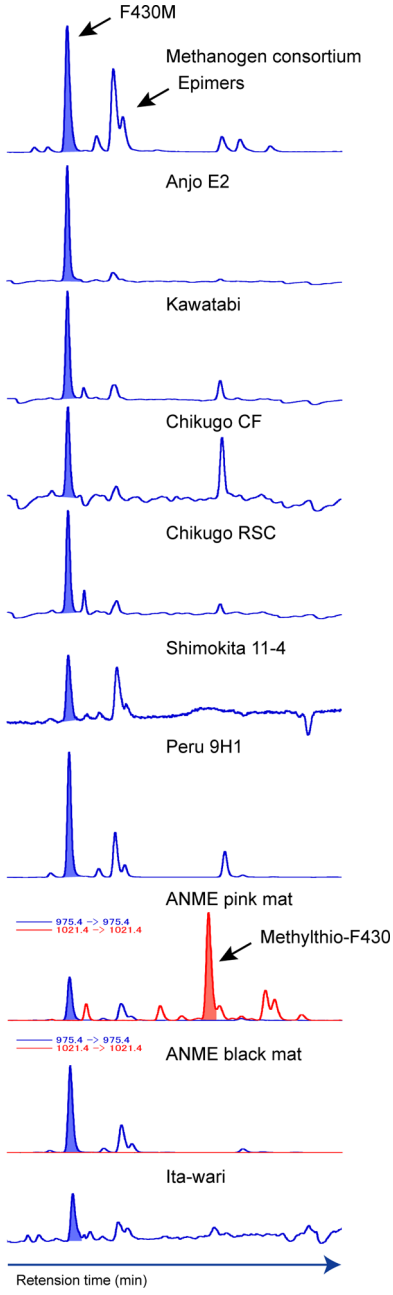

Figure 3. MRM chromatogram (975.4 → 975.4) of the F430M fractions from all samples. For the ANME pink (ANME-1 dominated) and black (ANME-2 dominated) mats, MRM chromatograms of (17^2S)-17^2-methylthio-F430 (1021.4 → 1021.4) are overlain.

sediment section (97 m depth; Figure 3) with a concentration of 63.3 fmol g^{-1} wet, which is consistent with a previous study (40 fmol g^{-1} wet),[12] although the offline analysis of this earlier study could not discriminate F430 from its epimers. In a sediment sample from Peru Margin (site 1229), methanogenesis locally occurs between double sulfate–methane interfaces, from which the presence of methanogens belonging to the orders Methanobacteriales and Methanosarcinales have been identified from analyses of rRNA and *mcrA*.[32] We detected 1915 fmol g^{-1} wet of F430 at 70 m depth. At this depth, the methane concentration reaches a maximum (2.6 mM).[18]

In the Black Sea, a chimneylike structure of a microbial mat is developed on the sea floor in which ANME is predominant.[33,34]

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The inner part of the structure (pink mat) is dominated by ANME-1, and the exterior (black mat) is dominated by ANME-2.[35] F430 and its homologue (17^2S)-17^2-methylthio-F430 associated with ANME were detected from all portions of this microbial mat.[8,9] We analyzed F430 separately from the inner pink and outer black mat. F430 concentrations were 0.83 nmol g^{-1} wet for the pink mat and 44 nmol g^{-1} wet for the black mat (Figure 3 and Table 1). Furthermore, (17^2S)-17^2-methylthio-F430 was only detected in the pink mat (Figure 3 and Figure S-8 of the Supporting Information). These findings strongly suggest that (17^2S)-17^2-methylthio-F430 is associated with ANME-1. The concentration of (17^2S)-17^2-methylthio-F430 in the pink mat was 2.3 nmol g^{-1}wet (Figure 3), which is consistent with a previously reported value (5 nmol g^{-1}wet).[8] The summed concentrations of F430 and (17^2S)-17^2-methylthio-F430 in both mats correspond with between 2×10^{10} and 3×10^{11} cells for the pink mat and between 3×10^{11} and 4×10^{12} cells for the black mat. These calculated cell numbers are broadly consistent with previously reported values obtained by FISH-based cell counting (4×10^{10} cells cm^{-3} for ANME-1; 1×10^{10} cells cm^{-3} for ANME-2).[11]

In the deep aquifer of Ita-wari, it has been reported that methanogens closely related to *Methanobacterium aarhusense*, *M. alcaliphilum*, and *Methanothermobacter thermautotrophicus* are dominant based on 16S rRNA analysis.[20] Active methanogenesis has also been indicated by anaerobic culture experiments.[20] The F430 concentration in this sample is 811 fmol L^{-1}, which corresponds to cell density from 5×10^3 to 8×10^4 cells mL^{-1} of methanogens. Although the cell density of methanogens in this sample was not determined, the calculated cell density is similar to the total microbial cell density of 3.0×10^5 cells mL^{-1} that was determined by the cell counting method using an epifluorescence microscope (see the Supporting Information).

CONCLUSIONS

We have developed a procedure for the quantitative analysis of coenzyme F430 by online LC−MS/MS. With zero-collision energy MRM analysis, subfemtomol detection of F430 was achieved. Application of the developed method enabled the detection of F430 from a wide range of environmental samples, including paddy soils, deep marine sediments, microbial mats, and anaerobic groundwater samples in which the presence of methanogens or ANME had been confirmed by previous studies. In particular, the detection of F430 from deep marine sediments indicates that our method is applicable to samples with low methanogen populations and complex inorganic and organic matrices. F430 concentrations in these samples are consistent with biomass associated with methanogens and ANME determined by other biological and geochemical techniques and demonstrates that F430 can be used as a diagnostic biomarker to understand the distribution and biomass of methanogens and ANME. Strictly, since F430 is a prosthetic group of MCR that catalyzes the last step reaction of methanogenesis, the F430 concentration represents the MCR concentration. Although we compared F430 concentration with biomass of methanogens and ANME, the concentration has the potential to reflect in situ methanogenic/anaerobic methane oxidation activity. Further study is required to clarify the relationship between F430 concentration and the rate of methanogenesis.

Because the artifacts produced during the experimental procedure is small, the measured F430 may represent the original concentration in the samples. However, we still know little of the F430 stability during the preservation for years at −20 °C, a typical storage temperature for geochemical samples. Appropriate condition for the sample preservation needs to be investigated in the future study.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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