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Spectrophotometric and Mass Spectrometric Properties of Lake Biwa Fulvic Acids, a Japanese Humic Substances Society Standard Sample

Masakazu Aoyama^{1*}, Yuko Sugiyama², Kosuke Ikeya³, and Nagamitsu Maie⁴

¹Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki 036-8561, Japan,

²Faculty of Science, Okayama University of Science, Okayama, Okayama 700-0005, Japan,

³Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601, Japan,

⁴School of Veterinary Medicine, Kitasato University, Towada, Aomori 034-8628, Japan.

Abstract

The fulvic acid (FA) sample isolated from the surface water of Lake Biwa has been authorized as a standard sample by the Japanese Humic Substances Society. The present paper aimed to provide information on their chemical characteristics, based on the diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy, ramp cross polarization/magic angle spinning (CPMAS) ¹³C nuclear magnetic resonance (NMR) spectroscopy with phase-adjusted spinning side bands (PASS) method, excitation-emission matrix (EEM) fluorescence spectroscopy in combination with statistical analysis using the parallel factor model (PARAFAC), and Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS). The DRIFT and ¹³C CP/PASS NMR spectra indicated high aliphaticity of the Lake Biwa FA. Its alkyl C accounted for 46% of total C with significant contribution of branched alkyl chains and aliphatic acids. On the contrary, the content of aromatic components such as phenolic compounds were smaller than soil FAs, which was also lined with a weaker fluorescence intensity compared to soil FAs. In EEM-PARAFAC, fluorescent component assignable to photorefractory or photo-degraded materials and that to microbially derived materials were relatively larger than those associated with soil FAs. In FTICR-MS using electrospray ionization (ESI) and negative mode detection, 2090 and 1369 peaks were detected and assigned to molecular formulas, respectively. Those were characterized by the large number of peaks including P and by the absence of molecular formulas similar to condensed aromatics and tannin. Although most of the molecular formulas had H/C and O/C ratios similar to lignin (67%) and aliphatics (26%), the number of molecular formulas similar to lignin was considered to be small because of the inclusion of N, P, or S in many of them and depending on the recent new criteria between lignin-like and aliphatic formulas.

Keywords: Aquatic humic substances, DRIFT, FTICR-MS, Fulvic acids, PARAFAC.

Introduction

Since 1991, the Japanese Humic Substances Society (JHSS) distributed two sets of humic acids (HAs) and fulvic acids (FAs) that were prepared from an Andosol (Inogashira, Shizuoka Prefecture) and a Cambisol (Dando, Aichi Prefecture) as standard samples. However, as aquatic FAs have frequently different chemical structures from soil FAs, the first series of standard HS samples were not necessarily appropriate to use as references for studies on aquatic HS. In 2010, the JHSS accredited an aquatic FA sample isolated from the surface water (at 50 cm depth) of Lake Biwa at Kitakomatsu in the north

basin as another standard sample. Lake Biwa (Shiga Prefecture) is the largest lake in Japan with surface area of 670 km² and lake volume of 275 km³. The water samples used for the isolation of the FA sample was collected in autumn, from the middle of October to the early November in 2001. Chemical properties including elemental composition, liquid-state ¹H and ¹³C NMR spectra, molecular size distribution based on high-performance size exclusion chromatography, and excitation-emission matrix (EEM) fluorescence spectra have been reported by Fujitake et al. (2009). Furthermore, Fujitake et al. (2012) investigated seasonal variation in the chemical properties of Lake Biwa FAs and observed that the O content, O/C

*Corresponding author: Faculty of Agriculture and Life Science
Hirosaki University, Hirosaki 036-8561, Japan. Tel. & Fax +81-172-39-3792, E-mail aoyamam@hirosaki-u.ac.jp

ratio, and relative abundance of methyl H and alkyl C with distinct alkyl C signals in the liquid-state ^{13}C NMR spectra decreased from spring to winter, when the absorbance at 600 nm per unit concentration was higher. The decreases in alkyl C and H, which were possibly originated from aquatic biota, could be attributed to their consumption during summer season. However, the quantity and quality of FAs and their seasonal variations are not likely identical throughout the basin (Aoki et al., 2004).

This paper is aimed at further characterizing the Lake Biwa FA sample using the latest popular spectrophotometric and mass spectrometric methods in this research field, including the diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy, ramp cross polarization/magic angle spinning (CPMAS) ^{13}C nuclear magnetic resonance (NMR) spectroscopy, EEM fluorescence spectroscopy in combination with statistical analysis using the parallel factor model (PARAFAC), and Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) with electrospray ionization (ESI).

DRIFT spectroscopy has a higher sensitivity than transmittance IR spectroscopy, because it has a longer optical path length due to repetitive interaction between diffuse reflectance and powdered sample. Quantitative spectra could be obtained by correcting the extended absorption intensity by minor functional groups using Kubelka-Munk transform (Childers and Palmer 1986) and deconvolution of overlapped peaks enhances the number of absorptions analyzed quantitatively (Kauppinen et al., 1981). Requirement of small amount of powder samples is also advantageous in analysis of fractionated samples (D'Orazio and Senesi, 2009; Karim and Aoyama, 2013; Aoyama, 2015). Quantitative detection of every C functional groups in HS in conventional solid-state ^{13}C NMR, ramp CPMAS, has been a problem for a long time. Recently, Johnson and Schmidt-Rohr (2014) proposed a new pulse sequence that can obtain spectra with similar quality to direct polarization by repeating short CP with ^1H spin-lattice relaxation periods (multiple CP). Another pulse sequence called "phase adjusted sideband suppressing (PASS)" in combination with ramp technique can also obtain quantitative 1-D spectra, in which spinning sidebands (SSBs) are separated completely in 2-D spectra and then their signal intensities were summed-up to those of inherent center peaks (Dixon, 1982; Antzutkin et

al., 1995).

EEM-PARAFAC and FTICR-MS are available only for liquid samples. EEM-PARAFAC was developed on the assumption that a set of EEM used for PARAFAC analysis is composed of common fluorescent components with different composition. Based on this assumption, EEM of each sample is deconvoluted using least-square method. In the studies of HS or dissolved organic matter (DOM), EEM are usually decomposed into four to ten fluorescent components and their variations are compared within the dataset. This method can take advantage of information in EEM which is otherwise difficult to use due to overlapping of several fluorophores, and is particularly useful for ecosystem studies (Jaffé et al., 2015). In contrast to EEM-PARAFAC, FTICR-MS can detect hundreds to thousands of HS components at the molecular formula level (Stenson et al., 2002). The data obtained are frequently plotted in H/C-O/C diagram, called van Krevelen diagram, and the composition is analyzed with comparing the H/C-O/C range of various organic matter groups (Hockaday et al., 2009).

Materials and methods

Samples used

The Lake Biwa FA sample prepared according to the method recommended by the IHSS (Fujitake et al., 2009) was distributed by the JHSS. As references, standard soil FA samples of JHSS, Inogashira Andosol FAs and Dando Cambisol FAs were also used in some of analyses.

DRIFT spectra

A 1 mg powdered sample was mixed with 100 mg KBr (FT-IR grade; Wako, Osaka, Japan) that was dried at 110°C for 2-3 h beforehand, mixed thoroughly on an agate mortar, and placed in an aluminum sample cup. The FTIR spectra were recorded on IR spectrometer (FT/IR-4100, JASCO, Tokyo, Japan) equipped with a diffuse reflectance accessory (DR-81, JASCO) in the range of 800-4000 cm^{-1} with resolution of 4 cm^{-1} . Data accumulation was repeated 100 times, and the averaged spectra were transformed into Kubelka-Munk units (Fery-Forgues and Lavabre, 1999). Fourier selfdeconvolution (FSD) was performed for the absorptions from 800 to 1800 cm^{-1} using JASCO FT-IR software provided with the spectrometer.

¹³C CP/PASS NMR spectrum

About 35 mg sample was packed in a 4 mmϕ Zirconium Oxide sample tube. ¹³C ramp CPMAS NMR spectrum with the conventional 2D-PASS sequence was recorded on an NMR spectrometer ECA700 (JEOL RESONANCE, Tokyo, Japan) with ¹³C resonating at 176 MHz under the following conditions: spinning rate, 9 kHz; pulse width, 5.8 μs for flip angle 90°; contact time, 1.0 ms; recycle delay time, 2 s; and total accumulation number, 10,400. Chemical shifts were adjusted based on the methyl peak (17.36 ppm) of hexamethylbenzene, an internal standard. The spectrum was then divided into 5 regions of 0-45 ppm (alkyl C), 45-110 ppm (*O*-alkyl C), 110-160 ppm (aromatic C), 160-190 ppm (carboxy C), and 190-220 ppm (ketone C), and the cumulated signal intensities in each region relative to total signal intensity was regarded as the relative content of each C functional group.

EEM-PARAFAC analysis

The EEM was measured at excitation (Ex) wavelength between 240-550 nm at an increment of 5 nm and emission (Em) wavelength between 290-600 nm at an increment of 2 nm using a spectrofluorometer (FluoroMax-4, Horiba Jobin Yvon, Longjumeau, France) equipped with a 150-W continuous output xenon arc lamp. Number of emission scans was 106. All the fluorescence spectra were acquired in the ratio mode, whereby the ratio of sample emission signals to reference excitation lamp output signals were calculated (Abe et al., 2011). The inner filter effect was corrected according to McKnight et al. (2001), and EEM was corrected for Raman scattering and background fluorescence by subtracting the spectra of an ultrapure water (Advantec RFU424CA, 18.3MΩ/cm). The EEM was normalized into Raman unit (RU) by Raman peak area of Milli-Q water at an excitation of 350 nm (Lawaetz and Stedmon 2009). PARAFAC analysis was performed for the region with excitation wavelength of 260 to 450 nm and emission wavelength of 290 to 518 nm with DOMFluor toolbox (Stedmon and Bro, 2008; Maie et al., 2014) on MATLAB software ver. 7.7 (MathWorks, Inc., Natick, MA, USA). The number of EEM dataset used in the PARAFAC analysis was 156, which were composed of 13 FA samples (8 samples were extracted with alkaline solutions from paddy soils and 5 samples were DOM) measured at different pH (from 1 to 12 by

one pH unit). The validity of the model was confirmed by split half analysis and Tucker's congruence coefficient (Stedmon and Bro 2008).

ESI FTICR-MS analysis

The FA sample was dissolved in water/methanol (1:1) containing 0.1% ammonium hydroxide (pH 8) and injected into an Apollo II ESI ion source (utilizing N₂ as the drying and nebulizing gas) of a 12 Tesla Apex Qe FTICR-MS (Bruker Daltonics, Billerica, MA, USA). The sample was introduced by a syringe pump at an infusion rate of 120 μL h⁻¹ and analyzed in negative ion mode with an *m/z* range of 200-2000. Ions were accumulated in a hexapole before being transferred to the ICR cell, where 300 transients collected with a 4 MWord time domain were co-added. Peaks were identified at an S/N ≥ 4. The mass spectra were externally calibrated with a polyethyleneglycol standard. The resulting mass spectra were internally calibrated using alkanolic acids and α,ω-alkanoic diacids naturally present in the samples, according to Sleighter et al. (2008). Molecular formulas were assigned to the peaks in the *m/z* range of 200-800 utilizing the Molecular Formula Calculator v.1.0 (©NHMFL) using C₁₋₁₀₀, H₁₋₂₀₀, N₀₋₇, O₀₋₅₀, S₀₋₃, and P₀₋₃ atoms, following rules outlined by Stubbins et al. (2010). The maximum error allowed between the actual and theoretically calculated *m/z* values was 1.0 ppm. If several formula candidates existed for a single peak, the formula having the least N, S, and/or P atoms was assigned (Kujawinski et al., 2009). Isotopic peaks containing ¹³C, which have a larger *m/z* value by 1.00335 (the mass difference between ¹³C and ¹²C), were removed prior to formula assignment. The double bond equivalent (DBE), which is the number of rings plus double bonds for a neutral molecular formula assigned to C_cH_nN_nO_sS_p, was calculated using the following equation (Bae et al., 2011):

$$\text{DBE} = c - h/2 + n/2 + p/2 + 1,$$

where the lowest valence state for all atoms is considered.

The assigned molecular formulas were examined using the van Krevelen diagram. The formulas plotted on the van Krevelen diagram were categorized by comparing seven square regions assignable to biomolecular compound groups: lipids (H/C = 1.5-2.0, O/C = 0-0.3), protein and other aliphatics (H/C = 1.5-2.2, O/C = 0.1-0.67), carbohydrates (H/C = 1.5-2.4, O/C = 0.67-1.2), unsaturated hydrocarbons

(H/C = 0.7-1.5, O/C = 0-0.1), lignin (H/C = 0.7-1.5, O/C = 0.1-0.67), tannin (H/C = 0.75-1.4, O/C = 0.67-0.85), and condensed aromatics (H/C = 0.2-0.7, O/C = 0-0.67) according to Hockaday et al. (2009) and Ohno et al. (2010).

Results and discussion

DRIFT spectroscopy

Figure 1a shows the DRIFT spectra of the JHSS standard FA samples. Absorptions at approximately 2850 and 2920 cm^{-1} due to aliphatic C-H were more conspicuous in the Biwa lake FAs compared to the soil FAs. In the spectra after deconvolution (Fig. 1b), the absorption peak due to carboxy C=O at approximately 1720 cm^{-1} was most intense in all the three samples, while the peaks at approximately 1670 and 1560 cm^{-1} related to amide group were more intense in the Lake Biwa FAs than in the soil FAs. In the Lake Biwa FAs, the peak at approximately 1600 cm^{-1} that could be assigned to aromatic C=C was scarce and the peaks at approximately 1510 and 1410 cm^{-1} that were considered to be derived from lignin and phenolic O-H, respectively, were weaker than the soil FAs. On the contrary, the peaks at approximately 1460 and 1380 cm^{-1} , which were derived from aliphatic C-H bending vibration and the C-H bending vibration of methyl group, were more conspicuous in the Lake Biwa FAs. Three absorption bands at around 1120, 1080, and

1030 cm^{-1} are possibly derived from polysaccharides. Although those were observed in all the samples, peak intensities were stronger in the Inogashira soil FAs and Lake Biwa FAs than in the Dando soil FAs. Tremblay and Gagné (2009) interpreted a higher absorption at 2960 cm^{-1} (CH_3) than that at 2930 cm^{-1} (CH_2) in the dissolved FAs in St. Lawrence estuary system, which was not observed in the dissolved HAs, as the reflection of the higher content of branched and relatively small aliphatic structures in the FAs. Similar trend was also observed in the Lake Biwa FAs in comparison with the soil FAs.

^{13}C CP/PASS NMR spectroscopy

The ^{13}C CP/PASS spectrum of the Lake Biwa FAs (Fig. 2) showed major peak maxima at 25 (methylene), 43 (methine or quaternary C), 79 and 84 (secondary alcohol or ether), 130 (aromatic C-C), and 174 (carboxy C=O) ppm. It was characterized by strong alkyl C signals and absence of methoxy and phenolic C suggesting lignin and/or tannin as a possible origin.

Table 1 shows the C composition as was estimated from ^{13}C CP/PASS NMR spectrum, together with that from liquid-state ^{13}C NMR spectra with inverse gated decoupling by Fujitake et al. (2009). Two data from different methods of ^{13}C NMR was not identical but similar to each other. Since the use of solid-state NMR is major in the study field of humic substances and other natural organic matter while liquid-state NMR is still useful in combination of various pulse sequences, the comparability of the results between the solid- and liquid-state NMR is of value to extend the knowledge on the FA structure in future studies. Major C component was alkyl C, which accounted for 46% of total C. On the contrary, the relative abundance of aromatic C was only 14%, which is smaller than those

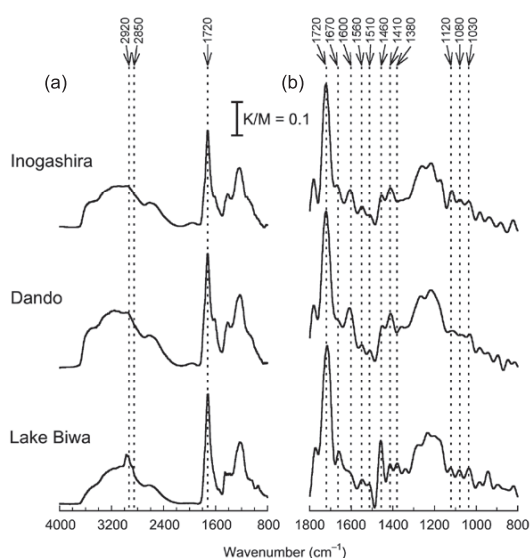


Fig. 1. DRIFT spectra of three fulvic acids before (a) and after (b) self-deconvolution.

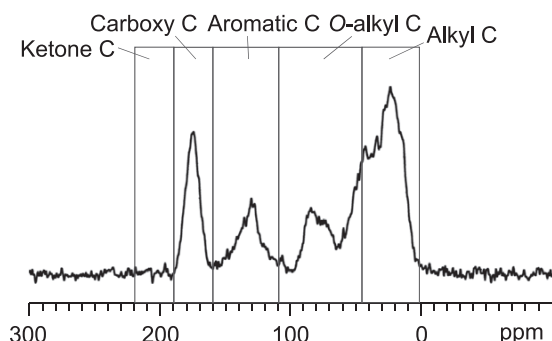


Fig. 2. ^{13}C CP/PASS NMR spectrum of Lake Biwa fulvic acids.

Table 1 Composition of C functional groups in the Lake Biwa fulvic acids as estimated from ^{13}C NMR (%)

Method	Alkyl C (0-45 ppm)	O-alkyl C (45-110 ppm)	Aromatic C (110-160 ppm)	Carboxy C (160-190 ppm)	Ketone C (190-220 ppm)
CP/PASS	45.7	24.3	13.6	16.0	0.4
IGD [†]	39.4	23.5	17.1 [‡]	14.3 [‡]	5.6

[†]Inverse gated decoupling in liquid-state ^{13}C NMR (Fujitake et al., 2009).

[‡]Aromatic C and carboxy C were divided at 165 ppm.

in the Inogashira and Dando FAs, 25-27%, estimated using the same method (Ikeya and Watanabe, 2016). The signal assignable to methylene C at around 25 ppm is generally small in soil FAs while those in 30-45 ppm derived from methine or quaternary C are common to soil FAs (Watanabe et al., 2004). Carboxyl rich alicyclic structures, of which presence in marine DOM and riberine DOM were suggested by Hertkorn et al. (2006) and Sleighter and Hatcher (2008), respectively, might contribute to the signals in 30-45 ppm. Such compounds have molecular formula similar to lignin, and the FTICR-MS result of the Lake Biwa FAs showed a number of molecular formula similar to lignin as described below. However, methoxyl C (50-60 ppm) and phenolic C (150-160 ppm) signals were lacking in the ^{13}C NMR spectrum, indicating least contribution of lignin to this FAs as was also suggested by Fujitake et al. (2009).

The data obtained by ^{13}C CP/PASS NMR analysis agreed to the results of DRIFT spectra. In addition, the C composition of the Lake Biwa FAs was quite similar to that of Pony Lake FAs (Brown et al., 2004; Cawley et al., 2013), one of which is accredited as the IHSS reference sample. This was outstanding even when the ^{13}C NMR spectra were compared among FAs from clear water rivers and lakes (Tsuda et al., 2010). Pony Lake is a coastal pond in Antarctica with a high dissolved organic matter content, and Pony Lake FAs are considered to be derived from microbial origin. Thottathil et al. (2013) suggested that FAs were produced in the deeper layers of Pony Lake and supplied to the surface layer based on the profile of concentration of humic-like fluorescent DOM.

EEM-PARAFAC analysis

Figure 3 shows the contour plots of 7 PARAFAC components decomposed by PARAFAC analysis. Peak position of each PARAFAC components and the relative abundance of each component are presented in Tables 2 and 3, respectively. Simply a lower value of the sum of 7 components in the Lake Biwa FAs,

11.5 RU $\text{mg}^{-1} \text{L}^{-1}$, than those in the Inogashira (41.2 RU $\text{mg}^{-1} \text{L}^{-1}$) and Dando (19.8 RU $\text{mg}^{-1} \text{L}^{-1}$) FAs, agrees to their aliphatic nature.

Fluorescence property of the Lake Biwa FAs was characterized by a higher contribution of C1 component assignable to photorefractory or photodegraded materials, followed by C7 component assignable to microbially derived materials. In contrast to the Lake Biwa FAs, the relative abundance of C7 component in the two soil FAs was very low. The opposite trend was observed for C3 and C5 components, which composed less than 10% of the sum of the 7 PARAFAC components in the Lake Biwa FAs. These two peaks are possibly associated with terrestrial FAs. Therefore, PARAFAC results demonstrated the difference in the major origins of the Lake Biwa FAs from the soil FAs.

Mostafa et al. (2005) suggested that a decrease in the FA-like fluorescence intensities of DOM in the lake epilimnion in summer was due to photodegradation of FAs in their 3-D EEM study of Lake Biwa DOM. Their suggestion was supported by a larger contribution of C1 component. Stubbins et al. (2014) investigated the relationship between each PARAFAC component and molecular formulas assigned in FTICR-MS for 22 DOM samples. Taking their results into account,

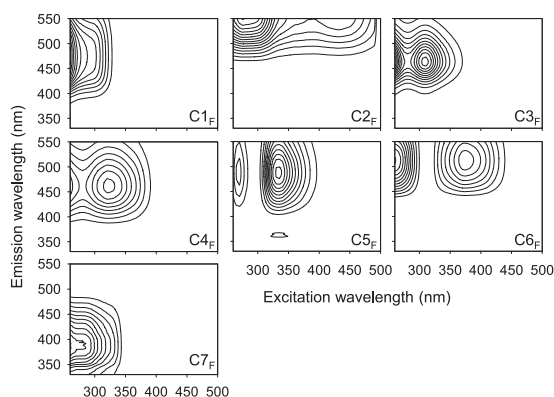


Fig. 3. Contour plots of seven fluorescence components decomposed by PARAFAC analysis.

Table 2 Seven PARAFAC components decomposed from fulvic acid dataset

Component	Excitation peak (nm)	Emission peak (nm)	Remarks
C1	<260	476	Peak A ¹⁾ ; Intermediate photo-degradation photoproducts ²⁾ ; Q2 (quinone-type) ³⁾
C2	<u>275</u> [*] , 430	550	Humic acid-type humic like peak ; SQ1 (Semi-quinone type) ³⁾
C3	<260, 310	464	Peak C ¹⁾
C4	<260, <u>320</u>	460	Peak C ¹⁾
C5	270, <u>335</u>	492	Peak C ¹⁾ ; C1 ³⁾
C6	<260, 375	512	Peak C ¹⁾
C7	<260	392	Peak M ¹⁾ ; C6 ³⁾

*Number with underline refers to peak with maximum peak intensity. ¹⁾ Coble 1996.

²⁾ Du et al. 2016, ³⁾ Cory and McKnight 2005

Table 3 Intensity (RU mg FA⁻¹ L⁻¹) and composition (%; in parentheses) of 7 PARAFAC components for the JHSS standard fulvic acid samples

Component	Inogashira	Dando	Lake Biwa
C1	14.49 (35.1)	3.67 (18.5)	2.50 (21.6)
C2	5.93 (14.4)	3.96 (20.0)	1.43 (12.4)
C3	6.31 (15.3)	4.13 (20.8)	1.13 (9.8)
C4	2.94 (7.1)	1.59 (8.0)	1.72 (14.9)
C5	5.17 (12.5)	2.49 (12.6)	0.80 (7.0)
C6	4.98 (12.1)	2.93 (14.8)	1.81 (15.7)
C7	1.41 (3.4)	1.05 (5.3)	2.15 (18.6)
Sum	41.2	19.8	11.5

C1 component, close to Peak A, may be associated with highly aromatic, high molecular weight, and low N molecular formulas, while C3 component, close to Peak C, may be associated with lignin-like compounds. The interpretation of Peak A may be reasonable because it was very strong in the Inogashira FAs from an Andosol. A smaller contribution of C3 to the Lake Biwa FAs also agrees to the results from the DRIFT and ¹³C CP/PASS NMR analyses.

ESI FTICR-MS

Figure 4a shows the ESI FTICR mass spectrum of the Lake Biwa FAs. Well resolution to assign peaks to chemical formulas was obtained as is shown in Fig. 4b. Peak intensities started to increase at m/z of around 240 to 370 and then decreased, with strong peaks at m/z of 255.2329 (C₁₆H₃₁O₂), 283.2642 (C₁₈H₃₅O₂), 368.9765 (unassignable), and 412.9661 (unassignable). The first 2 peaks could be assigned to palmitic and stearic acids, respectively.

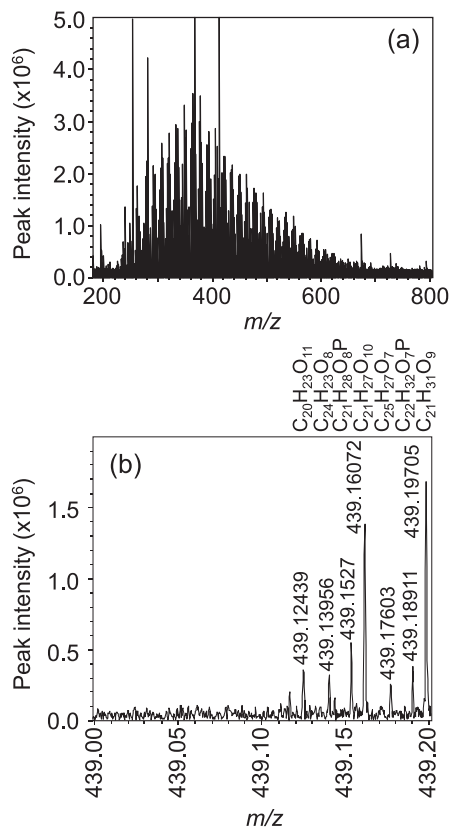
**Fig. 4.** Whole (a) and partial (b) of ESI-FTICR mass spectrum of Lake Biwa fulvic acids.

Figure 5a shows the van Krevelen diagram of the molecular formulas assigned and Table 4 summarizes the detection and assignment of peaks. Total number of peaks detected (2090) and that of formulas assigned (1369) were about two-thirds or half the number of them reported for the Dando and Inogashira soil FAs,

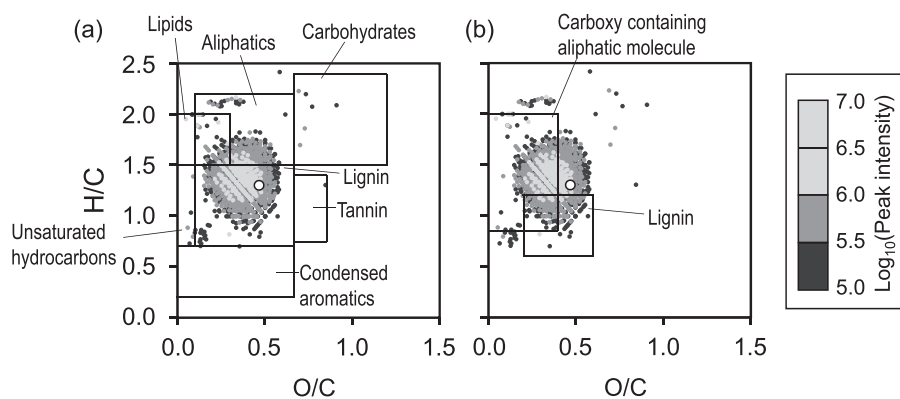


Fig. 5. van Krevelen diagram of the molecular formulas assigned to the FTICR mass spectrum of Lake Biwa fulvic acids. Open circle indicates the atomic H/C and O/C values (1.30 and 0.47) of the bulk sample according to Fujitake et al. (2009).

Table 4 Number of peaks/formula in the FTICR mass spectrum of Lake Biwa fulvic acids

Category	Number	Peak intensity ($\times 10^6$)
Total number of peaks ^a	2090	-
Number of peaks excluding contributions from ¹³ C and salt peaks ^b	1861	-
Number of formulas assigned	1369 ^c (74) ^d	n.c. ^e
Number of formulas consisting of C, H, O	922 ^c (50)	n.c.
Number of formulas consisting of C, H, O, N	104 ^c (5.6)	n.c.
Number of formulas consisting of C, H, O, N, S, P	343 ^c (18)	n.c.
Number of formulas similar to:		
Condensed aromatics	2 (0.2) ^f	0 (0) ^f
Lignin	920 (67)	594 (73)
Lipids, proteins, and other aliphatics	434 (32)	220 (27)
Carbohydrates	6 (0.4)	2 (0.3)
Tannin	1 (0.1)	< 1 (<0.1)
Others	8 (0.6)	3 (0.3)

^a In the m/z range of 200-750.

^b Peaks including ¹³C and salts were excluded because those are unassignable due to high mass defects.

^c Including the peaks having >1 and ≤ 2 ppm error between theoretical and observed m/z values.

^d Values in parentheses are proportions of the number of peaks belonging to each category to total number of peaks after ¹³C and salt peaks were excluded.

^e n.c., Not calculated.

^f Values in parentheses are proportions of the number of peaks or the sum of peak intensities belonging to each category to total of them assigned.

3358-3476 and 2549-2913, respectively (Ikeya et al., 2012). None of the peaks had molecular formulas similar to condensed aromatics, and only 1 peak had molecular formula similar to tannin. Number of formulas including N, 104, was one-fourth of the Dando and Inogashira soil FAs (419-470, revised as personal data). This result does not correspond to higher IR absorptions due to amide groups (Fig. 1).

However, the N content in the Lake Biwa FAs, 1.26 atomic-%, is not higher than that in the Inogashira soil FAs, 1.19 atomic-% (Fujitake et al., 2009), and fluorescent peak originated from aromatic amino acids was not detected in PARAFAC. Furthermore, it may be difficult to detect proteinaceous materials in FTICR-MS as well as polysaccharides. In fact, most of the formulas including N were distributed not in

the region of protein and other aliphatics but in the region of lignin in the van Krevelen diagram (data not shown). Thus, the content of proteinaceous materials in the Lake Biwa FAs may be higher only in the comparison to the soil FAs.

Most of the formulas assigned were concentrated in the area of the H/C-O/C combination of lignin and aliphatics including lipids in Fig. 5a and those occupied 67% and 32% of the total number of the formulas assigned, respectively (Table 3). However, these results do not indicate necessarily that the Lake Biwa FAs are rich in lignin-derived components, because the number of formulas similar to lignin was also almost two-third while that similar to aliphatics was 4 times of those in the Dando and Inogashira soil FAs (unpublished data) and some of them included N, P, or S. In addition, in some recent studies (e.g., DiDonate et al., 2016), 0.6-1.2 and 0.85-2.0 of H/C were used as the range indicating the molecular formulas similar to lignin-like and carboxy containing aliphatic molecules, respectively (Fig. 5b), and if followed this classification, many formulas in the lignin region in Fig. 5a are re-assigned to the carboxy containing aliphatic molecules. Thus, the Lake Biwa FAs could be strongly characterized by aliphatic molecular components. D'Andrilli et al. (2013) characterized the Pony Lake FAs by using FTICR-MS analysis comparing with the Suwannee River FAs as a reference and showed the higher proportion of N and S containing species and their assignment to protein-like and amino sugar-like components that corresponds to aliphatic region in Fig. 5a. They concluded that the Pony Lake FAs are consist of more labile and microbially derived material. In the Lake Biwa FAs, many P containing formulas were observed around a border of aliphatic and lignin region of the van Krevelen diagram (data not shown), which could be rather similar to the characteristic of the Pony Lake FAs than the Suwannee River FAs as shown by D'Andrilli et al. (2013).

Figure 6 shows the plots of carbon number and DBE for the Lake Biwa FAs, comparing with the plot area for the Suwannee River and Pony Lake FAs edited from D'Andrilli et al. (2013). The DBE values of the formulas in the Lake Biwa FAs dominantly ranged 0-14, with 20-21 as a minor group, while those in the Suwannee River and Pony Lake FAs ranged 0-17 and 0-22, respectively. The highr DBE values in the Lake Biwa and Pony Lake FAs were likely due

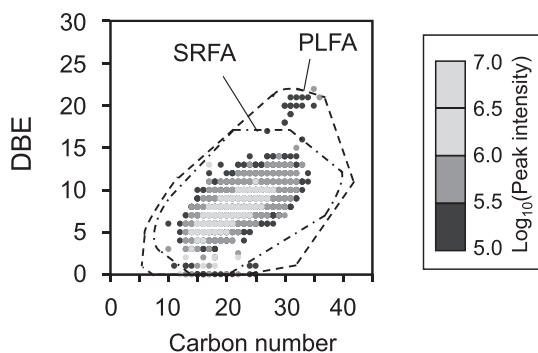


Fig. 6. The plots of carbon number and DBE for the Lake Biwa fulvic acids, comparing with the plot area for the Suwannee River (SRFA) and Pony Lake (PLFA) fulvic acids edited from D'Andrilli et al. (2013).

to unsaturated hydrocarbon or condensed aromatic compounds (Fig. 5a; D'Andrilli et al., 2013), but which is quite minor in the Lake Biwa FAs. In case of 2 soil FAs, DBE values ranged 0-24 and occurred mainly in condensed aromatic region (Ikeya et al., 2012). Therefore, molecular formulas with DBE values of >15 could have condensed aromatic components, but not in the Lake Biwa FAs.

Conclusion

The Lake Biwa FAs were characterized by high aliphaticity including branched alkyl chains and aliphatic acids. In association with small content of aromatic components, total fluorescence intensity was weak, for which the dominance of fluorescent components that are assignable to photorefractory or photo-degraded materials and microbially derived materials were suggested by EEM-PARAFAC. Few molecular formulas similar to condensed aromatics and tannin and a number of peaks including P in FTICR-MS agreed to the large contribution of microbially derived materials as source of the Biwa FAs. Although molecular formulas that have H/C and O/C ratios similar to lignin were also abundant as well as those from aliphatics, many of them were probably related not to lignin but to the acidic aliphatic molecules because of the presence of N, P, or S.

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