

## Article

# Ruthenium Tetroxide Oxidation Analysis of Soil Fulvic Acids

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## Abstract

Aromatic components in fulvic acids (FAs) prepared from a Dystric Cambisol (Dando) and an Umbric Andosol (Inogashira) were investigated using ruthenium tetroxide oxidation (RTO) technique. Frame of condensed aromatic components were estimated from the degradation products, mainly benzenepolycarboxylic acids (BPCAs), and compared with those from humic acids (HAs). Eight kinds of BPCAs with two to six carboxyl groups that correspond to naphthalene to coronene rings, and seven kinds of BPCAs having additional side chains were found in the degradation products from both the FAs and HAs. Sum of the yields of BPCAs from the FAs (20–21 and 41–42 mg g<sup>-1</sup>) was similar to that from the HAs (14–15 and 34–35 mg g<sup>-1</sup>) in the respective soils. Composition of polynuclear aromatic components based on that of BPCAs was similar between the two FAs and Inogashira HAs. Three monocarboxylic acids (C<sub>16</sub>, C<sub>18</sub>, and C<sub>24</sub>) were also detected in the degradation products from the FAs, while 24 or 25 compounds including C<sub>14</sub> to C<sub>30</sub> monocarboxylic acids and C<sub>16</sub> to C<sub>26</sub> dicarboxylic acids from the HAs. Sum of the yields of aliphatic compounds from the FAs (0.4–0.6 mg g<sup>-1</sup>) was ca. one-tenth of that from the HAs, suggesting that the polymethylene chains linked to a benzene ring and those bridging two benzene rings are minor structure in the FAs.

## Introduction

Humic substances are complex dark colored natural organic materials that are synthesized secondary from biomass constituents or their metabolites biochemically and/or chemically in environment. <sup>13</sup>C NMR investigations have indicated that aromatic components are one of major structural units of terrestrial humic substances (Mahieu et al., 1999; Watanabe et al., 2004). The aromatic C content in soil humic acids (HAs), the alkali-soluble and acid-insoluble humic substances, correlates to the degree of humification as assessed by the degree of darkening (Ikeya et al., 2004), which is probably related to the degradability of HAs in soil (Watanabe and Takada, 2006; Mao et al., 2007). Although structure of aromatic, especially condensed aromatic, components in humic substances has been clarified little, recently Kramer et al. (2004) suggested the presence of highly condensed and carboxylated aromatic structures in a highly-humified HAs using Fourier transform ion

cyclotron resonance mass spectrometry. Ikeya et al. (2007) suggested the presence of a series of polynuclear aromatic rings from naphthalene to perylene or coronene in various soil HAs using ruthenium tetroxide oxidation (RTO) technique.

RTO rapidly oxidizes aromatic rings substituted with electron-donating groups to CO<sub>2</sub>, while aromatic rings substituted with electron-withdrawing groups are unaffected (Ilsley et al., 1986). Polymethylene chains linked to benzene rings yield aliphatic acids (Murata et al., 2001). Condensed aromatic structures are changed into aromatic acids, mainly benzenepolycarboxylic acids (BPCAs), through the ring opening reaction followed by the production of carboxyl groups. Based on the number and position of carboxyl groups on BPCAs obtained upon RTO, the original polynuclear aromatic structures in coals (Blanc et al., 1991) and keogens (Yoshioka and Ishiwatari, 2003) were estimated. Ikeya et al. (2007) introduced this technique to soil HAs and reported that the yields of BPCAs correlated posi-

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tively to the degree of humification and aromatic C content, and that the degree of condensation of polynuclear aromatic components was also higher in the HAs with a higher degree of humification.

Although fulvic acids (FAs), the humic substances soluble in water at any pH, are brighter in color compared with HAs, the aromatic C content in FAs is within the range of that in HAs (Mahieu et al., 1999; Watanabe et al., 2004). Although pyrolytic (Ikeya et al., 2006) and classical oxidative degradation techniques (Griffith and Schnitzer, 1989) have yielded trace amounts of various substituted benzenes from FAs, kinds and amounts of polynuclear aromatic components are almost unknown (Hansen and Schnitzer, 1969). In the present study, we investigated structural units, including polynuclear aromatic components, of two soil FAs using RTO. The yield and composition of RTO products were compared with those from HAs in the same soils.

## Materials and methods

### Samples

The FAs and HAs accredited as standard samples by the Japanese Humic Substances Society were used. Those were prepared from the A-horizon of Inogashira Ando soil (Umbric Andosols; Fujinomiya, Japan) and Dando Brown Forest soil (Dystric Cambisols; Shitara, Japan) using IHSS method (Kuwatsuka et al., 1992).

### Ruthenium tetroxide oxidation

Fulvic or humic acids (15 mg) and 500 mg of sodium periodate were suspended in a mixture containing 2 ml of chloroform, 2 ml of acetonitrile, and 3 ml of water; 1–2 mg of ruthenium chloride  $\cdot n$ -hydrate was added and the suspension was stirred at 25°C for 24 hours. The mixture was then filtered through glass fiber filter (GB-140, ADVANTEC, Tokyo), and the residue on the filter was washed with dichloromethane (DCM), and then water. The filtrate and washings were combined and then separated into aqueous and organic phases.

The aqueous phase was dried at <40°C using a rotary evaporator, dispersed in diethyl ether, and methylated with diazomethane prepared from *p*-toluenesulfonyl-*N*-methyl-*N*-nitrosoamide. The methylation procedure was repeated for the residue until the yellow color of diazomethane could no longer be eliminated. The diethyl ether-soluble

products were combined and further treated with diazomethane. Then the methylated material was dehydrated, dried under N<sub>2</sub> gas flow, and weighed.

The organic phase was passed through a column packed with Celite 545 (mesh 30/60, GL Science, Fukushima, Japan) and anhydrous magnesium sulfate at a ratio of 1:1, dried under N<sub>2</sub> gas flow, and methylated with hydrochloric acid–methanol. Methylated material was extracted with *n*-hexane, dehydrated, dried, and weighed.

### Gas chromatography/mass spectrometry (GC/MS) and GC

The methylated derivatives of the water- and DCM-soluble materials were mixed with 70–80 µg of nonadecanoic acid methyl ester as internal standard and dissolved in 2 ml of DCM or *n*-hexane. An aliquot (1–2 µl) of the sample solution was injected (300°C) into a GC, HP5890 SERIES II (Agilent, Santa Clara, USA) with Ultra ALLOY<sup>+</sup>-5 column (Frontier Lab, Koriyama, Japan; 30 m length x 0.25 mm i.d. x 0.25 µm film thickness), or a GC/MS system, HP6890 (Agilent) with DB-5MS column (Agilent; 30 m length x 0.25 mm i.d. x 0.5 µm film thickness)–MStation-IMS700 (JEOL, Tokyo). The operating conditions were as follows: carrier gas, He; GC inlet mode, pulsed splitless (total flow, 8.0 ml minute<sup>-1</sup>; pulsed pressure, 250 kPa; pulsed time, 1 minute); column temperature, increasing from 50 to 300°C at a rate of 10°C minute<sup>-1</sup> while holding the final temperature for 20 minutes. Ionization mode in GC/MS was electron impact ionization (electron energy, 70 eV; ion source temperature, 250°C; mass range, *m/z* 50–500), and peaks were assigned by comparing the mass spectra and chromatograms with previous data (Ikeya et al., 2004, 2007). The detection mode in GC was flame ionization, and yields of the assigned compounds were determined using the internal standard method and normalized per gram of initial sample (mg g<sup>-1</sup>).

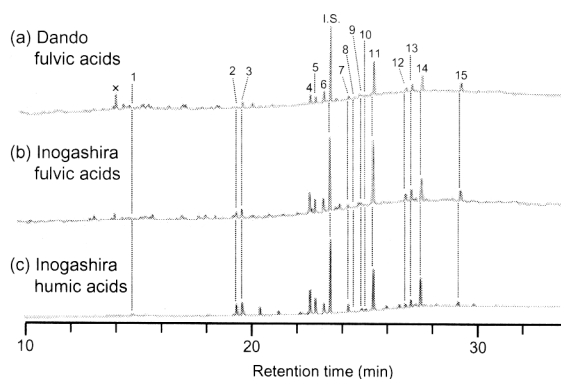
## Results and discussion

The yields of water- and DCM-soluble products from the FAs and HAs upon RTO following methylation are presented in Table 1. The yield of water-soluble products from Inogashira FAs, 744 mg g<sup>-1</sup>, was larger than that from Dando FAs, 451 mg g<sup>-1</sup>, as was the case of HAs (318 versus 229 mg g<sup>-1</sup>). Fulvic acids were initially water-soluble and probably had

**Table 1.** Yields of methylated derivatives of water-soluble and dichloromethane (DCM)-soluble products from fulvic and humic acids upon ruthenium tetroxide oxidation ( $\text{mg g}^{-1}$ )\*.

Fraction	--- Fulvic acids ---		--- Humic acids ---	
	Dando	Inogashira	Dando	Inogashira
Water-soluble	451	744	229	318
DCM-soluble	9	14	20	23

\* Ash free basis.



**Fig. 1.** Chromatograms of the water-soluble products obtained from Dando and Inogashira fulvic acids and Inogashira humic acids upon ruthenium tetroxide oxidation. Peak assignment is listed in Table 2. X, unknown. I.S., internal standard. Vertical scale is adjusted to the intensity of the internal standard, which is the same among the chromatograms.

**Table 2.** Yields of benzenepolycarboxylic acids (BPCAs) as their methyl esters (MEs) from fulvic and humic acids upon ruthenium tetroxide oxidation ( $\text{mg g}^{-1}$ )\*.

Peak No.	Compound	----- Fulvic acids -----		----- Humic acids -----	
		Dando	Inogashira	Dando	Inogashira
1	Benzene-1,2-dicarboxylic acid ME	0.6	0.4	0.1	0.4
2	Benzene-1,2,3-tricarboxylic acid ME	0.4	1.1	N.D.	1.9
3	Benzene-1,2,4-tricarboxylic acid ME	0.7	1.7	0.3	2.6
4	Benzene-1,2,3,4-tetracarboxylic acid ME	1.4	3.7	0.4	4.5
5	Benzene-1,2,4,5-tetracarboxylic acid ME	1.0	2.3	0.3	2.9
6	Benzene-1,2,3,5-tetracarboxylic acid ME	1.8	2.8	0.6	2.3
7	BPCA with additional side chains-1**	0.5-0.6	0.5-0.6	0.5-0.6	1.2-1.4
8	BPCA with additional side chains-2	0.3	N.D.	0.6-0.7	0.1
9	BPCA with additional side chains-3	1.0-1.1	1.9-2.2	0.7-0.8	0.6-0.8
10	BPCA with additional side chains-4	0.4-0.5	1.9-2.2	0.1	0.3-0.4
11	Benzenepentacarboxylic acid ME	5.2	12.2	1.8	7.8
12	BPCA with additional side chains-5	0.7-0.8	2.2-2.6	0.6-0.7	0.8-0.9
13	BPCA with additional side chains-6	1.2-1.3	2.3-2.7	0.9-1.0	1.4-1.7
14	Benzenhexacarboxylic acid ME	3.3	5.1	3.8	5.8
15	BPCA with additional side chains-7	1.6-1.9	2.4-2.8	3.1-3.6	1.4-1.7
Total		20-21	41-42	14-15	34-35

\* Ash free basis. \*\* BPCAs with a pair of methylene carboxyl and oxocarboxyl groups or three methylene carboxyl groups as side chains. N.D., not detected.

smaller molecular weights and larger carboxyl group content than HAs (Watanabe and Kuwatsuka, 1992). Thus, the high yield of water-soluble products following methylation from FAs may be affected by FA molecules/fragments that became soluble in DCM during the treatment with diazomethane. The yield of DCM-soluble products was smaller in FAs ( $9\text{--}14 \text{ mg g}^{-1}$ ) than in HAs ( $20\text{--}23 \text{ mg g}^{-1}$ ).

The FID-GC chromatograms of the water-soluble products upon RTO obtained from FAs are shown in Fig. 1. An overall peak intensity in the chromatogram of the water-soluble products from HAs was larger in Inogashira than in Dando sample. The former is also presented in Fig. 1. Assignment of peaks and yields of the assigned compounds are presented in Table 2, where peak number corresponds to that in Fig. 1. Eight kinds of BPCAs with two to six carboxyl groups and seven kinds of BPCAs with a pair of methylene carboxyl and oxocarboxyl groups or three methylene carboxyl groups as possible additional side chains (Ikeya et al., 2007) were observed. Kinds of aromatic compounds were similar between FAs and HAs. Potential origins with maximum numbers of aromatic nuclei for the eight BPCAs (Blanc and Albrecht, 1991; Mojelsky et al., 1992) are: naphthalene (benzene-1,2-dicarboxylic acid), 2-naphthyl benzene (benzene-1,2,4-tricarboxylic acid), benzonaphthene (benzene-1,2,3-tricarboxylic acid), anthracene (ben-

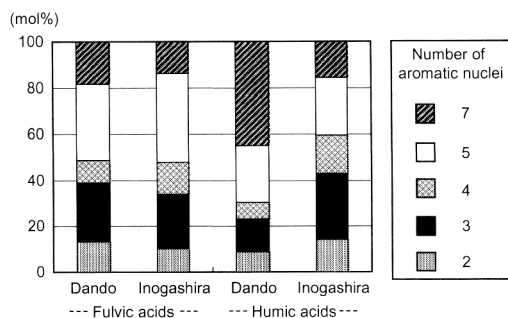
zene-1,2,4,5-tetracarboxylic acid), 2-benzonaphthenyl benzene (benzene-1,2,3,5-tetracarboxylic acid), pyrene (benzene-1,2,3,4-tetracarboxylic acid), benzo(cd) pyrene (benzenepentacarboxylic acid), and coronene (benzenehexacarboxylic acid). The sum of the yields of BPCAs including those with additional side chains, was larger in Inogashira FAs (41–42 mg g<sup>-1</sup>) than in Dando FAs (20–21 mg g<sup>-1</sup>; Table 2). The sum of the yields of BPCAs from the HAs, 34–35 (Inogashira) and 14–15 mg g<sup>-1</sup> (Dando), was similar to that from the FAs in each soil. Watanabe and Kuwatsuka (1991) suggested the presence of polynuclear aromatic structure in the Ando soil FAs based on a broad peak at a long wavelength in the pH12–7 ionization difference spectra. The difference in the yields of BPCAs between the two FAs agreed to their sugges-

tion.

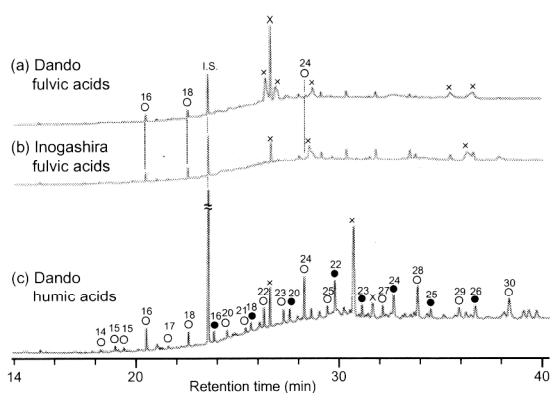
The composition of polynuclear aromatic components was calculated assuming that BPCAs were derived from condensed aromatic structures with maximum numbers of aromatic nuclei among the potential origins (Fig. 2). The estimated composition of polynuclear aromatic components was similar between the two FAs and Inogashira HAs, while the proportion of coronene was much higher in Dando HAs (47%) than in the other three samples (14–19%). Because the Dando HAs belong to Type P<sub>++</sub> that contain green fraction (Pg; Kumada, 1987), perylenequinone the chromophore of Pg may contribute as an origin of benzenehexacarboxylic acids.

Since the presence of carboxyl group on aromatic ring suppresses ring opening reaction in RTO (Ilsley et al., 1986), similar BPCAs to those derived from polynuclear aromatic rings without substitution by carboxyl group may be produced from aromatic acids with smaller numbers of aromatic nuclei. As FAs are abundant in carboxyl groups and BPCAs up to pentacarboxylic acids were detected from methylated derivatives of the Dando and Inogashira FAs by pyrolysis GC/MS (Ikeya et al., 2006), polycyclic aromatic acids might contribute to the high yield of BPCAs from FAs upon RTO. Although our results suggested the presence of condensed aromatic components having 2–7 aromatic nuclei in the FAs, there still be room for discussion about the similarity in their composition between FAs and HAs.

Figure 3 shows the FID-GC chromatograms of the DCM-soluble products from FAs. Only three peaks were assigned to C<sub>16</sub>, C<sub>18</sub>, and C<sub>24</sub> monocarboxylic acids with the amount of 0.1–0.3 mg g<sup>-1</sup> (Table 3), and none of dicarboxylic acids were detected. On the other hand, 25 (Inogashira) or 24 (Dando) peaks were assigned to the methylated counterparts of aliphatic monocarboxylic (C<sub>14</sub> to C<sub>30</sub>) and dicarboxylic (C<sub>16</sub> to C<sub>26</sub>) acids for the DCM-soluble products from HAs. The chromatogram of Dando sample is exhibited as representative in Fig. 3. The sum of the yields of aliphatic compounds was 4.9 (Inogashira) and 7.2 (Dando) mg g<sup>-1</sup> (Table 3), which accounted for 21 and 36% of the DCM-soluble products. As the possible original structures of the aliphatic monocarboxylic and dicarboxylic acids are polymethylene chains linked to a benzene ring and those bridging two benzene rings, respectively (Mojelsky et al., 1992; Yoshioka and Ishiwatari, 2003), such structures were considered to be minor in the FAs. Watanabe et al.



**Fig. 2.** Composition of polynuclear aromatic components estimated from the ruthenium tetroxide oxidation products of fulvic and humic acids.



**Fig. 3.** Chromatograms of the dichloromethane-soluble products from Dando and Inogashira fulvic acids and Dando humic acids upon ruthenium tetroxide oxidation. Numbers above peaks indicate the number of carbon in aliphatic acids. ○, aliphatic monocarboxylic acid methyl ester. ●, aliphatic dicarboxylic acid dimethyl ester. X, unknown. I.S., internal standard. Vertical scale is adjusted to the intensity of the internal standard, which is the same among the chromatograms.

**Table 3.** Yields of aliphatic acids as their methyl esters (MEs) from fulvic and humic acids by ruthenium tetroxide oxidation (mg g<sup>-1</sup>)\*.

Humic substances	Soil	- Monocarboxylic acids ME -				- Dicarboxylic acids ME -	Total
		C <sub>16</sub>	C <sub>18</sub>	C <sub>24</sub>	Others**	C <sub>16</sub> -C <sub>26</sub> ***	
Fulvic acids	Dando	0.1	0.2	0.1	N.D.	N.D.	0.4
	Inogashira	0.2	0.3	0.1	N.D.	N.D.	0.6
Humic acids	Dando	0.4	0.2	0.6	3.3	2.8	7.2
	Inogashira	0.5	0.3	0.3	2.5	1.4	4.9

\* Ash free basis.

\*\* Including C<sub>14</sub>-C<sub>15</sub>, C<sub>17</sub>, C<sub>20</sub>-C<sub>23</sub>, and C<sub>25</sub>-C<sub>30</sub> for Inogashira, and C<sub>14</sub>-C<sub>15</sub>, C<sub>17</sub>, C<sub>20</sub>-C<sub>23</sub>, C<sub>25</sub>, and C<sub>27</sub>-C<sub>30</sub> for Dando humic acids.\*\*\* Including C<sub>16</sub>, C<sub>18</sub>, C<sub>20</sub>, and C<sub>22</sub>-C<sub>26</sub>. N.D., not detected.

(2004) reported that the yields of aliphatic mono carboxylic and dicarboxylic acids released upon thermally-assisted hydrolysis and methylation tended to be higher in the HAs than in the FAs. Thus, the same trend was observed between the two degradation methods.

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