Article

Thermal treatment of mixtures of rice bran and casein: Influence of C/N ratio in starting materials on the structural features of humic acids in the compost-like materials

Hikari Kanno [1,2], **Junki Yonaiyama** [1], **Naoya Tachibana** [1], **Masami Fukushima** [1*]
and Shinya Kanno [2]

[1] Laboratory of Chemical Resources, Division of Sustainable Resources Engineering, Graduate School of Engineering, Hokkaido University, Sapporo 060-8628, Japan
[2] Doiso KET Institute Corporation, 9-joudori 2-chome 2346-1, Asahikawa 070-0039, Japan

Abstract

The C/N molar ratio of raw organic wastes (ROWs) was found to be an important factor in the production of a compost-like material (CLM) prepared using a thermal treatment system in conjunction with an organo-iron catalyst and a red loam as a bulking agent that includes 7.1% of iron, which can also serve as a co-catalyst. A mixture of rice bran and casein were used at the starting material. Using mixtures of these 2 components, starting materials samples with three different C/N ratios (10, 15 and 20) were prepared and their influences on the degree of humification of humic acids (HAs) in the resulting CLM were evaluated. The evaluations involved the determination of the levels of nitrogen-containing compounds and unsaturated carbon species, such as olefinic and aromatic moieties. In preparing the CLMs using the thermal treatment system, the starting material was incubated at 60 ºC for 5 days, and then heated at 170 ºC for an additional 3 days. A C/N ratio of 15 was found to be optimal for preparing a CLM comprised of HAs with higher levels of nitrogen-containing compounds, olefinic and aromatic moieties. While iron in the red loam (7.1 %) functioned as a catalyst for the humification process, adding the catalyst was advantageous for producing a CLM that contained higher levels of aromatized HAs.

Keywords: Rice bran, Casein, Thermal treatment, C/N ratio, Humic acid, Degree of humification

Introduction

During the maturation of compost, proteins, polysaccharides and lignins in organic raw wastes (ROWs) are converted into more stable dark-colored polymers, humic substances (Bernal et al., 2009). Humic substances play important roles in nitrogen fixation and supplying nutrients to plant in soil environments. In particular, humic acid (HA) fraction in humic substances can affect plant growth due to its large cation-exchange capacity (Eyheraguibel et al., 2008). The maturity of compost is related to the extent of humification for HAs, which can be evaluated by their chemical composition and the degree of darkening of the material (Tan, 2003). Although nitrogen contents in HAs increase in the early stage of humification, the oxygen contents increase with the extent of humification (Tsutsuki and Kuwatsuka, 1978; Kuwatsuka et al., 1978). In addition, the degree of darkening of HAs increases with the extent of humification (Kumada, 1955). An increase in the UV absorption of aqueous solutions of HAs with higher degree of humification can be attributed to the formation of chromophores, such as aromatic moieties, which are produced via the dehydration of carbohydrates (Fukushima et al., 2009a). In addition, structural investigations of HAs using FT-IR and ^{13}C NMR spectrometry can also provide useful information regarding the quality of compost and the degree of humification (Garcia et al.,

---

* Corresponding author: Tel. & Fax +81-11-706-6304, E-mail m-fukush@eng.hokudai.ac.jp

1991; Tomati et al., 2002; Makni et al., 2009; Zhu and Zhao, 2011). These types of data can be useful indices for evaluating the degree of humification for HAs in compost samples.

It has been reported that the compositions of ROWs, especially the C/N molar ratio, can affect the quality of the resulting compost (Nakasaki et al., 1992; Tomati et al., 1995; Huang et al., 2004; Kalamdhad and Kazmi, 2009; Silva et al., 2009). In microbial processes such as composting, the maturity of the compost increases with increasing nitrogen content in the starting material (*i.e.* ROW), because nitrogen is needed for microbial growth, while excess nitrogen in the starting material can cause a decrease in microbial activity as the result of the increase in pH caused by the generation of NH_4 (Nakasaki et al., 1992). Thus, in the composting technique, it is important to determine the optimum C/N ratio for producing compost that includes HAs with higher degrees of humification.

On the other hand, the authors developed a physicochemical method, based on a thermal treatment using red loam and an organo-iron catalyst to convert ROWs into compost-like materials (CLMs) (Kanno et al., 2011). This technique can immediately convert ROWs to CLMs including HAs with the higher degree of humification. In this method, the C/N ratio in the starting materials appears to affect the quality of CLM, the final product. However, the influence of the C/N molar ratio in the starting material on the quality of CLM has not been examined in the physicochemical treatments of ROWs. In the present study, a series of CLMs were prepared by mixing rice bran and casein as model ROWs for use in the thermal treatment process. The C/N molar ratios of the ROW were adjusted by altering the ratios of rice bran and casein. The influence of the C/N ratios of the starting materials on the degree of humification of HAs in the produced CLMs were then investigated by elemental analyses, pyrolysis-gas chromatography/mass spectrometry (py-GC/MS), UV-vis absorption, FTIR and solid-state CP-MAS ^{13}C NMR spectra.

Materials and Methods

Materials

Rice bran and casein were obtained from Asahikawa Syokuryo (Hokkaido, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. The red loam was purchased from Mori Sangyo, and was ground and then sieved through a stainless-steel sieve (2 mm). The carbon and nitrogen contents of these materials were as follows: rice bran, %C 45.9, %N 2.20; casein, %C 47.3, %N 13.9; red loam, %C 1.56, %N not detected. The organo-iron catalyst (Kanno 2000), which is like an iron(II)-acetate preparation including 4.0 % iron at pH 2.24, was obtained from the Daiso KET Institute (Hokkaido, Japan).

Preparation of CLM samples

Starting materials with three C/N molar ratios of 10, 15 and 20 were prepared by mixing appropriate amount of rice bran and casein. The starting materials (1 kg), the bulking agent (1 kg) and the accelerator (0.01 kg) were mixed. After the addition of water (2 L), the mixture was allowed to stand for 5 days at 60 ºC in an LC63-type incubator (Yamato Scientific Co., Ltd.). During the incubation, the mixture was stirred using a mixer twice daily. After incubating, the mixture was transferred to a commercial heating instrument to reduce the volume of wet waste (MS-N10, Panasonic Co., Ltd.). CLM samples were then prepared by heating at 170 ºC for 3 days. Because the mixture had dried during thermal treatment, water (1 – 2 L) was added daily, followed by mixing. After the heating process, dried CLM samples were obtained. Six CLM samples were prepared as follows: with the organo-iron catalyst, C/N = 10 (C/N10Fe), 15 (C/N15Fe), and 20 (C/N20Fe); without the catalyst, C/N = 10 (C/N10), 15 (C/N15), and 20 (C/N20).

Extraction of HAs

A dry CLM (10 g) was extracted with aqueous 0.05 M NaOH (100 mL) under a N_2 atmosphere. After a 1-day incubation with shaking, the mixture was centrifuged (10,000 rpm, 15 min), and the supernatant was then filtered through 5A filter paper (Whatman). The pH of the filtrate was adjusted to 1 by adding concentrated HCl, and the supernatant was then stirred for 24 h in the dark. The precipitated HA fraction was separated by centrifugation, and the precipitate was then dialyzed (molecular weight cut-off, 1 kDa) against pure water. The resulting slurry was freeze-dried, to give an HA sample in powdered form.

Analyses of HAs

Elemental compositions (C, H, N, S, and ash contents), FT-IR spectra and pyrolysis-gas chromatography/mass spectrometry (py-GC/MS) for the HA samples were carried out, based on previously reported procedures (Kanno *et al.* 2011). In the case of elemental analyses, the oxygen contents of the HA samples were calculated by subtracting the sum of C, H, N, S and ash contents from 100%.

UV-vis absorption spectra of HA samples (50 mg L^{-1}) in a buffer at pH 7 (0.1 M NaH_2PO_4/Na_2HPO_4) were obtained using a V-630 type spectrophotometer (Japan Spectroscopic Co. Ltd.) with a quartz cell (1 × 1 cm) at 25 °C. Solid-state CP-MAS ^{13}C NMR spectra were acquired on a MSL-300 (Bruker) spectrometer, equipped with a 4-mm CP-MAS probe. A 20 – 50 mg portion of powdered HA was packed into a 4-mm zirconium rotor. The acquisition parameters were as follows: spectral frequency, 75.47 MHz for ^{13}C and 300.5 MHz for ^{1}H; contact time, 1 ms; pulse delay, 6.0s; scan times, 8000; line broadening, 30 Hz.

Results and Discussion

Elemental compositions

Contents of carbon and HAs in the CLM samples are summarized in Table 1. The HA content for C/N10 was the largest value of all CLM samples. The content of HA, one of alkaline extractable fractions in the CLM sample, is not always relating to the qualities of CLMs (Veeken et al., 2000). Thus, structural features of HA samples were investigated by elemental analysis and spectroscopic methods.

The elemental composition of an HA is one of the indices used to determine the degree of humification. The results of the elemental analyses of the HA samples are summarized in Table 1. It is known that oxidation and polycondensation reactions of ROWs that occur during the humification processes can lead to a decrease in hydrogen and an increase in the oxygen and nitrogen (for early stage of humification) of HAs (Tsutsuki and Kuwatsuka, 1978; Kuwatsuka et al., 1978). The H/C, N/C and O/C molar ratios for the HA samples were calculated from the values in Table 1, and the relationships between N/C and H/C or O/C molar ratios were investigated (Fig. 1). The N/C ratio was larger and the H/C ratio was smaller in the HA samples prepared using a starting material with a C/N ratio of 15 (C/N15Fe and C/N15 in Fig.

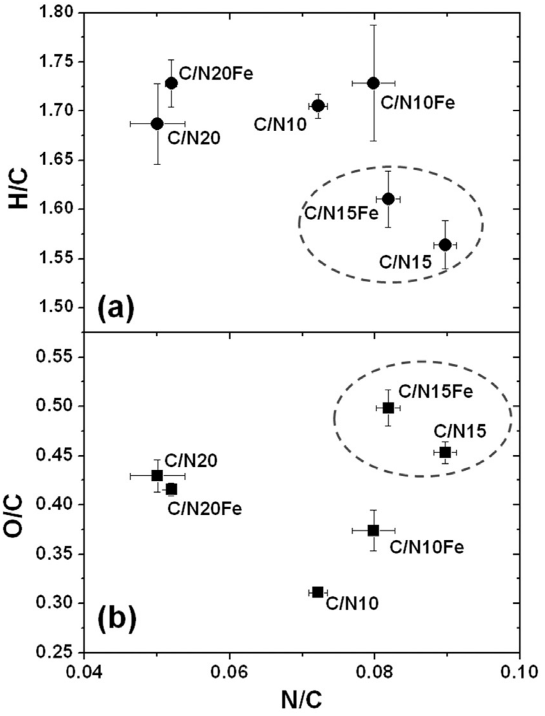

Figure 1. Relationships between N/C and H/C (a) or O/C (b) molar ratios in the HA samples.

Table 1 Carbon and HA contents in CLMs, and elemental compositions of HAs.

Samples	C in CLM (%)	HA in CLM (%)	Elemental composition of HA (%)					
			C	H	N	O	S	ash
C/N10Fe	56.14	23.8	55.0±1.1[a]	7.98±0.22[a]	5.12±0.16[a]	27.4±1.4[a]	0.41±0.01[a]	4.11±0.11[a]
C/N15Fe	50.67	29.7	50.6±0.4[a]	6.84±0.11[b]	4.83±0.09[b]	33.6±1.2[b]	0.34±0.02[b]	4.08±1.82[a]
C/N20Fe	49.77	17.5	49.5±0.3[b]	7.18±0.09[c]	3.00±0.04[c]	27.4±0.4[a]	0.54±0.01[c]	12.5±0.06[b]
C/N10	59.71	65.7	59.6±0.1[c]	8.53±0.06[d]	5.02±0.09[a]	24.7±0.4[c]	0.44±0.02[a]	1.75±0.47[c]
C/N15	52.21	25.1	51.5±0.8[a]	6.76±0.01[b]	5.39±0.04[d]	31.1±0.6[d]	0.35±0.01[b]	4.90±1.42[a]
C/N20	50.32	18.6	51.2±0.9[a]	7.25±0.12[c]	2.99±0.22[c]	29.3±1.0[e]	0.46±0.02[d]	8.84±0.25[d]

Superscripts represent significant differences, and "±" means standard deviations (*n* = 3).

1a). The increased level of nitrogen in the HA can be attributed to an enhanced incorporation of nitrogen-containing compounds, such as peptides and proteins, into unsaturated carbons via the nucleophilic addition reactions (Hsu and Hatcher, 2005). In addition, the decreased level of hydrogen indicates the formation of unsaturated carbons from carbohydrates via dehydration reactions (Fukushima et al., 2009a). On the other hand, in the HA samples for C/N15Fe and C/N15, the N/C and O/C ratios were both higher than those for C/N10Fe, C/N10, C/N20Fe and C/N20 (Fig. 1b). The increased levels of oxygen in the HA can be attributed to an increased level of oxygen-containing functional groups via the oxidation of carbohydrates (Tsutsuki and Kuwatsuka, 1978). These results suggest that an HA with a higher degree of humification can be produced, when the C/N molar ratio for the starting materials is 15.

Table 2 Spectroscopic parameters (absorptivity at 280 and 600 nm) of HAs.

Samples	E_{280}	E_{600}
C/N10Fe	$12.2±5.4^a$	$0.58±0.40^a$
C/N15Fe	$48.7±7.7^b$	$1.68±0.52^b$
C/N20Fe	$11.2±1.1^a$	$0.90±0.09^c$
C/N10	$16.9±3.5^c$	$0.93±0.11^c$
C/N15	$32.2±2.7^d$	$1.58±0.22^b$
C/N20	$16.0±2.2^c$	$1.16±0.39^c$

Superscripts represent significant differences, and "±" means standard deviations ($n = 3$).

The effect of the organo-iron catalyst was not so significant in the extent of humification based on elemental analysis data. As described previously (Kanno et al., 2011), in the case of CLMs that were prepared by the thermal treatment system in the presence of a red loam without the catalyst, resulted in the formation of HA samples with relatively higher levels of nitrogen and oxygen. The red loam, which is classified as a clay mineral, is an oxidation product of volcanic ash soil and contains transition metals (Bhakta and Munekage, 2010). The red loam used in the present study was originated from the loamy layer of the Kanto Region, and the compositions of inorganic elements were as follows: %Al 12.5, %Si 21.1, %Ca 0.36 and %Fe 7.06. It has been reported that soils derived from volcanic ash can enhance polycondensation reactions (*i.e.* humification) of humic precursors, such as catechol and glycine (Miura et al., 2009; Fukushima et al., 2009b). Transition metals in clay minerals, especially iron, can serve as Lewis acids and this would be expected to contribute to an enhanced humification (Miura et al., 2009; Fukuchi et al., 2010). Thus, iron in the red loam can serve as a Lewis acid in the production of CLMs that include HAs with a higher degree of humification.

Spectroscopic parameters

The increased absorbance in the UV region in the UV-vis absorption spectra of HAs can be attributed to the formation of chromophores, such as olefin and aromatic moieties, via the dehydration of carbohydrates. The absorptivity at 280 nm (E_{280}) is assigned to the UV absorption of the π-π* transition for C=C bonds and indicates the levels of olefinic and aromatic moieties (Traina et al., 1990; Chin et al., 1994). In addition, the degree of darkening in aqueous solutions of HA increased with the extent of humification (Kumada, 1955), and this can be indicated by the absorptivity at 600 nm (E_{600}). The degree of darkening increased with the extent of humification. The spectroscopic parameters, E_{280} and E_{600}, are summarized in Table 2. The E_{280} and E_{600} values were largest at a C/N ratio of 15 for the starting materials, suggesting the presence of higher levels of aromatic and olefin moieties and the progression of humification. In particular, the E_{280} value in the presence of the organo-iron catalyst (C/N15Fe) was significantly larger than that in its absence (C/N15), based on the standard deviations of analytical values ($n = 3$). These results suggest that the addition of the organo-iron catalyst was effective in the production of CLM including HAs with higher levels of unsaturated carbon species, such as olefinic and aromatic moieties.

Py-GC/MS

Information on HAs at the molecular level is critical for evaluating the quality of HAs in sediments. Py-GC/MS is a useful technique for obtaining detailed structural information related to organic molecules. The method is based on the thermal breakdown of HA macromolecules into a variety of subunits, which can then be separated by gas chromatography and identified by mass spectrometry. However, polar compounds such as

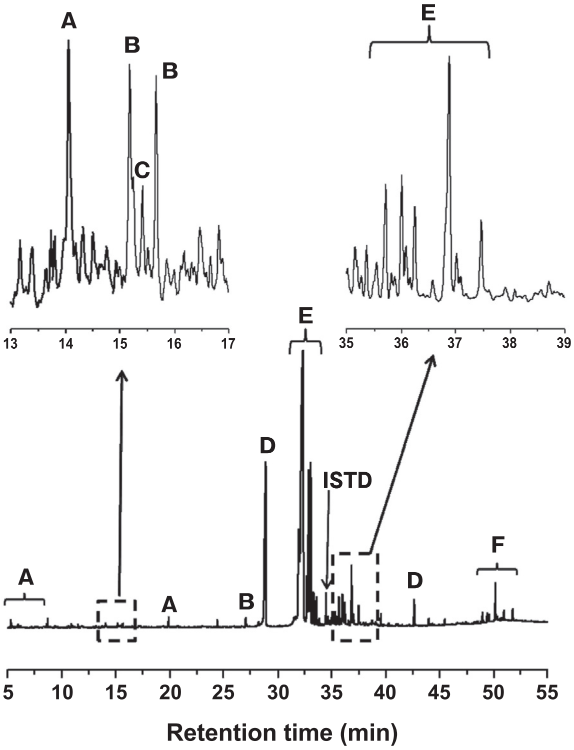

Figure 2. A typical pyrogram for a HA sample (C/N-15Fe). Groups of pyrolysate compounds: (A) nitrogen-containing compounds; (B) methoxy phenols and phenolic acids; (C) cyclic ether and alcohols; (D) saturated fatty acids; (E) unsaturated fatty acids; (F) sterols. ISTD means an internal standard, nonadecanoic acid methyl ester.

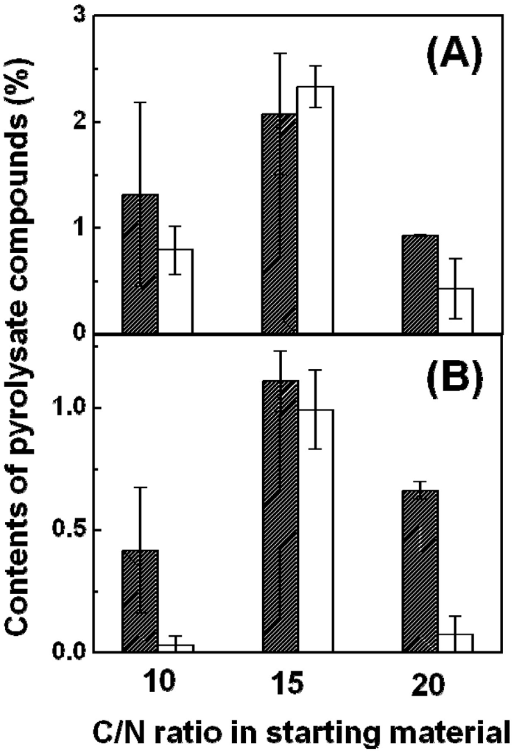

Figure 3. Contents of pyrolysate compounds (A) and (B) for HA samples.
▨ with catalyst, ☐ without catalyst.

phenols and carboxylic acids present in HAs are difficult to detect. To overcome this problem, TMAH may be used to cleave ester and ether linkages via thermochemolysis, with the formation of methyl esters of phenols and carboxylic acids (Fukushima et al., 2009c). Figure 2 shows a typical pyrogram of an HA sample (C/N15Fe). The pyrolysate compounds for each peak were identified by mass spectrometry, based on the previous reports on the py-GC/MS analysis of HAs from composts and sediments (Fukushima et al., 2009c; Fukushima et al., 2011). The pyrolysate compounds identified are summarized in Table 3. The pyrolysate compounds were further classified into groups as follows: (A) nitrogen-containing compounds; (B) methoxy phenols and phenolic acids; (C) cyclic ethers and alcohols; (D) saturated fatty acids; (E) unsaturated fatty acids; (F) sterols. As shown in Fig. 2, lipids (pyrolysate compounds, D – F) were the dominant structural moieties in the HAs (95 – 97% of all pyrolysate compounds) that were extracted from rice bran, as described in a previous report (Kanno et al., 2011). The largest peak for pyrolysate compound (E) appeared at a retention time of 32.3 min and was present in all HA samples (Table 3), and this peak was identified as the $C_{18:1\omega6}$ unsaturated fatty acid. The origin of this compound was likely unsaturated fatty acids in rice bran (Yoshida et al., 2011).

The nitrogen-containing pyrolysate compounds (A) can be attributed to the nucleophilic addition of nitrogen atoms in casein to olefinic carbons during the humification processes (Hsu and Hatcher, 2005). In general, the levels of nitrogen in HA increase in the early stage of humification (Tsutsuki and Kuwatsuka, 1978). Figure 3A indicates the contents of nitrogen-containing pyrolysate compounds (A) in HAs from CLMs that were prepared using a variety of C/N ratios. The levels of pyrolysate compound (A) at C/N = 15 were larger than those at C/N = 10 and 20. In addition, the levels of phenolic moieties

Table 3. Identified pyrolysate compounds, their base *m/z* values and classification.

R_t[a] (min)	Pyrolysate compounds	Base *m/z*	Classification[b]
5.76	*N,N*-dimethyl-3-octanamine	57, 86, 128	A
6.00	1-methoxy-4-methyl-benzene	91, 107, 122	B
8.83	6-(dimethylamino)-1-hexanol	58	A
9.50	buthylmethyl-carbamic acid methyl ester	58, 102	A
10.55	1,3-dimethyl-2,4(1*H*,3*H*)-pyrimidinedione	55, 83, 140	A
10.73	*N*-ethyl-*N*-nitroso-2-propanamine	116	A
11.00	4-methoxy-2,5-dimethyl-3(2*H*)-furanone	142	C
13.93	1-methyl-5-oxo-L-proline methyl ester	98	A
15.23	1,2,4-trimethoxybenzene	125, 153, 168	B
15.69	1,2,3,4-tetramethylmannose	71, 88, 101	C
15.81	1,2,3,4-tetramethoxybenzene	140, 183, 198	B
17.11	2-(1-oxopropyl)-benzoic acid methyl ester	163	B
19.60	n-$C_{12:0}$ fatty acid methyl ester	74, 87	D
20.63	3,4-dimethoxy-benzoic acid methyl ester	165, 181, 196	B
22.49	3-(4-methoxyphenyl)-2-propenoic acid methyl ester	133, 161, 192	B
24.43	n-$C_{14:0}$ fatty acid methyl ester	74, 87	D
27.08	3-(3,4-dimethoxyphenyl)-2-propenoic acid methyl ester	191, 207, 222	B
30.36	$C_{18:1\omega11}$ fatty acid methyl ester	74, 87, 98	E
31.53	$C_{18:1\omega9}$ fatty acid methyl ester	74, 87, 98	E
31.74	$C_{18:1\omega8}$ fatty acid methyl ester	74, 87, 98	E
32.30	$C_{18:1\omega6}$ fatty acid methyl ester	74, 87, 98	E
32.56	$C_{18:0}$ fatty acid methyl ester	74, 87	D
32.83	$C_{18:2\omega9,11}$ fatty acid methyl ester	81, 95, 109	E
32.98	$C_{18:2\omega9,12}$ fatty acid methyl ester	81, 95, 109	E
33.28	$C_{18:2\omega11,14}$ fatty acid methyl ester	81, 95, 109	E
33.79	$C_{23:1\omega13}$ fatty acid methyl ester	74, 87, 98	E
34.69	$C_{21:3\omega7,10,13}$ fatty acid methyl ester	69, 83, 97	E
35.71	$C_{21:1\omega11}$ fatty acid methyl ester	69, 98, 292	E
36.25	n-$C_{21:0}$ fatty acid methyl ester	74, 87	D
39.56	n-$C_{23:0}$ fatty acid methyl ester	74, 87	D
42.19	$C_{25:1\omega15}$ fatty acid methyl ester	74, 87	D
43.98	Farnesol isomers	81, 95, 109	F
44.08	n-$C_{26:0}$ fatty acid methyl ester	74, 87, 396	D
47.39	Gamma-sitsterol	381, 396, 414	F
48.96	Campesterol	367, 382, 400	F
49.39	Stigmasta-5,22-dien-3-ol	351, 369, 412	F
50.30	Fucosterol	271, 299, 314	F

[a] Retention time.

[b] Classification of pyrolysate compounds (A - H), as described in caption of Fig. 2.

[c] Internal standard.

(pyrolysate compound B) at C/N = 15 were slightly larger than those at C/N = 10 and 20 (Fig. 3B). The levels of phenolic moieties are related to the aromaticity of HAs, which can increase with the extent of humification. These results show that the degree of humification for HAs is optimal for a starting material with a C/N ratio of 15. It was noted, as shown in Fig. 3B, that the levels of phenolic pyrolysate compounds produced in the presence of the organo-iron catalyst were significantly larger than those in its absence. The phenolic moieties in HAs can be attributed to the products produced by the dehydration of carbohydrates during humification as well as phenolic moieties in the starting materials (Fukushima et al., 2009a). Thus, the presence of an organo-iron catalyst is effective in the sequestration of aromatic moieties to HAs.

Table 4 Compositions of carbon species and aliphatic (0 - 105 ppm)/aromatic (105 - 160 ppm) ratios, as estimated from solid-state CP-MAS ^{13}C NMR spectra of HA samples.

Chemical shift (ppm)	Assignments	Types of components	Composition of carbons (%)					
			C/N10Fe	C/N15Fe	C/N20Fe	C/N10	C/N15	C/N20
0 - 50	Aliphatic carbon atoms	Lipids	32.6	27.7	56.5	37.4	36.3	60.9
50 - 54	Methyl esters	Peptides	3.12	2.86	1.72	4.08	3.19	1.7
55 - 60	Methyl ethers	Lignin units	3.99	3.39	1.96	4.43	3.82	1.8
60 - 90	Aliphatic alcohols and ethers	Carbohydrates	33.7	35.4	16.2	24.5	28.4	13.4
90 - 105	Anomeric carbon atoms	Polysaccharides	4.85	6.58	2.99	4.18	4.28	2.22
105 - 135	Aromatic carbons attached to protons or other carbon atoms	Lignin units, tannins and flavonoids	8.6	10.2	10.1	10.8	9.05	8.85
135 - 160	Aromatic carbons attached to oxygen	Lignin units, tannins and flavonoids	3.91	5.09	3.42	5.05	4.36	4.14
160 - 180	Carbonyl carbons	Acids, esters and peptides	6.46	6.51	3.64	8.12	7.86	3.34
180 - 220	Carbonyl carbons	Quinones, ketones and aldehydes	2.82	2.28	3.44	1.58	2.72	3.7
Aliphatic/aromatic ratio			6.26	4.97	5.87	5.87	5.67	6.16

Solid-state CP-MAS ^{13}C NMR spectra

The compositions of carbon species in the HAs, as determined by solid-state CP-MAS ^{13}C NMR spectrometry, are summarized in Table 4. The spectral peaks were assigned, based on a previous report (Fukushima et al., 2007). The levels of aliphatic carbons (0 – 50 ppm) at C/N = 20 (C/N20Fe and C/N20 in Table 4) were much larger than those at C/N = 10 and 15, indicating that these HAs contained higher contents of lipids in HAs in the CLM produced in the presence of smaller amounts of casein. To evaluate the aromaticity of the HAs, the ratios of the sum of the peak integrations for the aliphatic region (0 – 105 ppm) to the aromatic region (105 – 160 ppm) were calculated. As shown in Table 4, the aliphatic/aromatic ratios for C/N15Fe and C/N15 were clearly smaller than those for the other samples. In addition, the aliphatic/aromatic ratio of the HA sample for C/N15Fe was much smaller than the corresponding values for the other samples. The lower aliphatic/aromatic ratio indicates a higher degree of aromaticity. Therefore, the starting material with a C/N ratio of 15 was optimal in terms of producing a HA with a higher aromaticity, and the presence of an organo-iron catalyst was effective in enhancing the aromatization of the HA. These results provide support for the trend for phenolic pyrolysate compounds shown in Fig. 3B and for the E_{280} values reported in Table 2.

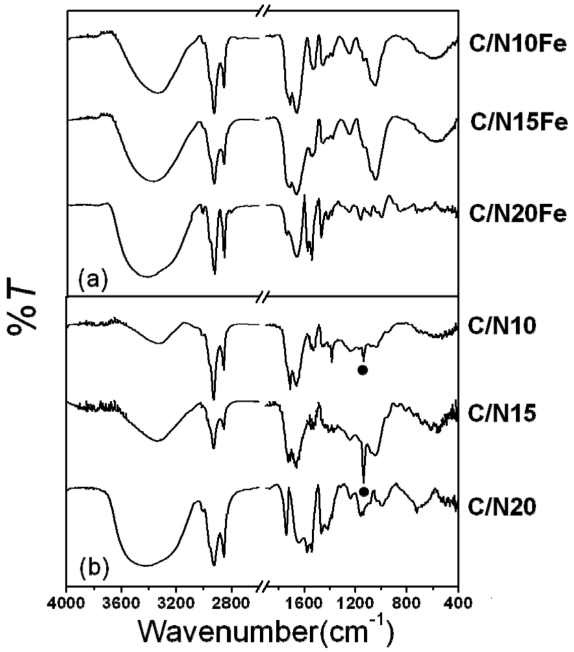

Figure 4. FT-IR spectra of HA samples.

FT-IR spectra

Figure 4 shows FT-IR spectra of the HA samples. The following peaks were assigned, based on previous assignments for HA samples from various origins (Fukushima et al., 1996; Yabuta et al., 2008):

the peak at 3400 cm^{-1} for phenolic O-H stretching and/or amine N-H stretching; two sharp peaks at 2900 – 2800 cm^{-1} for alkyl C-H stretching; the peak at 1600 cm^{-1} for aromatic C=C ring stretching; weak and broad peaks around 1450 cm^{-1} for aliphatic CH_3 and CH_2 bending; small peaks and shoulders at around 1220 cm^{-1} for C-O stretching and C-O-H deformation of COOH and phenols; broad peaks at 1200 – 1000 cm^{-1} for C-O stretching of alcohols and/or ethers. For the HA sample from the CLM produced in the absence of an organo-iron catalyst (C/N10 and C/N15), a sharp peak appeared at 1140 cm^{-1} (Fig. 4 ●). This peak, corresponding to the asymmetrical C-O-C stretching for aliphatic ethers (Silverstein and Webster, 1998), was observed as trace shoulders at 1200 – 1000 cm^{-1} for C/N10Fe and C/N15Fe, which were extracted from CLMs prepared in the presence of an organo-iron catalyst. These results indicate that the CLMs prepared using the red loam alone include HAs with a relatively aliphatic nature. These results support the conclusion that the use of an organo-iron catalyst is advantageous for preparing a CLM that contains HAs with a higher degree of aromatization.

In the present study, the indices of the structural features of HAs responsible for degree of humification were the levels of nitrogen and unsaturated carbon species. Aromatization of carbohydrates, such as polysaccharides in rice bran and the nucleophilic addition of nitrogen atoms in casein to unsaturated carbon species, such as olefinic and aromatic moieties, are well-known reactions that are associated with humification (Fukushima et al., 2009a; Hsu and Hatcher, 2005). In addition, the Maillard reaction, which involves the addition of nitrogen atoms to aldehyde carbons of polysaccharides in rice bran, also appears to be one of the important pathways in humification (Ikan, 1996). Casein contains amino acid residues, such as phenylalanine, tryptophan and tyrosine that contain aromatic groups (Alaimo et al., 1999). Thus, the levels of nitrogen and unsaturated carbon species in HAs may generally increase when the content of casein is increased (*i.e.* decreasing C/N ratio) in the starting materials. However, the nitrogen contents in the HA produced using a higher level of casein in the starting material (C/N10Fe and C/N10) were significantly lower than those for C/N15Fe and C/N15. That is, the optimal C/N ratio for the starting materials was determined for the production of CLMs by the thermal treatment system. Casein can strongly bind iron, and such chelation of metal ions can slow down the progress of the Maillard reaction (Ramonaityė et al., 2009; Guzun-Cojocaru et al., 2011). Thus, one of reasons for the decreased levels of nitrogen and unsaturated carbon species in HAs at C/N = 10 may be attributed to the deactivation of the organo-iron catalyst by forming chelates with casein. In addition, casein also adsorbs to iron on the surface of red-loam that can serve as catalytic sites for polycondensation reactions of humic precursors (Miura et al., 2009). This leads to the deactivation of surface iron on the red loam because of the coverage of catalytic sites.

Conclusions

The optimal C/N molar ratio in the starting material was determined, when the CLM containing HA with a higher degree of humification is prepared by a thermal treatment system using an organo-iron catalyst and a red loam. The degree of humification of the HA was evaluated by the levels of nitrogen-containing compounds and unsaturated carbon species such as aromatic and olefin moieties in the sample. Starting materials with three different C/N ratios (10, 15 and 20) were examined by varying the mixing ratios of rice bran and casein. When the nitrogen content in the starting material was the highest (C/N = 10), as well as the smallest nitrogen content (C/N = 20), the levels of nitrogen-containing compounds and unsaturated carbon species in HA samples were lower than those for C/N = 15. These results show that a C/N ratio of starting materials is one of important factors to prepare a CLM that contains HA with a higher degree of humification. It is noteworthy that, although the iron in the red loam served as the catalyst for the humification, the presence of an organo-iron catalyst was advantageous for preparing a CLM that contained HA with higher levels of aromatic and olefinic moieties. These results promise to be useful in the application of thermal treatment systems in the preparation of CLMs for the recycling of ROWs.

Acknowledgment

The authors are grateful for the financial support from the Mitsui & Co., Ltd. Environment Fund (R09-D033).

References

Alaimo, M.H., Farrell Jr., H.M. and Germann, M.W. (1999) Conformational analysis of the hydrophobic peptide α_{s1}-casein(136-196). *Biochim. Biophys. Acta* **1431**, 410-420.

Bernal, M.P., Alburquerque, J.A. and Moral, R. (2009) Composting of animal manures and chemical criteria for compost maturity assessment. A review. *Bioresource Technol.* **100**, 5444-5453.

Bhakta, J.N. and Munekage, Y. (2010) Mercury removal by some soils of Japan from aquatic environment. *Environ. Eng. Manage. J.* **9**, 503-510.

Chin, Y.-P., Aiken, G. and O'Loughlin, E. (1994) Molecular weight, polydispersity, and spectroscopic properties of aquatic humic substances. *Environ. Sci. Technol.* **28**, 1853-1858.

Eyheraguibel, B., Silvestre, J. and Morard, P. (2008) Effects of humic substances derived from organic waste enhancement on the growth and mineral nutrition of maize. *Bioresource Technol.* **99**, 4206-4212.

Fukuchi, S., Miura, A., Okabe, R., Fukushima, M., Sasaki, M. and Sato, T. (2010) Spectroscopic investigations of humic-like acids formed via polycondensation reaction between glycine, catechol and glucose in the presence of natural zeolites. *J. Mol. Struct.* **982**, 181-186.

Fukushima, M., Furubayashi, K., Fujisawa, N., Takeuchi, M., Komai, T., Ootsuka, K., Yamamoto, M., Kawabe, Y. and Horiya, S. (2011) Characterization of humic acids in sediments from dam reservoirs by pyrolysis-gas chromatography/mass spectrometry using tetramethylammonium hydroxide: Influence of the structural features of humic acids on iron(II) binding capacity. *J. Anal. Appl. Pyrolysis* **91**, 323-331.

Fukushima, M., Yamamoto, K., Ootsuka, K., Komai, T., Aramaki, T., Ueda, S. and Horiya, S. (2009a) Effects of the maturity of wood-waste compost on the structural features of humic acids. *Bioresource Technol.* **100**, 791-797.

Fukushima, M., Miura, A., Sasaki, M. and Izumo, K. (2009b) Effect of an allophanic soil on humification reactions between catechol and glycine: spectroscopic investigations of reaction products. *J. Mol. Struct.* **917**, 142-147.

Fukushima, M., Yamamoto, M., Komai, T. and Yamamoto, K., (2009c) Studies of structural alterations of humic acids from conifer bark residue during composting by pyrolysis-gas chromatography/mass spectrometry using tetramethylammonium hydroxide (TMAH-py-GC/MS). *J. Anal. Appl. Pyrolysis* **86**, 200-206.

Fukushima, M., Tanabe, Y., Morimoto, M. and Tatsumi, K. (2007) Role of humic acid fraction with higher aromaticity in enhancing the activity of a biomimetic catalyst, tetra(*p*-sulfonatophenyl)porphineiron(III). *Biomacromolecules* **8**, 386-391.

Fukushima, M., Tanaka, S., Nakamura, H. and Ito, S. (1996) Acid-base characterization of molecular weight fractionated humic acid. *Talanta* **43**, 383-390.

Garcia, C., Hernandez, T., Costa, F. and Polo, A. (1991) Humic substances in composted sewage-sludge. *Waste Manage. Res.* **9**, 189-194.

Guzun-Cojocaru, T., Koev, C., Yordanov, M., Karbowiak, T., Cases, E. and Cayot, P. (2011) Oxidative stability of oil-in-water emulsions containing iron chelates: Transfer of iron from chelates to milk proteins at interface. *Food Chem.* **125**, 326-333.

Hsu, P.-H. and Hatcher, P.G. (2005) New evidence for covalent coupling of peptides to humic acids based on 2D NMR spectroscopy: A means for preservation. *Geochim. Cosmochim. Acta* **69**, 4521-4533.

Huang, G.F., Wong, J.W.C., Wu, Q.T. and Nagar, B.B. (2004) Effect of C/N on composting of pig manure with sawdust. *Waste Manage.* **24**, 805-813.

Ikan, P. (1996) In: *The Maillard Reaction. Consequences for the Chemical and Life Sciences*. Wiley, New York, pp. 1-25.

Kalamdhad, A.S. and Kazmi, A.A. (2009) Rotary drum composting of different organic waste mixtures. *Waste Manage. Res.* **27**, 129-137.

Kanno, H., Tachibana, N. and Fukushima, M. (2011) Optimization of conditions for thermal treatment of rice bran using an accelerator including an organo-iron compound. *Bioresource Technol.* **102**, 3430-3436.

Kanno, S. (2000) Treating agent for converting garbage into soil, its production and treatment of garbage for conversion into soil. Japanese Patent (JP 2000-061427). The abstract is available via the internet at http://www.ipdl.inpit.go.jp/homepg.ipdl.

Kumada, K. (1955) Absorption spectra of humic acids. *Soil and Plant Food* **1**, 29-30.

Kuwatsuka, S., Tsutsuki, K. and Kumada, K. (1978) Chemical studies on soil humic acids I. Elementary composition of humic acids. *Soil Sci. Plant Nutr.* **24**, 337-347.

Makni, H., Ayed, L., Khedher, M.B. and Bakhrouf, A. (2010) Evaluation of the maturity of organic waste composts. *Waste Manage. Res.* **28**, 489-495.

Miura, A., Okabe, R., Izumo, K. and Fukushima, M. (2009) Influence of the physicochemical properties of clay minerals on the degree of darkening via polycondensation reactions between catechol and glycine. *Appl. Clay Sci.* **46**, 277-282.

Nakasaki, K., Yaguchi, H., Sasaki, Y. and Kubota, H. (1992) Effects of C/N ratio on thermophillic composting of garbage. *J. Fermentation Bioeng.* **73**, 43-45.

Ramonaitytė, D.T., Keršienė, M., Adams, A., Tehrani, K.A. and Kimpe, N.D. (2009) The interaction of metal ions with Maillard reaction products in a lactose-glycine model system. *Food Res. Int.* **42**, 331-336.

Silva, M.E., Lemos, L.T., Cunha-Queda, A.C. and Nunes, O.C. (2009) Co-composting of poultry mature with low quantities of carbon-rich materials. *Waste Manage. Res.* **27**, 119-128.

Silverstein, R.M. and Webster, F.X. (1998) In: *Spectroscopic Identification of Organic Compounds (6-th edition)*. Wiley, New York, pp.90-91.

Tan, K.H. (2003) In: *Humic Matter in Soil and the Environment. Principles and Controversies,* Dekker, New York, pp.75-126.

Tomati, U., Belardinelli, M., Andreu, M., Galli, E., Capitani, D., Proietti, N. and De Simone, C. (2002) Evaluation of commercial compost quality. *Waste Manage. Res.* **20**, 389-397.

Tomati, U., Galli, E., Pasetti, L. and Volterra, E. (1995) Bioremediation of olive-mill wastewater by composting.

Waste Manage. Res. **13**, 509-518.

Traina, S.J., Novak, J. and Smeck, N. (1990) An Ultraviolet absorbance method of estimating the percent aromatic carbon content of humic acids. *J. Environ. Qual.* **19**, 151-153.

Tsutsuki, K. and Kuwatsuka, S. (1978) Chemical studies on soil humic acids II. Composition of oxygen-containing functional groups of humic acids. *Soil Sci. Plant Nutr.* **24**, 547-560.

Veeken, A., Nierop, K., Wilde, V.D., Hamelers, B. (2000) Characterization of NaOH extracted humic acids during composting of a biowaste. *Bioresource Technol.* **72**, 33–41.

Yabuta, H., Fukushima, M., Kawasaki, M., Tanaka, F., Kobayashi, T. and Tatsumi, K. (2008) Multiple polar components in poorly-humified humic acids stabilizing free radicals: Carboxyl and nitrogen-containing carbons. *Org. Geochem.* **39**, 1313-1335.

Yoshida, H., Tanigawa, T., Yoshida, N., Kuriyama, I., Tomiyama, Y. and Mizushina, Y (2011) Lipid components, fatty acid distributions of triacylglycerols and phospholipids in rice brans. *Food Chem.* **129**, 479-484.

Zhu, Y. and Zhao, Y. (2011) Stabilization process within a sewage sludge landfill determined through both particle size distribution and content of humic substances as well as by FT-IR analysis. *Waste Manage. Res.* **29**, 379-385.