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# Structural changes in lipid-free humic acids during composting of sewage sludge

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## Abstract

Structural changes in humic acids (HAs), extracted after lipid removal from sewage sludge during composting, were investigated using various chemical methods (elemental analysis, Fourier transform infrared spectroscopy and <sup>13</sup>C-nuclear magnetic resonance (NMR) spectroscopy). Compared to non-purified HAs, lipid-free HAs (LFHAs) exhibit higher C and N contents and high absorbance around 1652, 1540 and 1230 cm<sup>-1</sup>, which indicates the intensity of the etherified aromatic structures and nitrogen-containing components. Less absorbance around 2920, 1600, 1414 and 1100 cm<sup>-1</sup> could be assigned to their low level of aliphatic compounds, mainly those with a carboxyl group. According to <sup>13</sup>C-NMR spectroscopy, almost 45% of aliphatic structures are removed by lipid extraction and these correspond mainly to long-chain fatty acids. During composting, significant decomposition of non-substituted alkyl structures and N-containing components occurred, increasing the relative intensity of etherified aromatic structures.

*Keywords:* Lipid-free humic acids; Sludge; Composting; Etherified aromatic structures

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## 1. Introduction

Composting is a useful method of producing from sludge waste a stabilized product that can be used as a source of nutrients and soil conditioner in the field. Compost has the advantage of improving soil structure and fertility by increasing the proportion of stable humic substances in the soil (Hsu and Lo, 1999). However, harmful effects can sometimes arise after application of unmaturing composts with incomplete stabilization or biotransformation of the organic fraction into stable humic substances (Mathur et al., 1993; Déportes et al., 1995). The content and structure of the humic substances in compost have been investigated in numerous studies as the main indicators for assessing compost maturity (Garcia et al., 1992; Ouatmane

et al., 2000; Tomati et al., 2000). However, the structure of humic material is still controversial. Some studies suggest the predominance of aromatic units in humic substances, whereas others have shown that many humic extracts contain largely aliphatic structures. Many factors, such as the origin of humic material, the extraction technique and the purification methods are responsible for discrepancies found (Gonzalez-Vila et al., 1983). Some studies are based on the hypothesis reported by Schnitzer and Khan (1972) and Schnitzer et al. (2000) that humic acids (HAs) could be assumed to be highly cross-linked aromatic polymers of high molecular weight with covalent carbon-carbon, ether and ester-linkages connecting the substituted aromatic moieties. Others support humic substances as groups of similar particles which are associated by weak covalent and non-covalent bonds to “homogeneous” aggregates, which in unfractionated humic substances form large mixed aggregates (Wershaw et al., 1977; Piccolo, 1997).

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In sludge humic structures, dicarboxylic and fatty acids were reported to be the most abundant compounds (Hernandez et al., 1990). Fatty acids or free lipids are not considered to be true constituents of humic structures by some authors, e.g. Réveillé et al. (2003). Lipids have been shown to be present as an admixture, or held by non-covalent bonds to humic macromolecules. Piccolo et al. (1990) and Preston and Schnitzer (1984) suggested that fatty acids occur in humic substances partly as phenolic esters and partly adsorbed by weaker forces such as H-bonding and van der Waals forces. They also disturb chemical and spectroscopic analysis of humic substances. The  $^{13}\text{C}$ -nuclear magnetic resonance (NMR) spectra of humic substances are usually reported to exhibit a good signal/noise ratio, but to be poorly resolved because of their heterogeneous nature and their tendency to aggregate or to form micelle-like structures. Other causes for poor resolution, such as the presence of paramagnetic, organic-free radicals or metal ions, were not found to have excessive influence on line broadening in humic substances spectra (Preston and Schnitzer, 1987). Therefore, purification is needed to eliminate the weakly bound long alkyl chains from the raw humic material. It removes the extractable monomers or fragments that interfere with the analysis of the main macromolecular structure of humic substances. Purification permits the isolation of the more homogenous structures and provides better representative information about the true changes of humic macromolecular structure when using various chemical and spectroscopic techniques (elemental analysis, Fourier transform infrared analysis [FTIR] and  $^{13}\text{C}$ -NMR spectroscopy). Gonzalez-Vila et al. (1999) suggest that a systematic study of organic material of compost should include the characterization of both the colloidal organic fraction (which conforms to the operationally defined compost humic substances) and the “extractives” (lipids and water-soluble products). Therefore, this study was carried out in order to compare the HAs extracted from uncomposted sludge after lipid removal, lipid-free HAs (LFHAs 0), or without lipid removal (HAs 0). The structural changes in LFHAs were also investigated during the course of composting. Elemental analysis, FTIR and  $^{13}\text{C}$ -NMR were used for analysis, because information gained from each analytical technique is important and because individual techniques complement each other. When used together, they provide an unbiased analysis rather than the selectivity that each individual technique may impart (Chefetz et al., 2002; Hafidi et al., 2005).

## 2. Materials and methods

### 2.1. Composting

Sewage sludge from an anaerobic lagoon in an experimental wastewater treatment plant in Marrakech

city (Morocco) was mixed (90:10) with straw and composted in a heap for 6 months on a purpose-built platform. To provide aerobic conditions the mixture was turned every 15 days. The progress of composting was followed by monitoring the temperature. This peaked at  $52\text{ }^{\circ}\text{C}$  after 7 days of the stabilization phase and then decreased to  $32\text{ }^{\circ}\text{C}$  during the maturation phase (Amir and Hafidi, 2001). Sampling was carried out at different stages during composting. These were at the initial mixing stage, after 1 month and after 6 months.

### 2.2. Purification pre-treatments

Free lipids were removed before extraction of humic substances using a 2:1 chloroform–methanol mixture (Lichtfouse et al., 1998). Lipid extraction was carried out three times at  $4\text{ }^{\circ}\text{C}$  using 15 g fresh samples with 120 ml solvent mixture.

These pre-treated samples were then subjected to evaporation to remove remaining solvent, and they were then washed three times with water to remove other non-humic water-soluble molecules, such as sugars and proteins, which might interfere with the analysis of the humic substances.

### 2.3. HA extraction

Extraction of humic substances from the purified samples in 0.1 M NaOH was repeated several times until colourless solutions were obtained. After centrifugation and filtration, the solutions were cooled and precipitated by acid treatment with 3 M  $\text{H}_2\text{SO}_4$  at  $4\text{ }^{\circ}\text{C}$  for 24 h. The precipitated HAs were separated from the fulvic acid solution by filtration and then re-dissolved in 0.1 M NaOH. The HA solutions obtained were freeze-dried after dialysis using a Spectra-por membrane (1000 Da) to eliminate excess salts. The HA content was calculated after freeze-drying a known volume.

### 2.4. Humic acid analysis

The chemical structure of the HAs was investigated by various techniques: elemental analysis was performed for C, H, O and N on a Carlo Erba EA 1112 analyser. E4/E6, the ratio of the absorbance of 2 mg freeze-dried HAs in 25 ml 0.025 M  $\text{NaHCO}_3$ , was measured at 465 and 665 nm using a U.V. Unicam SP 1800 spectrophotometer. FTIR spectra were recorded from KBr pellets (250 mg dried KBr and 2 mg freeze-dried HAs pressed under vacuum) with a FTIR Perkin Elmer 1600 spectrophotometer over the  $4000\text{--}400\text{ cm}^{-1}$  range, at a rate of  $16\text{ nm s}^{-1}$ .  $^{13}\text{C}$ -NMR spectra with 1H broadband decoupling were recorded at 75.469 MHz on a Bruker AM WB 300 MHz spectrometer. The solution was prepared by dissolving 100 mg HAs in 3 ml 0.5 M NaOD ( $\text{NaOH}/\text{D}_2\text{O}$ ). The spectra were obtained using inverse-gated-decoupling to suppress

nuclear Overhauser enhancement and provide quantitative results (Preston and Schnitzer, 1984). Acquisition time was 0.98 s, relaxation delay 1.8 s, pulse of 35°, total acquisition time 72 h. Free induction decays were processed by applying 50 Hz line broadening and baseline corrections (Wilson et al., 1983). Chemical carbon distribution was estimated from the ratio of integrated areas of the spectrum to the whole spectrum area: 0–55 ppm (alkyl carbon), 55–110 ppm (alkyl carbon substituted by oxygen or nitrogen), 110–165 ppm (aromatic carbon), and 165–200 ppm (carboxylic and amide carbon).

### 3. Results and discussion

#### 3.1. Elemental analysis

The elemental composition of unpurified HAs and LFHAs extracted at different stages of composting are illustrated in Table 1. HAs extracted from initial sludge after lipid removal, LFHAs 0, when compared with HAs extracted without lipid pre-purification (HAs 0), show relatively higher C and N contents and C:H ratios, but lower contents of O and H, and C:N and O:C ratios. This could be attributed to their high content of aromatic structures and nitrogen-containing components, owing to an increase of their intensity following the removal of lipids, mainly long-chain fatty acids. Piccolo et al. (1990) found that the percentage of N increased in the humic structures isolated after purification. The low content of O is attributed to loss of carboxyl groups of fatty acids. During composting, a decrease in C, H and especially N occurred with an increase of O, and of C:H, C:N and O:C ratios. This could be explained by the oxidative microbial degradation of N-containing components in the starting material, which results in an increase of more oxidized aromatic structures (Amir et al., 2004). Studies have also suggested a partial loss of N-containing structures and

aliphatic side chains during composting, and formation of more oxidized aromatic HAs (Garcia et al., 1992; Diaz-Burgos et al., 1994). In the raw material, the level of LFHAs 0 is less than the level of HAs 0 expressed as dry weight of raw sludge (Table 1). The calculated difference between contents of HAs 0 and LFHAs 0 implies that about 13.8% could represent the long-chain fatty acid removed by purification and which have been accounted for in the level of HAs 0. During composting, the level of LFHAs increased markedly compared with the level of unpurified HAs (Table 1). This difference could be attributed partly to an underestimation of unpurified HAs resulting from a decrease in the amount of free fatty acid isolated with them. Indeed, in the course of composting, microbial populations could use fatty acids readily as a source of energy. The high intensity of the condensed aromatic carbon in LFHAs could also explain this difference. The E4/E6 ratio of LFHAs is below those of HAs without lipid removal, which may be explained by the fact that the compounds remaining in LFHAs, after lipid removal, are mainly condensed aromatic structures. The slight increase in the E4/E6 ratio during composting could originate from reduction in the molecular weight of the HAs through loss of their aliphatic compounds and/or from the increase of their oxygen content (Amir et al., 2003).

#### 3.2. FTIR spectra

The FTIR spectra of HAs 0 and LFHAs from sludge at different stages of composting (Fig. 1) exhibited similar peak locations, but the relative intensity of the absorbances changed with lipid removal and between LFHAs isolated at different stages of composting. The assignments of the main bands, based on numerous studies (Gerasimowicz and Byler, 1985; Hernandez et al., 1990; Ricca and Severini, 1993; Gonzalez-Vila et al., 1999; Amir et al., 2004; Hafidi et al., 2005) are given in Table 2.

Table 1

Elemental composition and E4/E6 ratio of the unpurified humic acids (HAs) and of lipid-free humic acids (LFHAs) extracted from composted sewage sludge at different stages of treatment

Samples	C <sup>a</sup>	H <sup>a</sup>	N <sup>a</sup>	O <sup>a</sup>	Atomic ratio			E4/E6	HA amount (mg g <sup>-1</sup> ) <sup>b</sup>
					C/H	C/N	O/C		
LFHAs 0	51.14	6.45	6.10	36.31	0.66	8.39	0.53	1.62	8.57
LFHAs 1	49.86	5.87	4.46	39.81	0.71	11.17	0.60	1.79	15.00
LFHAs 6	50.08	5.90	4.77	39.26	0.71	10.50	0.59	1.94	17.57
HAs 0*	48.90	6.87	5.61	38.60	0.59	10.00	0.59	3.70	9.94
HAs 1*	49.20	6.42	4.46	39.90	0.64	12.86	0.60	3.93	8.80
HAs 6*	47.84	6.50	4.58	41.00	0.61	12.18	0.64	4.00	11.60

\*Amir et al. (2004).

<sup>a</sup>% calculated on organic matter basis.

<sup>b</sup>Dry weight basis.

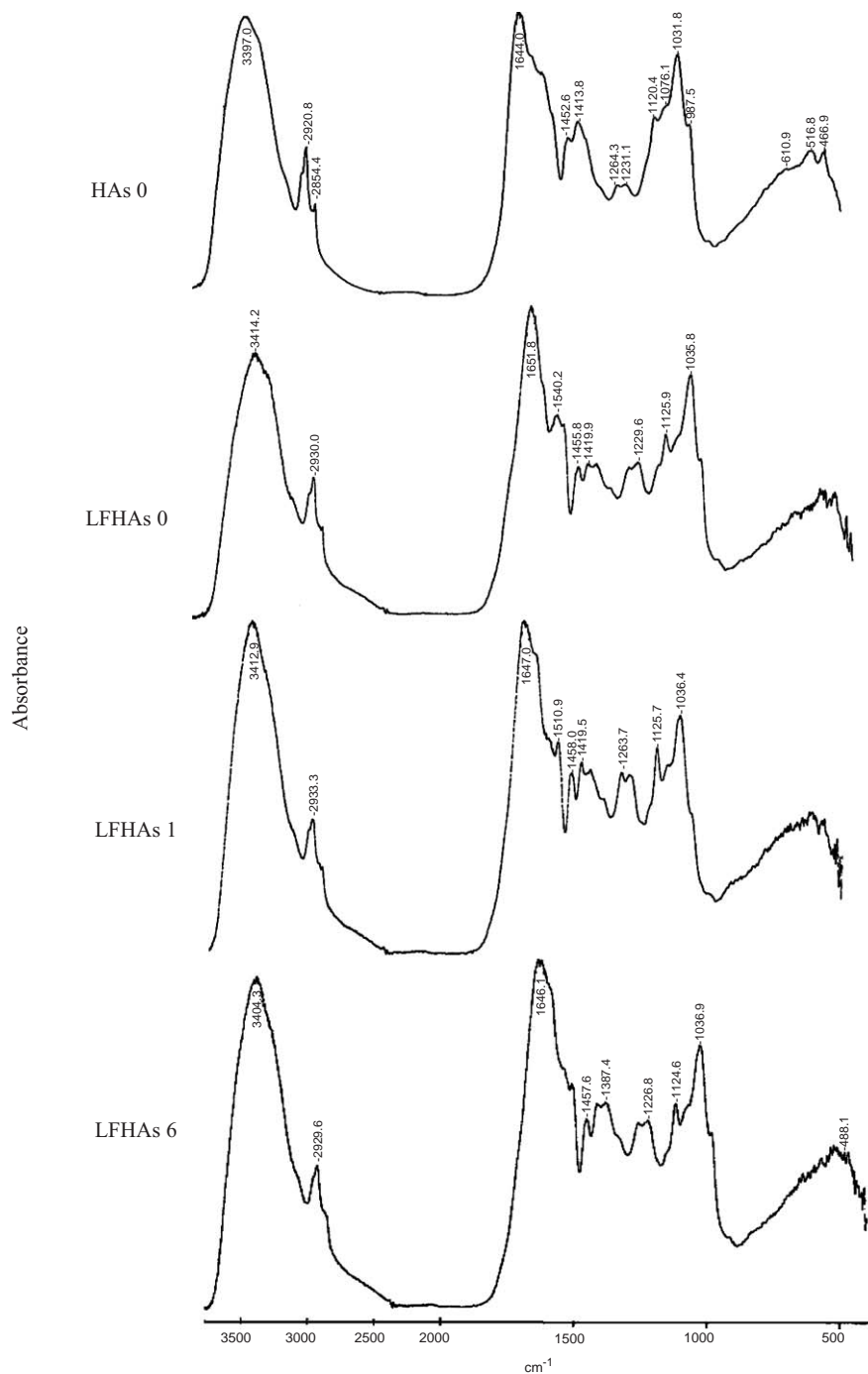


Fig. 1. FTIR spectra of the unpurified HAs extracted from the raw sewage sludge (HAs 0) and of lipid-free HAs extracted from composted sludge at different stages of treatment LFHAs (0, 1, 6 months).

Compared to unpurified HAs 0, LFHAs 0 showed a high incidence of structures absorbing around 1652, 1540 and 1230  $\text{cm}^{-1}$ , assigned to etherified aromatic structures and N-containing compounds. However, these LFHAs are less rich in compounds absorbing around 3400, 2920, 1600, 1450–1400 and 1100  $\text{cm}^{-1}$

corresponding to aliphatic structures such as long-chain fatty acids and carbohydrates with a carboxylic extremity.

During composting, there was a decrease in absorbance around 2930 and 1540  $\text{cm}^{-1}$  related to microbial oxidation of aliphatic and peptidic compounds, whilst

Table 2  
Main absorbance bands in FTIR spectra and their assignments

Bands and peaks (cm <sup>-1</sup> )	Assignments
3300–3400	H-bonded OH groups of alcohols, phenols and organic acids, as well as H-bonded N–H groups
2920–2930	C–H stretching of alkyl structures
1630–1650	Aromatic and olefinic C=C, C=O in amide (I), ketone and quinone groups
1600	Aromatic C=C, COO <sup>-</sup> , C=O
1540–1550	amide (II)
1400	OH of phenols, COO <sup>-</sup> , –CH <sub>3</sub>
1260–1200	amide III or aromatic ethers C–O–C
1170–1030	–C–O–C of carbohydrates, aromatic ethers, Si–O–C groups

the increase of absorbance around 3400, 1600, 1400 and 1230 cm<sup>-1</sup> is attributed to the increased relative intensity of phenolic and etherified aromatic structures.

### 3.3. <sup>13</sup>C-NMR spectra

The <sup>13</sup>C-NMR spectra of HAs 0 and of LFHAs at different steps of composting were interpreted (Fig. 2, Table 3) on the basis of spectra, in the studies of Preston and Schnitzer (1984), Inbar et al. (1991), Ricca and Severini (1993), Gonzalez-Vila et al. (1999), Amir et al. (2004) and Hafidi et al. (2004). Carbon distribution among various chemical groups was obtained by integration of the spectrum into four areas (Table 4). The spectra of LFHAs (Fig. 2) were better resolved and revealed a high intensity of aromatic structures. The lipid removal may reduce the tendency of humic molecules to form aggregates in solution, and diminish the complex stereochemistry of the high-molecular-weight humic material without purification. Aliphatic carbon decreased, while the aromaticity of extracts was generally enhanced by purification. Values for the carboxylic and amide carbon increased with lipid removal (Table 4). The purified LFHAs 0 had fewer aliphatic carbons, an average of 38.1% less than unpurified HAs 0, which had 69.6%. In contrast, aromatic carbons in LFHAs 0 showed higher values, an average of 40.5% compared to 12.4% in HAs 0. Réveillé et al. (2003) reported that the aliphatic character of the HAs of sludge is attributable to the presence of lipids presumably trapped in the humic structure.

Therefore, almost 45% of the aliphatic structures in HAs without previous lipid removal were composed of free lipids, mainly long-chain fatty acids. This level increased to 62% during the stabilization phase and decreased slightly to 58% in the last stage of composting. This increase in lipid release could be the result of the depolymerization of organic macromolecules through microbial decomposition and changes in the

physico-chemical properties of the sludge heap during composting.

The LFHAs showed, during composting, a significant decrease in C-alkyl or non-substituted aliphatic carbon with a marked increase of aromatic carbons (Table 4). The increase of aromaticity could have resulted from microbial synthesis, or as a result of a concentration effect brought about by the reduction of other classes of compounds. In the substituted alkyl area, although a decrease of signal of around 60 ppm occurred and is attributed to decomposition of amidic compounds, the methoxyl of etherified aromatic compounds around 56 ppm always showed a high intensity and appeared to be resistant to microbial decomposition (Amir et al., 2003, 2004). Carboxyl carbon showed a slight decrease during the stabilization phase (1 month), and then increased during the maturation phase. The decrease of resonance in the carboxyl area during the stabilization phase may originate from decomposition of aliphatic acids or amides. The subsequent increase during the maturation phase is probably related to the increase in the proportion of benzoic sub-units (Gerasimowicz and Byler, 1985; Garcia et al., 1992).

In the case of unpurified HAs, the signal in the carboxyl region of the spectrum shows important variations, which attributed to the loss of free lipid content during composting from 2.14% to 0.83% (expressed in dry weight). The variation in other aromatic and aliphatic areas is markedly less intense compared to the purified HA structures (LFHAs) in the course of composting. Therefore, it may be concluded that with purification more representative information about the change of structural humic macromolecules during composting has been obtained.

## 4. Conclusion

Using the chemical methods of elemental analysis, Fourier transform infrared spectroscopy and <sup>13</sup>C-NMR

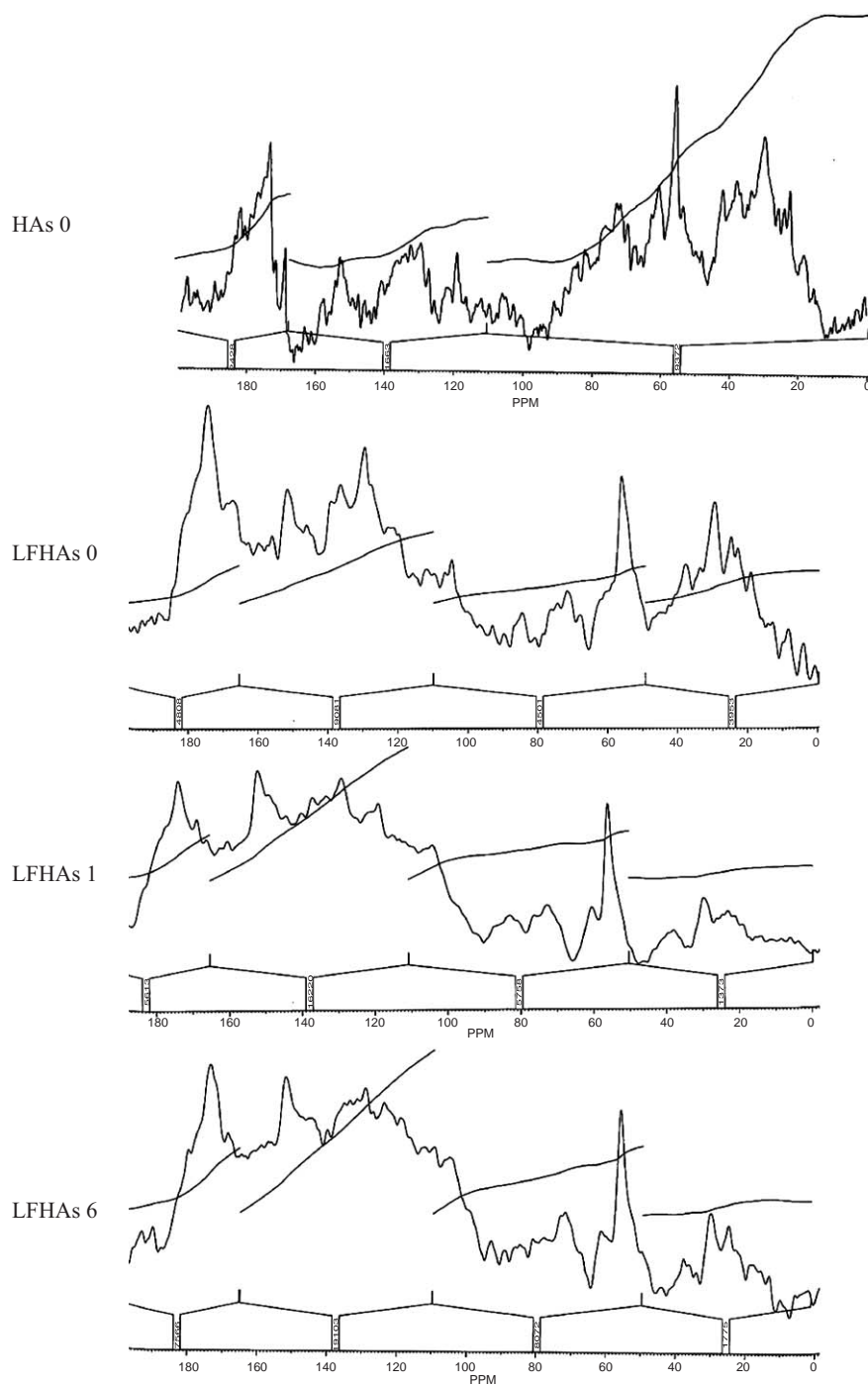


Fig. 2.  $^{13}\text{C}$ -NMR spectra of the unpurified HAs extracted from the raw sewage sludge (HAS 0) and of lipid-free HAs extracted from composted sludge at different stages of treatment LFHAs (0, 1, 6 months).

to compare HAs isolated from sludge after or without lipid removal, show that almost half the amount of aliphatic structures in HAs (LFHAs) without lipid removal was mainly of free fatty acids. The purified HAs were richer in aromatic structures, and low in aliphatic structures. During composting, the structural changes observed in the LFHAs were a marked increase

in etherified aromatic structures and a decrease in aliphatic structures. These variations are underestimated in the case of unpurified HAs owing to the occurrence of free fatty acids the levels of which fell during composting. As a result of lipid removal before HA isolation, best information about structural change in the humic during composting has been obtained.



Table 3  
Resonance signals in  $^{13}\text{C}$ -NMR spectra and attributed chemical groups

Signal (ppm)	Attributed chemical groups
0–50	Paraffinic C in alkyl chains
50–110	Aliphatic carbons substituted by oxygen and nitrogen, mainly around 56 ppm methoxyl groups of aromatic ethers
110–130	Unsubstituted aromatic C
130–145	Carbon-substituted aromatic carbons
145–160	Oxygen- or nitrogen-substituted aromatic carbons
160–200	Carboxylic carbons, ester or amide

Table 4  
Changes of carbon distribution in the unpurified humic acids (HAs) and of lipid-free humic acids (LFHAs) extracted from composted sludge at different stages of treatment

Samples	C-alkyl <sup>a</sup>	O, N-alkyl <sup>a</sup>	C-aliphatic <sup>a,b</sup>	C-aromatic <sup>a</sup>	C-carboxylic, C-amide <sup>a</sup>
LFHAs 0	17.61	20.50	38.11	40.46	21.42
LFHAs 1	4.74	19.88	24.62	56.00	19.38
LFHAs 6	4.86	22.11	26.97	52.31	20.72
HAs 0*	/	/	69.61	12.35	18.03
HAs 1*	/	/	64.43	24.28	11.29
HAs 6*	/	/	64.64	20.65	14.70

\*Amir et al. (2004).

<sup>a</sup>Values are expressed as percentages of the whole spectrum area.

<sup>b</sup>C-aliphatic = C-alkyl + O- or N-alkyl.

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