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Characterization of humic acids extracted from sewage sludge during composting and of their Sephadex[®] gel fractions

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Abstract – The molecular weight of humic acids extracted from sewage sludge at different stages of composting is estimated by separation on Sephadex gel. The two types of fractions obtained are characterized by Fourier Transform Infrared, Ultraviolet-Visible spectroscopy, and the E4/E6 ratio. The fraction of high molecular weight excluded from the gel largely prevails. This fraction consists of polycondensed lignin-like structures bearing peripheral lipidic and peptidic chains. The fraction retained on the gel is characterized by a low and medium molecular weight and is composed of low-condensed lignin constituents with high acidic functional group content. During composting, an increase in aromaticity and polycondensation degree of humic acids, and decrease in aliphatic, peptidic and lipidic compounds are observed. The results obtained show the continuity of the molecular weight gradient from small to very large humic macromolecules, and that humic macromolecules can be regarded as associations of molecular humic sub-units of a similar chemical nature.

humic acid / sewage sludge compost / gel chromatography / spectroscopic characterization

Résumé – Caractérisation des acides humiques extraits lors du compostage de boues de station d'épuration et de leurs fractions sur gel de Sephadex[®]. L'estimation du poids moléculaire des acides humiques extraits des boues résiduaires lors du compostage est réalisée à l'aide d'une séparation sur gel de Séphadex. Les deux types de fractions obtenues sont caractérisés par spectroscopie infrarouge à transformée de Fourier, ultraviolet-visible, et par le rapport E4/E6. La fraction de grand poids moléculaire exclue du gel prédomine largement. Elle est constituée de structures type lignine largement polymérisées sur lesquelles sont branchés des composés périphériques type peptidiques et lipidiques. La deuxième fraction, retenue sur le gel, caractérisée par des molécules de faible et moyen poids moléculaire, est composée de structures type lignine faiblement polymérisées avec une dominance de groupements fonctionnels. Lors du compostage, une augmentation du degré de polymérisation et d'aromaticité des acides humiques, et une diminution des composés aliphatiques, peptidiques et lipidiques ont été enregistrées. L'ensemble des résultats montre une continuité du gradient moléculaire des petites aux grosses macromolécules humiques, et la formation des macromolécules humiques à partir de sous-unités de nature chimique très semblable.

acide humique / compost de boue résiduaire / chromatographie sur gel / caractérisation spectroscopique

1. INTRODUCTION

Organic waste recycling in agriculture as soil amendments has increased greatly over the last few years, because it is a proper solution for resource-recovery, and avoids environmental and economic problems resulting from the disposal of large amounts of organic wastes [1]. Soil application of organic wastes and residues of any nature requires, however, that these materials be previously subjected to appropriate treatments. These treatments tend, on the one hand, to minimize or eliminate a number of probable adverse

effects related to this practice and, on the other hand, to optimize the efficiency of these materials once in the soil. The composting process is a suitable treatment used to produce a stable product, rich in humic-like substances that are environmentally safe and practicable at acceptable operational costs [28]. The agronomic interest of the final product is identified by the determination of the quantity and quality of humic-like acids present in it [2, 29, 30]. Thus, the aims of numerous studies have been the determination of the molecular size distribution of such materials and the characterization of the resulting fractions. According to Senesi

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and Brunetti [27], the composting treatment allows the transformation of organic wastes into humic-like compounds relatively homogeneous in composition, and chemical and structural properties. This process is accompanied by the loss of peptidic, aliphatic and polysaccharidic structures, possibly due to intense mineralization achieved by microbial activity during the composting process and formation of more polycondensed, oxidized, unsaturated and aromatic humic acids. Other authors, e.g., Zhigunov and Simakov [32], Inoko and Tamai [21] and Tomikawa and Oba [31] suggested the formation of more oxidized humic compounds, with a concomitant lowering of molecular weight, during the composting process.

The present work examines the spectroscopic characteristics (UV-visible and FTIR) of humic acids extracted from sewage sludge at different times of composting, and of the humic fractions obtained by Sephadex gel chromatography.

2. MATERIALS AND METHODS

2.1. Humic acid extraction

The humic acids were extracted from a mixture of sewage sludge (165 kg) and straw (20 kg) at various stages of composting (1, 3, 6 and 9 months). At first, the sample (30 g) was successively treated three times with 40 ml of distilled H₂O to extract the non-humic, water-soluble substances (sugars and proteins), in order to avoid interference between these compounds and humic compounds [9]. The extraction was then carried out on 30 g of the pre-treated sample with 40 ml of NaOH (0.1 N). Extraction was repeated several times until uncolored supernatants were obtained. Centrifugation was then carried out at 4000 *g* for 15 min, and filtration was done on Whatman paper (125 mm). The filtered solutions were precipitated by acid treatment with 3 N H₂SO₄ for 24 h at 4 °C. The precipitated humic acids were separated from fulvic acid solution by filtration on Whatman paper (125 mm) and then were solubilized by NaOH solution (0.1 N). The obtained humic acid solution was dialyzed with a Spectra Por MWCO membrane (7500 Dalton) to eliminate excess salts. The dosage of humic acids was done by KMnO₄ oxidation [12].

2.2. Gel fractionation

Fractionation of humic acids was realized by passing 2 ml of humic acid solution through Sephadex gels with different separation powers: G25 (17 cm × 4.9 cm, 5000 Da), G75 (17 cm × 4.9 cm, 50 000 Da) and G100 (14 cm × 4.9 cm, 100 000 Da). The obtained excluded fraction of G25 was lyophilized, redissolved in 2 ml of H₂O with the addition of a small drop of 0.1 N NaOH, and passed through G75. The excluded fraction G25-75 obtained by the latter separation was lyophilized, redissolved (2 ml of H₂O, and a drop of 0.1 N NaOH) and then passed through G100 to achieve the G25-75-100 fractionation.

The eluant used was distilled water; the fractions (about 2 ml) were collected every 3 min and the absorbance of these fractions was measured with a Unicam SP 1800

spectrophotometer at 350 nm. In order to obtain enough material for the spectroscopic analysis, each fractionation was repeated four times.

2.3. Fourier transform infrared spectroscopy (FTIR)

Potassium bromide pellets of each sample were prepared by pressing under vacuum a mixture of 250 mg of dried KBr and 1 mg of lyophilized sample (humic acid and separated fractions) [15]. The spectra were obtained using a FTIR Perkin Elmer 1600 spectrophotometer over a 4000 to 400 cm⁻¹ range, at a rate of 16 nm/s.

2.4. Ultraviolet-visible spectroscopy

Absorbance measurements of the humic acid solution obtained from sewage sludge at various stages of composting and of its fractions excluded on G25, G75 and G100, and their dilute solution of 1/2, 1/4, 1/8, 1/16 and 1/32 were made in 1 cm quartz cells over a 800 to 200 nm range using a Unicam SP 1800 spectrophotometer.

The E4/E6 ratio was measured as the ratio of absorbances at 465 nm and 665 nm after dissolution of the lyophilized sample (2 mg) of the humic acids and of their excluded fractions in 25 ml of 0.05 N NaHCO₃ [13].

3. RESULTS AND DISCUSSION

3.1. Gel permeation chromatography and E4/E6 ratio

Fractionation on Sephadex® gel is a very useful technique for estimating the molecular weight of humic acids [5, 23, 30, 31]. The chromatograms obtained on G25 Sephadex for the humic acids extracted from the material at various stages of composting show a similar pattern consisting of one symmetrical peak (Fig. 1), whereas the chromatograms on G75, G100, G25-75 and G25-75-100 also show one predominant, but asymmetrical peak, which could be divided into two main portions: I and II (Fig. 1). Portion I corresponds to the excluded fraction, and is the most abundant fraction, consisting of large molecules with high molecular weight. Portion II corresponds to the retained fraction that consists of molecules of medium and small sizes which are lower than the diameter of the gel pores. Thus, molecules of large molecular weight prevail in the humic acids of sewage sludge during composting, as previously observed by other authors [3, 24]. The second peak (Fig. 1) present in most chromatograms is very small and corresponds, according to Blondeau [10] and Bailly [6], to Na-humate complexes, whose solubility is influenced by their adsorption on the gel. The only significant difference between these chromatograms is the absorbance intensity during composting of the first peak, which correlates with the increase from 1.4 mg/ml after 1 month to 1.77 mg/ml after 9 months of humic matter concentration in the sample.

Moreover, with the fractionation and the increase in the gel separation power from G75 to G100, a parallel decrease in the E4/E6 ratio of the excluded fraction is observed at any stage of composting (Tab. I). According to Chen et al. [13] and Riffaldi et al. [26], the decrease in the E4/E6 ratio may be ascribed to either an increase in the molecular weight of the excluded

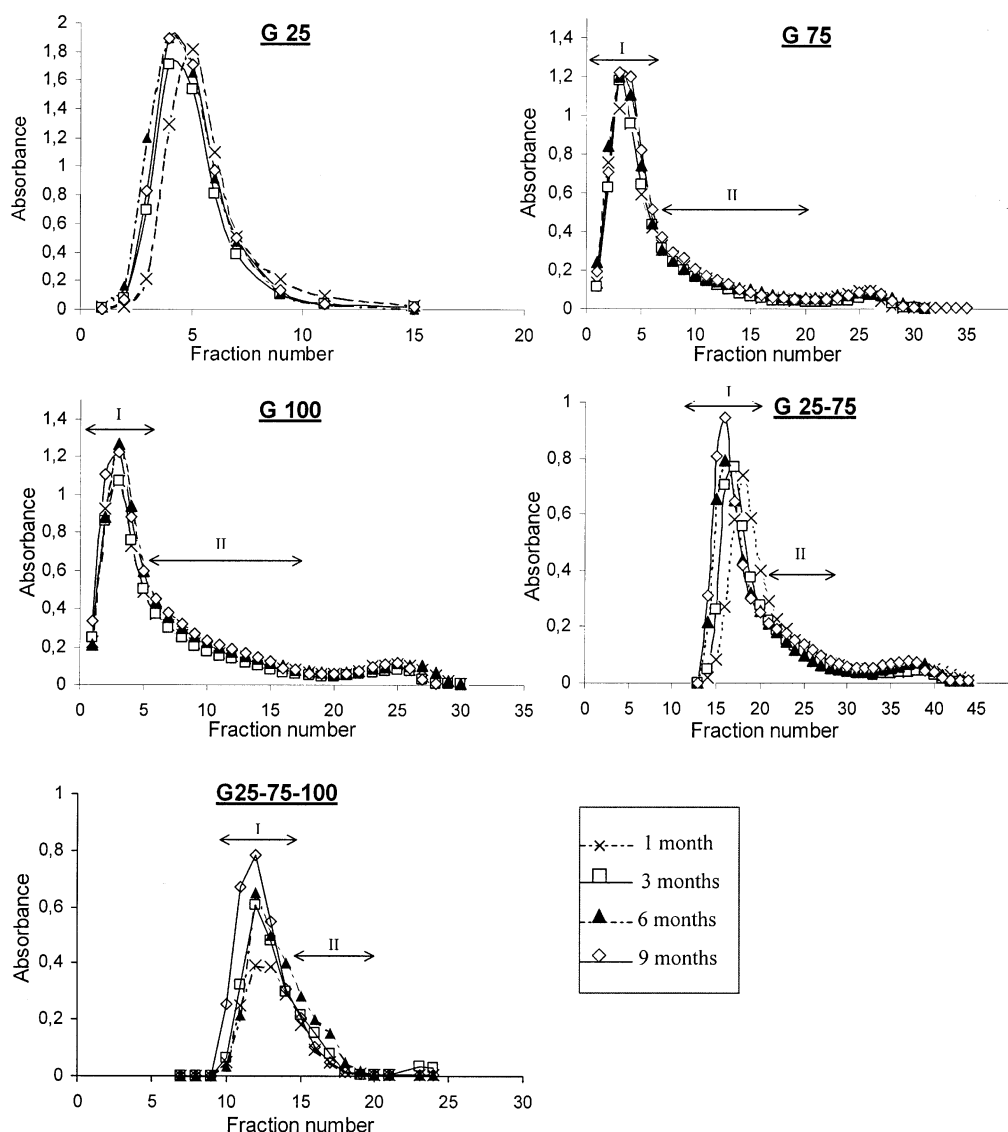


Figure 1. Gel permeation patterns of humic acids extracted from sewage sludge during composting on Sephadex gel G25; G75, G100 and G25-75-100.

fraction, or to a decrease in the total acidity of this fraction. Thus, by using gels with increasing separation power, a more efficient separation of the medium and small molecular weight molecules from the large weight fraction is achieved, and consequently a better selection of the molecules with a larger molecular weight in this fraction. These observations confirm the continuity of the molecular weight gradient from small to very large humic macromolecules, considered by many authors [6, 22].

3.2. FTIR spectroscopy

The FTIR spectra of unfractionated humic acids, G0, at various stages of composting, and of the two fractions separated

Table I. E4/E6 ratio of unfractionated humic acids at different stages of composting and of their fractions excluded from Sephadex gel G75 and G100.

E4/E6	HA	E-HA (G75)	E-HA (G100)
1 month	3.93	3.66	3.50
3 months	3.93	3.63	3.50
6 months	4.00	3.80	3.66
9 months	4.00	3.83	3.73

HA: unfractionated humic acids, E-HA: excluded humic acid fractions.

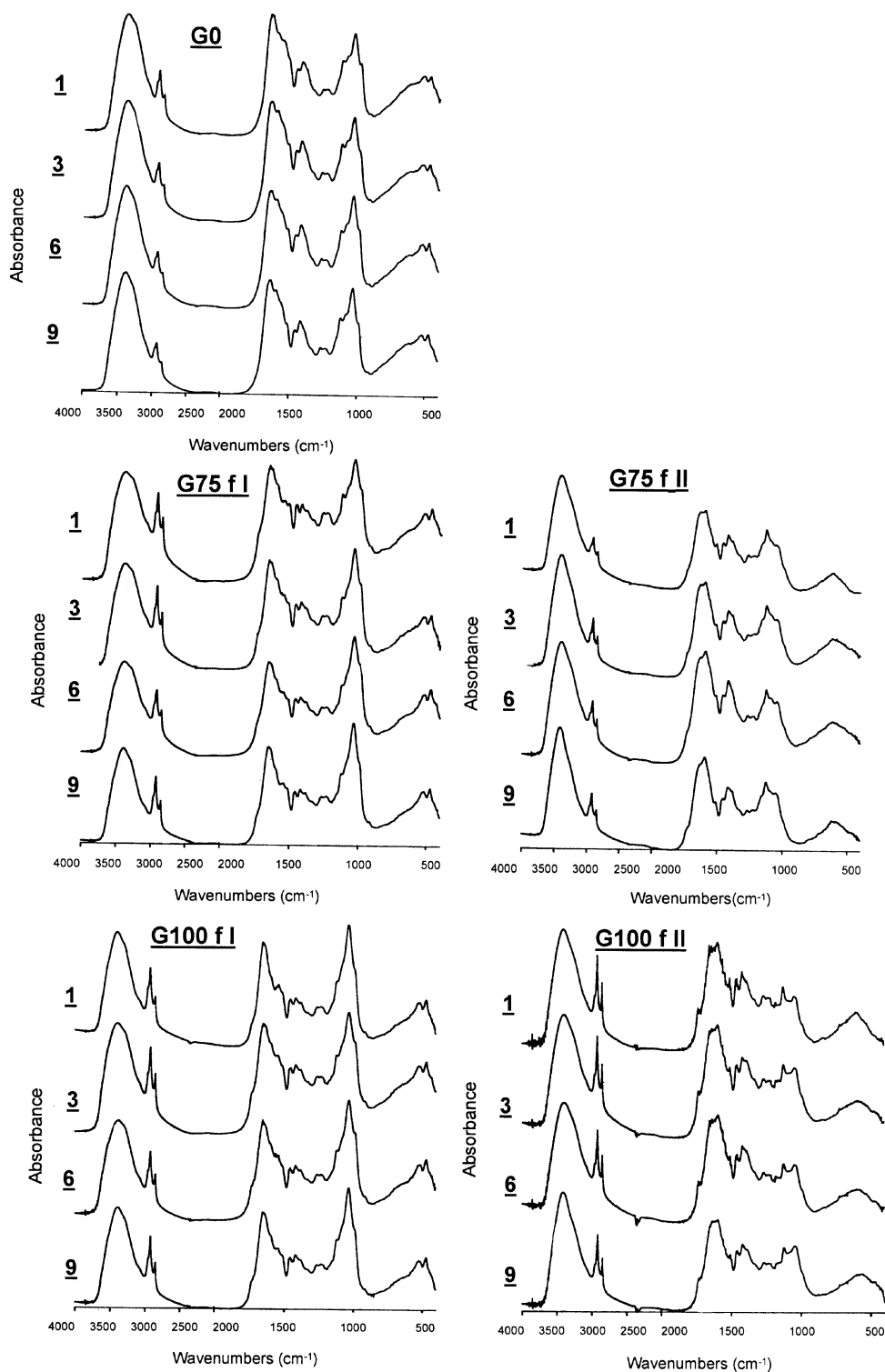


Figure 2. FTIR spectra of the unfractionated humic acids, G0, of sewage sludge at different stages of composting (1, 3, 6 and 9 months) and of their fractions (I and II) obtained from Sephadex gels G75 and G100.

by the gels G75 and G100 (Fig. 2), show a great similarity in some absorption bands with some differences in the intensity. This result indicates the maintenance of the basic chemical structure of the unfractionated humic acids and of their fractions during composting. The assignment and interpretation of

the bands were based on references [6–8, 14–17, 19] and are the following: (a) a broad band at $3300\text{--}3400\text{ cm}^{-1}$, assigned to H-bonded OH groups (alcohols, phenols and organic acids) and N-H groups; (b) an intense peak at $2920\text{--}2930\text{ cm}^{-1}$, due to asymmetrical aliphatic carbon, especially CH_2 ; (c) a less

intense peak at 2855 cm^{-1} , due to symmetrical aliphatic carbon CH_2 ; (d) a broad band at $1630\text{--}1650\text{ cm}^{-1}$ ascribed to olefinic and aromatic $\text{C}=\text{C}$, $\text{C}=\text{O}$ (amide I) and quinone groups with a shoulder at 1600 cm^{-1} corresponding to aromatic $\text{C}=\text{C}$, $\text{C}=\text{O}$, and/or $\text{C}=\text{O}$ of bonded conjugated ketones, quinone, and $\text{C}=\text{O}$ stretch of amide (I) and a second less marked shoulder at $1540\text{--}1550\text{ cm}^{-1}$, due to amide II and/or aromatic $\text{C}=\text{C}$; (e) a small band at $1460\text{--}1380\text{ cm}^{-1}$ consisting of a weak peak at 1455 cm^{-1} corresponding to CH_3 and CH_2 , and a second, more pronounced peak near 1420 cm^{-1} corresponding to several groups including phenolic OH , COO^- asymmetric stretch, stretching of ortho-disubstituted aromatic rings; (f) a weak band at $1265\text{--}1223\text{ cm}^{-1}$ due to amide III or aromatic ethers; (g) a broad band at $1120\text{--}980\text{ cm}^{-1}$ consisting of a large peak centered near 1034 cm^{-1} characteristic of aromatic ethers, and possibly polysaccharides and Si-O of silicates; and (h) two peaks at 520 and 470 cm^{-1} due to para-di-substituted aromatic ring bend.

Comparison between the spectra of the excluded (f I) and retained (f II) fractions on G75 and G100 (Fig. 2) shows the presence of the same basic FTIR bands, in agreement with Ouattmane et al. [22], who suggested a similar chemical structure between fractions with various molecular weights. However, some differences in the absorption intensity of some bands were observed between fractions I and fractions II.

Spectra of the excluded fractions I show well-pronounced peaks at 1034 cm^{-1} , 2925 cm^{-1} and 2855 cm^{-1} ; peaks with relatively high intensity are also observed near 1650 cm^{-1} , 527 and 470 cm^{-1} , while the absorption intensity near 1600 cm^{-1} and of the peak at 1420 cm^{-1} are weak.

In contrast, the retained fractions II are characterized by the disappearance of the peaks centered at 1034 cm^{-1} , 527 and 470 cm^{-1} and the decrease in absorption near 1650 cm^{-1} . However, these fractions maintain the peaks at 1600 and 1420 cm^{-1} , and show the appearance of new weak peaks at 1734 and 1180 cm^{-1} specific to COOH [16, 20], and at $1505\text{--}1510\text{ cm}^{-1}$ which corresponds either to amides II [11] or to aromatic ring stretch in lignine-like structures [18]. The G100-retained fractions exhibit a greater intensity of the peaks at 2920 and 2850 cm^{-1} and a stronger reduction in the peak centered at 1034 cm^{-1} , in comparison with the G75-retained fraction.

Thus, in agreement with Tomikawa and Oba [31], the humic acid fractions characterized by a high molecular weight consist of polycondensed aromatic structure lignin-like constituents bearing peripheral lipidic and peptidic chains. In contrast, the fractions of low or medium molecular weight are composed of low condensed lignin-like structures with a high content of acidic functional groups.

The changes in the aromaticity and polycondensation degree during composting are reflected by the differences in the ratios of the main FTIR peaks of humic acids and of excluded fractions G75 and G100 recorded between the first stage (1 month) and the final stage (9 months) (Tab. II). During composting an increase in the aromaticity degree ($1650/2925$ and $1650/2855$) and in the polycondensation degree ($1034/2925$, $1034/2855$ and $1034/1540$) of humic acids and their excluded fractions from G75 is shown together with lowering of aliphatic, lipidic and peptidic compounds. In contrast, the fractions excluded from G100 present only a weak increase in these ratios. Thus, during the humification

Table II. Increase in the ratios of the main FTIR absorption peaks of unfractionated humic acids and of their fractions excluded on Sephadex gels G75 and G100 between the first stage (1 month) and final stage (9 months) of composting.

Ratio	HA (1)	E-HA (G75) (1)	E-HA (G100) (1)
1650/2925	20	8	0.20
1650/2855	20	22	2
1034/2925	30	29	7.20
1034/2855	30.50	45	9.54
1034/1540	6	31.50	0.01

(1) the results are expressed in %.
HA: unfractionated humic acids, E-HA: excluded humic acid fraction on Sephadex gel.

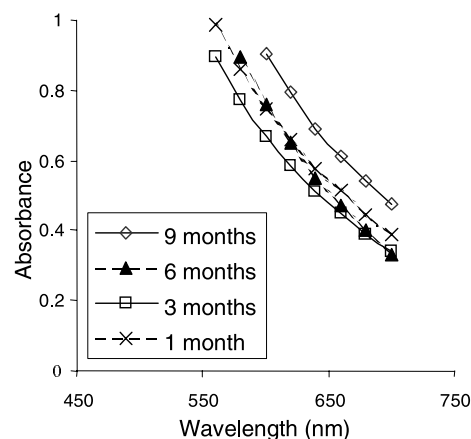


Figure 3. Ultraviolet-visible absorbance curves of unfractionated humic acids extracted from the sewage sludge at different stages of composting.

process, aliphatic, lipidic and peptidic compounds of large molecular weight are decomposed, and more oxidized and polycondensed humic compounds appear with relatively low molecular weight.

Results similar to those described here were reported by Zhigunov and Simakov [32], who found a higher proportion of the high molecular weight fraction in humic acids separated from decomposing plant residues during the first stages of decomposition, with respect to the later stages. Inoko and Tamai [21] also suggested that many animal and plant residues entering the soil are decomposed and converted into humic compounds with a concomitant lowering of molecular weight. Similarly, Tomikawa and Oba [31] found that with the progress of humification, the relative absorbance of large particle components and the polydispersion of particle weights are decreased, and that the degree of humification was maximum for the fraction with medium or small particle weight.

3.3. UV-visible spectroscopy

UV-visible spectra of humic acids extracted at various stages of composting show similar patterns (Fig. 3), which confirms the resemblance of the basic chemical structures of

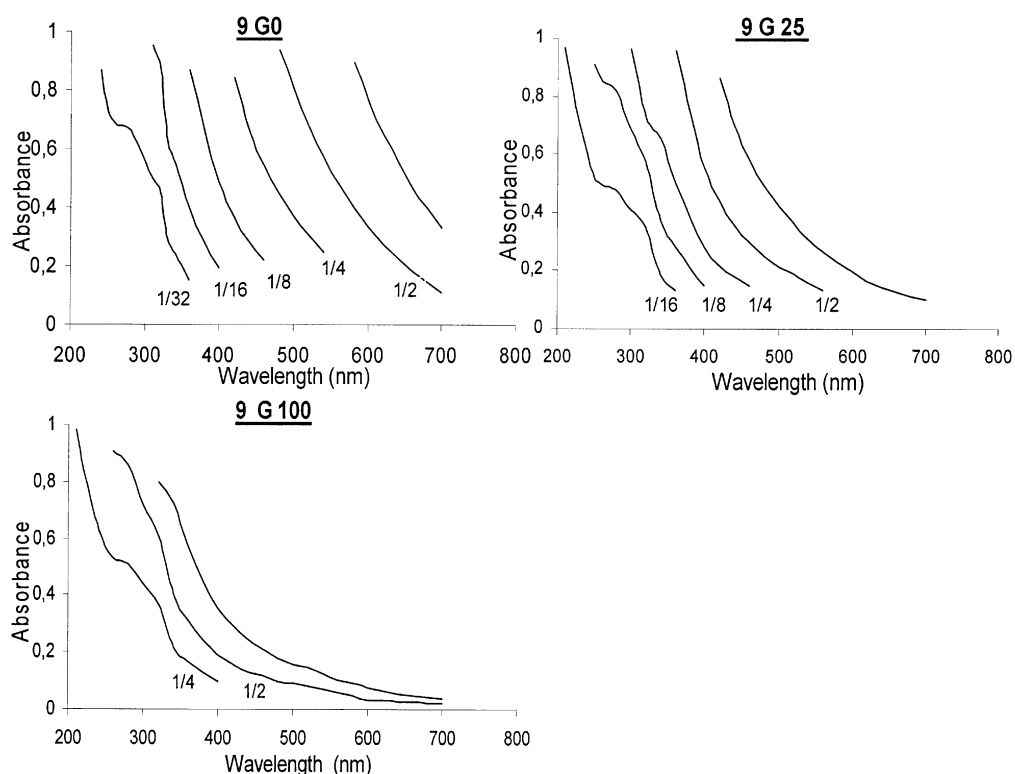


Figure 4. Ultraviolet-visible absorbance curves at different dilutions (1/2, 1/4, 1/8, 1/16 and 1/32) of unfractionated humic acids extracted from the sewage sludge at 9 months of composting and of their G25 and G100 excluded fractions on Sephadex.

humic acids at any stage of composting [4]. The decrease in absorbance of humic acid observed during the first month, and its following increase to the final stage after 9 months of composting, may be due to the initial mineralization phenomenon and final resynthesis of complex humic molecules during composting, as suggested by Senesi et al. [28].

On the other hand, spectra obtained by dilutions (1/2, 1/4, 1/8, 1/16 and 1/32) of these humic acids and their excluded fractions, show a dilution effect, indicated by a maximum ultraviolet absorbance at 260–340 nm; the case of 9 months of composting is shown in Figure 4. This is attributed to more exposed C=O, COOH and OH groups following separation by dilution of the molecules or micelles previously bound, or unfolding of molecular links, which are masked in the concentrated solutions [4]. This dilution effect increases with the increase in the gel separation power from G25 to G100 (Fig. 4). According to Piccolo [25], during gel permeation, the presence of COO⁻ groups inside humic acid micelles induces electrostatic repulsion of negative charges and disruption to smaller aggregates from the original structures. The electrostatic repulsion appears to momentarily overcome the attractive hydrophobic forces that hold together the humic macromolecules. The small sub-units formed are progressively separated from the large fraction, and diffuse through the small gel pores; thus their elution is delayed. The humic sub-units are finally eluted for volumes typical of low molecular weight compounds.

4. CONCLUSION

Humic acids extracted from sewage sludge at different stages of composting were analyzed by various chemical methods (chromatography on Sephadex gel, ultraviolet-visible and FTIR spectroscopy). The results obtained show a predominance of the fraction excluded from the gel of high molecular weight, which consists of polycondensed aromatic lignin-like structures, bearing peripheral lipidic and peptidic chains. In contrast, the fractions retained on the gel of low or medium molecular weight are composed of low condensed lignin-like structures with a high content of acidic functional groups.

During gel permeation, the presence of COO⁻ groups inside humic acid micelles induces electrostatic repulsion of negative charges, which overcome the humic hydrophobic forces and then the disruption to smaller aggregates from the original structures. The small sub-units formed are progressively separated from the large fraction, and diffuse through the small gel pores; thus their elution is delayed for volumes typical of low molecular weight compounds. This dilution effect increases with the increase in the gel separation power.

During composting, the aliphatic, lipidic and peptidic compounds of large molecular weight are decomposed, and more oxidized and polycondensed humic compounds appear with relatively low molecular weight. Thus, with composting appear the humic macromolecules consisting mainly of molecular humic sub-units of a similar chemical nature.

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